

# Effects of Angiotensin Receptor Neprilysin Inhibitors on Inducibility of Ventricular Arrhythmias in Rats with Ischemic Cardiomyopathy

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### Summary

The aims of the present study were to investigate the effects of angiotensin receptor neprilysin inhibitors (ARNi) on the susceptibility of ventricular arrhythmias (VAs) in rats with myocardial infarction (MI) and to explore the related mechanisms.

A total of 32 adult male Sprague-Dawley rats were divided into 3 groups: a control group, MI group, and MI+ARNi group. MI was generated by ligation of the left anterior descending coronary artery. ARNi was given at 68 mg/kg/day for 4 weeks after MI surgery. At 4 weeks after MI, electrical programmed stimulation (EPS) was performed in all groups for the evaluation of VAs, and echocardiography was used to evaluate cardiac function. Indicators of sympathetic neural remodeling and cardiac remodeling were detected to further explore the related mechanisms.

Four weeks after MI, rats in the ARNi group exhibited low susceptibility of VAs in comparison with that in the MI group, which was coincident with the attenuation of sympathetic nerve remodeling, amelioration of cardiac fibrosis, and regulation of Cx43 expression.

ARNi is effective in reducing VAs in rats with ischemic cardiomyopathy, which is associated with attenuating sympathetic nerve remodeling and myocardial fibrosis.

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**Key words:** Myocardial infarction, Cardiac fibrosis, Sympathetic nerve remodeling, Connexin43

It is widely acknowledged that myocardial infarction (MI) is one of the most common causes of human death.<sup>1)</sup> Arrhythmias, especially ventricular arrhythmias (VAs), contribute the most to death from MI.<sup>2)</sup> Despite emergency revascularization strategies for MI that develop rapidly, mortality from malignant VAs remains high. Hence, any approaches to reduce the susceptibility of VAs might also have potential effects on reducing mortality in patients with ischemic cardiomyopathy.

In cardiovascular disease, especially ischemic cardiomyopathy, cardiac remodeling with autonomic dysregulation plays a vital role in its generation, progress, and prognosis.<sup>3)</sup> After MI, cardiac remodeling and sympathetic remodeling could together promote the occurrence of VAs.<sup>4)</sup> In the subacute period after acute MI, autonomic dysregulation occurs after myocardial hypoxia. It shows up as a series of pathophysiological changes including myocardial denervation, nerve sprouting, sympathetic over-regeneration, and high domination,<sup>5)</sup> ultimately developing into electrophysiological heterogeneity.<sup>6)</sup> After the subacute period, fibrous and scar tissue replaces ischemic

necrotic myocardium. The fibrous tissue could develop into regions of conduction block and form nonuniform anisotropy and slow conduction which may finally result in reentry substrate for sustained VAs.<sup>7)</sup> Therefore, suppression of these pathological changes after MI could be effective to reduce the occurrence of malignant VAs.

Angiotensin receptor neprilysin inhibitors (ARNi), beyond blocking angiotensin II signaling, augment natriuretic peptides by inhibiting their breakdown by neprilysin.<sup>8)</sup> Both basic and clinical studies demonstrated that ARNi could attenuate hypertension<sup>9)</sup> and improve ventricular remodeling<sup>10)</sup> in heart failure. The PARADIGM-HF (Prospective Comparison of ARNi with ACEI to Determine Impact on Global Mortality and Morbidity in Heart Failure) study showed that ARNi is more effective in reducing sudden cardiac death in patients with reduced ejection fraction heart failure compared to angiotensin inhibition.<sup>11)</sup> But the effect of ARNi on arrhythmias has been little studied until recently. Diego, *et al*<sup>12)</sup> reported that ARNi could effectively decrease arrhythmia in myocardial ischemia patients with reduced ejection

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fraction. However, little is known of the mechanisms underlying the phenomena. Thus, the purpose of the present study was to investigate the effects of ARNi on inducibility of VAs in a rat model of MI-induced ischemic cardiomyopathy and explore the possible underlying mechanisms.

## Methods

**Animals and Experimental Protocols:** All procedures were approved by the Ethics Committee of Nanjing Medical University. The animal experiments were performed to conform with the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health publication 8th edition, 2011). Thirty-two male Sprague-Dawley (SD) rats (200–220 g) were provided by Nanjing Medical University Laboratory Animal Center. After a one-week adaption, 24 rats underwent ligation of the left anterior descending (LAD) coronary artery to induce MI, while 8 rats that underwent sham surgery served as the control. Subsequently, 23 surviving MI rats were randomly divided into 2 groups by a random number method: MI with ARNi (ARNi group,  $n = 11$ ), and MI with vehicle (MI group,  $n = 12$ ). ARNi (Novartis Pharma Schweiz AG, Chinese national medicine permission number J20171054) was administered intragastrically at a dose of 68 mg/kg body weight for 4 weeks starting on day 1 after MI surgery. The medication method for ARNi was mainly determined from its dose-dependent therapeutic effect in SD rats according to a previous pharmacokinetic and pharmacodynamic study<sup>13)</sup> as well as recent research on ARNi in MI rats.<sup>10,14)</sup> All data in this research was collected using a double-blind experimental method.

**Myocardial infarction model:** The MI model was created at 0 weeks to induce ischemic cardiomyopathy as described previously. In brief, after being anesthetized by intraperitoneal injections of 2% sodium pentobarbital (50 mg/kg), the rats were endotracheally intubated and mechanically ventilated. Thoracotomy was performed at the fourth intercostal space, and then the left anterior descending coronary artery was ligated with a 7-0 silk suture at about 1–2 mm below the starting point of the branch. After a local pale area could be seen at the surface of the heart, the thoracic cavity and skin were sutured. All rats that underwent MI were given penicillin intramuscularly to prevent infection. Rats in the control group only underwent thoracotomy without ligation. All MI procedures were performed by the same operators.

**Echocardiography:** Echocardiography was performed at 4 weeks. After the rats were anesthetized with isoflurane, the structure and function were evaluated using a Vevo 2100 (VisualSonics, Canada) system equipped with a MS-250, 16.0–21.0 MHz imaging transducer. The investigators were blinded to the treatment of the rats.

**Electrical programmed stimulation:** After echocardiography, all rats underwent ventricular electrical programmed stimulation before being sacrificed. After being anesthetized by intraperitoneal injection with 2% sodium pentobarbital (50 mg/kg), electrocardiography was performed using 3 needle electrodes placed on the right upper limb and legs. Next, electrical programmed stimula-

tion (EPS) was used to stimulate the left ventricular apex of the heart through a bipolar electrode and the incidence of VAs was investigated. By a cycle length of 140 ms, the threshold potential for stable pacing was achieved. Pacing was started with twice as much as the threshold and a cycle length of 140 ms, which was the interval of 8 stimulus (S1). An extra stimulus (S2) was applied until it failed to induce ventricular depolarization, while the interval between S1 and S2 was progressively shortened by 10 ms. The operators were blinded to the treatment of the rats.

**Histological analysis:** Rats were sacrificed immediately after EPS. The hearts were excised after perfusion with phosphate-buffered saline and washed with phosphate buffered saline. The heart was cut horizontally along the long axis at the pale area of the infarct and fixed with 4% paraformaldehyde for 24 hours. After being fixed, dehydration, and paraffin embedding, the hearts were made into pathological sections. The hearts were stained with Masson's trichrome stain, tyrosine peroxidase (TH) stain, and growth associated protein 43 (GAP43) stain. After staining, the hearts were observed under a normal light microscope and 6 representative fields were randomly selected for analysis by Image-Pro Plus 6.0.

**Immunofluorescence labeling:** Immunofluorescence labeling was used to investigate the distribution of Connexin 43 (Cx43) in the infarcted border zone. The samples were evaluated under a fluorescence microscope (Nikon, Japan).

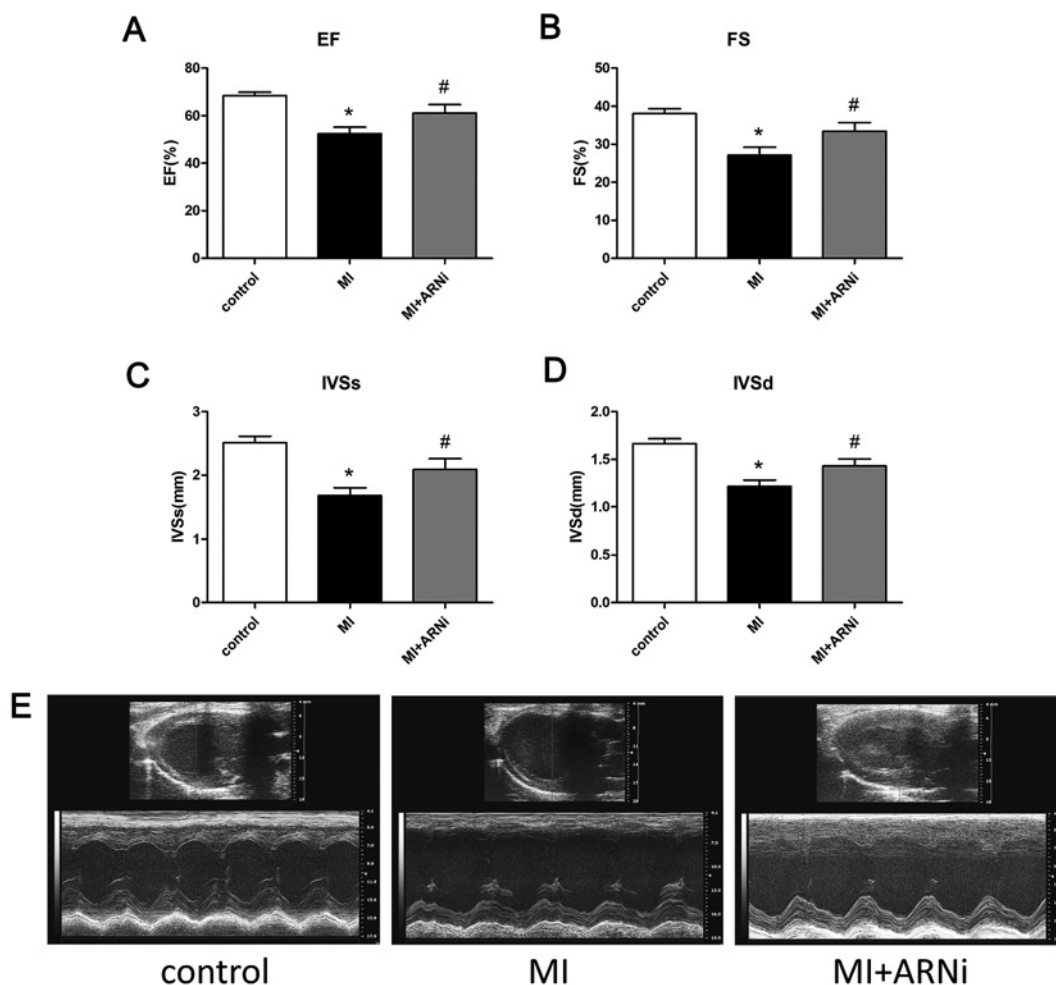
**Western blot:** Protein expression of nerve growth factor (NGF) (Abnova, China) and increased tissue neuropeptide-Y (NPY) (Abnova, China) in myocardial tissue was detected by Western blotting. After the heart samples were lysed in lysis buffer (Abnova, China), the protein concentrations were determined using the BCA method as previously described. GAPDH (Abnova, China) was used to normalize protein levels.

**Statistical analysis:** SPSS 16.0 software was used for statistical analysis, and GraphPad Prism 5 software was used for mapping. Quantitative data are shown as the mean  $\pm$  SEM, and for two-group comparisons, data were analyzed with two-tailed unpaired *t* tests, while for multiple-group comparisons, data were performed using one-way ANOVA followed by the LSD test. Qualitative data were analyzed with Fisher's exact test.  $P < 0.05$  was considered statistically significant.

## Results

### ARNi ameliorated MI-induced cardiac dysfunction:

Four weeks after MI, the control, MI, and ARNi groups had 8, 9, and 10 surviving rats, respectively, and echocardiography was performed to analyze the cardiac function. MI rats exhibited significantly decreased EF% (MI  $52.36 \pm 2.14\%$  versus Control  $68.46 \pm 1.44\%$ ,  $P = 0.0002$ ) and FS% (MI  $27.09 \pm 2.79\%$  versus Control  $38.06 \pm 1.31\%$ ,  $P = 0.0007$ ), compared with those in the control group (Figure 1), suggesting cardiac dysfunction happened. However, ARNi obviously ameliorated these cardiac dysfunctions induced by MI, analyzed by EF (MI + ARNi  $62.10 \pm 2.59\%$  versus MI  $52.36 \pm 2.14\%$ ,  $P = 0.02$ ), FS (MI + ARNi  $34.45 \pm 1.43\%$  versus MI  $27.09 \pm 2.79\%$ ,  $P$



**Figure 1.** ARNi partly restored cardiac dysfunction at 4 weeks. Echocardiography assessment of EF (A), FS (B), IVSs (C), and IVSd (D). Representative tracings of echocardiography (E).  $n = 8, 9, 10$  in Control, MI and MI + ARNi group. Data are mean  $\pm$  SE. \* $P < 0.05$  versus Control group; # $P < 0.05$  versus MI group.

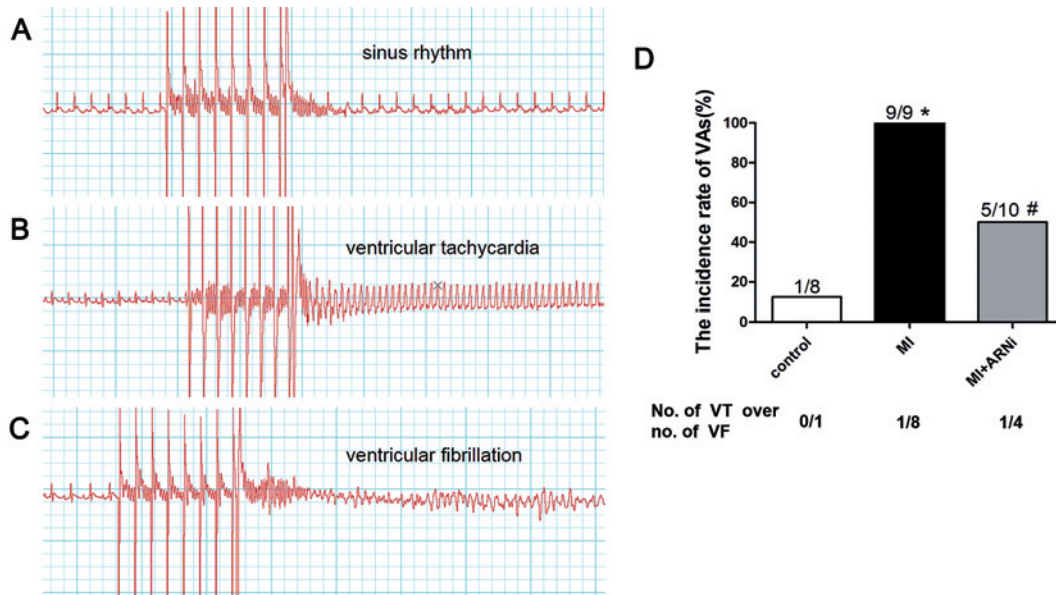
= 0.0097), IVSd (MI + ARNi  $1.43 \pm 0.07$  mm versus MI  $1.21 \pm 0.06$  mm,  $P = 0.0434$ ) and IVSs (MI + ARNi  $2.12 \pm 0.15$  mm versus MI  $1.67 \pm 0.12$  mm,  $P = 0.0374$ ) (Figure 1).

**ARNi inhibited the occurrence of ventricular arrhythmias:** Next, electrical programmed stimulation (EPS) was performed in all groups in order to induce VAs. The mean voltage level of each group was similar (Control group  $2.75 \pm 0.22$  V versus MI group  $2.97 \pm 0.26$  V versus MI + ARNi group  $2.90 \pm 0.26$  V,  $P > 0.05$ ). The incidence of pacing-induced VAs were significantly higher in the MI group than that in the control group (MI 9/9, Control 1/8,  $P < 0.05$ ), respectively. In the ARNi group, however, the susceptibility of VAs was greatly reduced (MI + ARNi 5/10, MI 9/9,  $P < 0.05$ ) (Figure 2).

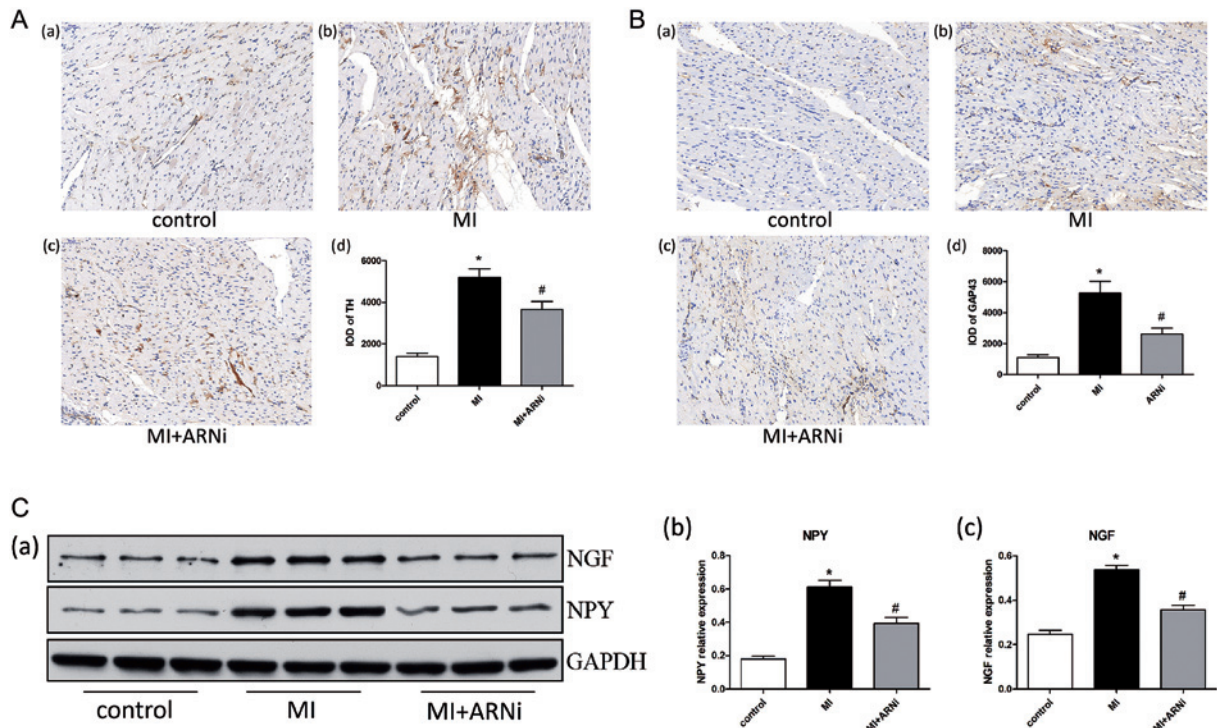
**Effects of ARNi on sympathetic neural remodeling:** The sympathetic activity in the peri-infarct zone was evaluated by immunocytochemical staining and Western blotting. Tissue samples were taken from the peri-infarct zone. Tyrosine peroxidase (TH) is an important rate-limiting enzyme for the synthesis of adrenergic neuro-

transmitter and can specifically show the distribution of sympathetic nerves.<sup>15,16</sup> Growth associated protein 43 (GAP43) is a neuron-specific protein synthesized in neurons, serving as a marker for eruption of nerves.<sup>17</sup> The density of nerves positive for TH (MI  $5203 \pm 416.4$  versus Control  $1378 \pm 173.7$ ,  $P < 0.0001$ ) and GAP43 (MI  $5293 \pm 724.4$  versus Control  $1093 \pm 190.9$ ,  $P < 0.0001$ ) were both significantly higher in MI rats than in control rats, while these increases in TH (MI + ARNi  $3651 \pm 388.2$  versus MI  $5203 \pm 416.4$ ,  $P = 0.0143$ ) and GAP43 (MI + ARNi  $2622 \pm 375.0$  versus MI  $5293 \pm 724.4$ ,  $P = 0.0036$ ) were effectively reversed by ARNi (Figure 3A, B).

As a crucial regulator, nerve growth factor (NGF) initiated neural sprouting and induced sympathetic regeneration.<sup>18</sup> Increased tissue neuropeptide-Y (NPY) abundance was previously associated with increased sympathetic activity.<sup>19,20</sup> NGF and NPY protein expression was assessed in cardiac peri-infarct zone tissues of rats by Western blotting. NGF and NPY expression was significantly greater in the MI group compared to the control group (NGF: MI

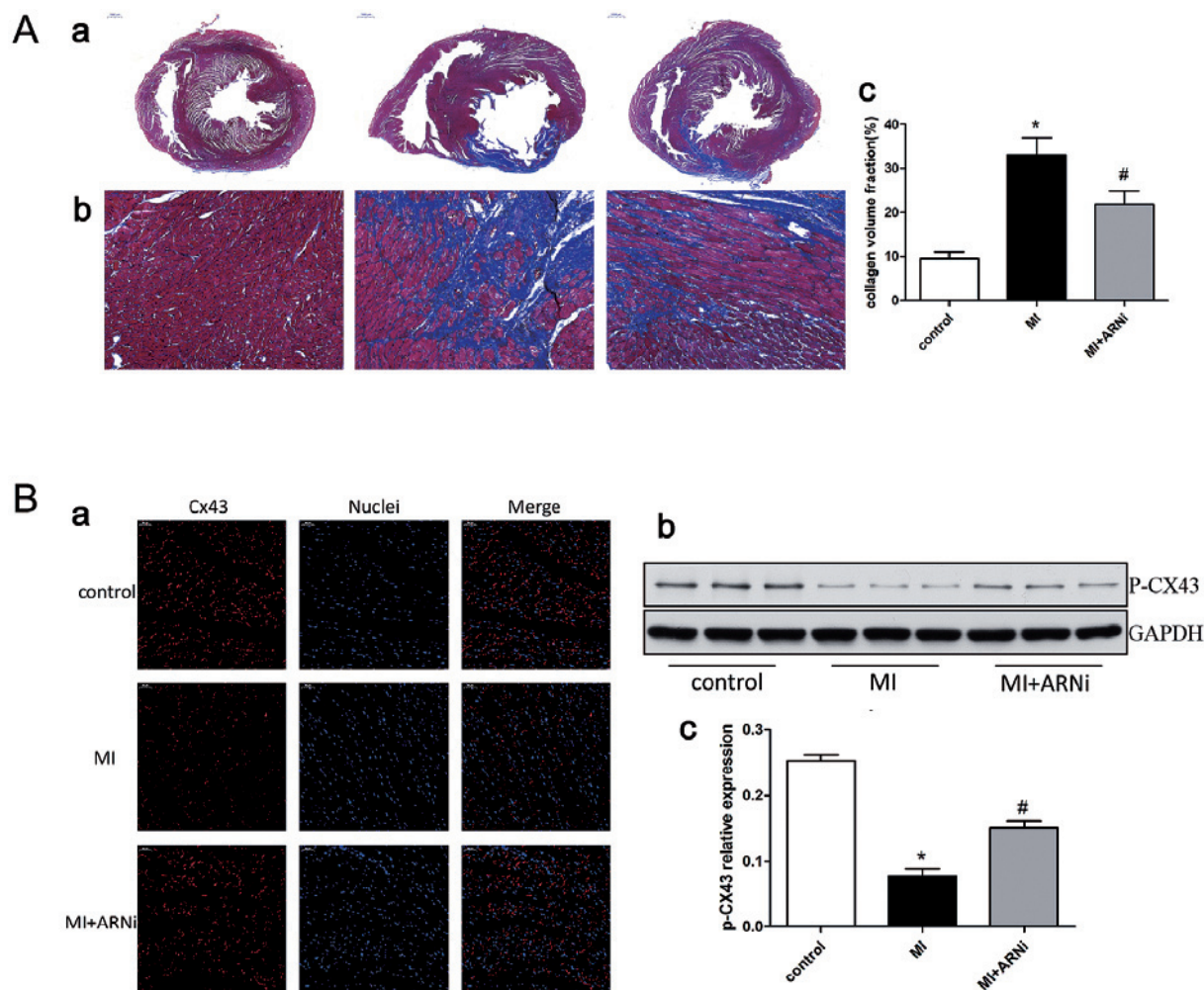


**Figure 2.** ARNi significantly reduced the inducibility of VAs. Representative ECG of electrical stimulation, including sinus rhythm (A), ventricular tachycardia (B), and ventricular fibrillation (C). VAs were more easily induced in the MI group rather than in the MI + ARNi group (D).  $n = 8, 9, 10$  in Control, MI and MI + ARNi group,  $*P < 0.05$  versus Control group;  $#P < 0.05$  versus MI group.



**Figure 3.** ARNi significantly attenuated sympathetic neural remodeling. **A:** ARNi significantly reduced cardiac expression of TH. **a-c:** Representative images of immunohistochemical staining of cardiac TH protein expression (magnification  $\times 200$ ). **d:** Quantitative analysis of TH expression. **B:** ARNi significantly reduced cardiac expression of GAP43. **a-c:** Representative images of immunohistochemical staining of cardiac GAP43 protein expression (magnification  $\times 200$ ). **d:** Quantitative analysis of GAP43 expression. **C:** ARNi significantly reduced protein expression of NPY and NGF. **a:** Representative cropped Western blot of NPY, NGF and GAPDH in heart. **b, c:** Quantitative analysis of NPY and NGF in heart by Western blot. Data are mean  $\pm$  SEM.  $*P < 0.05$  versus Control group;  $#P < 0.05$  versus MI group.





**Figure 4.** ARNi significantly attenuated cardiac fibrosis and regulated Cx43. **A:** ARNi significantly attenuated cardiac fibrosis. **a, b:** Representative ventricular fibrosis (in blue) by Masson's staining of samples. **c:** Quantitative analysis of **b** by CVF. **B:** ARNi significantly improved distribution and expression of Cx43 in the infarcted border zone. **a:** The distribution of Cx43 was disrupted in the border-zone of the infarcted area and the chaos of Cx43 was attenuated by ARNi. **b:** Representative cropped Western blot of p-Cx43 and GAPDH in heart. **c:** Quantitative analysis of **b**. Data are mean  $\pm$  SEM. \* $P < 0.05$  versus Control group; # $P < 0.05$  versus MI group.

$0.52 \pm 0.024$  versus Control  $0.24 \pm 0.016$ ,  $P < 0.0001$ ; NPY: MI  $0.61 \pm 0.049$  versus Control  $0.18 \pm 0.020$ ,  $P < 0.0001$ , but these upregulations were significantly decreased in the ARNi group rats (NGF: MI + ARNi  $0.35 \pm 0.022$  versus MI  $0.52 \pm 0.024$ ,  $P = 0.0001$ ; NPY: MI + ARNi  $0.39 \pm 0.035$  versus MI  $0.61 \pm 0.049$ ,  $P = 0.0026$ ) (Figure 3C).

**Effects of ARNi on cardiac remodeling in ventricle:** Masson staining was used to assess cardiac fibrosis in the left ventricle. Collagen volume fraction (CVF), an indicator of the degree of fibrosis, was significantly increased in the MI rats (MI  $33.01 \pm 3.99\%$  versus Control  $9.53 \pm 1.48\%$ ,  $P < 0.0001$ ) while ARNi effectively attenuated this increase caused by MI (MI + ARNi  $21.83 \pm 3.09\%$  versus MI  $33.01 \pm 3.99\%$ ,  $P = 0.03$ ), suggesting that ARNi could reverse the myocardial fibrosis after MI (Figure 4A).

Connexin 43 (Cx43) is an important gap junction which is located between cardiomyocytes and facilitates conduction through normal ventricular muscle in a con-

tinuous process.<sup>21)</sup> The localization and expression of Cx43 was evaluated by immunofluorescence and Western blotting, respectively. In the MI group, the expression and distribution of Cx43 in infarcted myocardial tissue become irregular and inhomogeneous (MI  $0.073 \pm 0.012$  versus Control  $0.253 \pm 0.009$ ,  $P < 0.0001$ ), while in the ARNi group, the chaos of Cx43 was significantly attenuated and the expression of p-Cx43 was markedly increased (MI + ARNi  $0.151 \pm 0.011$  versus MI  $0.073 \pm 0.012$ ,  $P = 0.0003$ ) (Figure 4B).

## Discussion

In the present study, we induced ischemic cardiomyopathy in our rat MI model to evaluate the protective effect of ARNi on the occurrence of postinfarction VAs, and then we investigated the related mechanism. The main findings of this study are as follows: (1) ARNi significantly reduced VAs inducibility after MI; (2) ARNi at-

tenuated sympathetic neural remodeling; and (3) ARNi decreased myocardial fibrosis and regulated Cx43 expression.

ARNi is the combination of angiotensin receptor antagonism and neprilysin inhibition.<sup>22)</sup> Previous studies have confirmed its therapeutic effect on hypertension and heart failure.<sup>10,23,24)</sup> However, very few studies have focused on the role of ARNi against VAs. Recently, Diego, *et al.*<sup>12)</sup> described interesting observations from a cohort of 120 patients implanted with ICDs in which ARNi decreased arrhythmias and appropriate ICD shocks, revealing the potential association between ARNi and VAs. In line with that report, we also found that ARNi effectively decreased the susceptibility of VAs in rats with ischemic cardiomyopathy, which strongly supports the findings of Diego, *et al.*

Many factors are involved in the pathogenesis of postinfarction VAs. In the subacute period after acute MI, sympathetic remodeling due to autonomic dysregulation provides essential substrates.<sup>25)</sup> After ischemic myocardial injury, degeneration and death of sympathetic nerve fibers formed in the infarcted area with significant inflammatory cell infiltration. These inflammatory cells such as macrophages and myofibroblasts could synthesize and release NGF and then initiate sympathetic nerve sprouting in the heart.<sup>26)</sup> With autonomic dysregulation, this kind of inflammatory response eventually develops into sympathetic over-regeneration and high domination,<sup>16)</sup> that is, sympathetic remodeling. This particular pathological change is closely related to VAs. On the one hand, sympathetic hyperinnervation can reduce the ventricular refractory period and VF threshold.<sup>3)</sup> On the other hand, it could also promote triggered activity and automaticity,<sup>27)</sup> which together creates the substrate for VAs. Consistent with these findings, we observed cardiac sympathetic neural remodeling at 4 weeks after MI, and these changes coincided with the higher occurrence of VAs. Due to the strong association between sympathetic remodeling and arrhythmia, many clinical treatments target autonomic dysregulation in order to reduce the occurrence of malignant arrhythmia, such as traditional left stellate ganglion block.<sup>28)</sup> Similarly, Jackson, *et al.*<sup>29)</sup> recently reported that renal denervation (RDN), a new technique to reduce sympathetic activity,<sup>30)</sup> leads to a reduction of postinfarction VAs by attenuating sympathetic nerve remodeling. Intriguingly, Polhemus, *et al.*<sup>31)</sup> recently further found that RDN could also initially increase natriuretic peptide concentrations and have effects similar to those of ARNi in heart failure rats. These mechanistic findings reveal a relationship between the sympathetic system and neprilysin activity might have been “hidden” in the pathophysiology of heart disease.<sup>32)</sup> In the present study, we found that ARNi could significantly suppress sympathetic remodeling induced by MI, as evidenced by decreased expression of TH, GAP43 and NGF, which strongly supports the hypothesis that neprilysin activity and the sympathetic system might be intrinsically related.<sup>32)</sup> And this effect of ARNi on sympathetic tone may also form the basis for a potential therapeutic role of ARNi in postinfarction VAs.

Except for sympathetic nerve remodeling, cardiac structure remodeling also plays a critical role in the occur-

rence of arrhythmias after MI,<sup>33)</sup> especially beyond the subacute period after MI.<sup>4)</sup> Myocardial remodeling, characterized by death of normal myocardial cells and formation of fibrosis and scar tissue, is the response to variable environment stimuli including myocardial infarction.<sup>34)</sup> This kind of structural change not only contributes to heart failure, but is also closely linked to the progression of sustained VAs.<sup>35,36)</sup> After MI, reparative fibrosis formed in the infarcted zone due to the myocardial cell loss, while reactive fibrosis formed in perivascular tissue due to inflammation.<sup>37)</sup> These fibrous tissues are intertwined with surviving cardiomyocytes, developing into regions of conduction block and form nonuniform anisotropy and slow conduction, which finally results in reentry substrate for sustained VAs.<sup>38)</sup> In addition, cardiac remodeling also has great influence on the quantity and distribution of gap junctions. Under physiological conditions, gap junctions locate in intercalated discs and facilitate conduction through normal myocardial cells in a continuous process.<sup>39)</sup> After necrosis of normal myocardial cells, the number of connexins decreases with the loss of cardiomyocytes, and the distribution of connexins among surviving cardiomyocytes tends to be disordered.<sup>40)</sup> These changes of connexins could alter the spread of impulse in ventricular myocardium and directly influence the electrophysiology of cardiomyocytes, further increasing the risk of VAs.<sup>41)</sup> In fact, recent clinical studies also showed an increasing tendency to link myocardial remodeling with the genesis of VAs. A meta-analysis of Scott, *et al.*<sup>42)</sup> demonstrated that the formation of ventricular scar was closely related to the occurrence of VAs. Similarly, Disertori, *et al.*<sup>43)</sup> and Gulati, *et al.*<sup>44)</sup> also confirmed the predictive value of late gadolinium enhancement (LGE) for VA risk by assessment of ventricular fibrosis. Hence, any approach to ameliorate structural remodeling might also serve as a potential therapy for VAs. In the present study, ARNi indeed improved structural remodeling, manifested as attenuation of fibrosis and amelioration of the reduction and disorder of Cx43, which could be an explanation for the lower incidence of VAs.

At present, research on ARNi's prevention on VAs is still ongoing. In clinical studies, although ARNi has no significant effect on electrocardiogram (ECG) parameters in healthy people,<sup>45)</sup> it did reduce the incidence of VAs in myocardial ischemia patients with reduced ejection fraction.<sup>12)</sup> Accordingly, combined with our present study, we speculate that the direct preventive effect of ARNi on VAs is likely to be based on the abnormal neurohumoral activation after MI. It is well recognized that excessive activation of the neurohumoral systems is the vital cause leading to deleterious myocardial effects after MI, including VAs, while ARNi is the most effective oral drug for the regulation of multiple neurohumoral systems.<sup>8,46)</sup> At an effective therapeutic dose, ARNi could markedly inhibit the over-activated RAAS system and increase the level of natriuretic peptide in circulation, which may further reverse heart damage caused from interconnections between neurohumoral systems. Thus, in ischemic cardiomyopathy, its protective role is embodied in obvious attenuation of cardiac fibrosis, prevention of sympathetic remodeling, as well as decreases in myocardial stretch and myocardial

wall stress, which are vital foundations for the induction of VAs after MI. Accordingly, at this effective therapeutic dose, ARNi can play a preventive role in the occurrence of VAs after MI.

## Conclusion

In conclusion, we have demonstrated that ARNi ameliorates postinfarction VAs in rats with ischemic cardiomyopathy, which could be associated with protective effects on MI-induced sympathetic nerve remodeling and structural remodeling. Future investigation is still required to elucidate the signaling mechanisms underlying the protective effect of ARNi.

**Limitations:** Several limitations of the present study should also be acknowledged. First, the extent of change in LVEF after MI was significant but not overly huge. In order to ensure the survival of rats beyond the initial MI, coronary occlusion was performed at a relatively distal part of the LAD. Thus, with relatively small resultant lesions, the MI rats showed a modest but still significant decrease in LVEF. However, combined with the obvious myocardial fibrosis, this significant change in cardiac function might still indicate the successful generation of MI. And this modest decrease might also be of clinical relevance as many patients with MI underwent early percutaneous coronary intervention (PCI) and ended up with only a modest reduction in EF. Second, the present study lacks effective refractory period (ERP) data for each group, which would better show the correctness of the hypothesis. Our PES program (S1S2 program) was mainly aimed to induce VAs, and then the occurrence between groups was compared for further conclusion. This kind of PES method that induces VAs by shortening the interval between S1 and S2 will already cause VAs before the S1S2 spacing could reach ERP. Thus, for most rats, especially MI rats, ERP cannot be recorded. Future studies should take this into consideration.

## Disclosures

**Conflicts of interest:** None.

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