*Research Article*

# Lipoxin A$_4$ Preconditioning and Postconditioning Protect Myocardial Ischemia/Reperfusion Injury in Rats

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This study aims to investigate the pre- and postconditioning effects of lipoxin A$_4$ (LXA$_4$) on myocardial damage caused by ischemia/reperfusion (I/R) injury. Seventy-two rats were divided into 6 groups: sham groups (C$_1$ and C$_2$), I/R groups (I/R$_1$ and I/R$_2$), and I/R plus LXA$_4$ preconditioning and postconditioning groups (LX$_1$ and LX$_2$). The serum levels of IL-1$\beta$, IL-6, IL-8, IL-10, TNF-$\alpha$, and cardiac troponin I (cTnI) were measured. The content and the activity of Na$^+$-K$^+$-ATPase as well as the superoxide dismutase (SOD), and malondialdehyde (MDA) levels were determined. Along with the examination of myocardium ultrastructure and ventricular arrhythmia scores (VAS), connexin 43 (Cx43) expression were also detected. Lower levels of IL-1$\beta$, IL-6, IL-8, TNF-$\alpha$, cTnI, MDA content, and VAS and higher levels of IL-10, SOD activity, Na$^+$-K$^+$-ATPase content and activity, and Cx43 expression appeared in LX groups than I/R groups. Besides, H&E staining, TEM examination as well as analysis of gene, and protein confirmed that LXA$_4$ preconditioning was more effective than postconditioning in preventing arrhythmogenesis via the upregulation of Cx43. That is, LXA$_4$ postconditioning had better protective effect on Na$^+$-K$^+$-ATPase and myocardial ultrastructure.

## 1. Introduction

Myocardial ischemia/reperfusion (I/R) injury, a general health problem, is due to blood restoration after a critical period of coronary artery obstruction, which is associated with clinical interventions such as thrombolysis, angioplasty, and coronary bypass surgery. This reperfusion injury involves the activation of an inflammatory cascade and is manifested as functional impairment, arrhythmia, and accelerated progression of cell death in certain critically injured myocytes. The major mediators of reperfusion injury are oxygen radicals, calcium loading, and neutrophils [1–3]. Currently, great progress has been made in the basic research about the mechanism of myocardial I/R, but symptomatic treatment still remains as the primary therapeutic plan. With the development of molecular biology, pharmacy, and other related disciplines, it will be an important task for scientists in the future to find a safer, more effective therapeutic method.

Lipoxins (LXs) are the most recent addition to the family of bioactive products generated from arachidonic acid [4]. These molecules have both proinflammatory and anti-inflammatory actions [5] and inhibit neutrophil/granulocyte infiltration and reactive oxygen species (ROS) production [6, 7]. Lipoxins are short-lived endogenously produced nonclassic eicosanoids, whose appearance in inflammation signals the resolution of inflammation [8, 9]. Recent studies showed that LX could protect several organs such as heart, brain, lung, kidney, and stomach from I/R injury [6, 10–13]. However, there has been little research about their effect on myocardial systemic inflammation, ultrastructure changes, and arrhythmia caused by I/R injury.

Therefore, we aimed to investigate the effects of LXA$_4$ preconditioning and postconditioning on myocardial I/R injury in rats. In particular, we studied the effects of LXA$_4$ on oxidative stress, inflammatory reaction, Na$^+$-K$^+$-ATPase, and connexin 43 (Cx43) alternations caused by I/R injury.

## 2. Materials and Methods

*2.1. Rat Model of Myocardial Ischemia/Reperfusion Injury.* All animal procedures were approved by Wenzhou Medical College Animal Care and Use Committee, which is certified by the Chinese Association of Accreditation of Laboratory Animal Care (SYXK, Zhejiang 2010-0150). Sprague-Dawley (SD) male rats (eight weeks old, 200 to 250 g) were fed a standard diet and maintained in controlled environment at $25 \pm 1°C$ under a 12 h light-dark cycle. Briefly, rats were anesthetized by an intraperitoneal injection of 10% chloral hydrate (300 mg·kg$^{-1}$ body weight) and placed in a supine position. Blood was collected by femoral venipuncture. Next, the animals were intubated for artificial ventilation with 100% oxygen using a small animal breathing machine (tidal volume 5 mL, frequency 70 per min) and monitored by ECG. Thoracotomy was performed between the sternum and left costa, and then the pericardium was gently opened. Myocardial ischemia was induced by ligating the left anterior descending coronary artery (LAD) using a 3–0 silk suture with a section of PE tubing placed over the LAD and 1 mm from the tip of the normally positioned left atrium, and the coronary artery was occluded by pulling on the suture tightly 10 min later. After 30 min of myocardial ischemia, reperfusion started by releasing the ligature and removing the tube for 120 min. The chest wall was closed, the animal extubated, and body temperature maintained using a 37°C warm plate.

The indications of successful LAD occlusion included elevated ST segment (0.1 mv) or sharp rise of T wave, wider and higher QRS wave on ECG, and visual cyanosis of myocardial discoloration. The indications of successful reperfusion were ST segment depression ($\geq 1/2$) of the ECG, gradual falling of T wave, and QRS wave, and myocardial color turning to pink [14–16].

*2.2. Animal Treatment and Grouping.* LXA$_4$ (C$_{20}$H$_{32}$O$_5$, see Supplementary Figure 1 in the Supplementary Material available online at http://dx.doi.org/10.1155/2013/231351) was purchased from Cayman Chemical Company, Ann Arbor, USA (cat. number 90415). Seventy-two SD rats were used in the study and were randomly divided into 6 groups as follows: (1) applying LXA$_4$ before I/R group 1 (LX$_1$): femoral vein injection of LXA$_4$ (100 $\mu$g/kg, dissolved in normal saline 2 mL/kg) before the I/R procedure; (2) applying LXA$_4$ after I/R group 2 (LX$_2$): after 30 min of myocardial ischemia, reperfusion lasted for 30 min before LXA$_4$ (100 $\mu$g/kg, dissolved in normal saline 2 mL/kg) was injected through femoral vein. After that, reperfusion was resumed for an additional 90 min; (3) ischemia/reperfusion group 1 (I/R$_1$): femoral vein injection of normal saline (2 mL/kg) before I/R; (4) ischemia/reperfusion group 2 (I/R$_2$): after 30 min of myocardial ischemia, reperfusion lasted for 30 min, and then normal saline (2 mL/kg) was injected through femoral vein. After that, reperfusion was resumed for an additional 90 min; (5) sham group 1 (C$_1$): rats underwent a similar operation without myocardial I/R, and other treatment was the same as I/R$_1$ group; (6) sham group 2 (C$_2$ group): rats underwent a similar operation without myocardial I/R, and other treatment was the same as I/R$_2$ group.

*2.3. Blood Collection and Tissue Harvest.* Blood samples were collected in each group immediately before thoracotomy and after anaesthetization (T$_1$) or after experiments (T$_2$). In groups C$_1$ and C$_2$, T$_2$ was obtained after 150 min of placing surgical suture under LAD. For all other groups, T$_2$ blood samples were obtained after 120 min of reperfusion. The heart was removed after obtaining blood samples (T$_2$), and a portion of myocardial tissue was fixed in 4% formalin. Pathological examination of paraffin-embedded sections was performed. A separate portion of myocardial tissue was frozen in liquid nitrogen and was kept in the freezer at $-70°C$.

*2.4. Assessment of Arrhythmias.* ECG was recorded throughout the experiment and at a fast speed throughout ischemia and reperfusion. Ventricular arrhythmias during ligation of LAD to reperfusion (T$_3$) were determined in accordance with the guidelines of the Lambeth Convention [17]. Ventricular tachycardia (VT) was defined as a run of four or more consecutive ventricular premature beats. Ventricular fibrillation (VF) was defined as a ventricular rhythm without recognizable QRS complex, and the signal morphology changed from cycle to cycle. The ECG records for the incidence and total duration of VT and VF during reperfusion were analyzed. In order to evaluate the severity of arrhythmias, ventricular arrhythmia scores (VAS) were used by giving a certain grade to each rat as follows: 0 = less than 5 times VT; 1 = more than 5 times VT or other arrhythmias, no VF; 2 = 11~30 s VT or other arrhythmias, no VF; 3 = 31~90 s VT or other arrhythmias, no VF; 4 = 91~180 s VT or other arrhythmias, and/or less than 10 s reversible VF; 5 = more than 180 s VT or other arrhythmias, and/or more than 10 s reversible VF; 6 = irreversible VF [18].

*2.5. Cytokine and Cardiac Troponin I (cTnI) Levels.* For cytokine immunoassay, blood samples were collected by femoral venipuncture at set time points, before thoracotomy (T$_1$) and after reperfusion (T$_2$), and serum levels of IL-1$\beta$, IL-6, IL-8, IL-10, TNF-$\alpha$, and cTnI were measured using a rat ELISA kit (Shanghai Boyun Biotech, China) in accordance with the manufacturer instructions. Cytokine levels were expressed as ng/L.

*2.6. Malondialdehyde (MDA) Content Determination.* MDA content was determined on frozen myocardial tissue by thiobarbituric acid assay kit (Nanjing Jiancheng Bioengineering Institute, China). The optical density at 532 nm was measured using a spectrophotometer (UV-2000, UNICO, Shanghai). 1,1,3,3-Tetramethoxypropane was used as an external standard. The level of MDA was expressed as nanomoles per milligram protein.

*2.7. Superoxide Dismutase (SOD) Activity.* Myocardial SOD activity was determined on frozen myocardial tissue using Xanthine Oxidase Assay Kits (Nanjing Jiancheng Bioengineering Institute, China) and expressed as units per milligram protein.

*2.8. Na$^+$-K$^+$-ATPase Activity.* The activity of Na$^+$-K$^+$-ATPase was assessed by measuring the release of inorganic

phosphate (Pi) from ATP according to the kit protocol (Nanjing Jiancheng Bioengineering Institute, China). Briefly, frozen myocardial tissue was weighted and normal saline was added at a ratio of $1 : 9$. Then, the homogenates were centrifuged to obtain the supernatant, followed by the measurement of protein concentration using Bradford method. Finally, $Na^+$-$K^+$-ATPase activity was determined by measuring the amount of inorganic phosphate with malachite green dye method and expressed as micromoles per milligram protein.

*2.9. Histology and Immunohistochemistry.* The myocardial tissue 2 mm inferior to the ligation of LAD was fixed in paraformaldehyde and embedded in paraffin. Heart tissues were cut into 5 $\mu$m sections for histopathological examination by hematoxylin-eosin (H&E) staining and for immunohistochemical detection of Cx43. Briefly, rehydrated sections were pretreated with pepsin solution for 15 min at 37°C. The following protocols were according to the manual of the S-P kit (ZSGB-BIO, Beijing). The myocardium near LAD was incubated for 10 min with a peroxidase-blocking solution and goat serum solution for 30 min and then incubated with primary antibody (rabbit anti-rat Cx43) (Cell Signaling Technology, USA) overnight at 4°C. After three rinses with PBS, the sections were incubated with biotinylated secondary antibody (goat anti-rabbit IgG, included in the S-P Kit) for 30 min and further developed with 3,30-diaminobenzidine tetrahydrochloride (DAB).

*2.10. Transmission Electron Microscopy (TEM).* For TEM examination, samples containing a 2 mm portion from the edge of the incision were immediately fixed in 0.1 M phosphate buffer containing 2.5% glutaraldehyde and 2% paraformaldehyde for 4 h. The samples were then fixed with 1% osmium tetroxide for 2 h, dehydrated through a graded ethanol series, and embedded in epoxy resin. Resin-embedded blocks were cut into 60~80 nm ultrathin sections with an ultramicrotome (PT-XL, RMC, USA). The ultrathin sections were placed on carbon coated nickel grids and examined with an H-7500 transmission electron microscope (H-7500, Tokyo, Japan) operating at 80 kV.

*2.11. Real-Time Quantitative PCR (RT-qPCR).* Total RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA (1 ng) was reversed with the cDNA synthesis Kit (Invitrogen, USA). Real-time polymerase chain reaction (PCR) was performed using the SYBR green system (Bio-Rad, USA). Primer sequences were as shown in Supplementary Table 1. The relative quantification of a target gene was normalized to GAPDH and calculated using the absolute quantification standard curve method. Melting curve profiles were produced at the end of each PC to confirm the specificity amplifications. Each sample was analyzed in triplicate.

*2.12. Western Blotting.* Equal amounts of protein (50 $\mu$g) were subjected to SDS-PAGE. Proteins were then transferred to polyvinylidene fluoride (PVDF) membrane. Membrane was blocked with 5% nonfat dry milk in Tris-buffered saline, 0.1%

TABLE 1: The ventricular arrhythmia score (VAS), the duration time of VT (VTt) and VF (VFt) in different groups at $T_3$ ($n = 12$, mean ± SD).

| Group | VAS | VTt (s) | VFt (s) |
|---|---|---|---|
| $C_1$ | $0.25 \pm 0.45$ | 0 | 0 |
| $I/R_1$ | $4.67 \pm 0.49^*$ | $73.50 \pm 28.40^*$ | $16.67 \pm 14.62^*$ |
| $LX_1$ | $2.33 \pm 0.49^{*\#}$ | $22.67 \pm 7.51^{*\#}$ | 0 |
| $C_2$ | $0.33 \pm 0.49$ | 0 | 0 |
| $I/R_2$ | $4.83 \pm 0.39^*$ | $69.67 \pm 24.83^*$ | $22.00 \pm 16.03^*$ |
| $LX_2$ | $3.50 \pm 0.80^{*\#\Delta}$ | $35.42 \pm 14.92^{*\#\Delta}$ | $3.58 \pm 8.50^\#$ |

Note: $T_3$: during ligation of the left anterior descending coronary artery to reperfusion.
$I/R_1$ and $LX_1$ groups were compared with $C_1$ group, and $I/R_2$ and $LX_2$ were compared with $C_2$ group, $^*P < 0.05$.
$LX_1$ goup was compared with $I/R_1$ group, and $LX_2$ group was compared with $I/R_2$ group, $^\#P < 0.05$.
$LX_2$ group was compared with $LX_1$ group, $^\Delta P < 0.05$.

Tween 20 (Sigma, USA), and immunoblotting was performed using rabbit anti-rat $Na^+$-$K^+$-ATPase ($1 : 500$, Cell Signaling Technology, USA) as described by the manufacturer. Anti-$\beta$-Actin antibody ($1 : 200$, Santa Cruz, USA) was used as loading control. Blots were then developed by incubation with biotinylated anti-rabbit antibody ($1 : 2000$; Vector Laboratories), followed by incubation with ABC reagent (GE, USA). Signal was detected using an ECL luminescence kit (GE, USA) and X-ray film.

*2.13. Statistical Analysis.* Data were expressed as mean ± standard deviation. Statistical analysis was performed by one-way ANOVA to compare more than two groups or with paired *t*-test to compare two groups. All statistical analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). The significance level was set at $P < 0.05$.

## 3. Results

*3.1. The Effects of LXA$_4$ Preconditioning and Postconditioning on I/R-Induced Arrhythmias.* The effects of different treatments on I/R-induced arrhythmias are shown in Table 1. In $C_1$ and $C_2$ groups, neither VT nor VF was observed and only few ventricular premature beats appeared during the whole procedure. In contrast, almost all rats in $I/R_1$ and $I/R_2$ groups experienced obvious ST segment elevation, occurring VT, and high frequency of VF. Furthermore, the frequency of arrhythmias was significantly reduced in $LX_1$ and $LX_2$ groups with the exception of 2 rats showing VF in $LX_2$ group. Most of the arrhythmias observed occurred in the ischemia (30 min) and in the first 30 min of the reperfusion periods.

In addition, the durations of VT and VAS were markedly higher in $I/R_1$ and $LX_1$ compared with $C_1$ group, and the same was seen for $I/R_2$ and $LX_2$ groups when compared with $C_2$ group, ($P < 0.05$ in both cases). Finally, VAS and the VT durations of $LX_1$ and $LX_2$ groups were significantly ($P < 0.05$) lower than those in $I/R_1$ and $I/R_2$ groups. Interestingly, these parameters in $LX_1$ group were significantly lower than those

in $LX_2$ group ($P < 0.05$), indicating that $LXA_4$ preconditioning was more effective in preventing I/R-induced arrhythmias than $LXA_4$ postconditioning.

### 3.2. The Effect of $LXA_4$ Preconditioning and Postconditioning on Serum Levels of IL-1β, IL-6, IL-8, IL-10, TNF-α, and cTnI.
Serum levels of IL-1β, IL-6, IL-8, IL-10, TNF-α, and cTnI were determined using ELISA kits. We found that there was no statistical difference at $T_1$ among all the groups. In contrast, cytokines levels at $T_2$ in $I/R_1$ and $LX_1$ groups were significantly ($P < 0.05$) elevated compared to $C_1$ controls, and similar results were seen for $I/R_2$ and $LX_2$ groups compared with $C_2$ controls. In the meantime, reduced levels of proinflammatory cytokines such as IL-1β, IL-6, IL-8, and TNF-α and elevated level of the anti-inflammatory cytokine IL-10 were observed in $LX_1$ and $LX_2$ groups compared with $I/R_1$ and $I/R_2$ groups, respectively. These findings indicate that both $LXA_4$ preconditioning and postconditioning could induce the expressions of anti-inflammatory cytokines and inhibit the induction of proinflammatory cytokines. Moreover, these changes were more significant in $LX_2$ compared with $LX_1$ group ($P < 0.05$), further confirming that $LXA_4$ postconditioning was more effective in preventing I/R injury than $LXA_4$ preconditioning (Figure 1).

### 3.3. The Effect of $LXA_4$ Preconditioning and Postconditioning on MDA Levels and SOD and $Na^+$-$K^+$-ATPase Activities.
The production of MDA, SOD, and $Na^+$-$K^+$-ATPase activities in myocardial tissues in response to I/R injury were investigated. Compared with $C_1$ group, MDA production and SOD activity were increased and $Na^+$-$K^+$-ATPase activity was reduced in $I/R_1$ and $LX_1$ groups, and the same results were obtained in $I/R_2$ and $LX_2$ groups compared to $C_2$ group ($P < 0.05$). Furthermore, decreased MDA production and increased activities of SOD and $Na^+$-$K^+$-ATPase in $LX_1$ and $LX_2$ groups were observed compared with $I/R_1$ and $I/R_2$ groups, respectively. Similar results were observed when $LX_2$ group was compared with $LX_1$ group, indicating that $LXA_4$ postconditioning was more protective against I/R injury than $LXA_4$ preconditioning (Figure 2).

### 3.4. The Effect of $LXA_4$ Preconditioning and Postconditioning on the Ultrastructural Changes of the Myocardium.
TEM images of ultrathin sections of myocardial tissue are shown in Figure 3. Cardiomyocytes were clearly seen in a well-arranged myofilament and intercalated disc manner in sham groups ($C_1$ and $C_2$), as well as abundant normal mitochondria with no swelling, normal matrix density, and intact cristae. The round endoplasmic reticulum can also be seen between the myofilaments and the cytoplasm (Figures 3(a) and 3(b)). However, in $I/R_1$ and $I/R_2$ groups, myocardial I/R produced remarkable ultrastructural damages associated with irregularities and edematous separation of myofilaments, hypercontraction, and shortening of sarcomeres. Large area of cytoplasmic vacuolization and mitochondrial swelling were evident with decreased matrix density and distortion of cristae (Figures 3(c) and 3(d)). Treatment with $LXA_4$ showed clear protection with relatively parallel arrangement of myofilaments and normal sarcomeres. Mitochondria were normal

with mild swelling and normal matrix density but slightly damaged cristae. However, mild cytoplasmic rarefaction with mild edema could still be seen (Figures 3(e) and 3(f)).

### 3.5. The Effect of $LXA_4$ Preconditioning and Postconditioning on Pathological Changes.
To further assess the effect of $LXA_4$ on I/R injury, we analyzed the pathological changes it induced (Figure 3). In the sham groups ($C_1$ and $C_2$), myocardial fibers were normal with clear striations. No epimorphosis, necrosis, or neutrophil infiltration was observed (Figures 3(g) and 3(h)). In contrast, nontreated I/R groups ($I/R_1$ and $I/R_2$) showed local swelling, myocardial necrosis, disorganized myocardial fibers, and ruptured cells with large number of inflammatory cells in the cytoplasm (Figures 3(i) and 3(j)). In the $LXA_4$-treated groups ($LX_1$ and $LX_2$), myocardial cells with normal structure and shape can be seen, though the myocardial fibers were mildly swollen or partially ruptured and slight edema can be seen in the interstitial tissues with small amount of inflammatory cells (Figures 3(k) and 3(l)).

### 3.6. The Effect of $LXA_4$ Preconditioning and Postconditioning on Cx43 Expression.
The gap junction protein Cx43 is the major component of gap junctions between cardiomyocytes of mammalian ventricular myocardium, including human and rodent hearts. Using an immunohistochemical approach, we wanted to know whether $LXA_4$ preconditioning and postconditioning affected Cx43 expression. As shown in Figure 4, Cx43 expression, demonstrated by a pervasive brown/yellow color in cardiac muscle cells, was observed in myocardial tissue samples from all the groups. The areas of Cx43 expression in myocardial tissue samples from sham groups ($C_1$ and $C_2$) were larger than those detected in the I/R groups, which was also confirmed by the quantitative analysis of integral optical density (IOD) (Figure 4). Cx43 was well arranged together at both ends of the intact cardiac myocyte in $I/R_1$ and $I/R_2$ groups, and Cx43 staining was significantly reduced when compared with the sham groups. However, in $LXA_4$-treated $LX_1$ and $LX_2$ groups, most Cx43 staining was located at the ends of intact cardiac myocytes (intercalate disc) and redistributed in both sides of the cell. Compared with nontreated $I/R_1$ and $I/R_2$ groups, the IOD value was statistically higher in $LX_1$ and $LX_2$ groups, respectively. Additionally, Cx43 expression of $LXA_4$ preconditioning ($LX_1$ group) was significantly ($P < 0.05$) higher than $LXA_4$ postconditioning ($LX_2$ group) (Figure 4).

### 3.7. The Effects of I/R and $LXA_4$ Treatment on $Na^+$-$K^+$-ATPase and Cx43 at mRNA Level.
The $Na^+$-$K^+$-ATPase and Cx43 mRNA levels were examined using real-time quantitative PCR to determine if I/R injury influences the $Na^+$-$K^+$-ATPase and Cx43 expressions and whether this effect is blocked by the different $LXA_4$ treatment (Figure 5). Compared with the sham groups ($C_1$ and $C_2$), mRNA levels of $Na^+$-$K^+$-ATPase and Cx43 in $I/R_1$, $LX_1$, $I/R_2$, and $LX_2$ were remarkably reduced. Meanwhile, $Na^+$-$K^+$-ATPase and Cx43 expressions in $LXA_4$-treated $LX_1$ and $LX_2$ groups were higher than the saline-treated $I/R_1$ and $I/R_2$ groups, respectively ($P < 0.05$). Furthermore, $Na^+$-$K^+$-ATPase mRNA level of $LXA_4$
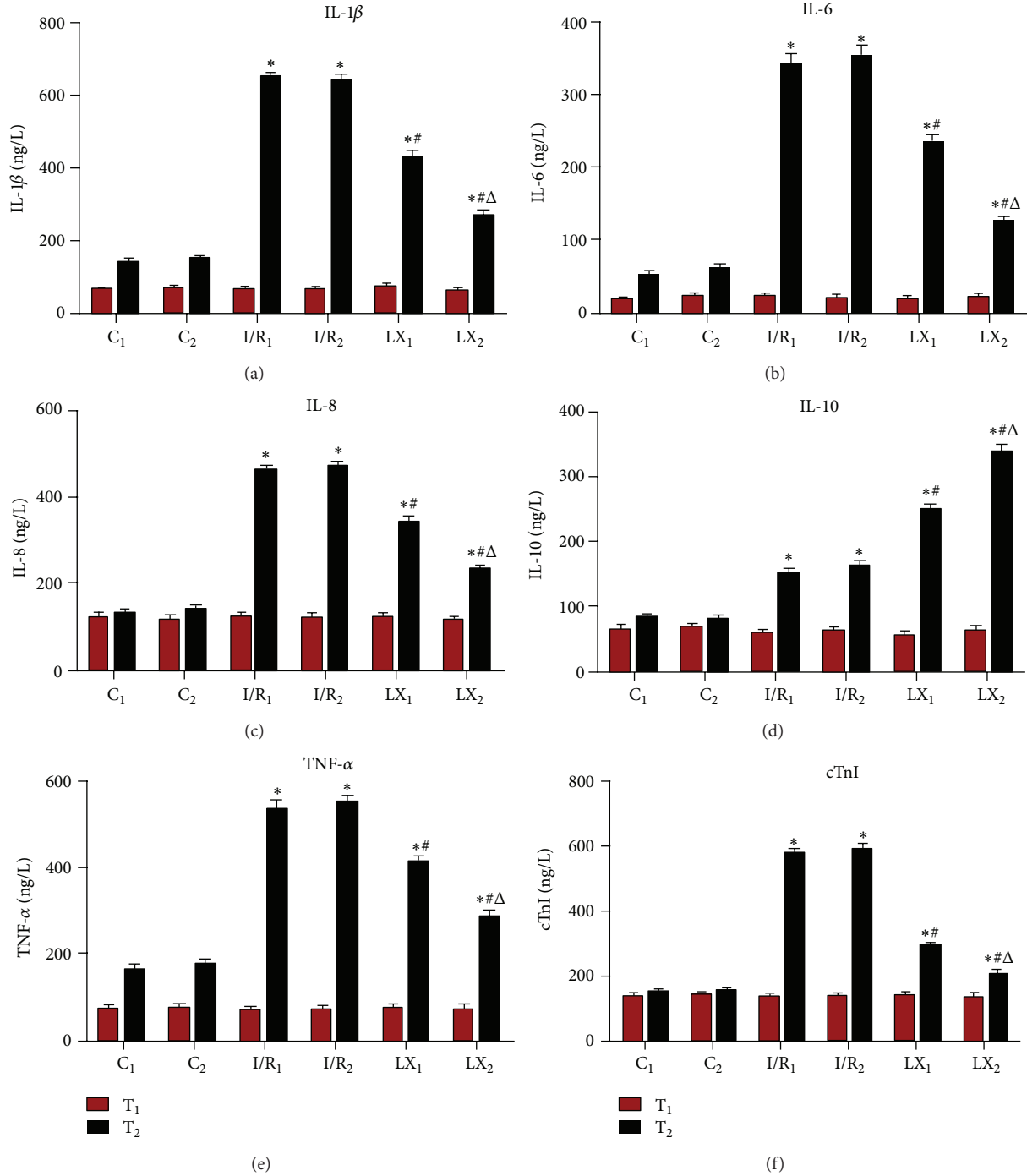
FIGURE 1: Comparison of concentrations of serum IL-1$\beta$, IL-6, IL-8, IL-10; TNF-$\alpha$;, and cTnI at $T_1$ and $T_2$ among all groups ($n$ = 12 for each group). At time point $T_1$, blood was collected immediately before thoracotomy. At time point $T_2$, blood was collected right after the I/R procedure was over. (a) IL-1$\beta$; (b) IL-6; (c) IL-8; (d) IL-10; (e) TNF-$\alpha$; (f) cTnI. $^*P$ < 0.05 for comparisons of I/R$_1$ and LX$_1$ groups with C$_1$ group, $P$ < 0.05 for comparisons of I/R$_2$ and LX$_2$ with C$_2$ group; $^\#P$ < 0.05 for comparisons of LX$_1$ group with I/R$_1$ group, $P$ < 0.05 for comparisons of LX$_2$ group with I/R$_2$ group; $^\Delta P$ < 0.05 for comparisons of LX$_2$ group with LX$_1$ group.

preconditioning (LX$_1$) was lower than that of LXA$_4$ postconditioning (LX$_2$). However, Cx43 expression was higher in LX$_1$ compared to LX$_2$ group ($P$ < 0.05).

*3.8. Further Analysis of the Effects of LXA$_4$ Treatment on Na$^+$-K$^+$-ATPase Isoform Expression.* The results of

Na$^+$-K$^+$-ATPase isoforms analyzed by western blotting are shown in Figure 6. Compared to the sham C$_1$ group, the expressions of Na$^+$-K$^+$-ATPase isoforms $\alpha_1$, $\alpha_2$, $\alpha_3$, and $\beta_1$ were markedly reduced in I/R$_1$ and LX$_1$ groups and similar results were obtained in I/R$_2$ and LX$_2$ groups, compared to C$_2$ group ($P$ < 0.05). However, Na$^+$-K$^+$-ATPase isoform
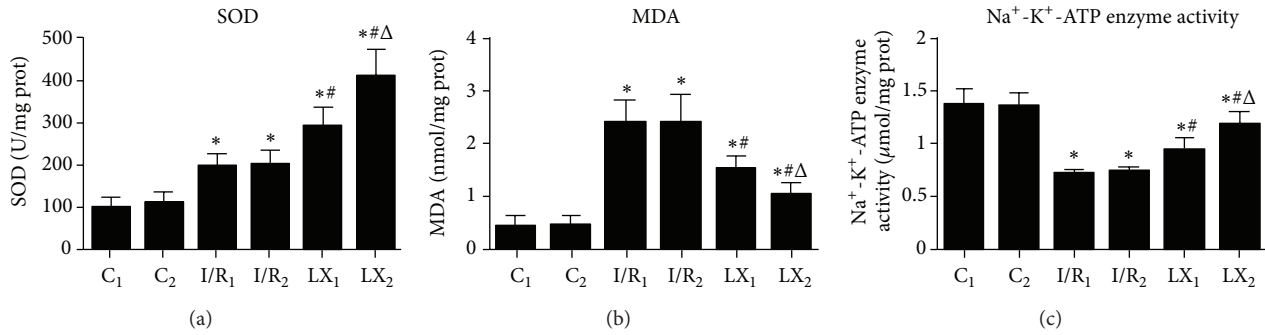
FIGURE 2: Comparison of cardiac MDA content and SOD and $Na^+$-$K^+$-ATPase activities at $T_2$ among all groups ($n = 12$ for each group). At time point $T_2$, myocardial tissue was collected right after the I/R procedure was over and kept frozen in liquid nitrogen. MDA content and SOD and $Na^+$-$K^+$-ATPase activities were measured as described in the section of Materials and Methods. (a) SOD; (b) MDA content; (c) $Na^+$-$K^+$-ATPase activity. *$P < 0.05$ for comparisons of I/$R_1$ and LX$_1$ groups with C$_1$ group, $P < 0.05$ for comparisons of I/$R_2$ and LX$_2$ with C$_2$ group; #$P < 0.05$ for comparisons of LX$_1$ group with I/$R_1$ group, $P < 0.05$ for comparisons of LX$_2$ group with I/$R_2$ group; $\Delta$$P < 0.05$ for comparisons of LX$_2$ group with LX$_1$ group.

levels in LXA$_4$-treated LX$_1$ and LX$_2$ groups were higher than the saline-treated I/$R_1$ and I/$R_2$ groups respectively ($P < 0.05$). Additionally, protein levels of $Na^+$-$K^+$-ATPase in LXA$_4$ preconditioning (LX$_1$) were significantly ($P < 0.05$) lower than those measured in LXA$_4$ postconditioning (LX$_2$).

## 4. Discussion

With the widespread use of cardiac surgery for congenital heart disease and coronary artery bypass grafting under extracorporeal circulation, thrombolytic therapy, percutaneous transluminal coronary intervention, organ transplantation, and cardiopulmonary resuscitation, it has currently become a hot topic for cardiovascular scientists to find safe and effective drugs to treat myocardial I/R injury [10]. In this study, we used rats to induce I/R injury because of the little variation in their cardiovascular system, similarity to human coronary artery territory, less myocardial collateral circulation, and the high probability of occurrence of I/R injury. Due to the smaller size of coronary artery in rats, more than 30 min of blocking time can easily lead to permanent occlusion of coronary artery [14, 19, 20]. Therefore, we chose 30 min of myocardial ischemia followed by 120 min reperfusion. LXA$_4$ is usually dissolved in ethanol. However, we found that ethanol affected the health of experimental animals. Therefore, LXA$_4$ was dissolved in saline as suggested by the manufacturer's manual.

Currently, the effect of I/R injury on myocardial ultrastructure is not entirely clear. El Kebir et al. [21] reported that LX can inhibit neutrophil aggregation at inflammatory sites and regulate the balance of proinflammatory/anti-inflammatory cytokines. In addition, LXA$_4$ can suppress vascular-endothelial-growth-factor- (VEGF-) induced inflammatory reaction through the regulation of IL-6, TNF-$\alpha$, IL-8, ICAM-1, and IL-10 expressions in human umbilical vein endothelial cells [22]. Souza et al. [13] found that reduced production of TNF-$\alpha$ following LXA$_4$ treatment of intestinal I/R injury may be due to the increased IL-10 expression, and LXA$_4$

could not prevent reperfusion injury in IL-10-deficient mice. In our study, we confirmed that I/R resulted in myocardial inflammatory injury through analyzing serum components before and after I/R. Although both proinflammatory/anti-inflammatory cytokines were upregulated, the expressions of proinflammatory cytokines were much higher than the anti-inflammatory ones. However, with LXA$_4$ treatment, the expressions of proinflammatory cytokines (IL-1$\beta$, IL-6, IL-8, and TNF-$\alpha$) were reduced while the anti-inflammatory cytokine IL-10 was upregulated, leading to an anti-inflammatory state (Figure 1).

The results of H&E staining confirmed that LXA$_4$ could alleviate I/R injury as evidenced by reduced swelling, well-organized cardiac muscle, clear striations, and less inflammatory cell infiltration. In accordance with the pathological changes, we found that LXA$_4$ protected the ultrastructures of myocardial tissue as shown by normal matrix density, relatively parallel arrangement of myofilaments, and normal sarcomere mitochondria by TEM examination (Figure 3).

There has been report showing that LXA$_4$ can inhibit the generation of ROS [7]. We found that I/R groups had increased MDA levels and SOD activity, which indicated enhanced oxidative stress. After LXA$_4$ treatment, SOD activity was significantly improved and MDA levels were markedly reduced (Figure 2), suggesting that the alleviated I/R injury seen after LXA$_4$ treatment might be due to decreased ROS generation. Mitochondria are the main organelle maintaining the cell respiration and oxidation. Close to 90% of cellular oxygen intake is used in oxidative phosphorylation reactions in the mitochondria, but 1% to 2% of oxygen can escape to form ROS. ROS can decrease the efficiency of mitochondrial performance and cause swelling of mitochondria by attacking the mitochondrial inner membrane. Indeed, mitochondria are both a primary source and target of ROS [23]. As we can see from the TEM images (Figure 3), the swelling condition of mitochondria has been remarkably alleviated after LXA$_4$ treatment, with clear cristae and well-arranged cardiac fiber ultrastructure. This agrees with the MDA levels and the SOD
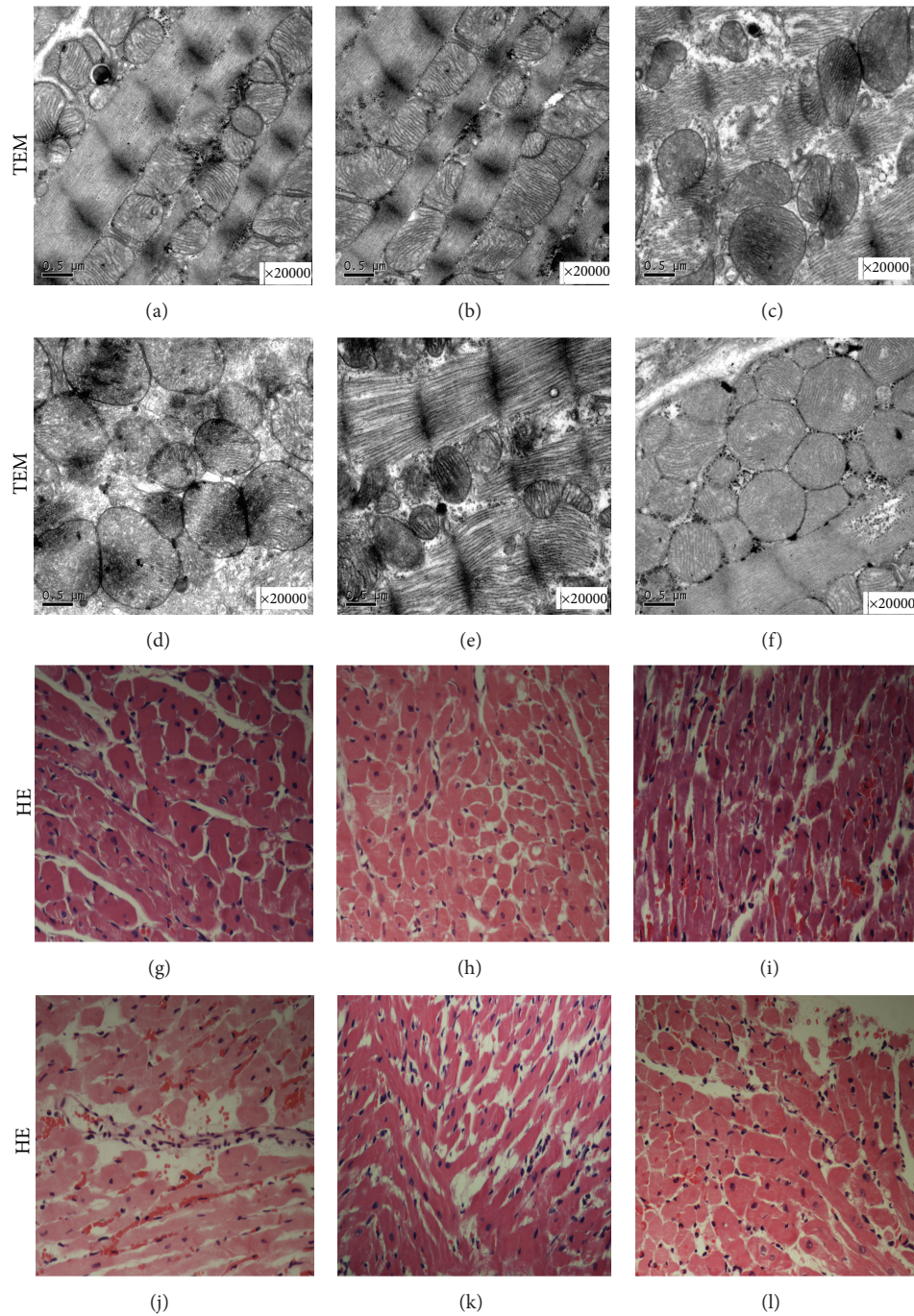
FIGURE 3: Transmission electron microscopy ((a)–(f)) and histological study ((g)–(l), He ×400) of cardiac tissues from rats of different groups at $T_2$ time point. (a) Group $C_1$ and (b) group $C_2$: regular myocardial myofilament arrangement, clear intercalated disk structure, normal mitochondrial morphology, and structure with endoplasmic reticulum embedded in myofilament matrix; (c) group $I/R_1$ and (d) group $I/R_2$: disordered arrangement of myofilament, a large number of myofilament fractured, intercalated disk structure unclear, and abnormal mitochondrial morphology (a high degree of swelling, membrane lysis, fuzzy ridge structure, formation of vacuoles, and endoplasmic reticulum vacuolization); (e) group $LX_1$ and (f) group $LX_2$: myofilament arrangement is a little sparse, the intercalated disk structure is slightly fuzzy, and slightly swollen mitochondria; (g) group $C_1$ and (h) group $C_2$: normal size cardiomyocyte, no hemorrhage, or neutrophil granulocyte infiltration; (i) group $I/R_1$ and (j) group $I/R_2$: Cardiomyocyte degeneration, hemorrhage, edema, and significant interstitial neutrophil granulocyte infiltration; (k) group $LX_1$ and (l) group $LX_2$: no significant cardiomyocyte degeneration, no obvious bleeding, slight edema between the myocardial fibers, and mild neutrophil granulocyte infiltration.
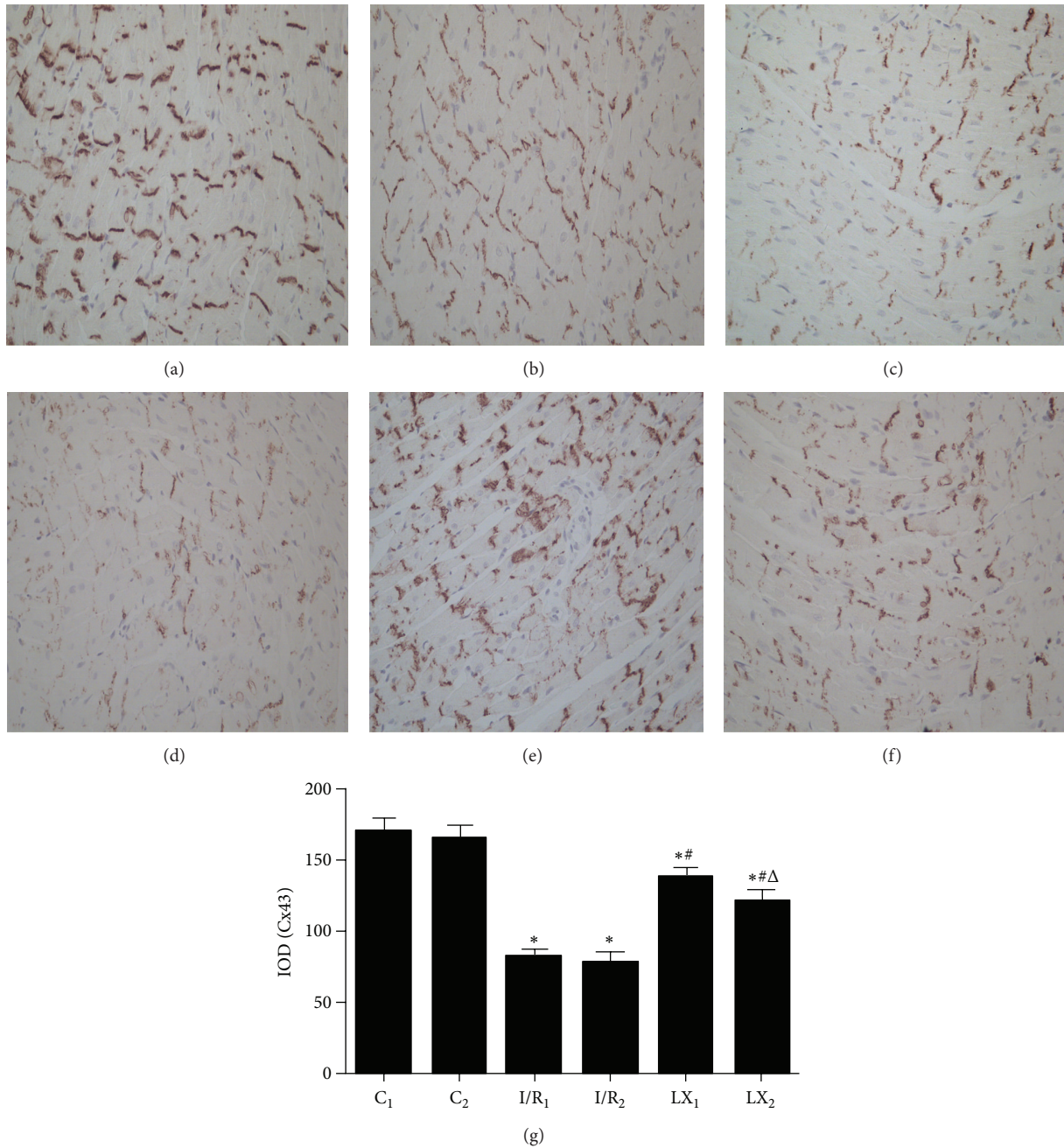
(a)

(b)

(c)

(d)

(e)

(f)

(g)

FIGURE 4: Immunohistochemical staining and IOD of Cx43 in cardiac tissues from rats of different groups at $T_2$. (a) Group $C_1$ and (b) group $C_2$: the Cx43 positive particle intensively and regularly was distributed and was clustered in the intercalated disk; (c) group $I/R_1$ and (d) group $I/R_2$: sparsely distributed Cx43 positive particles; (e) group $LX_1$ and (f) group $LX_2$: Slightly decreased Cx43 positive particles, mostly still clustered in the intercalated disk. (g) For IOD of Cx43. $*P < 0.05$ for comparisons of $I/R_1$ and $LX_1$ groups with $C_1$ group, $P < 0.05$ for comparisons of $I/R_2$ and $LX_2$ with $C_2$ group; $^{\#}P < 0.05$ for comparisons of $LX_1$ group with $I/R_1$ group, $P < 0.05$ for comparisons of $LX_2$ group with $I/R_2$ group; $^{\Delta}P < 0.05$ for comparisons of $LX_2$ group with $LX_1$ group.

activity data and further confirms that reduced I/R injury may be due to the inhibition of oxygen-free radical targeting of mitochondrial membranes.

The $Na^+$-$K^+$-ATPase is a heterodimer that plays an important role in the maintenance of cell homeostasis by regulating membrane potential and cation transport across the sarcolemmal membrane, which is a reliable indicator

of metabolic disorders and tissue injury. I/R injury could affect mitochondrial function and inhibit $Na^+$-$K^+$-ATPase activity, leading to intracellular calcium overload, activation of proteases, and release of oxygen-free radicals, which in turn aggravate I/R injury. Belliard et al. [24] reported that the maintenance of $Na^+$-$K^+$-ATPase cell surface abundance was crucial in myocyte survival after an ischemic attack treated
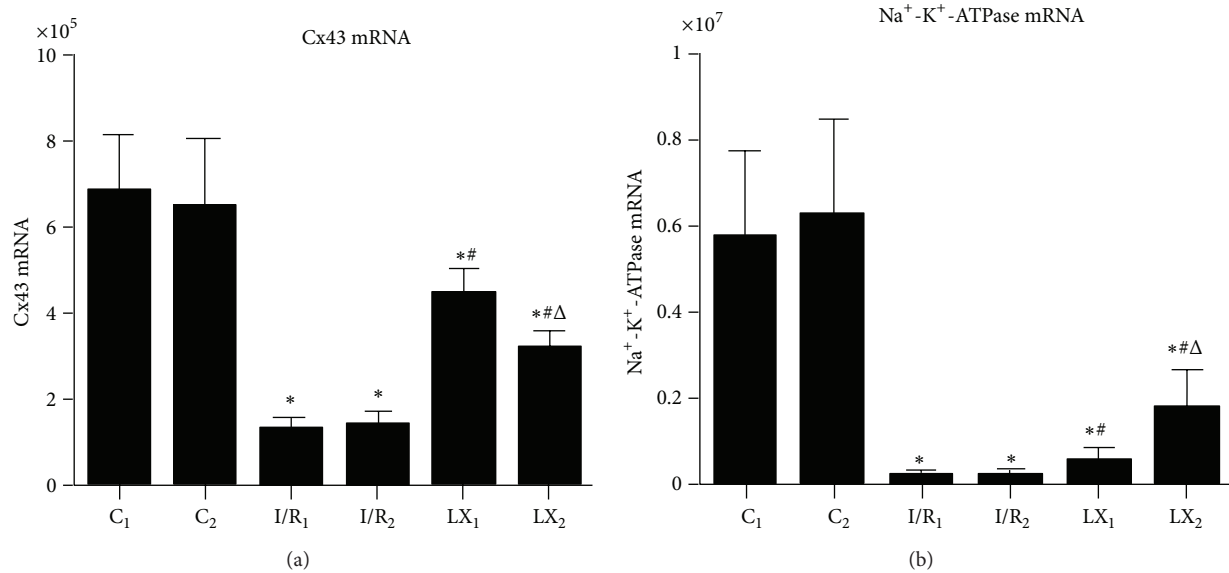
FIGURE 5: mRNA levels of Cx43 and $Na^+$-$K^+$-ATPase of cardiac tissues from rats of different groups at $T_2$. Samples were collected right after I/R procedure. (a) Cx43 mRNA; (b) $Na^+$-$K^+$-ATPase mRNA. $^*P < 0.05$ for comparisons of $I/R_1$ and $LX_1$ groups with $C_1$ group, $P < 0.05$ for comparisons of $I/R_2$ and $LX_2$ with $C_2$ group; $^\#P < 0.05$ for comparisons of $LX_1$ group with $I/R_1$ group, $P < 0.05$ for comparisons of $LX_2$ group with $I/R_2$ group; $^\Delta P < 0.05$ for comparisons of $LX_2$ group with $LX_1$ group.

by ouabain preconditioning, and the protection conferred by increased surface expression of $Na^+$-$K^+$-ATPase may be independent of ion transport. Meanwhile, Zheng et al. [25] found that the protection from cardiac injury may be determined by the $Na^+$-$K^+$-ATPase activity, involving ERK1/2 and PI3 K/Akt signal pathways. Furthermore, continued intermittent hypobaric hypoxia can reduce the reperfusion injury in guinea pig by increasing myocardial $Na^+$-$K^+$-ATPase activity [26]. The $Na^+$-$K^+$-ATPase consists of $\alpha$ and $\beta$ subunits; the $\alpha$ subunit is responsible for the catalytic activity of the enzyme and includes three isoforms: $\alpha_1$, $\alpha_2$, and $\alpha_3$ in the rat heart. The $\beta$ subunit is responsible for the proper localization and insertion of $Na^+$-$K^+$-ATPase in the sarcolemmal membrane. In this study, the expressions of $\alpha_1$, $\alpha_2$, and $\alpha_3$ proteins decreased after ischemia/reperfusion injury, which affected the active center and further reduced the activity of the ATPase enzyme. The expression of $\beta$ subunit also declined after I/R injury, and this could cause damage to the cytoskeleton, lead to detachment of $Na^+$-$K^+$-ATPase from the cell membrane, and further reduce its activity [27]. The effect of ischemia/reperfusion on the expressions of $Na^+$-$K^+$-ATPase $\alpha_1$, $\alpha_2$, and $\alpha_3$ subunits were slightly different from earlier report [28], which may be due to differences in experimental and detection methods. The content and the activity of $Na^+$-$K^+$-ATPase can reflect myocardial injury to a certain extent, and I/R injury can also be alleviated by increasing its content and activity. Figtree et al. [29] suggested that cardiomyocytes could be protected potentially by reversing the oxidation inhibition of $Na^+$-$K^+$-ATPase. And from the our study, we know that $LXA_4$ enhanced $Na^+$-$K^+$-ATPase subunits expressions (Figures 5 and 6) and reduced cTnI concentration, indicating that the cardioprotective effect of $LXA_4$ could be due to increased $Na^+$-$K^+$-ATPase content and activity.

The gap junction (GJ) plays key roles not only in electrical coupling of cardiomyocytes but also in intercellular transport of biologically active substances. GJ is mainly located in the intercalated disc of the cardiac tissue [30] and participates in decision making for cell survival versus cell death in various types of cells. It has been suggested that reperfusion injury to the heart is partially mediated by gap junctions. Cx43 forms gap junctions that facilitate electrical cell-cell coupling and unopposed/nonjunction hemichannels which provide a pathway for the exchange of ions and metabolites between cytoplasm and extracellular milieu [31]. It has also been known that Cx43 participates in the process of myocardiac ischemia/reperfusion injury [32, 33]. During I/R injury, alterations in Cx43 expression, its localization and its phosphorylation status, and changes in GJ properties collectively contribute to myocardial infarction and arrhythmogenesis [34–36]. From our research, we found that Cx43 was well arranged together in both ends of intact cardiac myocytes (intercalate disc) in the sham group, while in the I/R group, Cx43 expression was reduced which was associated with damaged cellular ultrastructures (Figures 4 and 5). In addition, increased VAS, VTt, and VFt duration and reduced Cx43 mRNA and protein expressions after injury strongly indicate that I/R injury could induce arrhythmia through Cx43 pathway.

The mechanism of arrhythmogenesis caused by I/R injury has not yet been illustrated clearly. Cabo et al. found that this may be due to the structural and functional alternations of Cx43 after injury [37], and Danik reported that Cx43 knockout mice showed complicated tachyarrhythmia [38]. Recently, two lipoxins have been identified, lipoxin $A_4$ ($LXA_4$) and lipoxin $B_4$ ($LXB_4$). $LXA_4$ treatment in our study showed that this molecule can alleviate Cx43 degradation and protect its distribution that limited the damage to the
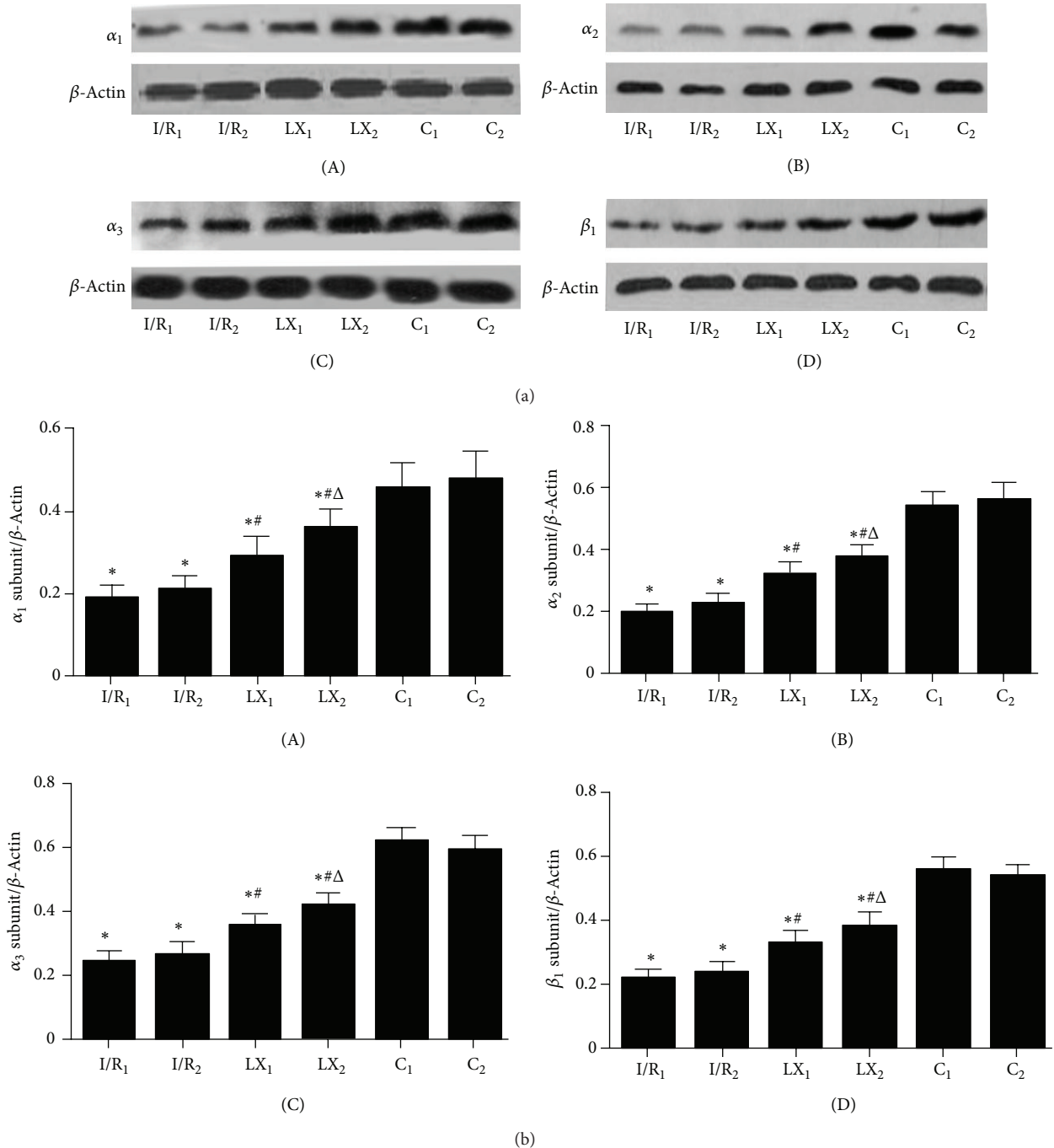
(a)



(b)

FIGURE 6: Protein levels of $\alpha_1$, $\alpha_2$, and $\alpha_3$ and $\beta_1$ subunits of $Na^+$-$K^+$-ATPase of cardiac tissues from rats of different groups at $T_2$. Samples were collected right after I/R procedure was over and western blot was performed as described in the section of Materials and Methods, and $\beta$-Actin was used as a loading control. Representative blots of three independent experiments were shown. (a) Western blotting: (A) $\alpha_1$ subunit of $Na^+$-$K^+$-ATPase; (B) $\alpha_2$ subunit; (C) $\alpha_3$ subunit; (D) $\beta_1$ subunit. (b) Quantitative data of western blot: (A) $\alpha_1$ subunit of $Na^+$-$K^+$-ATPase; (B) $\alpha_2$ subunit; (C) $\alpha_3$ subunit; (D) $\beta_1$ subunit. $^*P < 0.05$ for comparisons of $I/R_1$ and $LX_1$ groups with $C_1$ group, $P < 0.05$ for comparisons of $I/R_2$ and $LX_2$ with $C_2$ group; $^\#P < 0.05$ for comparisons of $LX_1$ group with $I/R_1$ group, $P < 0.05$ for comparisons of $LX_2$ group with $I/R_2$ group; $^\Delta P < 0.05$ for comparisons of $LX_2$ group with $LX_1$ group.

ultrastructure of cardiac tissue caused by I/R injury. At the same time, the mRNA transcriptional levels of Cx43 were notably increased, which was in accordance with the immunohistochemical results. This indicates that $LXA_4$ plays a protective role in regulating the expressive levels of Cx43 protein and gene. In addition, reduced arrhythmia frequency and VAS were observed, indicating that the protective effect of $LXA_4$ may be associated with Cx43 remodeling, an

observation consistent with a previous study showing that cardiac function could be preserved through Cx43 remodeling [39].

The pharmacological preconditioning and postconditioning with $LXA_4$ can both attenuate myocardial I/R injury in rats, with $LXA_4$ preconditioning being more effective in preventing the arrhythmogenesis than the $LXA_4$ postconditioning. An effect associated with higher Cx43 expression and lower VAS. However, $LXA_4$ postconditioning had a better protective effect on $Na^+$-$K^+$-ATPase and the ultrastructure of the myocardium, which may be related to the physico-chemical property, duration of the treatment, and the dose of $LXA_4$. We also observed that most arrhythmias happened in the ischemia (30 min) and the first 30 min of reperfusion, which may be due to the activated compensatory mechanism of the injured body and increased tolerance to the drug. Cx43 is easy to be degraded when I/R injury happens [40]. Cx43 is also involved in pathological and physiological processes other than arrhythmias during I/R injury [41, 42], and it may cause uncontrolled opening of hemichannels in the plasma membrane [43] and may be deleterious to the myocardium [44]. Blocking hemichannels may confer cardioprotection by preventing ionic imbalance, excessive entry of $Na^+$ and $Ca^+$, ATP leakage, cell swelling, and loss of critical metabolites [40, 45, 46]. Therefore, Cx43-mediated intercellular communication of the injured tissue can be enlarged into other cells, and this may explain that $LXA_4$ preconditioning induced higher Cx43 expression but caused more serious damage to myocardium. At the same time, it indicated that the up-regulation of Cx43 is helpless in the myocardial energy metabolism, systemic inflammation, and ultrastructure. The application of pharmacological preconditioning has been limited largely by the unpredictability of I/R injury, and this implicates that $LXA_4$ postconditioning could have a great potential for clinical application in I/R injury in the future.

Although there have been reports showing that $LXA_4$ treatment was protective in cerebral I/R injury in rats [12] and myocardial I/R injury in mice [47] and rabbits [48], ours is the first study to report a protective effect of $LXA_4$ preconditioning and postconditioning on myocardial I/R injury in rats. Further studies are needed to optimize the dose, the timing, and the route of administration of $LXA_4$.

## 5. Conclusions

$LXA_4$ preconditioning and postconditioning in myocardial I/R injury can attenuate the metabolic disturbance in the myocardium through upregulating $Na^+$-$K^+$-ATPase expression. Meanwhile, both $LXA_4$ preconditioning and postconditioning protected the ultrastructure of the myocardium by inhibiting the inflammatory reaction and oxidative stress. In addition, the upregulation of Cx43 expression had a positive effect in preventing arrhythmogenesis caused by I/R injury. All the results showed that $LXA_4$ has great potential for protecting the myocardium from myocardial I/R injury.

## Conflict of Interests

The authors declare no conflict of interests.

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