

# Artemisinin prevents electric remodeling following myocardial infarction possibly by upregulating the expression of connexin 43

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**Abstract.** Artemisinin has been demonstrated to exert beneficial effects on ventricular remodeling. The present study investigated whether artemisinin was able to decrease the ventricular fibrillation threshold (VFT) in rats following myocardial infarction (MI) and aimed to determine the possible underlying mechanisms. The rats were subjected to surgery to induce MI by ligation of the left anterior descending artery and were randomly allocated to receive vehicle or artemisinin (75 mg/kg/day) treatment for four weeks. Programmed electrical stimulation demonstrated a significantly increased VFT in the artemisinin-treated group compared with the vehicle-treated group. The electrophysiological improvement of the VFT was accompanied by increased immunofluorescence-stained connexin 43 (Cx43), myocardial Cx43 protein and mRNA levels in artemisinin-treated rats. The present study also demonstrated that artemisinin significantly decreased tissue tumor necrosis factor (TNF)- $\alpha$  levels at the infarcted border zone. Thus, artemisinin demonstrated a protective effect on ventricular arrhythmias following MI. Although the precise mechanism by which artemisinin modulates the dephosphorylation of Cx43 is unknown, it is likely that artemisinin increased the expression of Cx43 via the inhibition of TNF- $\alpha$ .

## Introduction

It is well known that a lethal ventricular tachyarrhythmia, including ventricular fibrillation, is one of the most common reasons for sudden cardiac death following myocardial

infarction (MI). Multiple factors may contribute to the genesis of ventricular arrhythmia (VA); the electrical remodeling generates a proarrhythmogenic substrate following MI. The proarrhythmogenic substrate may be involved in altered ionic currents, action potentials, cardiac fibrosis and cell-to-cell coupling. Therefore, novel approaches that prevent electrical remodeling following MI are required.

Artemisinin is the active component of *Artemisia annua* L. and is approved worldwide for the treatment and prevention of malaria (1). In addition to its antimalarial properties, previous studies have demonstrated that artemisinin significantly inhibits ventricular remodeling. The present study demonstrated that artemisinin attenuates ventricular remodeling and neural remodeling following MI by exhibiting anti-inflammatory effects (2,3). However, whether artemisinin is able to attenuate electrical remodeling following MI remains unclear. In order to assess our hypothesis, a rat MI model was used to determine whether artemisinin prevents electrical remodeling following MI.

## Materials and methods

**Animal preparation.** All experiments were approved by the Institutional Animal Care and Use Committee of Wuhan University (Wuhan, China) and were conducted in accordance with the Guideline for the Care and Use of Laboratory Animals.

Adult male Sprague-Dawley rats (Center for Animal Experiments, Wuhan University, Wuhan, China) weighing 250-300 g were anesthetized with an intraperitoneal injection of 3% pentobarbital sodium (30 mg/kg). A left intercostal thoracotomy was performed to expose the heart and the left anterior descending (LAD) artery was ligated at the origin. The sham group underwent thoracotomy and pericardiotomy; however, not LAD ligation (4). The chest was then closed and the animals were allowed to recover in a warm, clean cage. Fatalities within 24 h of surgery were excluded from the present study.

**Reagents.** Artemisinin was purchased from the Guilin Pharmaceutical Factory (Guangxi, China). The drug was dissolved in 0.5% carboxymethyl cellulose (CMC) immediately prior to use. The drug safety of artemisinin up to 28 days

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was presented in a previous study (5). The vehicle (0.5% CMC) was used as a control.

**Treatment protocol.** The rats surviving 24 h following MI were randomly assigned to two treatment groups as follows: 75 mg/kg/day artemisinin by oral gavage three times a day for four weeks (MI + artemisinin group; n=21); the same volume of 0.5% CMC liquid vehicle by oral gavage three times a day for four weeks after MI (MI + vehicle group; n=21) and the same treatment as the MI + vehicle group in the sham operation group 24 h after sham operation (thoracotomy with LAD isolation, however, without ligation; n=10) for four weeks.

**Measurement of tumor necrosis factor (TNF)- $\alpha$  levels.** Blood (0.75 ml) was collected into chilled EDTA tubes one day prior to MI surgery and subsequently on days 1, 3, 5, 7, 14, 21 and 28 after surgery. The blood samples were centrifuged at 1,100  $\times$  g, 4°C and the plasma samples were separated and stored at -70°C until they were assayed for TNF- $\alpha$ . Red blood cells were suspended in an equal volume of heparinized saline and reinfused.

The TNF- $\alpha$  levels in the rats were measured using an ultrasensitive rat TNF- $\alpha$  ELISA kit (Biosource International, Inc., Camarillo, CA, USA) according to the manufacturer's instructions. The details of the methodology were described in a previous study (6).

**Electrophysiological evaluation.** To assess the inducibility of VAs, the burst stimuli was used (2 ms pulses at 50 Hz, 2 sec burst duration) and burst pacing was used for up to 3 min of pacing in the infarcted border zone (IBZ).

To assess the ventricular fibrillation threshold (VFT), electrical stimulation was supplied with 100 Hz to the right ventricle. Each stimulation was administered for 30 sec. The interval between each episode of stimulation was 1 min. The initial pacing voltage was 1 V and progressively increased by 0.5 V. VFT was defined as the lowest voltage at which ventricular fibrillation was induced and sustained for at least 20 sec (7).

**Quantitative polymerase chain reaction (qPCR).** Total RNA was extracted from the non-infarcted zone (NIZ) and the IBZ of the left ventricle using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. qPCR was performed using the ABI Prism 7000 (Applied Biosystems, Foster City, CA, USA). cDNA was amplified under the following conditions: 94°C for 10 min and then for 45 cycles at 94°C for 10 sec and 56°C for 30 sec. The primer sequences of connexin 43 (Cx43) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown in Table I. For quantification, Cx43 expression was normalized to GAPDH. The reactions were programmed on a computer linked to the detector (ABI Prism 7000 Sequence Detection system; Applied Biosystems) for 40 cycles of the amplification step. Experiments were replicated three times and the results are expressed as the mean value.

**Western blot analysis of Cx43.** The peri-infarcted zone of the left ventricle and the same zone of the left ventricle in the sham

Table I. Primer sequences for Cx43 and actin.

Gene	Primer sequence
Cx43	
Forward	ACAGCGCAGAGCAAAATCG
Reverse	ATGGCTGGAGTTCATGTCCAG
Actin	
Forward	GCTCCTCCTG AGCGCAAGTA
Reverse	CCTGCTTGCTGATCCACATCT
Cx43, connexin 43.	

group were used for western blot analysis. Equal quantities of protein were loaded and separated by SDS-PAGE, transferred onto a nitrocellulose membrane and incubated with primary antibodies (anti-Cx43, polyclonal antibody; dilution 1:1,000; Abcam, Cambridge, MA, USA) and anti-GAPDH (1:2,000; Abcam) overnight at 4°C. Horseradish peroxidase-conjugated anti-rabbit immunoglobulin IgG (1:1,500; Beyotime Institute of Biotechnology, Shanghai, China) was applied as the secondary antibody for 1 h at room temperature. Finally, the blots were visualized using an enhanced chemiluminescence kit (Beyotime Institute of Biotechnology) and the signals were analyzed using a Bio-Rad image system (Bio-Rad, Hercules, CA, USA).

**Immunofluorescent studies of Cx43.** Immunofluorescence staining was performed to investigate the localization and the distribution of Cx43 at the peri-infarct zone of the left ventricle. Fresh tissue was fixed in 4% paraformaldehyde for 24 h, then dehydrated in 30% sucrose for 48 h. The tissue was then embedded at -25°C (frozen section inside the machine) and then frozen sectioning was performed. Following inhibition with goat serum for 30 min, the frozen slices (5  $\mu$ m thick) were incubated with rabbit polyclonal anti-Cx43 antibody (Zymed Life Technologies, Carlsbad, CA, USA) overnight at 4°C and then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (Zymed Life Technologies) for 1 h at room temperature. The specimens were examined under a Leica M205 FA fluorescence microscope (Leica, Wetzlar, Germany).

**Statistical analysis.** All values are expressed as the mean  $\pm$  standard deviation. Comparisons between groups were performed using one way analysis of variance and the least significant difference test was used for post hoc multiple comparisons.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Animal survival rate and infarct size.** At 28 days after MI, the survival rate in the artemisinin treatment group (68.88%; 31/45) was significantly higher than that in the vehicle-treated group (42.22%, 19/45;  $P < 0.05$ ). The infarct size four weeks after MI was similar between the MI + artemisinin group and the MI + vehicle group ( $34.32 \pm 1.68\%$  and  $33.18 \pm 1.42\%$ , respectively;  $P > 0.05$ ).

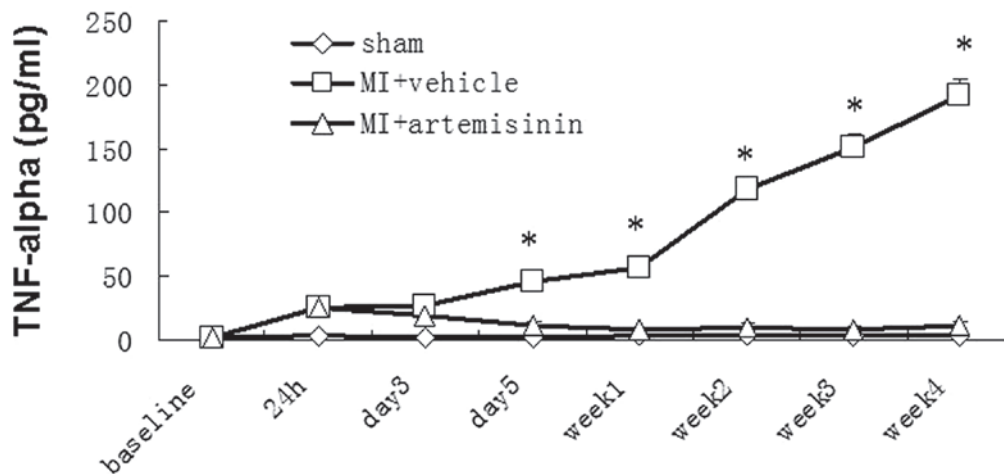


Figure 1. Plasma TNF- $\alpha$  levels among three groups following MI in rats with vehicle or artemisinin treatment for 4 weeks. TNF- $\alpha$  levels were comparable among the three groups at baseline. In the vehicle-treated group, TNF- $\alpha$  levels increased within 24 h after MI and remained elevated throughout the study. In the artemisinin treatment group, there was a similar early increase in TNF- $\alpha$  within 24 h; however, TNF- $\alpha$  was reduced to baseline levels by day 4 of artemisinin treatment and remained low throughout the rest of the study. In the sham-operation group, the TNF- $\alpha$  levels remained at or around baseline throughout the study. \* $P < 0.05$  vs. the sham group and the artemisinin treatment group. TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; MI, myocardial infarction.

**Plasma TNF- $\alpha$  levels.** As shown in Fig. 1, the baseline plasma TNF- $\alpha$  level (pg/ml) in the three groups observed were comparable across groups ( $P > 0.05$ ). In the sham group, TNF- $\alpha$  levels remained at or around baseline during the entire study. By contrast, in the MI + vehicle group the TNF- $\alpha$  level was increased within 24 h after MI ( $25.6 \pm 4.2$ ) and remained elevated ( $26.71 \pm 4.11$ ,  $46.18 \pm 4.7$ ,  $56.42 \pm 3.47$ ,  $117.52 \pm 8.72$ ,  $150.8 \pm 11.31$ ,  $191.08 \pm 12.68$  on days 3, 5, 7, 14, 21, 28 after MI, respectively) throughout the study. The TNF- $\alpha$  level in the MI + artemisinin group also increased within 24 h ( $24.46 \pm 6.15$ ), however, decreased ( $18.24 \pm 3.24$ ) on day 3, returned to baseline by day 5 and then remained low ( $10.31 \pm 3.09$ ,  $7.72 \pm 2.08$ ,  $9.95 \pm 2.37$ ,  $8.08 \pm 2.49$ ,  $10.33 \pm 3.08$ ) throughout the study.

**Inducibility of VAs and VFT.** The VA was induced by burst stimuli, compared with the sham group, the inducibility was significantly higher in the MI + vehicle group; however, the inducibility was reduced in the artemisinin treatment group ( $P < 0.05$ ). VFT, reflects the vulnerability of ventricular fibrillation and VFT was significantly decreased in the MI + vehicle group and only marginally decreased in the MI + artemisinin group (Fig. 2).

**qPCR of Cx43.** The Cx43 mRNA levels 4 weeks after MI demonstrated a significant downregulation at the IBZ in the vehicle-treated group compared with the sham group. However, the reduction of Cx43 mRNA expression in the artemisinin-treated group following MI was significantly smaller than the reduction observed in the vehicle-treated group (Fig. 3).

**Western blot analysis.** The level of Cx43 protein was investigated by western blotting. Representative blots and quantitative results are presented in which Cx43 band intensities are normalized to  $\beta$ -actin. Two predominant forms of Cx43 were detected, the phosphorylated form of Cx43 (P-Cx43;

43 kDa) and the non-phosphorylated form of Cx43 (NP-Cx43; 41 kDa). Compared with the sham group, the P-Cx43/ $\beta$ -actin was significantly reduced in the MI + vehicle group. By contrast, the decrease in P-Cx43/ $\beta$ -actin was reversed in the MI + artemisinin group (Fig. 4).

**Immunofluorescent studies of Cx43.** The connexin localization was evaluated using an immunofluorescent technique. Cx43 protein was only located at the intercalated disk area in the left ventricle of the sham group or normal tissue far from the infarcted zone. However, the distribution of Cx43 on the IBZ was disrupted. In the areas of the IBZ, Cx43 was distributed on the lateral side of the myocyte, however, not in the intercalated disk area. The disarray of Cx43 was ameliorated in the IBZ of the artemisinin treatment group, where its distribution was relatively normal. Quantitative analysis of the Cx43-signal intensity demonstrated that the Cx43-signal in the vehicle-treated group was markedly reduced in the IBZ, however, was partially attenuated by artemisinin treatment (Fig. 5).

## Discussion

To the best of our knowledge, the present study demonstrated for the first time that artemisinin is able to ameliorate electrical remodeling at the IBZ following MI. Firstly, artemisinin treatment significantly inhibited the reduction of total Cx43 and phosphorylated Cx43. Secondly, the ischemia-induced disarrangement and distribution were reversed following artemisinin treatment. Thirdly, artemisinin treatment reduced the inducibility of VAs and increased VFT in rats with MI.

Artemisinin is the active component of *Artemisia annua* L. and is approved worldwide for the treatment and prevention of malaria (1). Previous studies have indicated that artemisinin is important in cardioprotection, and that it is able to attenuate ventricular remodeling and neural

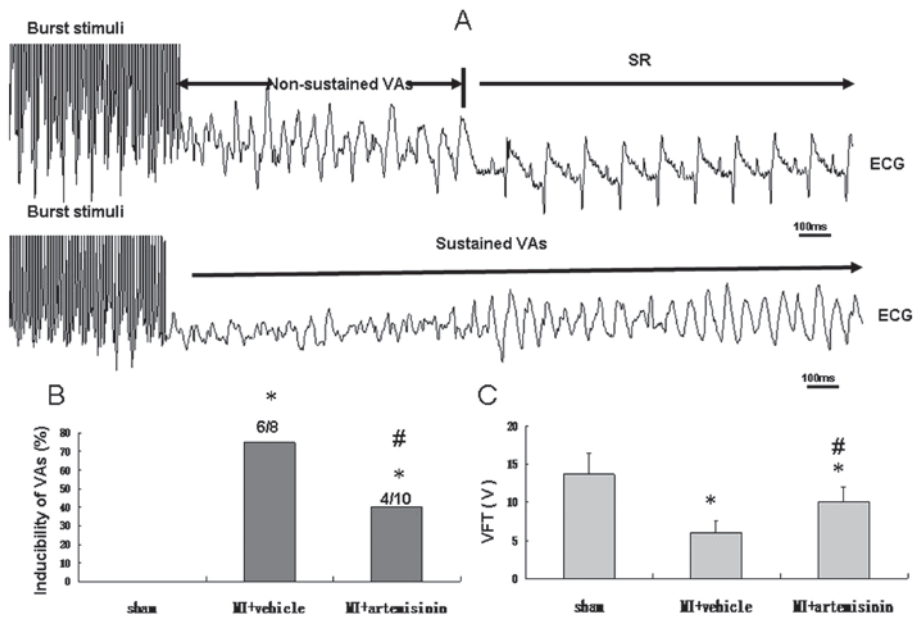


Figure 2. Inducibility of VAs by burst stimulation and the VFT by burst stimulation with increased pacing voltage. (A) Representative sustained ventricular arrhythmias induced by ventricular pacing. (B) The inducibility of ventricular arrhythmias (VAs) was significantly lower in the artemisinin-treated group than the vehicle-treated group. (C) VFT was reduced in the MI + vehicle group and marginally reduced in the MI + artemisinin group. VFT, ventricular fibrillation threshold; VAs, ventricular arrhythmias.

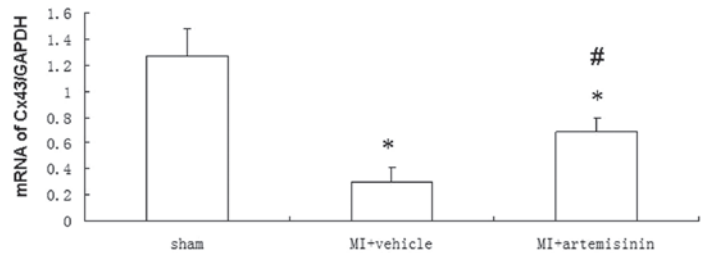


Figure 3. qPCR analysis of Cx43 following MI. Each mRNA was normalized to the mRNA level of GAPDH. Each column and bar represent the mean  $\pm$  standard deviation. \* $P < 0.05$ , compared with the sham group, # $P < 0.05$ , compared with the MI + vehicle group. Cx43, connexin 43; MI, myocardial infarction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; qPCR, quantitative polymerase chain reaction.

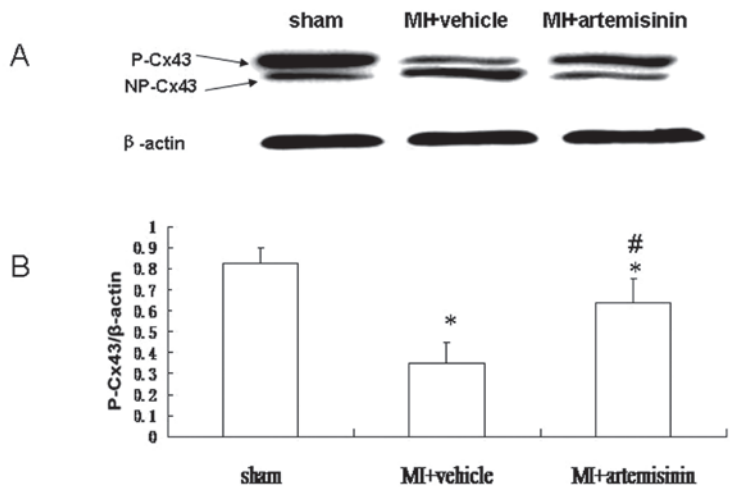


Figure 4. Western blot analysis of Cx43 protein from the infarcted border zone of the left ventricle 4 weeks after MI. (A) Representative immunoblots from the sham group, the MI + vehicle group and the MI + artemisinin group, probed with polyclonal Cx43 antibody. Arrows indicate the position of the phosphorylated isoform of Cx43 (43 kDa) and the nonphosphorylated isoform of Cx43 (41 kDa) bands, respectively. (B) Quantitative densitometric analysis of the phosphorylated isoform of Cx43. Data are expressed as the mean  $\pm$  standard deviation. \* $P < 0.05$ , compared with the sham group, # $P < 0.05$ , compared with the MI + vehicle group. Cx43, connexin 43; MI, myocardial infarction.



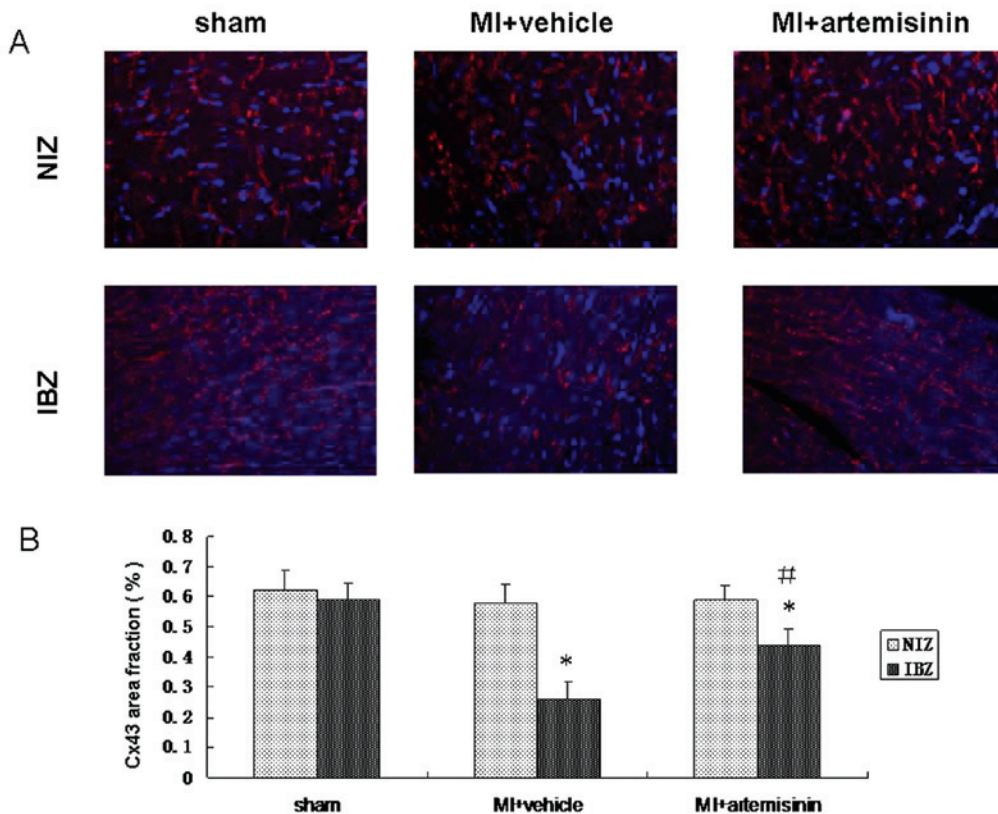


Figure 5. Immunofluorescent studies of Cx43 following MI. (A) Cx43 signals were mainly located at end-to-end apposition among the neighboring cells in the sham group. By contrast, MI markedly decreased the expression of Cx43 in the IBZ; however, not in the NIZ. Following administration of artemisinin, Cx43 signals were increased in the IBZ. Blue fluorescence indicates nuclei (DAPI) and red indicates Cx43 (rabbit polyclonal anti-Cx43 antibody). (B) Proportion of the total cell area occupied by Cx43 immunoreactive signals at the border zone. Data are expressed as the mean  $\pm$  standard deviation. \* $P < 0.05$ , compared with the sham group, # $P < 0.05$ , compared with the MI + vehicle group. Cx43, connexin 43; MI, myocardial infarction; IBZ, infarcted border zone; NIZ, non-infarcted zone.

remodeling (2-3). The present study demonstrated the anti-arrhythmic effect of artemisinin.

It is well known that Cx43, which is the predominant ventricular gap junction protein, is critical for maintaining normal cardiac electrical conduction. Cardiac-restricted knockout of Cx43 causes a slowed ventricular conductive velocity and spontaneous VAs (8-9). Apart from reducing conduction, Cx43 remodeling may be associated with action potential duration dispersion in the failing heart (10). Furthermore, increased Cx43 by either Cx43 gene transfer or transplantation of embryonic cardiomyocytes decreases the spatial heterogeneity of repolarization and has the potential to reduce life-threatening post-infarct arrhythmias (11-12). Post-MI, the epicardial border zone (EBZ) demonstrated a marked disruption in gap-junctional distribution, with Cx43 disarrayed along the lateral surfaces of the cells (13) and selectively reduced in the EBZ (14). The present study observed that the disarray of Cx43 was ameliorated in the IBZ of the artemisinin-treated heart, displaying a relatively normal distribution. In addition, the present study demonstrated that the Cx43 mRNA level and the quantity of Cx43 protein were significantly increased at the IBZ by artemisinin therapy. The beneficial effects on Cx43 may improve electrical conduction and ameliorate repolarization dispersion in the IBZ. It may provide a rational explanation for why artemisinin prevented the induction of VAs in the present study.

Although the present study suggests that the mechanisms by which artemisinin produces anti-arrhythmic effects are associated with increases in Cx43 expression, other potential mechanisms require investigation. Firstly, our previous studies demonstrated that artemisinin attenuates interstitial fibrosis (2) and sympathetic reinnervation in the IBZ (3), which contributes to VAs and sudden cardiac death. Secondly, previous studies have confirmed that artemisinin exerts a direct effect on the ion channels (15-16). Therefore, it was hypothesized that artemisinin produces anti-arrhythmic effects by directly regulating the cardiac ionic channel.

Several potential mechanisms may be involved in the ability of artemisinin to inhibit Cx43 degradation and increase Cx43 expression following MI. It has been demonstrated that myocardial ischemia with sympathetic nerve stimulation promoted the degradation of the Cx43 protein (17) and vagal nerve stimulation protects the heart against ischemic-induced arrhythmias by preserving Cx43 protein (18), suggesting that autonomic nerve activity correlates with the survival time of Cx43. Our previous study revealed that artemisinin inhibits neural remodeling and sympathetic hyperinnervation following MI in rats (3). One mechanism by which artemisinin inhibits the degradation of Cx43 following MI may be by directly attenuating sympathetic tone. Secondly, artemisinin is able to upregulate the mRNA level of Cx43, indicating that increasing Cx43 protein at the transcriptional

level may serve as another mechanism. Thirdly, inflammatory factors, including TNF- $\alpha$ , which is able to aggravate the dephosphorylation and degradation of Cx43 (19), were increased significantly following MI. Artemisinin is able to inhibit the levels of TNF- $\alpha$  following MI, which contributes to the upregulation of the Cx43 protein.

There are several limitations in the present study. Firstly, the transmural heterogeneity of Cx43 expression was not evaluated in the present study, such an analysis is required to clarify the precise mechanisms for the anti-arrhythmic effects of artemisinin. Secondly, although artemisinin was demonstrated to attenuate the reduction of Cx43 possibly by reducing TNF- $\alpha$  levels in the IBZ, the present study did not provide direct evidence by evaluating the effect of artemisinin on Cx43 expression following inhibition of TNF- $\alpha$  expression. Finally, the cardiac morphology and hemodynamics were not measured at the end of the study, although the results were demonstrated in our previous study (2).

In conclusion, artemisinin reduces the vulnerability to VAs and increases the VFT following MI. The cardioprotective effects of artemisinin may occur by reducing TNF- $\alpha$  levels and preventing gap junction remodeling. The results suggest that artemisinin is a potential therapeutic candidate for the prevention of ventricular electrical remodeling following MI.

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