

## Original Research Article

# Effect of pinocembrin pre-treatment on expressions of Cx43 protein and claudin 1 in myocardial ischemia cardiomyocytes of arrhythmic rats

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### Abstract

**Purpose:** To investigate the effects of pinocembrin on ventricular rhythm and the expression of cardiomyocyte ligament junction protein (Cx43) and claudin 1 (ZO-1) in ischemia/reperfusion (I/R) rats.

**Methods:** Ischemia/reperfusion (I/R) model rats ( $n = 15$ ) were divided into 5 groups: IR group, control group, and 3 pinocembrin groups (3, 10 and 30 mg/kg). The serum levels of creatine kinase-MB isoenzyme (CK-MB) and troponin I (cTnI) were measured by enzyme-linked immunosorbent assay (ELISA). Changes in myocardial tissue were detected by H & E staining, while mRNA and protein levels of Cx43, ZO-1 and Kir2.1 were measured by reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting, respectively.

**Results:** In pinocembrin groups, heart rate (HR), mean arterial pressure (MAP) and rate-pressure product (RPP) levels were significantly higher compared with IR group ( $p < 0.05$ ). Moreover, the extent of arrhythmia and the levels of CK-MB and cTnI in pinocembrin groups were lower relative to IR group, while  $\text{Na}^+/\text{K}^+$ -ATPase and  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase activities, as well as Cx43 mRNA, ZO-1 mRNA, and protein levels of Cx43, ZO-1 and Kir2.1 were significantly higher than the corresponding values for IR group ( $p < 0.05$ ).

**Conclusion:** These results suggest that pinocembrin reduces ventricular arrhythmias in I/R rats by up-regulation of expressions of Cx43, ZO-1 and Kir21, and inhibition of re-distribution of ZO-1 and Cx43. These findings provide the basis for the clinical application of pinocembrin in the treatment of arrhythmia.

**Keywords:** Pinocembrin, Ventricular arrhythmia, Ligament junction protein, Recombinant human Kir2.1 protein, Arterial pressure, Protein levels, Claudin, Cardiomyocyte

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## INTRODUCTION

Acute myocardial infarction is an important cause of sudden cardiac death. Early acute myocardial ischemia and myocardial injury can result in

unstable electrical activity in the heart, as well as ventricular re-polarization, which may lead to malignant arrhythmia and sudden death [1]. Myocardial ischemia is mitigated by rapid and effective recovery, but myocardial ischemia and

reperfusion can aggravate heart failure caused by ischemia and myocardial cell damage, leading to intracellular calcium overload, apoptosis and inflammatory reactions [2].

Pinocembrin, a major flavonoid derived from propolis, plays a role in the treatment of myocardial ischemia and reperfusion injury through its antioxidant effect, reduction of calcium overload, as well as inhibition of inflammation and myocardial cell apoptosis [3]. Consistent with its myocardial protective role, studies have demonstrated that pinocembrin reduced myocardial infarct area and the incidence of ventricular fibrillation in rats with ischemia reperfusion, and increased the expression of phosphorylation gap junction proteins in myocardial tissue [4]. However, the mechanism underlining the anti-myocardial ischemia reperfusion injury of pinocembrin has not been fully elucidated. Intracellular calcium overload is a key cause of arrhythmia.  $\text{Na}^+\text{-K}^+\text{-ATPase}$  and  $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$  play important roles in maintaining intracellular calcium homeostasis. It has been reported that the activities of  $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$  and  $\text{Na}^+\text{-K}^+\text{-ATPase}$  decreased in arrhythmia patients [5,6]. The action potential in cardiomyocyte tissue takes place at gap junctions, and influences intercellular electrical coupling and impulse conduction. The main gap junction regulatory protein expressed in ventricular working muscle cells is Cx43 [7,8]. In myocardial pathology, the abnormal level of humoral factors affects the expression, transport and distribution of connexin; it changes the structure and function of gap junction, increases the rate of myocardial dislocation, and induces arrhythmia [9].

In this study, the effects of pinocembrin pretreatment on ventricular rhythm, and on the expression of cardiomyocyte ligament junction proteins (Cx43) and Claudin 1 (ZO-1), were determined in rat myocardium I/R model. This was with a view to unraveling the basis for its clinical use in the treatment of arrhythmia.

## EXPERIMENTAL

### Animals and treatment

Healthy 10-week-old male sprague dawley (SD) rats, weighing 350 - 400 g were used for this study. The rats were obtained from the Animal Experimental Center of the Chinese Academy of Medical Sciences in Beijing (SPF class animal laboratory, license number SYXK-2013-0025). The animals were provided feed and clean drinking water *ad libitum* during the acclimatization period, in line with experimental

animal standards. The male SD rats were randomly divided into IR group, control group, and three graded pinocembrin dose groups (3, 10 and 30 mg/kg).

I/R model was established as previously described [10]. The left anterior descending coronary artery was subjected to reperfusion for 30 min. The rats were fixed in supine position, and anaesthetized intraperitoneally with 5 % mebumalnatrium. This was followed by recording a normal II lead ECG to exclude abnormal rat. HR, MAP, RPP were recorded 10 min before ischemia, 30 min after ischemia, and 30, 60, 120 min after reperfusion, to observe the arrhythmia. Rats in the three pinocembrin groups were injected intravenously at 10 min before ischemia. The IR and control groups received equivalent volume of saline. The animal experiments were approved by the Animal Ethic Committee of the Second Hospital of Hebei Medical University (approval ref no. 2015103) and were in compliance with the Principles of Laboratory Animal Care (NIH publication no. 83-23 revised 1985) [11].

### Chemicals and reagents

Mebumalnatrium and pinocembrin were from Sigma (San Francisco, USA) and Chengdu Best Special Reagent Co., Ltd (Sichuan, China) respectively. The immunohistochemistry kits for CK-MB, cTnl (troponin), Cx43 and ZO-1 were purchased from Beijing Zhongjin Jinqiao Biotechnology Co., Ltd., Beijing, China. Trizol kits and reverse transcription kits were supplied by Invitrogen (Carlsbad, America). Rabbit anti-rat Cx43, ZO-1, Kir2.1 monoclonal antibody, secondary antibody, and DAB kit were provided by Wuhan Boster Biological Engineering Co., Ltd., (Hebei, China).  $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$  and  $\text{Na}^+\text{-K}^+\text{-ATPase}$  use were provided by Nanjing Institute of Bioengineering, Jiangsu Province, China. Primers were synthesized by Shanghai Bioengineering Co., Ltd. (Shanghai, China).

### Assay of $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$ and $\text{Na}^+\text{-K}^+\text{-ATPase}$ activities

The activities of  $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$  and  $\text{Na}^+\text{-K}^+\text{-ATPase}$  in myocardial tissue were assayed spectrophotometrically in myocardial tissue homogenates as directed in the kit instructions. The protein contents of the homogenates were determined by Coomassie brilliant blue method.

### Enzyme-linked immunosorbent assay (ELISA)

Serum levels of CK-MB and cTnl were determined by ELISA according to instructions in

the ELISA kits. Absorbance was read at 450 nm in a microplate reader.

### Histological examination of myocardial tissue

Myocardial tissue sections were processed for light microscopy. The tissues were immersed in paraformaldehyde and embedded in paraffin. Sections (5  $\mu$ m) were cut with microtome, stained with hematoxylin-eosin (H & E), and observed under the light microscope.

### Reverse transcription- polymerase chain reaction

Total RNA was extracted from the myocardium and reverse-transcribed into cDNA. RT-PCR was performed according to Trizol and quantified by UV spectrophotometer. The primers used are shown in Table 1. The amplified product was analyzed three times by agarose gel electrophoresis. The relative expression was expressed as the ratio of the gray value of the gene to the GAPDH gray value. BIO-RAD gel imaging system and Quantity OneV4.31 image analysis system were used for analysis.

### Immunohistochemistry

The myocardial tissue of each rat was immersed in paraformaldehyde and made into 5- $\mu$ m paraffin sections, and antigenized after de-waxing. Then, H<sub>2</sub>O<sub>2</sub>, primary antibody (Cx43, ZO-1 1: 100/1: 100), polymer enhancers and enzyme-labeled anti-mouse/rabbit polymers were added in turn. The samples were then stained with DAB, stained after re-dyeing, and sealed. The positive expression of Cx43 and ZO-1 in cardiomyocytes was analyzed by image-pro plus image. This involved selection of 5 different visual fields in the surrounding area of the tumor tissue under 40 magnification, and recording the number of positive expression cells in the field of view.

**Table 1:** Sequences of primers used

Gene		Series (5'-3')	Fragment size (bp)
Cx43	Upstream primer	GCGGCTTGCTGAGAACC	295
	Downstream primer	TTGCGGCACGAGGAATT	
Zo-1	Upstream primer	GCGCTGGAGAGAGACAAGAT	179
	Downstream primer	ATTGACGTTTCCCCACTCTG	
Kir2.1	Upstream primer	GGAGCGTGGCAACAGATAG	167
	Downstream primer	TCTCCCCCTTATTTGCTTCTTC	
GADPH	Upstream primer	TCCCTCAAGATTGTCAGCAA	308
	Downstream primer	AGATCCACAACGGATACATT	

### Western blotting

Total protein was separated by polyacrylamide gel electrophoresis. Then, poly (vinylidene fluoride) was used to derivatize the protein, followed by treatment with skim milk powder. Cx43, ZO-1 and Kir2.1 protein monoclonal antibody (1: 500 dilution) and GAPDH primary antibody (1: 1000 dilution) were added. The mixture was incubated overnight, and thereafter rinsed with TBST for 1 h. Then GAPDH secondary antibody (1: 2000 dilution) was added. Finally, Quantity One image analysis software was used for the protein color band analysis in a dark room.

### Statistical analysis

Measurement data were expressed as mean  $\pm$  standard deviation (SD). SPSS 19.0 was used for statistical analysis. Chi-square test was used to compare rates. Normality test was used to evaluate the measurement data. Multiple groups were compared by one-way ANOVA. Differences between groups were deemed statistically significant at  $p < 0.05$ .

## RESULTS

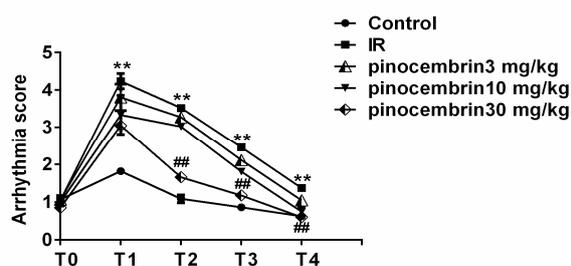
### Effect of pinocembrin pretreatment on hemodynamics and arrhythmia

The levels of HR, MAP and RPP in I/R rats decreased with time in the three pinocembrin pretreatment groups ( $p < 0.05$ ). However, the levels of HR, MAP and RPP in the control group and pinocembrin groups (3, 10 and 30 mg/kg) were significantly higher, while the arrhythmia score was significantly lower than those in IR group ( $p < 0.05$ ). The changes observed in the pinocembrin groups (3, 10 and 30 mg/kg) were dose-dependent. These results are shown in Table 2 and in Figure 1.

**Table 2:** Effect of pinocembrin pre-treatment on hemodynamics in I/R rats

Parameter	Group	Before Ischemia (10 min; T <sub>0</sub> )	Ischemia (30 min; T <sub>1</sub> )	Reperfusion (30 min; T <sub>2</sub> )	Reperfusion (60 min; T <sub>3</sub> )	Reperfusion (120 min; T <sub>4</sub> )
MB (mmHg)	Control	120±11	117±13	123±15	119±11	113±8
	IR	116±12	103±6**	98±5**	93±6**	92±10**
	pinocembrin3	118±13	106±9**#	101±6**#	98±7**##	94±12**#
	pinocembrin10	121±11	107±11**##	105±6**##	101±7**##	101±12**##
	pinocembrin30	116±10	110±18*##	109±8*##	105±10*##	104±14*##
HR (beats/min)	Control	420±29	413±23	405±23	401±26	394±23
	IR	417±17	378±13**	367±8**	361±9**	355±10**
	Pinocembrin 3	423±23	383±12**#	372±10**#	366±11**#	359±12**#
	Pinocembrin 10	410±21	391±14**##	385±12**##	379±13**##	371±15**##
	Pinocembrin 30	411±19	407±17*##	399±18*##	387±14*##	383±17*##
RPP (beats/min mmHg)	Control	44057±2204	43876±2134	44323±2351	44112±2495	45213±3079
	IR	43077±1988	36487±1892**	35427±1833**	34437±2028**	34751±1992**
	Pinocembrin 3	42978±2301	38645±1983**##	38121±1936**##	35983±1672**#	35723±1687**#
	Pinocembrin 10	43659±1890	40127±2012**##	38961±1856**##	37624±1692**##	37743±1769**##
	pinocembrin30	42355±2140	42521±1780**##	41262±1916**##	40256±1777**##	41980±1899**##

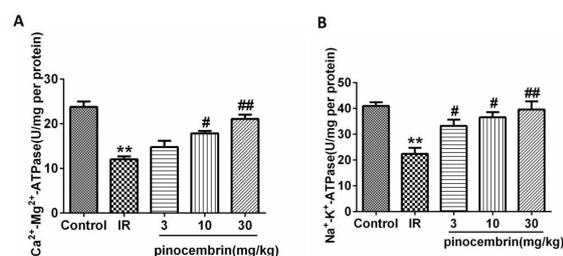
**Note:** Compared with control, \* $p < 0.05$ ; \*\* $p < 0.01$ ; vs IR, # $p < 0.05$ , ## $p < 0.05$



**Figure 1:** Effect of pinocembrin pretreatment on arrhythmia score in I/R rats. \* $p < 0.05$ ; \*\* $p < 0.01$  compared with control; # $p < 0.05$ , ## $p < 0.05$  compared with IR

### Effect of pinocembrin pre-treatment on Na<sup>+</sup>-K<sup>+</sup>-ATPase and Ca<sup>2+</sup>-Mg<sup>2+</sup>-ATPase levels

The levels of Na<sup>+</sup>-K<sup>+</sup>-ATPase and Ca<sup>2+</sup>-Mg<sup>2+</sup>-ATPase in the control group were higher than their corresponding levels in the other four groups, while those in the pinocembrin groups (3, 10, 30 mg/kg) were higher than values in IR group ( $p < 0.05$ ). These changes were dose-dependent, as shown in Figure 2.

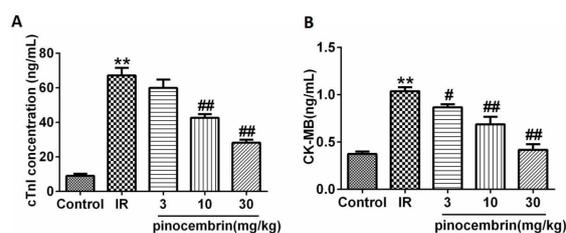


**Figure 2:** Effect of pinocembrin on activities Ca<sup>2+</sup>-Mg<sup>2+</sup>-ATPase and Na<sup>+</sup>-K<sup>+</sup>-ATPase in myocardial

tissue. (A) Ca<sup>2+</sup>-Mg<sup>2+</sup>-ATPase. (B) Na<sup>+</sup>-K<sup>+</sup>-ATPase; \* $p < 0.05$ , \*\* $p < 0.01$  compared with control; # $p < 0.05$ , ## $p < 0.01$  compared with IR

### Effects of pinocembrin pretreatment on the expression of CK-MB and cTnl in I/R rats

The levels of CK-MB and cTnl in IR group and pinocembrin groups (3, 10, 30 mg/kg) were significantly higher than those in the control group, while their levels in the IR group were significantly and dose-dependently higher than in the three pinocembrin groups (Figure 3).

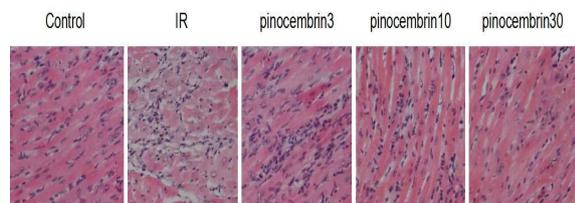


**Figure 3:** Effect of pinocembrin on serum concentrations of CK-MB and cTnl. (A) cTnl. (B) CK-MB; \* $p < 0.05$ , \*\* $p < 0.01$  compared with control; # $p < 0.05$ , ## $p < 0.01$  compared with IR

### Histopathological changes in myocardial tissue

The rats in the control group had no myocardial abnormalities; the arrangement of myocardial fibers was neat and normal (Figure 4). On the other hand, the myocardial fibers in the IR group were disordered, and were infiltrated by inflammatory cells. The myocardium of the

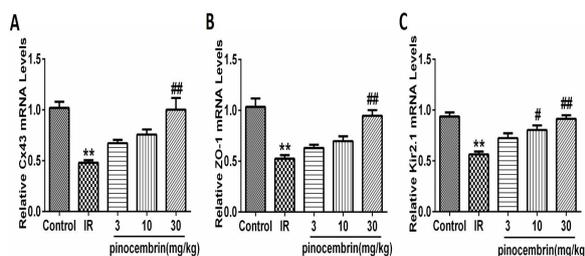
pinocembrin group was significantly and dose-dependently improved, when compared with the IR group.



**Figure 4:** Photomicrograph of myocardial tissues of rats in the various groups (H & E, × 200)

**Effect of pinocembrin on expressions of Cx43, ZO-1 and Kir2.1 mRNA in I/R rats**

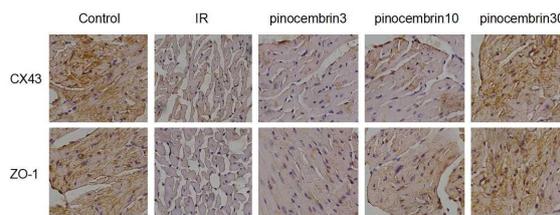
The results in Figure 5 show that the control group had significantly higher levels of Cx43, ZO-1 and Kir2.1 mRNA than in the IR group, while values of these parameters in the pinocembrin groups (3, 10 and 30 mg/kg) were significantly and dose-dependently higher than in the IR group ( $p < 0.05$ ).



**Figure 5:** Effect of pinocembrin on levels of Cx43, ZO-1 and Kir2.1 mRNA in I/R rats. (A) Cx43. (B) Kir2.1 mRNA. (C) ZO-1; \* $p < 0.05$ , \*\* $p < 0.01$ ; vs IR, # $p < 0.05$ , ## $p < 0.01$  compare with control

**Effect of pinocembrin pretreatment on distribution of Cx43 and ZO-1 proteins in I/R rats**

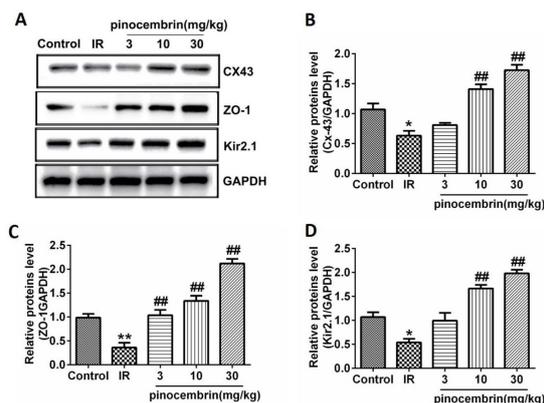
The expression of Cx43 and ZO-1 proteins in the control group was strongly positive. The proteins were distributed mostly in the long axis of myocardial fibers or the side-side junction or cytoplasm of the cardiomyocytes, with a few in the intercalary disk. In the IR group, there were decreases in the expressions of Cx43 and ZO-1 proteins, and the proteins were unevenly distributed. There were some dose-dependent improvements in the expression of histone in the pinocembrin groups (3, 10 and 30 mg/kg). These results are shown in Figure 6.



**Figure 6:** Effect of pinocembrin on the protein expressions of Cx43 and ZO-1 in the myocardium of I/R rats (× 200)

**Effects of pinocembrin pretreatment on expression of Cx43, ZO-1 and Kir2.1 proteins in I/R rats**

The expressions of Cx43, ZO-1 and Kir2.1 proteins were strongly positive in the control group, but were decreased in IR group (Figure 7). The expressions of those proteins in control group were higher than in the IR group, and dose-dependently higher in the pinocembrin groups than in the IR group.



**Figure 7:** Effect of pinocembrin on the expressions of Cx43, ZO-1 and Kir2.1 proteins in I/R rats; \* $p < 0.05$ , \*\* $p < 0.01$ ; vs IR, # $p < 0.05$ , ## $p < 0.01$  compared with control

**DISCUSSION**

Myocardial ischemia may cause degeneration and necrotic dissolution of cardiomyocytes. Reperfusion injury increases membrane permeability of cardiomyocytes and levels of myocardial injury markers in blood. The degree of myocardial injury is related to levels of CK-MB and cTnl [12,13]. In this study, the serum levels of CK-MB and cTnl in the ischemia-reperfusion model were significantly higher than those in the control group, but significantly decreased in the pinocembrin pretreatment groups. These results suggest that pretreatment with pinocembrin can relieve myocardial ischemia-reperfusion injury in rats.

The pathogenesis of arrhythmia is not yet fully elucidated. However, studies have indicated that it may be associated with degeneration of conductive fibers and autonomic cells, abnormal ion channels, vagal tone abnormalities, and ion transport on both sides of cardiomyocytes [14].  $\text{Na}^+\text{-K}^+\text{-ATPase}$  is a transmembrane, ATP-driven ion pump which is responsible for transmembrane transport of  $\text{Na}^+$  and  $\text{K}^+$ . It maintains electrochemical gradient across the cell membrane through asymmetric distribution of  $\text{Na}^+$  and  $\text{K}^+$  across the membrane. The main function of  $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$  is to actively transport  $\text{Ca}^{2+}$  into the sarcoplasmic reticulum from the cytoplasm. This result could be attributed to the release of  $\text{Ca}^{2+}$  obstacles in calcium pool which caused the reduction of  $\text{Ca}^{2+}$  permeability in sarcoplasmic reticulum and the calcium flow reduced consequently, leading further to deceleration of spontaneous depolarization of sinus node pacing cells.

Ultimately, slow arrhythmia can be induced.  $\text{Na}^+\text{-K}^+\text{-ATPase}$ ,  $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$  are involved in cardiomyocyte membrane ion channels and ion transport [15]. Ion transport on both sides of the cardiomyocyte membrane is the basis of generation of action potential. Abnormalities in cardiac ion channels are the main causes of slow arrhythmia [16]. The results of this study show that pretreatment with pinocembrin can significantly enhance the activities of  $\text{Na}^+\text{-K}^+\text{-ATPase}$  and  $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$  in myocardium of ischemia-reperfusion rats. This implies that the drug can maintain normal  $\text{Na}^+$  and  $\text{K}^+$  ion concentration gradient and electrochemical gradient needed for normal cellular energy metabolism. Cx40, Cx43 and Cx45 are the gap junction proteins in human tissue. Cx43 is the main connexin of cardiomyocytes. It is encoded by GJA1 gene cluster at the junction of cardiomyocytes; it maintains communication between cells, energy exchange and transmission of chemical information.

Decreased expression of Cx43 results in prolonged duration of action potential and repolarization process, leading to blockage of conduction. Cx43 plays important roles in the normal development of the heart, and is also involved in the synchronization of electrical activity of the body and the coordination of secretion [17]. Abnormal expression and distribution of Cx43 lead to decreased electrical coupling and intercellular conduction, and induction of re-entrant arrhythmia.

It has been shown that the heart structure and systolic function of homozygous mice with knock-out Cx43 gene were normal, but after two

months the rats developed spontaneous ventricular arrhythmias and died [18]. The use of gene therapy to increase the expression of Cx43 can exert a protective effect against fatal arrhythmia [19]. The structure and function of Cx43 in the gap junction of the myocardium are closely related to arrhythmia.

The cardiac inwardly rectifying K current ( $I_{K1}$ ) mediated by Kir2.1 protein maintains the stability of resting membrane potential. This protein is produced by chromosome 17 in KCNJ2 gene. Dysfunctional or decreased expression of Kir2.1 protein significantly reduces the action potential, leading to rapid repolarization of the current intensity, extension of QT interval and duration of action potential, and arrhythmia [20]. ZO-1 is coupled to Cx43 at the intercalation disk by binding to the Cx43 carboxyl terminus, thereby anchoring the Cx43 at the intercalation disk [21]. In the present study, the expression of ZO-1 was decreased in myocardial ischemia-reperfusion, Cx43 was intercalated with ZO-1, and Cx43 and ZO-1 were redistributed, leading to calcium overload and arrhythmia. The expressions of Cx43 and Kir21 proteins decreased in the myocardium of IR group, and were significantly lower than corresponding values in the pinocembrin group.

The up-regulation of Cx43 and Kir2.1 proteins was related to the expressions of their respective encoding genes GJA1 and KCNJ2. A variety of factors influence the opening and closing of gap junctions. These include intracellular  $\text{Ca}^{2+}$  levels, intracellular cytosolic pH, cross-channel voltage, and the phosphorylation status of gap junction protein [22]. Decreases in intracellular  $\text{Ca}^{2+}$  levels lead to decreases in electrical conduction in gap junctions. Pinocembrin exerted an anti-arrhythmic effect by increasing the activity of  $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$ , thereby maintaining cardiac channels and upregulating the expression of Cx43 protein.

## CONCLUSION

The results obtained in the present study suggest that pre-treatment with pinocembrin can alleviate arrhythmia in rats with myocardial ischemia and reperfusion. The mechanism of action of this drug is related to enhancement of  $\text{Na}^+\text{-K}^+\text{-ATPase}$  and  $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$  activities; up-regulation of protein expressions of Cx43, ZO-1 and Kir2.1, and suppression of the redistribution of ZO-1 and Cx43. The specific mechanism of regulation of Cx43, ZO-1 and Kir2.1 proteins may be related to expressions of GJA1 and KCNJ2 genes. These findings provide the basis for the clinical application of pinocembrin in the treatment of arrhythmia.

## DECLARATIONS

### Conflict of Interest

No conflict of interest is associated with this work.

### Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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