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Original Article

Tongxinluo Ameliorates Myocardial Ischemia-Reperfusion Injury Mainly via Activating Parkin-Mediated Mitophagy and Downregulating Ubiquitin-Proteasome System*

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ABSTRACT **Objective**: To investigate the protective effects and mechanism of Chinese herbal compound Tongxinluo Capsule (通心络胶囊, TXL) on the Parkin-mediated mitophagy and the ubiquitin-proteasome system in a rat model of myocardial ischemia-reperfusion injury (MIRI). **Methods**: Seventy adult male Sprague-Dawley rats were randomly divided into 7 groups: sham group, MIRI group, low- and high-dose TXL (0.5 and 1 $g \cdot kg^{-1} \cdot d^{-1}$, respectively) groups, atorvastatin (ATV) group (7.2 $g \cdot kg^{-1} \cdot d^{-1}$), chloroquine (CQ) group (10 $g \cdot kg^{-1} \cdot d^{-1}$), and high-dose TXL + CQ group. After pharmacological administration for 7 days, rats underwent left anterior descending artery ligation surgery to establish the MIRI models with 50 min ischemia followed by 4 h reperfusion. Blood was taken for cardiac troponin I (cTnI) detection and hearts were harvested for infarct staining and apoptosis detection. The autophagy or mitophagy proteins and ubiquitinated proteins were detected by Western blotting. **Results**: Compared with the sham group, the MIRI group exhibited a larger infarcted area (27.13% ± 0.01%, $P<0.01$), a higher apoptotic index (34.33% ± 2.03% *vs.* 1.81% ± 0.03%, $P<0.01$), and higher cTnI expression (14.18 ± 1.01 *vs.* 7.96 ± 0.32, $P<0.01$). The mitochondrial integrity was damaged in the MIRI group, while TXL and ATV alleviated the damage of MIRI. More autophagosomes were observed in the high-dose TXL group than in the MIRI group (7.00 ± 0.58 *vs.* 4.33 ± 1.15, $P<0.05$). More amounts of PTEN-induced putative kinase protein 1 (PINK1) and Parkin translocated onto the mitochondria were detected in the high-dose TXL group than in the MIRI group ($P<0.05$). The ubiquitin response was significantly downregulated in the high-dose TXL group relative to the MIRI group ($P<0.05$). CQ administration abolished the activation of autophagy flux and the PINK1/Parkin pathway induced by high-dose of TXL. **Conclusions**: TXL ameliorates MIRI via activating Parkin-mediated mitophagy in rats. The downregulation of the ubiquitin-proteasome system is also involved.

KEYWORDS Chinese medicine, mitophagy, ischemia-reperfusion injury, Parkin, ubiquitin

Myocardial ischemia-reperfusion injury (MIRI), noted with a high morbidity and mortality rate, represents a fatal challenge in coronary heart disease.(1) Rapid reperfusion is still the current standard treatment for ischemic myocardium after the ischemic phase of ischemic heart disease.(2) Nevertheless, reperfusion itself results in additional injury in the early period.(3) Among the causative mechanisms of MIRI, which include oxidative stress, apoptosis, calcium overload, inflammation, and mitochondrial permeablity transition pore (mPTP) opening,(4) growing evidence indicates that autophagy is intimately implicated in MIRI.(5,6)

Autophagy, generally referred to as macroautophagy,(5) is an evolutionarily conserved process through which cells sequester, degrade, and recycle cytoplasm.(6) The process uses specialized vesicles that ultimately fuse with lysosomes to ensure cellular homeostasis and to respond to metabolic stress or cellular energy crises.(5) It was reported that autophagy is induced by ischemia and further enhanced after reperfusion in the heart *in vivo*.(7) While, the argument that MIRI impairs autophagosome clearance, thereby blocking autophagy flux, has

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made the role of autophagy during reperfusion more ambiguous,[8] because whether the abnormal accumulation of autophagic vacuoles is properly recognized as a hypofunction or a hyperfunction is too difficult to be shed light on among the whole autophagic process. Therefore, it is increasingly urgent to illuminate the effect of autophagy in MIRI and the underlying mechanism. Autophagy can be classified as selective or nonselective according to whether the substrates degraded are specific. Mitophagy is the selective autophagy of mitochondria, a dynamic organelle that plays a central role in complex processes within cells including ATP-production for myocardial contractility, cellular homeostasis, oxidative stress induction and apoptosis that involved in many aspects of MIRI.[9] Mitophagy has been reported to be intimately related to MIRI.[10] However, evidence from *in vivo* experiments is still inconclusive for the relationship between the PTEN-induced putative kinase protein (PINK1)/Parkin pathway, a well-known pathway involved in mitophagy, and myocardial protection during MIRI.

The ubiquitin-proteasome system (UPS) also participates in MIRI.[11,12] UPS is the primary mechanism for the proteolysis of short-lived or misfolded proteins, whereas autophagy degrades long-lived proteins and aberrant organelles. There is a complementary association between UPS and autophagy in cellular stress and protection.[13] Moreover, ubiquitin plays important roles in selective autophagy.[14] Therefore, the interrelation between the two intracellular degradation systems warrants deeper research.

Tongxinluo Capsule (通心络胶囊, TXL), a Chinese herbal compound, is reported to have benefits in alleviating myocardial infarction, improving symptoms,[15,16] protecting microvasculature from acute ischemic heart disease,[17] ameliorating acute coronary syndrome with high platelet reactivity,[18] and inducing secondary prevention after acute myocardial infraction.[19] However, whether TXL directly confers cardioprotection through modulating mitophagy of cardiomyocytes that function as the dominant perfusion object needs further investigation in MIRI. This will be helpful to unveil the potential mechanisms of Chinese medicine in maintaining mitochondrial homeostasis and to improve cardiac drug development. The aim of the present study was to investigate the effect of TXL on cardiomyocytes in a rat model undergoing coronary artery ligation and the corresponding mechanism of mitophagy during MIRI.

METHODS

Drug Preparation

TXL superfine powder (Shijiazhuang Yiling Pharmaceutical Co., Ltd., No. SY1605001, China; 10 g/pouch) was dissolved in saline solution via ultrasonication and was administered at a low dose of 0.5 g•kg^{-1}•d^{-1} and a high dose of 1 g•kg^{-1}•d^{-1}. The solutions were stored in a refrigerator at 4 ℃ before intragastric administration. Atorvastatin (ATV; Pfizer, USA, H20051408; 20 mg/pill) and chloroquine (CQ; Sigma, USA, C6628; autophagy inhibitor) were dissolved in saline solution and administered at doses of 7.2 and 10 mg•kg^{-1}•d^{-1}, respectively.

Animals

Seventy specific-pathogen-free adult male Sprague-Dawley rats [200–230 g; certificate No. SCXK-(army) 2012-0004] were procured from the Laboratory Animal Center of the Military Medical Research Institute, Chinese PLA Military Academy of Sciences, Beijing, China. The rats were reared under a 12-h light/dark cycle at 25±2 ℃, and were provided with sufficient water and standard chow. The experiments conformed to the guidelines for the care and use of laboratory animals published by the US National Institutes of Health. All animal experiments were approved by the Animal Ethics Committee of the Military Medical Research Institute, Chinese PLA Military Academy of Sciences, Beijing, China (No. IACUC-DWZX-2018-007).

Grouping and Administration

The rats were randomly divided into 7 groups according to the method of random number table: sham, MIRI, low-dose TXL, high-dose TXL, positive drug (ATV), CQ, and high-dose TXL+CQ groups. Both low- and high-dose TXL group rats were administered with the corresponding concentration of TXL solution and the ATV group was administered with ATV solution, a statin with atheroprotective effects that was reported to protect against MIRI via autophagy modulation,[21] for 7 days before experiments. In the groups receiving CQ, a well-known autophagy inhibitor, each rat was administered by intraperitoneal injection (i.p.) with 0.2 mL/d CQ solution for 7 days. The rest of the rats were given normal saline. All the rats were anesthetized with 10% chloral hydrate (3 mL/kg, i.p.). A classical MIRI model was established in rats as previously

described.[20] The MIRI group and drug-administered groups had their left anterior descending artery ligated for 50 min, followed by loosening the ligature for 4 h, whereas the sham group was threaded but no ligation was performed. Successful ligation of the artery was verified by immediate regional cyanosis or paleness of the anterior ventricular wall below the ligation site, and confirmed further by electrocardiography.

Infarct Size Measurement

The infarct size of the myocardium was measured with 2,3,5-triphenyltetrazolium chloride (TTC) staining. The rats were sacrificed by cervical dislocation, then the heart tissue was harvested and immediately stored at –20 ℃ in a freezer for 40 min. The tissue was sliced perpendicular to the vertical axis into 5 uniform sections per heart. Every 2-mm-thick section was incubated in 1% TTC solution at 37 ℃ for 30 min in the dark, and then immersed in 4% paraformaldehyde overnight. The area of the infarction was determined by computerized planimetry and the area percentage was calculated.

Determination of Cardiomyocytes Apoptosis via TdT-Mediated dUTP Nick-End Labelin Assay

TdT-mediated dUTP nick-end labeling (TUNEL) staining was performed to detect cell apoptosis in myocardial tissue. The ischemic myocardial tissue was sliced along a cross section, embedded in paraffin, and cut into 5-μm-thick slices. TUNEL staining was conducted after antigen retrieval with a commercial kit (11684817910; ROCHE; USA) following the manufacturer's instructions. Apoptotic nuclei appeared yellow-brown, whereas normal nuclei appeared light blue. Ten non-overlapping areas (×400 magnification) from every slice were observed stochastically. The myocardial apoptotic ratio was calculated as apoptotic number/entire cell number ×100%.

Detection of Autophagosomes Using Transmission Electron Microscopy

The cardiac tissue of rats was fixed with 2.5% glutaraldehyde at 4 ℃ for 3 h, and then cut into 1 mm^3 cubes. The tissue samples were taken for further handling and observed by transmission electron microscopy (TEM).

Myocardial Enzymes in cTnI Assay

Blood was collected from the rats' aorta ventralis after 4 h of reperfusion and centrifuged to obtain the serum to measure cardiac troponin I (cTnI).

Isolation of Mitochondrial Protein

Mitochondrial protein isolation was performed with a cell mitochondria isolation kit (C3606; Beyotime Biotechnology; China) according to the manufacturer's instructions. Hearts were excised immediately after reperfusion, washed with phosphate buffered saline (PBS), ground and lysed for 2 min with an automatic grinder, and then centrifuged at 600×*g* for 20 s. The supernatant was carefully removed and discarded, and the solids were mixed well and centrifuged for 20 s. Mitochondria isolation reagent A from the kit was added, the tube was vortexed at medium speed for 5 s, and then kept on ice for exactly 2 min. Mitochondria isolation reagent B was added and the tube was vortexed at maximum speed for 5 s. The tube was kept on ice for 5 min and vortexed at maximum speed every minute. Mitochondria isolation reagent C was added and the tube was inverted several times. The tube was centrifuged at 11000×*g* for 10 min at 4 ℃. Then the supernatant was transferred to a new tube and centrifuged at 12,000×*g* for 15 min at 4 ℃. The cytosol fraction was transferred to a new tube. Mitochondria isolation reagent C was added to the pellet and the mixture was centrifuged again at 12,000×*g* for 10 min. The supernatant was discarded. The remaining pellet consisted of the isolated mitochondria. The mitochondria were lysed with RIPA buffer on ice for 30 min. The mixture was centrifuged at 12,000×*g* for 10 min, and then the supernatant containing the mitochondrial protein was transferred to a new tube for further analysis.

Western Blotting Analysis

The rat heart tissue was washed with PBS, and then lysed in lysed buffer containing protease inhibitor cocktail at 1:100 dilution on ice for 30 min. The mixture was cleared by centrifugation at 12,000×*g* for 10 min at 4 ℃, and the protein concentration was determined with a bicinchoninic acid (BCA) protein assay kit (Kang Wei Company, lot. No. CW0022S, China) according to the manufacturer's protocol. The resulting homogenate was mixed with PBS and an equal volume of loading buffer (Kang Wei Company, lot. No. CW0027S), giving a total volume of 100 μL, and the mixture was boiled for 10 min. Equal amounts (100 μg) of protein were loaded on 12% or 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels for electrophoretic transfer to 0.45 μm polyvinylidene difluoride (PVDF) membranes for 1 to 2 h. The membranes were blocked in 5% non-fat milk in 1×Tris-buffered saline Tween 20 (TBS-T) at room

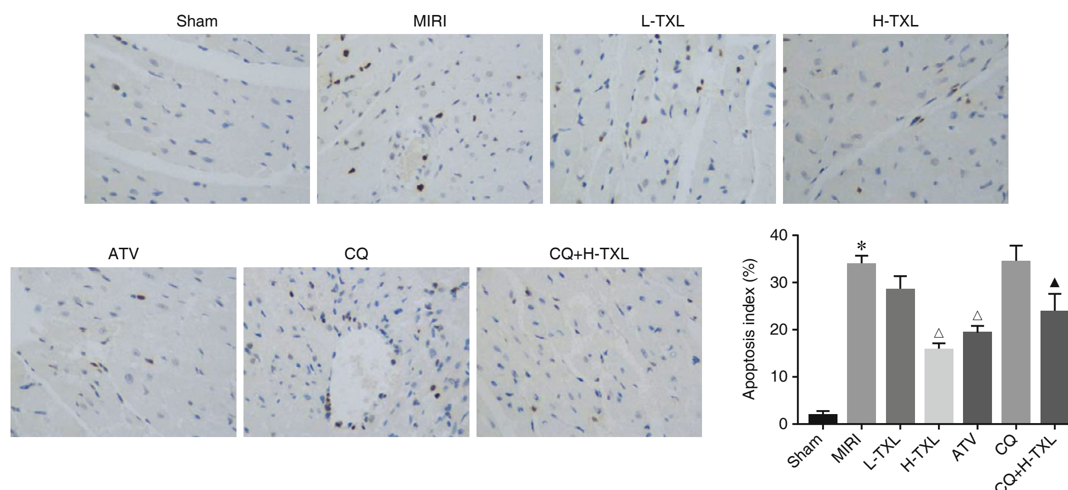

Figure 1. Ischemia-Reperfusion Induced Myocardial Apoptosis of Rats Detected by TUNEL Staining (×400)

Notes: TXL: Tongxinluo, L: low-dose; H: high-dose, ATV: atorvastatin, CQ: chloroquine; bar graph shows $\bar{x}\pm s$ of 3 independent experiments, the same below. *P<0.01 *vs*. sham group, △P<0.05 *vs*. MIRI group, ▲P<0.05 *vs*. TXL high-dose group.

temperature for 2 h and sequentially incubated with the primary antibodies of glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:2500, lot. No. RG000100, Solarbio, China), microtubule-associated protein light chain 3B (LC3B, 1:2000, lot. No. ab48394, Abcam, UK), p62 (1:500, lot. No. 55274-1-AP, Proteintech, China), beclin 1 (1:1000, lot. No. 3738S, CST, USA), PINK1 (1:1000, ab216144, Abcam), ubiquitin (1:1000, lot. No. ab134953, Abcam), COXIV (mitochondrial protein marker, 1:1000, lot. No. 4844, CST, USA), and Parkin (1:1000, lot. No. ab77924, Abcam) overnight at 4 ℃. The membranes were washed with TBST 3 times (5 min each) and incubated with a horseradish peroxidase-conjugated secondary antibody (HRP anti-rabbit 1:4000, SE134, Solarbio, China) for 1 h at room temperature followed by washing with TBST 3 times (5 min each). The antibody complexes were visualized with enhanced chemiluminescence solution (CW0049S, Kang Wei Company, China) and a Western blot detection system (Image Quant, LAS500, GE Company, USA). The protein expression levels were quantified with a relative grayscale and normalized to GAPDH as an internal control.

Statistical Analysis

All the data were analyzed with statistical software SPSS 20.0, and the values were expressed as mean ± standard deviation ($\bar{x}\pm s$). Statistical analyses among groups were evaluated via one-way analysis of variance (ANOVA) comparison. Tukey post-hoc tests were used to identify significant differences between means. P<0.05 was considered statistically significant.

RESULTS

TXL Alleviated MIRI in Dose-Dependent Manner and High-Dose TXL Had Similar Effect to ATV

As shown in Figures 1 and 2, compared with the sham group, the MIRI group had a higher cardiomyocyte apoptotic index (P<0.01), a larger infarct size (P<0.01), and more increased cTnI levels (P<0.01). The low- and high-dose TXL groups (P<0.05) showed decreased apoptosis index, heart infarcted area, and cTnI compared with the MIRI group. In contrast, CQ abrogated the protection effect

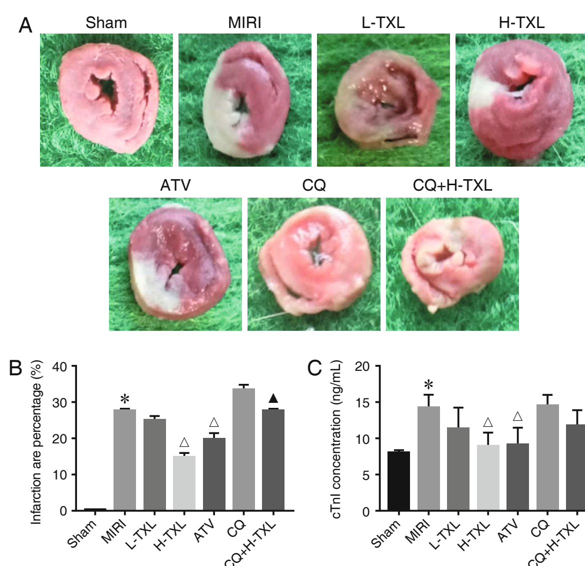

Figure 2. TXL Alleviated Myocardial Ischemia-Reperfusion Injury in Rats

Notes: A: infarcted area of groups; B: infarcted area percentage of heart subjected to ischemia and reperfusion injury; C: concentration of cTnI of groups. *P<0.01 *vs*. sham group and △P<0.05 *vs*. MIRI group, ▲P<0.01 *vs*. TXL high-dose group.

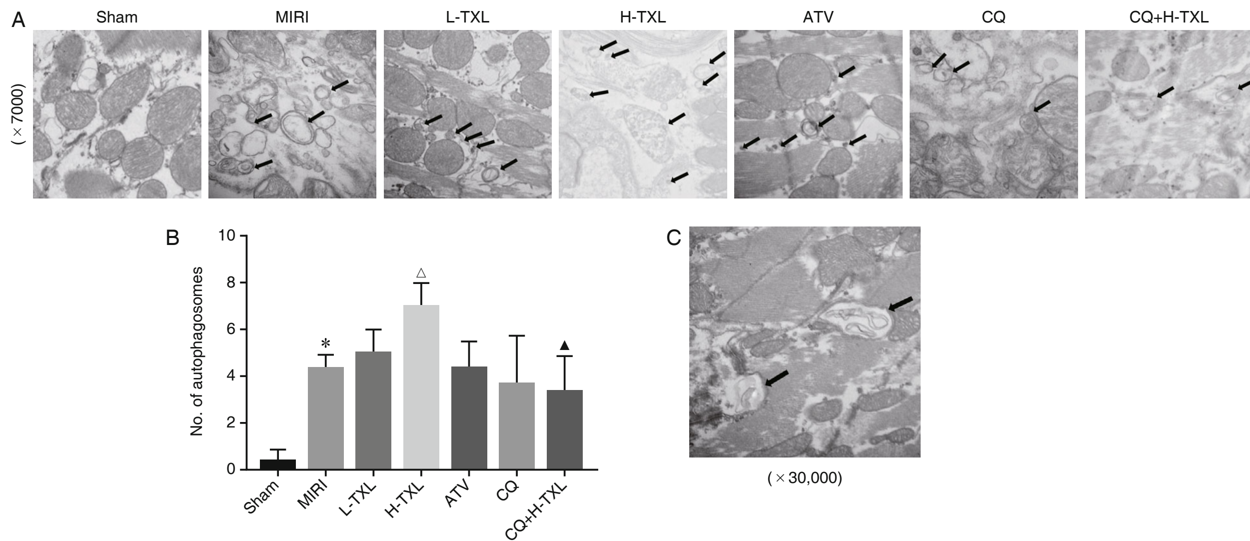

Figure 3. Pictures of Transmission Electron Microscope Detection of Mitochondrial in Rats

Notes: Black arrows represent autophagosomes or autolysosomes in A, autophagosomes engulfing suspected misfolded proteins of H-TXL group in D. *P<0.05 *vs*. sham group, △P<0.05 *vs*. MIRI group, ▲P<0.05 *vs*. TXL high-dose group.

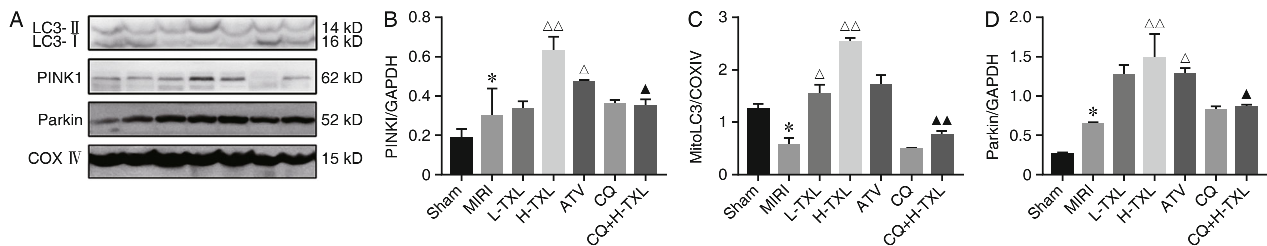

Figure 4. Expressions of LC3，PINK1 and Parkin Protein at Mitochondria by Western Blot in Rats

Notes: COX Ⅳ represents mitochondrial loading protein. *P<0.05 *vs*. sham group; △P<0.05, △△P<0.01 *vs*. MIRI group; ▲P<0.05, ▲▲P<0.01 *vs*. TXL high-dose group.

of TXL (P<0.05). The cardioprotection of high-dose TXL group was similar to that of ATV.

TXL Alleviated Mitochondrial Damage and Restored Mitochondrial Integrity

As shown in Figure 3, MIRI severely deformed the mitochondria, causing swelling, disappearance of the mitochondrial cristae, and the destruction and disintegration of the mitochondrial membrane. In contrast, the mitochondrial damage was mitigated and mitochondrial integrity was restored in the high-dose TXL group. ATV exerted a similar effect on the mitochondria and also showed similar cardioprotection in high-dose TXL group. However, administration with CQ abolished the protective effect of TXL.

TXL Upregulated Mitophagy and Stimulated Autophagy Flux to Protect Cardiac Cells from MIRI via PINK1/Parkin Pathway

As shown in Figure 4, the amounts of PINK1 and Parkin showed the same trend and were significantly higher in the high-dose TXL group than in the MIRI group (P<0.01 or P<0.05), but they were markedly decreased by CQ (P<0.05). Furthermore, mitochondrial LC3-Ⅱ expression was substantially higher in both the low- (P<0.05) and high-dose (P<0.01) TXL groups than in the MIRI group, although this was reversed by CQ (P<0.01).

Additionally, the TEM images showed that there were more autophagosomes in the high-dose TXL group than in MIRI group (P<0.05, Figure 3), and the number of autophagosomes decreased after CQ administration (P<0.05). Western blotting analysis of LC3-Ⅱ showed a significantly higher level in the high-dose TXL group than in the MIRI group (P<0.05, Figure 5B), and the increase was abolished by CQ (P<0.05). In contrast, the level of another autophagic protein, p62, was higher in the MIRI group relative to the sham group (P<0.01, Figure 5C), but decreased in the high-dose TXL group (P<0.01). Notably, there was no significant difference in the levels of beclin 1

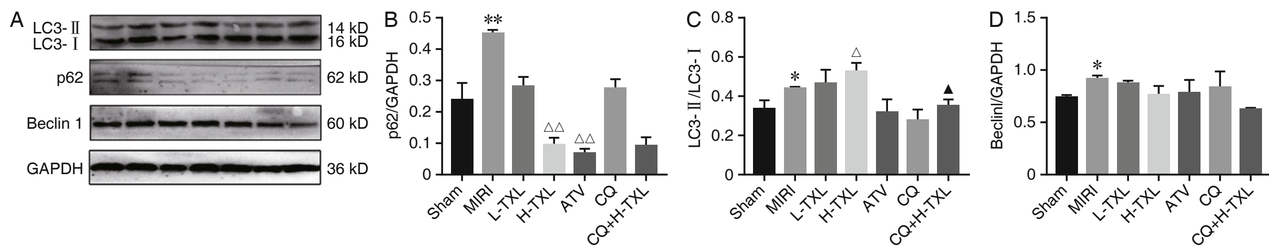

Figure 5. Expressions of Autophagic Proteins by Western Blot in Rats

Notes: $^{*}P<0.05$, $^{**}P<0.01$ *vs*. sham group; $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$ *vs*. MIRI group; $^{\blacktriangle}P<0.05$ *vs*. TXL high-dose group.

between the MIRI group and the high-dose TXL or ATV groups ($P>0.05$, Figure 5D).

Activation of Ubiquitination by MIRI was Alleviated by TXL in Dose-Dependent Manner

UPS purges misfolded proteins to maintain cellular homeostasis and is closely connected with autophagy in protein quality control.[22] An image of autophagosomes was captured engulfing suspected misfolded proteins in the high-dose TXL group (Figure 3C). The ubiquitinated proteins were overexpressed during MIRI compared with the sham group (Figure 6, $P<0.05$). As TXL activated mitophagy in a dose-dependent manner, ubiquitinated protein accumulation was accordingly alleviated with a TXL's dose-dependent trend compared with the MIRI group ($P<0.05$).

As for p62, it showed the same trend as ubiquitinated proteins. The levels of p62 were high in the MIRI group, but were decreased by TXL in a dose-dependent manner (Figure 5C and 6). In contrast to the accumulation of p62 in the MIRI group, LC3-Ⅱ binding to the defective mitochondria was lower in the MIRI group compared with the sham group (Figure 4C, $P<0.05$).

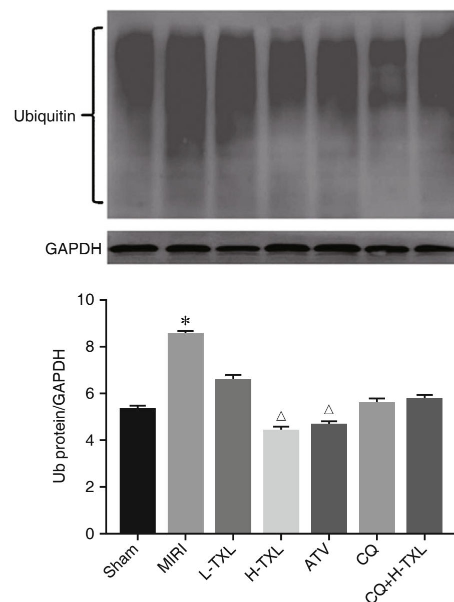

Figure 6. Expression of Ubiquitin Protein by Western Blot

Notes: $^{*}P<0.05$ *vs*. sham group; $^{\triangle}P<0.05$ *vs*. MIRI group

DISCUSSION

MIRI is the most challenging problem in the treatment of ischemic heart disease. Studies are increasingly exploring more efficient treatments based on the causative mechanisms, including autophagy, which has attracted intense interest. In the present study, we established a classical MIRI model in rat hearts by ligating the coronary artery. The badly infarcted area of the myocardium, apoptosis of cardiomyocytes, and high expression of cTnI confirmed the success of the MIRI model.

In high-energy-demand cells like cardiomyocytes, mitochondria account for about 30% of the cell volume.[23] Myocardial energy metabolism mainly depends on fatty acid substrates, which can only be oxidized by mitochondria. The stress resulting from mitochondrial metabolic imbalance, excessive reactive oxygen species production, mitochondrial membrane potential decrease, accelerating mitochondrial fission, and the release of apoptosis factors, such as cytochrome c, usually leads to apoptosis.[24] Therefore, it is vital to protect mitochondria to maintain cardiac intracellular homeostasis. The TEM images obtained in the present study showed swollen or degraded mitochondria in the MIRI group, confirming that mitochondrial integrity was severely damaged by MIRI. Selective autophagy of mitochondria, known as mitophagy, is an important mitochondrial quality control mechanism that eliminates damaged mitochondria and is usually initiated quickly to protect cardiomyocytes. Upregulation of mitophagy was reported to play a significant role in the gold standard of cardioprotection in ischemic heart disease.[25]

Four mitophagy pathways, including the most well-known PINK1/Parkin pathway, have been identified for detecting dysfunctional mitochondria and the recruitment of autophagosomes for degradation.[9] In healthy mitochondria, the mitochondrial kinase, PINK1, originating in the cytoplasm, is integrated into the inner mitochondrial membrane via insertion into the inner mitochondrial membrane translocase and is rapidly processed, and ultimately degraded by the mitochondrial membrane peptidase.[9] However, when mitochondria are depolarized, PINK1 can no longer be imported into the mitochondria but accumulates on the depolarized mitochondria, where it recruits and activates Parkin, an E3 ubiquitin ligase, to translocate onto the defective mitochondria to interact with LC3-Ⅱ and sequester defective mitochondria, thereby inducing mitophagy to execute mitochondria quality control.

Parkin has also been identified as a regulator of mitochondrial degradation in cardiac myocytes[26] and is recruited to mitochondria in the infarct border zone 4 h after myocardial infarction.[27] The elimination of dysfunctional mitochondria restores adenosine triphosphate generation to attenuate cardiac cellular stress, which is important for postmitotic cells, such as myocardial or neural cells, that cannot separate to dilute intracellular metabolic waste. In this study, TXL rescued autophagy flux at least partly via the PINK1/Parkin pathway to protect mitochondrial integrity and mitigated mitochondria disorders in MIRI via promoting mitophagy. Thus, TXL reduced the myocardial infarcted area and cardiomyocyte apoptosis.

TXL is a compound preparation that was reported to reduce myocardial infarct size and ameliorate heart function after reperfusion in acute myocardial infarction.[17] TXL consists of *Radix ginseng*, *Scorpio*, *Hirudo*, *Eupolyphaga seu steleophaga*, *Scolopendra*, *Periostracum cicadae*, *Radix paeoniae rubra*, *Semen ziziphi spinosae*, *Lignum dalbergia odorifera*, *Lignum santali albi*, and *Borneolum syntheticum*.[28] Experiments and clinical studies[29-32] have verified TXL's myocardial benefits converging on the protection of myocardial microvascular endothelial cells in ischemic heart disease. However, cardiac cells predominating in the whole myocardium reperfusion unit[33] deserve further mechanistic investigation. In our present study, TXL administration decreased the infarct size, reduced cTnI levels and cardiomyocyte apoptosis in a dose-dependent manner, and consequently protected the myocardium from MIRI. The beneficial effect of high-dose TXL was similar to that of ATV, which also confers a cardioprotective effect via mitophagy/autophagy modulation. CQ, which inhibits autophagy via preventing the fusion of autophagosomes and lysosomes, abolished the cardioprotection of TXL, confirming the important role of autophagy flux during MIRI. Notably, the expression of beclin 1, an autophagy marker protein, was higher in the MIRI group than in the sham group; however, there was no significant difference in its expression between the TXL groups and the MIRI group. This result is in agreement with other study on the effect of TXL on autophagy,[34] even though Matsui, et al[8] claimed that the further upregulation of autophagy during reperfusion is through a beclin 1-dependent mechanism. Indeed, special situations do exist in which autophagy is activated in a beclin 1-independent way.[35,36] In particular, some mitophagy is independent of beclin 1 and occurs when mitochondria have been damaged.[37] Interestingly, consistent with this point, we found that the mitophagy inactivated during MIRI was dramatically restored by TXL via the PINK1/Parkin pathway.

Apart from targeting specific organelles, autophagy also plays a role in cellular protein quality control via targeting the removal of protein aggregates and long-lived proteins. This is because disposing of misfolded/damaged proteins and handling protein homeostasis in cardiomyocytes are essential for maintaining cellular function and require precise control of protein synthesis, processing, and degradation. The degradation of terminally misfolded/damaged proteins is the last line of defense and is mainly performed by the UPS and autophagy.[38] In our present study, we found chain-like, suspected misfolded or unfolded proteins engulfed by autophagosomes in the TEM image of the high-dose TXL group. Autophagy and the UPS are activated in parallel as clearance mechanisms for eliminating aggregating and aggregated proteins[39] and there is overlap between the two mechanisms. For example, ubiquitinated protein is a common substrate recognition signal and a link between the two degradation systems. Furthermore, Parkin, an E3 ubiquitin ligase that eliminates pathological protein aggregation identified as the marker of Parkinson's disease, is located in the cytosol and translocates specifically to uncoupled mitochondria to induce mitophagy. We detected ubiquitinated protein by Western blotting analysis and found that the MIRI

group showed marked activation of ubiquitinated proteins relative to the sham group. In contrast, the high-dose TXL group showed inactivation of ubiquitinated protein compared with the MIRI group, consistent with the conclusion that inducing autophagy attenuated the accumulation of ubiquitinated proteins in cultured cardiomyocytes.[40] p62, a substrate adaptor protein that binds both ubiquitin and autophagy-specific machinery modifiers, links the UPS and autophagy in the heart during defense against proteotoxic stress.[38] In the present study, the accumulation of p62 in the MIRI group was strikingly decreased by TXL in a dose-dependent manner, sharing the same trend as ubiquitinated proteins. In contrast, LC3-Ⅱ was inactivated by defective mitochondria and the total increase in LC3-Ⅱ was not large as the accumulation of p62 in the MIRI group compared with the sham group. Given that UPS and autophagy share a compensatory mechanism in response to proteotoxic stress in cardiomyocytes, and UPS is essential for Parkin-mediated mitophagy,[14] we speculate that p62 may serve as the substrate adaptor for UPS rather than participating in autophagy in the MIRI group. While the activation of mitophagy by TXL via the PINK1/Parkin pathway compromised the UPS triggered by MIRI and exerts the cardioprotective effects.

In conclusion, our research suggests that autophagy flux was defective and the UPS was activated during MIRI. TXL elicited a protective effect on the myocardium via activating Parkin-mediated mitophagy, and thereby restoring autophagy flux. The downregulation of UPS was also involved in the process, which provides new insights into the cardioprotective effects of TXL.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

Author Contributions

All authors participated in the review of the manuscript. Yang MH and Li SD designed the experiments. Yang HX and Wang P performed the experiments with Wang NN recording and acquiring data. Yang MH guided the writing and revision of the paper. Yang HX analyzed data and wrote the manuscript.

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