Lipoxin A4 protects rat skin flaps against ischemia-reperfusion injury through inhibiting cell apoptosis and inflammatory response induced by endoplasmic reticulum stress

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Background: The ischemia-reperfusion (I/R) injury of skin flap is a complex pathophysiological process involving many cells and factors. Although endoplasmic reticulum (ER) stress-induced cell apoptosis and inflammatory response are of immense importance in the skin flap ischemia, the treatment for I/R injury induced by ER stress is barely reported.

Methods: Healthy male Wister rats were randomly divided into three groups: sham-operated group, I/R model group and I/R + LXA4 group. I/R-induced injury in skin flaps with or without pre-treatment of Lipoxin A4 (LXA4, 100 μg/kg) was tested by using HE and TUNEL staining. Related factors associated with oxidative stress, apoptosis, inflammatory response, and ER stress were tested by ELISA, biochemical assay, and western blotting, respectively.

Results: Our results showed that LXA4 treatment significantly promotes skin flap survival and attenuates I/R injury by inhibiting oxidative stress, apoptosis, and inflammatory factor release, evidenced by the decreased expression of malondialdehyde (MDA), lactate dehydrogenase (LDH), NF-κBp65, tumor necrosis factor α (TNF-α), ET, active Caspase-3 and Bax and up-regulated superoxide dismutase (SOD), glutathione (GSH) level and Bcl-2 expression. Moreover, LXA4 treatment also reverses the increased expression of GRP78, p-PERK, p-eIF2α, ATF4, and CHOP induced by I/R injury.

Conclusions: In conclusion, we showed that ER stress causes cell apoptosis and inflammatory response, resulting in the skin flaps injury. LXA4 exhibits a protective effect on skin flaps against I/R injury through the inhibition of ER stress.

Keywords: Ischemia-reperfusion (I/R); endoplasmic reticulum stress (ER stress); Lipoxin A4 (LXA4); apoptosis

Introduction

Flap transplantation is the most common, useful, and irreplaceable means for repairing tissue defect and abnormality and organ reconstruction during orthopedic surgery, and the survival of the skin flaps after transplantation is the key to the success of the operation. During the transplantation, the flap is wholly or partly necrosis through ischemia-reperfusion (I/R) injury, which

seriously affects the surgical outcome (1,2). Many studies have shown that the I/R injury of skin flap is a complex pathophysiological process involving many cells and many factors (3,4). The specific mechanism has not been fully explained.

Apoptosis is an essential way of cell death after I/R injury associated with the severity of tissue ischemia and the time of reperfusion (5). Apoptotic cells during I/R injury may

increase secondary necrosis of the flap tissue. ROS produced during I/R injury can destroy cell DNA and mitochondria, and the lipid peroxidation induced by cell membrane can affect signal transduction (6). Also, protein cross-linking results in the loss of protein function and affects nuclear gene transcription, inducing apoptosis. Calcium overload can also induce apoptosis through causing inactivation of Bcl-2, activating various calcium-dependent endonucleases and neutral proteases, degrading DNA, and mediating the TNF signaling pathway (7,8). Many cytokines, including TNF, IL-6, IL-8, and TGF and growth factors including VEGR, PDGR, EGF, and MCSF, are produced during I/R injury and induce apoptosis (9,10).

Endoplasmic reticulum (ER) is the site of protein synthesis and folding, synthesis of lipids and sterols, and maintenance of calcium homeostasis. Genetic or environmental damage can cause an imbalance of intracellular calcium homeostasis, oxidative stress, nutritional deficiency, glycation inhibition, and protein misfolding, breaking the ER function and inducing ER stress (11,12). Various factors including ischemia and hypoxia can cause the dysfunction of ER and cause ER stress, involved in the multistep process of pathological changes in I/R (13). ER stress induces apoptosis by activating downstream apoptotic factors, including CHOP/GADD153, ASK1/JNK, Caspases, and Bcl-2 (14). ER stress activates inflammatory response through unfolding protein response (UPR), leading to the activation of the NF-κB signaling pathway, which promotes the expression of pro-inflammatory cytokines (15).

Lipoxin A4 (LXA4) is one of the most important members of the Lipoxin family, which are endogenous lipoxygenases derived from eicosanoids and have significant anti-inflammatory and pro-resolution properties (16). It has been proved that LXA4 can protect the brain (17), lung (18), heart (19), kidney (20), stomach (21), and intestine (22) from I/R injury through inhibiting expression of pro-inflammatory factors, ROS production and ER stress apoptotic pathway. However, little is known about the role and underlying mechanisms of LXA4 protects skin flap against I/R injury. In the present study, we use the I/R model in rat skin flaps to investigate the effects of LXA4 and the underlying mechanisms associated with ER stress-induced apoptosis and inflammatory response and provide a promising therapeutic method of skin flap I/R injury. We present the following article in accordance with the ARRIVE reporting checklist (available at http://dx.doi.org/10.21037/atm-20-5549).

Methods

Experimental I/R model

The present study was performed in strict accordance with the guidelines on ethical care for experimental animals and approved by the Animal Research Committee of Xiaoshan Traditional Chinese Medical Hospital. A total of fifty-four healthy male Wister rats (10-week-old; 280–300 g) obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) were in line with the specific pathogen-free (SPF) conditions and housed in groups of three and given five days to acclimate to the housing facility. They have a dark/light cycle of 12/12 hours in 595×380×200 mm cages (Techniplast UK, 1354G Eurostandard Type IV) at a fixed temperature of 22–23 ℃ with water and food freely. During housing, animals were monitored twice daily for health status. No adverse events were observed. Rats were randomly divided into three groups: sham-operated group (n=18), I/R model group (n=18) and I/R + LXA4 (Haoran Biological Technology CO., LTD., Shanghai, China) group (n=18).

The preparation of abdominal island skin flap, which size was 6 cm × 3 cm, was created according to the method of Petry's (23). There are no other treatments after creating the skin flap according to the method above in the sham-operated group. In the I/R model group, after the skin flap formed according to the method above, the proximal end of the point that the superficial epigastric artery arose from the femoral artery was occluded by a vascular clamp and the vascular clamp was taken out in another operation after 8 hours as previously described (24). In the I/R + LXA4 group, 24 hours before surgery, intravenous tail injection of rats with LXA4 (100 µg/kg) once every 8 hours and the rest of the operation as the I/R model group of experimental. In each group, a 1.0×0.5×0.2 cm skin flap tissue sample was removed at 12, 24 and 48 hours following the surgical procedure or I/R for following experiments, except for the transmission electron microscope assay, in which a 1.0×2.0×2.0 cm skin flap tissue sample was removed (n=6 per time point).

Histological assessment

When the experiment ended, these rats got anesthetized with overdosed 10% chloral hydrate (450 mg/kg) via intraperitoneal injection before euthanasia by cervical dislocation with no signs of peritonitis being observed. The harvest was conducted to the skin flaps of the rat, which was

fixed within 10% formalin, got dehydration, and then was embedded into paraffin. Then, sections with a thickness of 4 μm are cut and then stained with hematoxylin and eosin (HE). The Olympus BX51 microscope with equipment of a camera of Olympus DP71 CCD from Olympus Corporation in Japan was used to capture digital images (magnification ×200). Also, samples were fixed within 3% glutaraldehyde for 3 hours and 1% osmic acid for 1 hour, got dehydration, and then were embedded into paraffin. Then, sections with a thickness of 70 nm were cut, and then the transmission electron microscope was used (JEM1230; JEOL LTD, Japan) to capture digital images (magnification ×10,000). A blind manner was used to perform analysis on all images.

TUNEL staining

Sectioned slides were digested for 40 minutes, followed by the incubation with 50 μL TUNEL buffer at 37 ℃ for 1 hour and 50 μL peroxidase at 37 ℃ for 30 minutes, respectively. The slides were stained with 3,3'-diaminobenzidine (DAB) for 10 minutes. Samples were visualized by using a microscope, and the apoptotic cells were counted using the imaging mass spectrometry (IMS) cell imagine analysis system software version 6.0 [JRDUN Biotechnology (Shanghai) Co., Ltd., China].

Measurement of lactate dehydrogenase (LDH), glutathione (GSH), malondialdehyde (MDA), superoxide dismutase (SOD), tumor necrosis factor α (TNF-α) and endothelin (ET) level

The contents of LDH, GSH, MDA, SOD, TNF-α and ET were measured by the Lactate dehydrogenase assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), Reduced glutathione (GSH) assay kit (Nanjing Jiancheng Bioengineering Institute), MDA assay kit (TBA method; Nanjing Jiancheng Bioengineering Institute), SOD assay kit (Nanjing Jiancheng Bioengineering Institute), rat TNF-α ELISA Kit (Bio-swamp Life Science, Shanghai, China), and the Endothelin Radioimmunoassay Kit (Beijing North Institute of Biological Technology, Beijing, China) following protocols of the manufacturer, respectively.

Western blot assay

Whole protein was isolated out of snap-frozen aorta samples, RIPA lysis buffer supplemented with protease inhibitors. Proteins are quantified with a BCA protein quantification kit (BCA, Thermo, Shanghai, China) and run on 12% SDS-PAGE gel and blocked with 5% skim milk for 1 hour at 25 ℃. The membrane was immunoblotted overnight at 4 ℃ with the first antibodies against Caspase-12, JNK, p-JNK, Bax, Bcl-2, active Caspase-3, NF-κBp65, GRP78, p-PERK, p-eIF2α, ATF4, CHOP, and GAPDH. Horseradish peroxidase-conjugated second antibodies were used to incubate membranes after they were washed (Beyotime Institute of Biotechnology, Inc., Shanghai, China) for 1 hour at 37 ℃. TBST, with 20% Tween 20, was used to wash these membranes, and blots were observed visually through enhanced chemiluminescence (ECL, Thermo Scientific, Shanghai, China) and exposed to X-ray film and quantified in Chemi Doc XRS Imaging System, Bio-Rad (Hercules, CA, USA).

Statistical analysis

Results are expressed as mean the SD from 3 independent experiments. All statistical analysis was completed by using the GraphPad Prism software version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Further, the statistical differences were calculated using a one-way ANOVA followed by Tukey's post hoc test or unpaired, two-tailed Students' t-tests. When P was less than 0.05, it had statistical significance.

Results

LXA4 promotes skin flap survival and attenuates I/R injury

The survival rate of skin flaps was markedly decreased by 14.9% in the I/R group compared with the sham group (*Figure 1A,B*). However, after pre-treatment of rats with LXA4, the survival rate was significantly increased by 11.6% and 16.6% in rats 24 and 48 hours post-I/R, respectively. These results suggest that LXA4 promotes skin flap survival in I/R-induced rats.

Histopathological changes of skin flaps during I/R and LXA4 pre-treatment showed that most of the cell bodies in the I/R group were shrank, pyknosis, and deep cytoplasm staining (*Figure 1C*). The morphological structure of skin flaps in the sham group is standard without the above changes. The abnormal cells in the LXA4 treatment groups were significantly lower than those in the I/R group, and the damage was mild (*Figure 1C*). Most of them showed slight
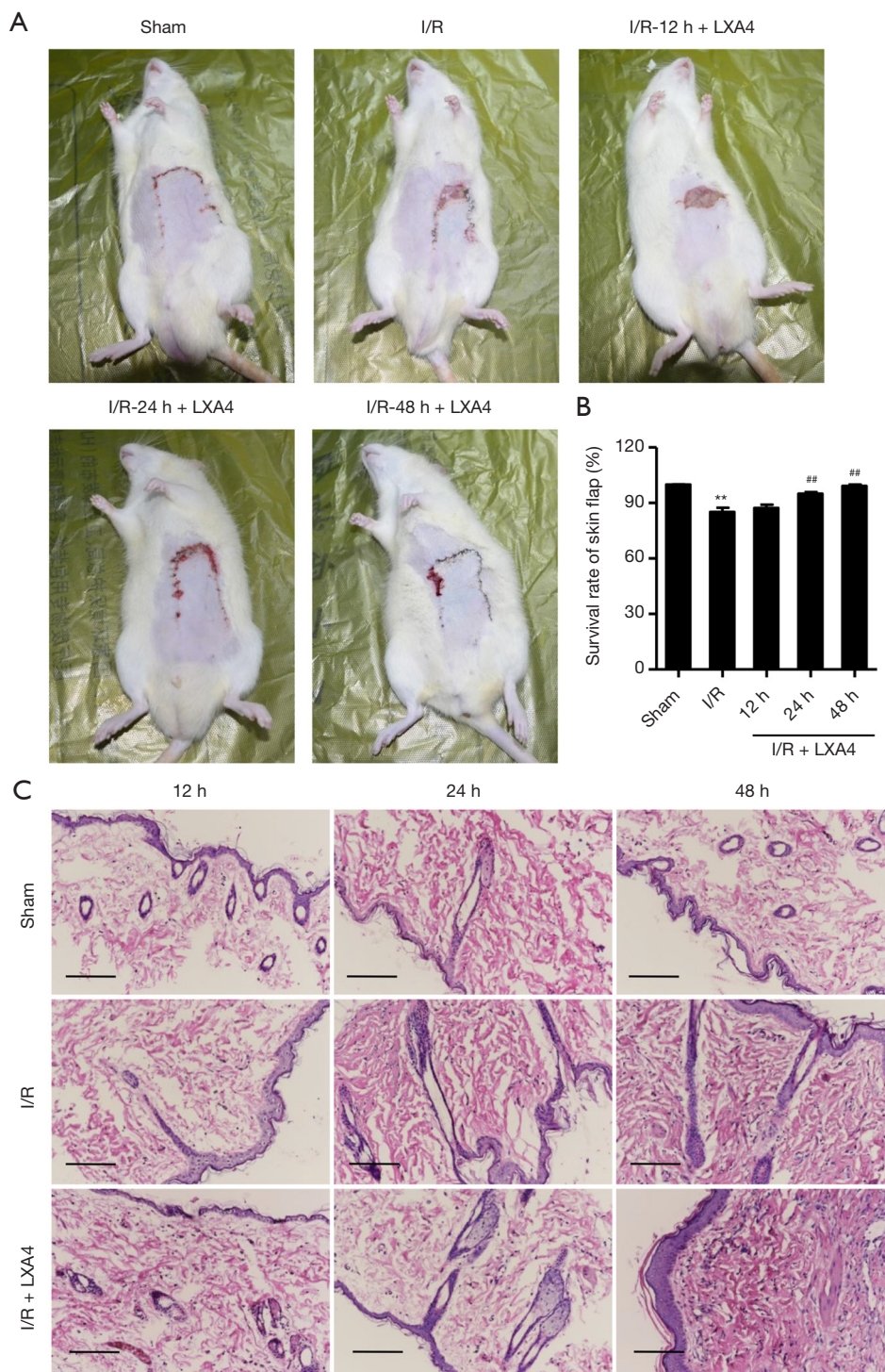
Figure 1 LXA4 promotes skin flap survival and attenuates I/R injury. The survival rate (A,B) and tissue injury (C) of skin flaps in rats after I/R with or without LXA4 pre-treatment were measured by the Image-Pro Plus 6.0 assay system, and HE stains. Scale bar: 100 μm. **, $P<0.01$ compared with the sham group; ##, $P<0.01$ compared with the I/R group. LXA4, Lipoxin A4; I/R, ischemia-reperfusion.

cytoplasm staining, pyknosis, and nucleoli disappearance, and the damaged and inflammatory cells were rarely observed. These results suggest that LXA4 reduces skin flap injury in I/R-induced rats.

LXA4 inhibits I/R-induced expression of LDH, GSH, MDA, SOD, TNF-α and ET

The concentration of LDH in the I/R group was markedly increased by 1.86-, 2.82- and 3.44-fold at 12, 24 and 48 hours post-I/R compared with the sham group (*Figure 2A*). The concentration of GSH in the I/R group was significantly decreased by 44.0%, 61.6% and 73.4% at 12, 24 and 48 hours post-I/R compared with the sham group (*Figure 2B*). The concentration of MDA in the I/R group was markedly increased by 1.04-, 2.36- and 2.52-fold at 12, 24 and 48 hours post-I/R compared with the sham group (*Figure 2C*). The concentration of SOD in the I/R group was significantly decreased by 44.6%, 54.7% and 68.2% at 12, 24 and 48 hours post-I/R compared with the sham group (*Figure 2D*). The concentration of TNF-α in the I/R group was significantly increased by 0.65-, 1.94-, and 2.06-fold at 12, 24, and 48 hours post-I/R compared with the sham group (*Figure 2E*). The concentration of ET in the I/R group was significantly increased by 0.94-, 2.14-, and 3.39-fold at 12, 24, and 48 hours post-I/R compared with the sham group (*Figure 2F*). However, the concentration of LDH, GSH, MDA, SOD, TNF-α and ET was significantly reversed by LXA4 treatment in rats at 12, 24, and 48 hours post-I/R compared with the I/R group (*Figure 2A,B,C,D,E,F*).

LXA4 inhibits I/R-induced apoptosis in skin flaps

The apoptotic rate in the I/R group was significantly increased by 1.05-, 3.75- and 4.61-fold at 12, 24 and 48 hours post-I/R compared with the sham group (*Figure 3A,B*). However, the apoptotic rate in LXA4 groups was significantly decreased by 19.9%, 15.8%, and 10.5% in rats at 12, 24 and 48 hours post-I/R compared with the I/R group. The apoptosis-related proteins, including Bax, Bcl-2 and Caspase-3, and NF-κBp65 were also evaluated by western blotting. The expression of active Caspase-3 and NF-κBp65 and Bax/Bcl-2 ratio in the I/R group was significantly increased compared with the sham group in a time-dependent manner, as shown in *Figure 3C,D*. However, the expression of active Caspase-3 and NF-κBp65 and Bax/Bcl-2 ratio in the LXA4 group was significantly decreased

compared with the I/R group in a time-dependent manner.

LXA4 inhibits I/R-induced ER stress in skin flaps

Morphological structure of cells during I/R and LXA4 pre-treatment showed incomplete cell structure and membrane, destroyed organelle structure and mitochondrial cristae, ER swelling, chromatin condensation, and plenty of vacuoles in the cytoplasm in the I/R group (*Figure 4A*). The structure of the cell in the sham group is still fine, with partly mass agglutination of chromatin, and organelle structure and mitochondrial cristae are partly destroyed. However, the morphological changes of the I/R group were significantly suppressed by LXA4 pre-treatment at 12, 24, and 48 hours post-I/R (*Figure 4A*). Since ER stress mediates three apoptotic pathways (14), Caspase-12, CHOP and JNK, these three markers of ER stress were therefore tested firstly. As shown in *Figure 4B,C*, the expression of Caspase-12, CHOP and p-JNK in the I/R group was increased compared with the sham group in a time-dependent manner. However, the expression of CHOP but not Caspase-12 and p-JNK in the LXA4 group was markedly decreased compared with the I/R group. Therefore, the ER stress-related proteins, including GRP78, p-PERK, p-eIF2α, and ATF4, were further evaluated by western blotting. As shown in *Figure 4B,D*, the expression of GRP78, p-PERK, p-eIF2α and ATF4 in the I/R group was increased compared with the sham group in a time-dependent manner. However, the expression of GRP78, p-PERK, p-eIF2α and ATF4 in the LXA4 group was markedly decreased compared with the I/R group at 48 hours post-I/R.

Discussion

I/R injury is one of the major factors leading to the increase of chronic rejection after skin flap transplantation. Previous studies showed that the success rate of free flap transplantation was 90–95% (25), and the necrosis rate of pedicle flap was 20–33% (26). The exact pathogenesis of I/R injury is not entirely clear, a complex pathophysiological process, and cell apoptosis plays a vital role in skin flap necrosis. LXA4 plays a vital role in inflammation, promoting inflammation, dissipation, and anti-proliferation (27). In the present study, LXA4 treatment inhibited rat skin flaps I/R injury, shown by inhibiting apoptosis, inflammatory response, and ER stress.

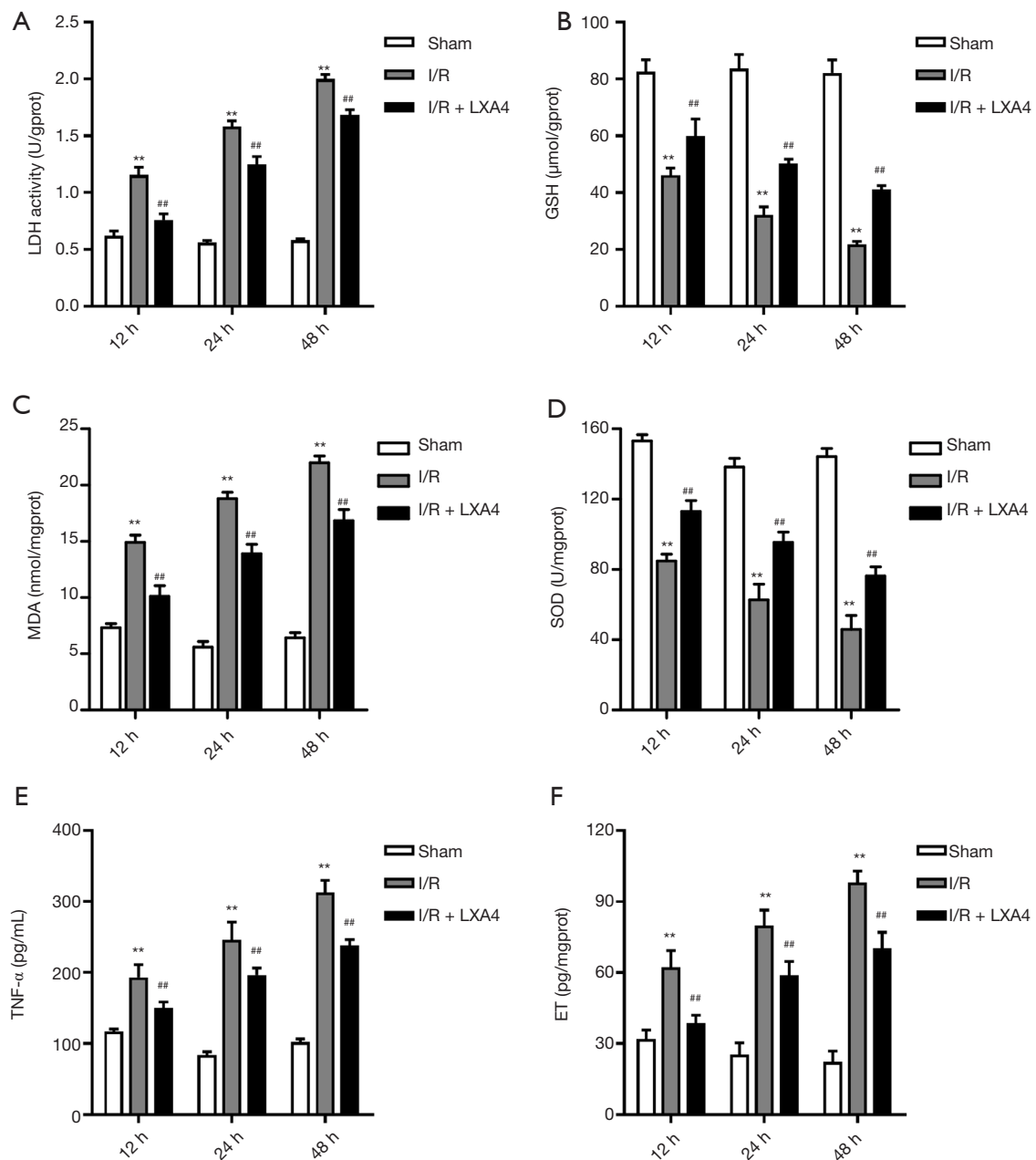Compared with the I/R group, LXA4 treatment showed

Figure 2 Effects of LXA4 on LDH, GSH, MDA, SOD, TNF-α and ET concentration in I/R-induced rats. The concentration of LDH (A), GSH (B), MDA (C), SOD (D), TNF-α (E), and ET (F) in skin flaps in rats after I/R with or without LXA4 pre-treatment was measured by biochemical assay, ELISA and radioimmunoassay, respectively. **, $P<0.01$ compared with the sham group; ##, $P<0.01$ compared with the I/R group. LXA4, Lipoxin A4; LDH, lactate dehydrogenase; GSH, glutathione; MDA, malondialdehyde; SOD, superoxide dismutase; TNF-α, tumor necrosis factor α; ET, endothelin; I/R, ischemia-reperfusion.
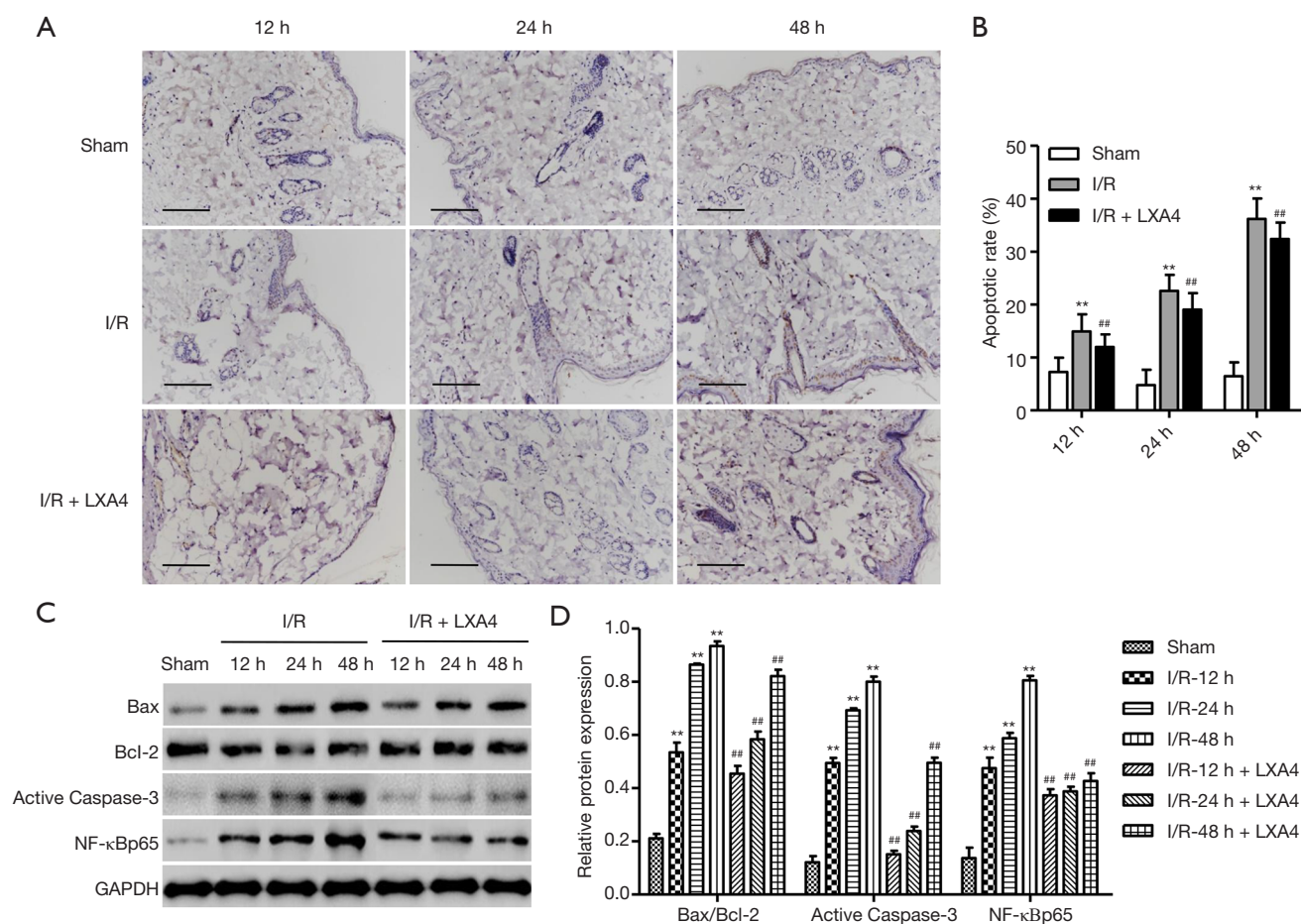
Figure 3 LXA4 inhibits I/R-induced apoptosis in skin flaps. (A,B) The apoptotic rate in skin flaps in rats after I/R with or without LXA4 pre-treatment was measured by TUNEL assay. Scale bar: 100 mm. (C,D). The expression of Bax, Bcl-2, active Caspase-3, and NF-κBp65 was measured by western blotting. **, P<0.01 compared with the sham group; ##, P<0.01 compared with the I/R group. LXA4, Lipoxin A4; I/R, ischemia-reperfusion.

higher survival of skin flaps, slight cytoplasm staining, pyknosis, and nucleoli disappearance. In line with our findings, previous studies have shown the protective effect of LXA4 on I/R injury (19,22). LXA4 has been found to decrease the serum concentration of TNF-α and MDA in the myocardial I/R injury model, suggesting that LXA4 reduces the myocardial reperfusion injury through inhibiting the production of oxygen free radicals (19). Our results also found that LXA4 inhibited ET and NF-κBp65 expression, and increased SOD and GSH level in skin flap I/R rats. Increasing ET release during I/R led to obvious vasoconstriction, induced tissue ischemia, aggravated vascular dysfunction, and caused irreversible damage to cells. LXA4 inhibits NF-κB activation and downregulation

of activated pro-inflammatory cytokines, including TNF-α, IL-1β, IL-6 and IL-8, suggesting that LXA4 may reduce inflammatory mediators and play a protective role by inhibiting phosphorylation of NF-κB (28).

In the I/R rats, we found significant morphological changes in apoptotic cells, including incomplete cell structure and membrane, destroyed organelle structure and mitochondrial cristae, ER swelling, chromatin condensation, and plenty of vacuoles in cytoplasm, which were consistent with the previous study (29). However, LXA4 treatment significantly inhibited cell apoptosis by decreasing the expression of the Bax/Bcl-2 ratio and Caspase-3. There is evidence showing that the family of Caspases and Bcl-2 was involved in the cell apoptosis during I/R injury, showing an
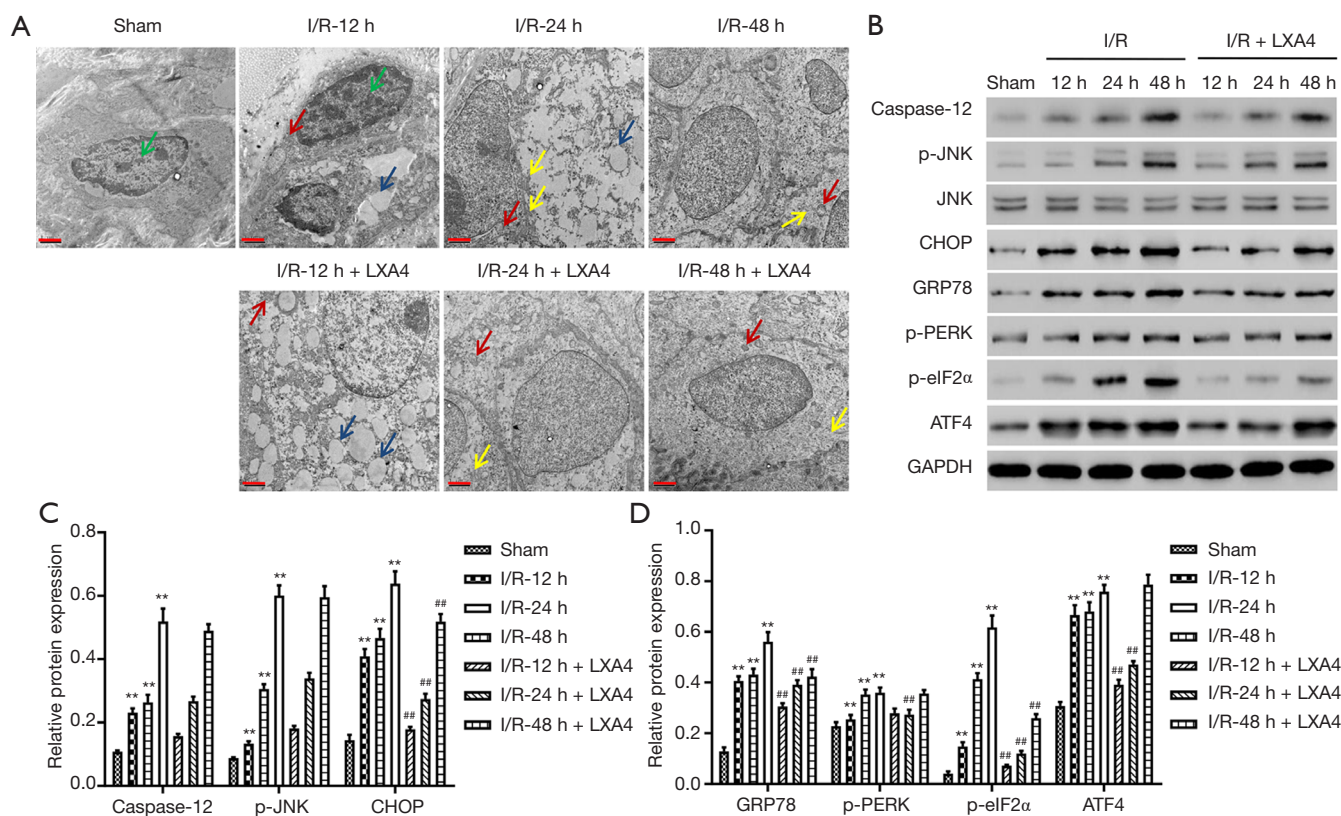
Figure 4 LXA4 inhibits I/R-induced ER stress in skin flaps. (A) The morphological structure of cells in skin flaps in rats after I/R with or without LXA4 pre-treatment was measured by transmission electron microscope assay. Scale bars: 1 μm. Chromatin condensation indicated by green arrows, vacuoles indicated by blue arrows, destroyed (I/R group) and improved (LXA4 pre-treatment group) mitochondrial cristae indicated by red arrows, significant (I/R group) and slight (LXA4 pre-treatment group) ER swelling indicated by yellow arrows. (B,C,D) The expression of Caspase-12, p-JNK, JNK, GRP78, p-PERK, p-eIF2α, ATF, and CHOP were measured by western blotting. **, $P<0.01$ compared with the sham group; ##, $P<0.01$ compared with the I/R group. LXA4, Lipoxin A4; I/R, ischemia-reperfusion; ER, endoplasmic reticulum.

essential role of those in the I/R injury (30,31).

Unfold protein response (UPR) can inhibit protein secretion in the ER, fold proteins and clear misfolded proteins involving GRP78, PERK and IRE1, increased in myocardial I/R models *in vivo* and *in vitro* (32,33). PERK is a transmembrane chaperone that activates eIF2α phosphorylation after activation, followed by increased ATF4 mRNA level and activation of CHOP, activating apoptotic signaling pathways in I/R injury (34). High expression of CHOP can inhibit the expression of Bcl-2 and cause the translocation of Bax from the cytoplasm to mitochondria (35). In the present study, LXA4 significantly inhibited increased expression of GRP78, p-PERK, p-eIF2α, ATF4, and CHOP in I/R rats. ER stress also activates NF-κB signaling and induces TNF-α expression

in I/R model (36). These findings suggest that the anti-apoptosis and anti-inflammatory effect of LXA4 may occur from the inhibition of ER stress. In addition to ER stress mediated CHOP apoptotic pathway, Caspase-12 and JNK are other apoptotic pathways mediated by ER stress (14). LXA4 attenuates myocardial I/R injury via ER stress mediated downregulation of Caspase-12 (37), and exerts a neuroprotective effect in cerebral I/R injury via a JNK-independent signaling (16). In the present study, LXA4 attenuates skin flaps I/R injury via ER stress mediated CHOP apoptotic pathway but not Caspase-12 or JNK apoptotic pathway in rats. However, on a cautionary note, there are some limitations to our current study that should be pointed out. First, other apoptotic pathways and major factors mediated by ER should be further investigated in

our future research. Second, the protective effects of LXA4 in clinical application demand further investigation.

Conclusions

In conclusion, we showed that ER stress causes tissue damage through induced cell apoptosis and inflammatory response in skin flap I/R rats. Besides, LXA4 presented a protective effect on skin flap tissues through inhibition of ER stress, leading to the reduced apoptotic cells and pro-inflammatory factor production. Our research indicates an important role of LXA4 in the treatment of skin flaps against I/R injury. Therefore, understanding the pharmacology of LXA4 is a key to developing new and more effective therapeutic strategies for treating skin flaps I/R injury.

Acknowledgments

Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at http://dx.doi.org/10.21037/atm-20-5549

Data Sharing Statement: Available at http://dx.doi.org/10.21037/atm-20-5549

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (http://dx.doi.org/10.21037/atm-20-5549). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The present study was performed in strict accordance with the guidelines on ethical care for experimental animals and approved by the Animal Research Committee of Xiaoshan Traditional Chinese Medical Hospital (No. XSZYY-2015-007).

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