Original Article
Effect of autophagy on cardiomyocyte membrane Cx43 acute remodeling in rats with ischemia-reperfusion

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Abstract: Background: To investigate the impact of autophagy on cardiomyocyte membrane connexin 43 (Cx43) expression, distribution, and phosphorylation in myocardial ischemia-reperfusion injury (MI/RI). Methods: Twenty-four male SD rats were randomly divided into a sham operation group, a chloroquine (CQ) + sham operation group, an I/R group, and a CQ + I/R group. The MI/RI model was established by reversible ligation of the left anterior descending coronary artery to induce ischemia for 30 min and reperfusion for 2 h. The left ventricular infarct size was measured by TTC (2,3,5-triphenyltetrazolium chloride) and Evans blue double staining. Cardiac troponin I (cTnI) content was detected by automatic biochemical analyzer. Autophagy related gene Beclin1, Cx43, and p-Cx43 protein expressions were tested by western blot. Cx43 and p-Cx43 distributions in ventricular myocardium were observed by immunofluorescence analysis. Results: Compared with the I/R group, the left ventricular infarct size, serum cTnI content, reperfusion arrhythmia severity, and in vivo induced ventricular fibrillation threshold, and Beclin-1 protein expression were significantly reduced in CQ + I/R group (P < 0.05). Compared with the SH group, Beclin-1 protein expression was significantly enhanced, while Cx43 and p-Cx43 levels were obviously downregulated in the I/R group. Beclin-1 protein declined, whereas Cx43 and p-Cx43 levels enhanced in CQ + I/R group compared with the I/R group. Conclusion: Autophagy may reduce myocardial ischemia-reperfusion injury and malignant arrhythmia by improving the acute remodeling of myocardial cell membrane Cx43.

Keywords: Ischemia-reperfusion injury, autophagy, connexin 43, acute remodeling, reperfusion arrhythmia

Introduction

The incidence of coronary heart disease in China exhibits an increasing and younger trend. Following the development of percutaneous transluminal coronary intervention (PCI), the emergence of myocardial ischemia/reperfusion injury (MI/RI) gradually attracts attention [1]. For the treatment of acute myocardial infarction, the most important way is to make the blood re-infuse into cells. In most cases, ischemia reperfusion tissues and organ function can be restored and structural damage can be repaired; but sometimes ischemia/reperfusion actually worsens ischemic tissues damage and causes organ dysfunction and structural damage [2]. The phenomenon of reperfusion after ischemia exaggerated tissue injury, and even caused irreversible damage known as ischemia-reperfusion injury.

Coupling between cells, one of the main ways of cardiac electrical impulse conduction, is mediated by the cell membrane gap junction protein (GJ). Connexin 43 (Cx43), an important protein that constitutes GJ in the ventricle, shows the highest content in the ventricular muscle. Cx43 protein is a single polypeptide chain molecules formed by 382 amino acids, the molecular weight is 43 kd. Cx43 mainly expresses in ventricular cells but also in the endothelial cells and atrial cells [3]. Cardiomyocyte membrane Cx43 occurs acute structural remodeling during MI/RI, manifested as abnormal changes in number, distribution, and phosphorylation, resulting in myocardial cell decoupling and the occurrence of malignant arrhythmia [4].

Autophagy is a conserved process for bulk degradation and recycling of cytoplasmic proteins and organelles in lysosomes, providing free fatty acids and amino acids for maintaining

energy production and protein synthesis. In order to accomplish this work orderly, autophagy related proteins including microtubule associated protein light chain 3 (LC3), LAMP-1, Beclin-1 and p62 were all indispensable for cytoplasm-to-lysosome delivery [5]. It is a self-catabolic process that degrades long-periodic proteins and can occur under pathological conditions, such as hunger, ischemia, and hypoxia [6]. At present, it was shown that autophagy participates in MI/RI process, whereas the effect and mechanism of autophagy on the cell membrane Cx43 protein are still unclear. The purpose of this study was to investigate the effect of autophagy on cardiomyocyte membrane Cx43 acute remodeling to provide an important experimental basis for the prevention and treatment of malignant arrhythmia caused by MI/RI.

Materials and methods

Experimental animals

Twenty-four male Sprague-Dawley rats weighted 230 ± 20 g were provided by the Hubei Experimental Animal Research Center [license number: SCXX (E) 2015-0018].

Main reagents and instruments

Chloroquine diphosphate salt (Sigma), pentobarbital sodium (Merck), Evans Blue (Shanghai Harling Biotechnology Co., Ltd.), TTC powder (Shanghai Harling Biotechnology Co., Ltd.), rat cTnI ELISA kit (R&D), anti-Cx43 antibody (Abcam), anti-GAPDH antibody (Abcam), anti-p-Cx43 antibody (Santa Cruz), anti-Beclin-1 antibody (Abcam), and horseradish peroxidase labeled secondary antibody (ASPEN). Animal ventilator ALC-V8 (Alcott) and automatic biochemical analyzer (HITACHI, Japan).

Methods

Animal modeling: The rats were fasted for 12 hours before surgery and anesthetized by 1% barbital sodium (30~40 mg/kg) intraperitoneal injection. The tracheal intubation was connected to the ventilator (tidal volume 4~5 ml/100 g, breathing ratio 1:1, and respiratory rate 80~90 beats/min). The 3rd and 4th ribs were cut at the 0.15 cm from the left edge of the sternum and the thoracic cavity was exposed using a thoracotomy device. After opening the pericardium, a 3/8 round 6 × 10 stainless steel needle

and a 6-0 silk thread were used to sew a needle under the left anterior descending coronary artery. Ischemia-reperfusion was performed by push-tube method. The two ends of the wire were passed through a hard plastic tube with a diameter of 1.5 mm and length of 1.5 cm, and lightly lifted and ligated. The pattern plier was adopted to push the hard plastic tube down to the coronary artery. The wire was clamped to block the coronary blood flow and relaxed to cause perfusion. The left ventricular anterior purple or grayish white, together with ST segment gyrus elevation of > 0.02 mv in the synchronized ECG II lead showed an upward, and high T wave was considered as a sign of successful ischemia. After the ligature was relaxed, the left ventricular anterior wall ischemic area disappeared and the up-regulated ST segment decreased by 1/2 or more were treated as successful reperfusion. The reperfusion time was 2 h.

Animal grouping and administration: Twenty-four male SD rats were randomly divided into a sham operation group, a chloroquine (CQ) + sham operation group, an I/R group, and a CQ + I/R group (n = 6). The feeding conditions in each group were the same. The sham operation group received opening the chest without left coronary artery ligation. The CQ + SH group and CQ + I/R group were intraperitoneally administered at 1 h before surgery. The SH group and I/R group were both given equal amounts of distilled water before surgery.

Arrhythmia scoring method: The rats were connected with the II lead electrocardiogram to record arrhythmia which occurred within 30 min of ischemia and 30 min of reperfusion. The ventricular arrhythmia (VA) score was performed according to the method of Ravingerova T *et al* [7]. Score 0 point for no VA or < 10 times of VPB, score 1 point for ≥ 10 times of VPB, score 2 points for only one burst of ventricular tachycardia (VT) < 120 seconds of, score 3 points for one VT ≥ 120 seconds or multiple arrays of cumulative VT < 120 seconds, score 4 points for multiple arrays of cumulative VT > 120 seconds, score 5 points for ventricular fibrillation (VF), and score 6 points for VF lasted for more than 5 minutes or died during observation.

Determination of ventricular fibrillation threshold: On the 40th day after surgery, the heart of the rat was exposed. At the apex of the anterior
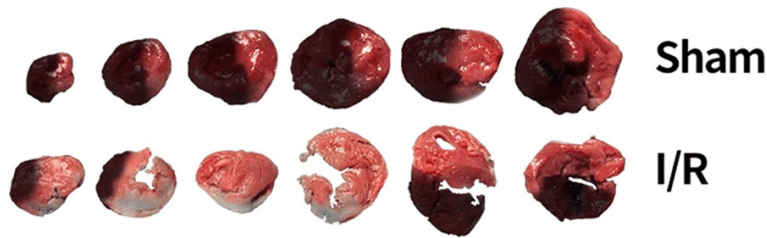
Figure 1. Myocardial infarction after I/R. Red referred to ischemic dangerous area, blue referred to normal blood flow area, and white referred to infarction area.

chamber, the bipolar pacing electrode (diameter 0.3 mm, two needles 2 mm apart) was inserted for 2 mm to reach the endocardium. S1S1 increasing stimulation with a pacing voltage of 5V and a pulse width of 10 ms was applied with a DF-5A electrophysiological stimulator. From 260 beats/min, each increment was 20 beats/min and lasted for 30 s with interval for 1 min, causing ventricular fibrillation and the lowest stimulation frequency to cause VF was defined as the ventricular fibrillation threshold.

TTC staining: After 2 hours of I/R, the blocked left coronary artery was ligated again. 1 ml of 1% Evans blue normal saline solution was slowly injected into the right internal jugular vein. After the blue pigmentation of the rat's lips, the heart was removed and quickly froze at -20°C for 20 min. The left ventricle was cut into 2 mm thickness in a parallel atrioventricular groove and stained with TTC at 37°C for 15 min.

Sample collection: The rats were reperfused 2 hours and the abdominal aorta blood was taken. The heart was quickly removed and washed with pre-cooled saline. The ventricular muscle tissue in the infarct area was cut out on ice. After weighing, it was quickly placed at -80°C.

ELISA: The blood was extracted and separated at 3000 r for 15 min to collect serum. The level of myocardial injury marker cTnI was measured by automatic biochemical analyzer according to the ELISA kit.

Western blot: Total protein was extracted from the infarct area of myocardial tissue and quantified by BCA method. 40 μg protein was separated by electrophoresis and transferred to PVDF membrane. After blocked by 5% skim milk for 1 h at room temperature, the membrane was incubated overnight in primary anti-

body at 4°C. After washed with TBST for 3 times, the membrane was incubated in HRP-labeled goat anti-rabbit secondary antibody (1:10000) for 1 h at room temperature. At last, the membrane was added with ECL luminescent solution for 10 s to 5 min and developed.

Immunofluorescence: The fresh apical tissue was fixed for 24 hours and embedded. The slice was incubated in mouse anti-rabbit CX43 antibody (1:200) at 4°C overnight. After washing, the slice was incubated in secondary antibody at 37°C for 50 min. After stained with DAPI avoid of light at room temperature for 5 min, the slice was added with appropriate amount of anti-fluorescence quencher and observed under a fluorescence microscope.

Statistical analysis

All data analyses were performed using SPSS 22.0. The measurement data were expressed as mean ± standard deviation and compared one-way ANOVA, Bonferroni, or Dunnett'S T3 methods. $P < 0.05$ was considered statistically significant.

Results

Cardiomyocyte I/R model establishment

As shown in **Figure 1**, Evans blue staining confirmed the area of left ventricular ischemia. TTC staining exhibited the area of myocardial infarction. Compared with SH group, the myocardial infarction area was obvious aggravated in I/R group. The myocardial injury marker cTnI was significantly increased in the I/R group ($P < 0.01$) (**Table 1**), indicating successfully I/R modeling.

VA severity comparison

According to the VA score scale at the initial reperfusion (within 30 min) (**Table 2**), the results showed that compared with the SH group, VA occurred in all of 6 rats in the I/R group, mainly within 5 to 20 minutes after reperfusion. The occurrence of VPB, VT, and VF were significantly increased in the initial stage of reperfusion ($P < 0.05$). Compared with the I/R group, the number of VA occurrences in the

Table 1. cTnI expression changes (± s)

Group	No.	cTnI (ng/mL)
SH	6	0.36 ± 0.14
CQ + SH	6	0.33 ± 0.08
I/R	6	1.30 ± 0.06[a]
CQ + I/R	6	1.26 ± 0.07[a]

[a]P < 0.01, compared with SH group.

Table 2. VA severity comparison

Group	No.	VA score
SH	6	0[b]
CQ + SH	6	1.17 ± 0.14[b]
I/R	6	6.74 + 2.60[a]
CQ + I/R	6	2.10 + 0.02[a,b]

[a]P < 0.05, compared with SH group. [b]P < 0.05, compared with I/R group.

Table 3. Ventricular fibrillation threshold in various groups (Mean ± SD)

Group	n	PV (v)	Ipm (Impulse/min)
SH	6	5	683.33 ± 60.88*
CQ + SH	6	5	633.33 ± 43.20*
I/R + CQ	6	5	645.00 ± 30.82*
IR	6	5	470.00 ± 90.99

*P < 0.05 vs. MI group.

early reperfusion in the CQ + I/R group reduced (P < 0.05), mainly on VPB and short VT. Occasional premature ventricular contractions were observed in the SH group and the CQ + SH group (P > 0.05).

Ventricular fibrillation threshold comparison

Ventricular fibrillation threshold was tested at 30 min after reperfusion. It was observed that the ventricular fibrillation threshold in I/R group (470.00 ± 90.99 Ipm) was significantly lower than SH group (683.33 ± 60.88 Ipm) (P < 0.05). Its level in CQ + SH group (633.33 ± 43.20 Ipm) and I/R + CQ group (645.00 ± 30.82 Ipm) was obviously higher than that of the I/R group (P < 0.05). There was no statistical difference among CQ + SH group, I/R + CQ group, and SH group (P > 0.05) (**Table 3**).

The impact of autophagy on Cx43, p-Cx43, and Beclin-1 protein expressions in I/R myocardium

Compared with the SH group, the phosphorylation status of Cx43 and the ratio of p-Cx43/CX43 were markedly decreased, while Beclin-1
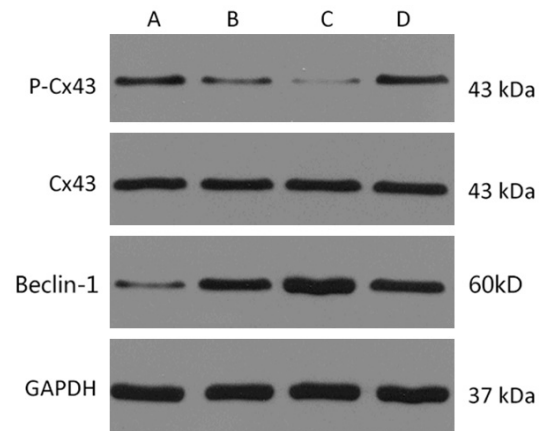

Figure 2. The impact of autophagy on Cx43, p-Cx43, and Beclin-1 protein expressions in I/R myocardium. A. SH group; B. CQ + I/R group; C. I/R group; D. CQ + SH group.

protein expression was apparently upregulated in the ventricular myocytes of the I/R group (P < 0.01). Compared with the I/R group, the I/R + CQ treatment significantly up-regulated p-Cx43 and the ratio of p-Cx43/CX43 (P < 0.05), whereas declined Beclin-1 protein level (P < 0.05) (**Figure 2**; **Table 4**).

Cx43 distribution in ventricular myocardium

In the I/R group, Cx43 was mainly expressed in the cardiomyocyte lateral-lateral junction. In the SH group, the CQ + SH group, and the CQ + IR group, Cx43 was mainly located in the junction between the two ends of the myocardium (ie, the disc), which was perpendicular to the long axis of the cell and arranged in line [7]. The lateral distributed Cx43 was relatively small. As shown in **Figure 3**, p-Cx43 distribution was similar to Cx43 in each group. Moreover, compared with the SH group, p-Cx43 distribution in the I/R group was significantly reduced due to myocardial ischemia-mediated dephosphorylation of connexin, while interventional autophagy after dephosphorylation of Cx43 was reversed, the p-Cx43 of the I/R + CQ group (**Figure 4**) was significantly increased.

Discussion

In this study, we established rat myocardial I/R model and confirmed acute remodeling of ventricular myocyte membrane Cx43 occurs during I/R. Cx43 expression and phosphorylation were down-regulated, together with abnormal spatial distribution, leading to reduced end to end distribution and increased lateral distribution in myocardial cells. The expression level of

Table 4. Cx43, p-Cx43, and Beclin-1 protein expressions in I/R myocardium (± s)

Group	No.	Cx43	p-Cx43	p-Cx43/Cx43	Beclin-1
SH	6	0.717 ± 0.023	0.261 ± 0.034[b]	0.361 ± 0.044[b]	0.14 ± 0.02[b]
CQ + SH	6	0.733 ± 0.001	0.230 ± 0.020[b]	0.362 ± 0.019[b]	0.16 ± 0.05[b]
I/R	6	0.719 ± 0.002	0.128 ± 0.012[a]	0.160 ± 0.010[a]	0.41 ± 0.04[a]
CQ/IR	6	0.728 ± 0.001	0.258 ± 0.017[a,b]	0.383 ± 0.032[a,b]	0.31 ± 0.07[a,b]

[a]$P < 0.05$, compared with SH group. [b]$P < 0.05$, compared with I/R group.
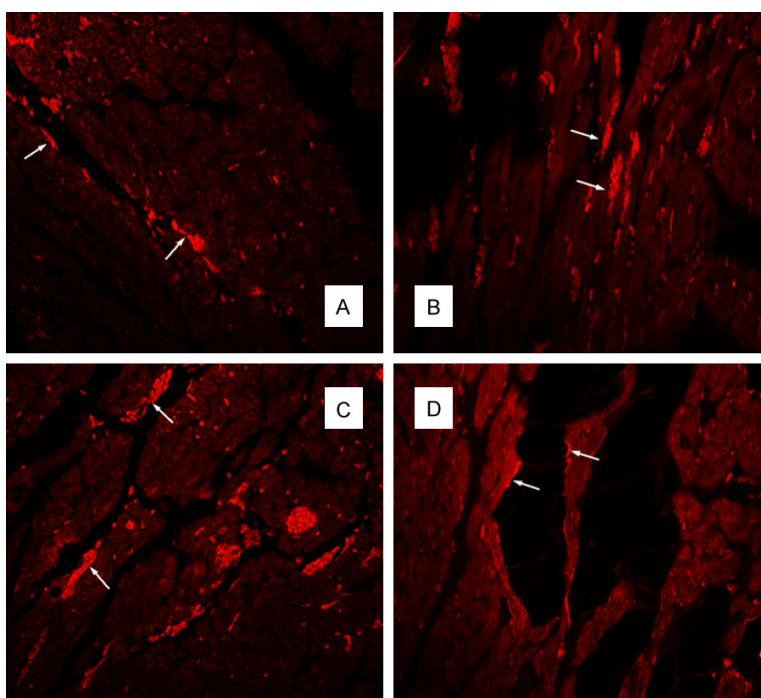

Figure 3. Immunofluorescence detection of Cx43 distribution in the myocardium. (A) In CQ + SH and CQ + I/R group, a plenty of end-to-end appositions (arrows) were preserved among the neighboring cells (B, C). The end-to-end appositions were significantly decreased in the I/R group induced by ischemia-reperfusion, replaced by side-to-side appositions (arrows) (D).

high temperature, and abnormal components [8]. Autophagy can be triggered by multiple stressors, such as nutrient deprivation, oxidative stress, and hypoxia, acting as a survival mechanism by restoring the intracellular pool of amino acids and ensuring energy demand. Additionally, autophagy can represent a process of protein quality control, by selectively degrading damaged or obsolete cellular components [9]. Accordingly, in the heart, an active autophagy pathway is required in different conditions, such as myocardial infarction, in order to maintain intracellular homoeostasis and heart function. In some circumstances, an exacerbated up-regulation of autophagy with an uncontrolled degradation of vital cellular components has been implicated in cardiomyopathy, thus attributing to autophagy a detrimental role [9, 10]. This contradictory dual role of autophagy seems to rely on the severity of the stimuli and the autophagy players involved {Matsui, 2007 #39009}. Cardiomyocytes can up-regulate autophagy after I/R [11]. Especially after reperfusion, a large amount of autophagy activation led to excessive degradation of important proteins and cardiomyocytes death. Beclin-1 protein is one of the key factors regulating autophagy. The intervention of Beclin-1 expression can regulate autophagy activity, especially during reperfusion [12]. Cx43 is a crucial component of the gap junction channel and is involved in the formation of functional gap junction channels after phosphorylation. GJ closed and adjacent
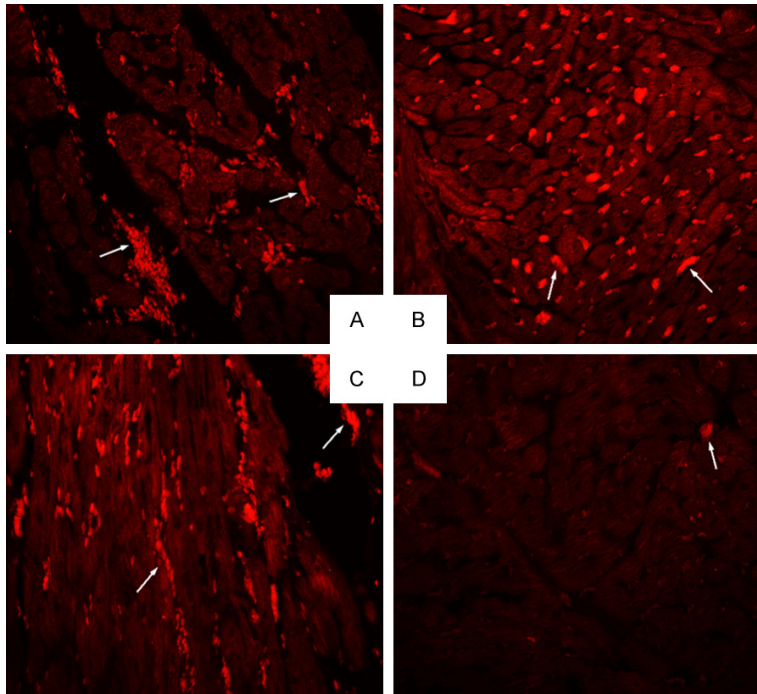
autophagy-related gene Beclin-1 was significantly increased during I/R, resulting in over-activated autophagy, aggravated myocardial injury, and increased reperfusion arrhythmia. Inhibition of autophagy decreased Beclin-1 expression, up-regulated Cx43 expression and phosphorylation, and enhanced Cx43 end to end distribution in cardiomyocytes increased significantly to decrease reperfusion arrhythmia.

Autophagy is a kind of cell degradation pathway in eukaryotes that is involved in maintaining the normal physiological activities. It can also occur under pathological conditions, such as oxidative stress, starvation, ischemia, hypoxia,

used to block the fusion of autophagosomes and lysosomes. It was found that Beclin-1 protein expression was decreased, p-Cx43 protein degradation was reduced, p-Cx43 and Cx43 levels were upregulated, the spatial distribution of Cx43 tended to be normal by increasing its distribution on the end-to-end side, and the severity of reperfusion arrhythmia was alleviated in ventricular myocytes after MI/IR treated by chloroquine. It was indicated that inhibition of autophagy may improve the acute remodeling of cell membrane Cx4 to reduce the occurrence of malignant rhythm in MI/RI.

Figure 4. Immunofluorescence detection of p-Cx43 distribution in the myocardium. p-Cx43 signals were mainly located at end-to-end apposition (arrows) among the neighboring cells in the sham group (A). Both the area and the intensity were decreased in the I/R group (arrows) (D). The distribution and the intensity of p-Cx43 were preserved among the neighboring cells in CQ + SH and CQ + I/R group (B, C).

Abnormal enhancement and remodeling of Cx43 degradation in cardiomyocyte membranes are often associated with various heart diseases. However, the mechanism of degradation in MI/RI remains unclear. As one of the most important gap junction protein in cardiac GJ, investigation of Cx43 degradation contributes to the development of new strategies for protecting GJ and cardiac function in myocardial I/R injury [18, 19]. The function of Cx43, which form gap junctions, can be impacted by degradation, because of their short half-life [20]. GJ internalization is known to occur in a highly efficient manner in response to natural mediators of inflammation in pathological conditions. Therefore, I/R may not only activate autophagy but also may promote Cx43 internalization into the cytoplasm and then cause Cx43 degradation through autophagy. This study confirmed the extensive dephosphorylation of Cx43 in MI/RI, which was closely related to the activation of autophagy, thus providing a theoretical basis to elucidate the role of Cx43 in cardiac electrical conduction. However, we did not explore the mechanism of excessive activation of autophagy in affecting Cx43 dephosphorylation.

cells decoupled in the early stage myocardial ischemia [13, 14], leading to ECG heterogeneity and decreased electrical conduction rate, which was easy to form reentry arrhythmia and related to the dephosphorylation of Cx43 [15]. This study showed that Beclin-1 protein expression was significantly elevated and the content of p-Cx43 was obviously decreased after ischemia-reperfusion. Meanwhile, increased number of reperfusion arrhythmia declined myocardial ventricular fibrillation threshold and elevated serum myocardial enzyme content, resulting in cardiomyocytes severe injury.

Beclin-1 protein was significantly increased in myocardial cells after I/R, indicating that autophagy participates in the MI/IR injury process. Connexins can be degraded through autophagy. Inhibition of autophagy-related gene expression may increase the total amount of Cx43 [16, 17]. Autophagic bodies form autophagic lysosomes with bilayer membrane structure by continuous endocytosis, which in turn degrade gap junction proteins [12]. In this study, the autophagy inhibitor chloroquine was

To sum up, autophagy may reduce myocardial ischemia-reperfusion injury and malignant

arrhythmia by improving the acute remodeling of myocardial cell membrane Cx43.

Acknowledgements

Disclosure of conflict of interest

None.

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