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Original Article

Anti-arrhythmic effects of atrial ganglionated plexi stimulation is accompanied by preservation of connexin43 protein in ischemia-reperfusion canine model

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Abstract: Background: Vagal nerve stimulation (VNS) has been shown to provide a protective effect against ischemia/reperfusion (I/R)-related arrhythmias by preventing the loss of Connexin43 (Cx43). Our previous studies showed that atrial epicardial ganglionated plexus stimulation (GPS) might exert a VNS-like effect on ventricular electrophysiology. Objectives: To investigate whether GPS could preserve Cx43 and reduce I/R induced ventricular arrhythmia. Methods: Sixteen dogs were randomly divided into GPS group (N = 8, receiving GPS) and Sham group (N = 8, receiving sham GPS). Ventricular effective refractory period (ERP) and heart rate variability (HRV) were measured at baseline and 1 h after GPS. Myocardial I/R was then performed. Ventricular arrhythmia occurred during the first hour after reperfusion was measured and myocardial tissue from the peri-infarct zone was excised for immunohistological analysis. In another 4 dogs (Control group, receiving sham GPS and sham I/R), myocardial tissue from the corresponding area was also excised. Results: Compared with the Sham group, GPS caused a significant increase in ventricular ERP and HRV, and a significant decrease in I/R-induced ventricular arrhythmias. Western blotting revealed a marked reduction in the amount of phosphorylated Cx43 and total Cx43 in the Sham group, whereas no significant change was observed in the GPS group compared with the Control group. Immunohistochemistry results confirmed that the myocardial I/R-induced loss of phosphorylated Cx43 from the intercellular junctions was prevented by GPS. Conclusion: GPS protects against I/R induced ventricular arrhythmias, accompanied by preserving Cx43.

Keywords: Ganglionated plexus stimulation, connexin43, ventricular arrhythmia

Introduction

In patients with coronary artery disease, sudden death is the most common pattern for ischemia-induced lethal ventricular arrhythmias (VAs) during the acute phase of myocardial infarction [1]. Myocardial reperfusion therapy, such as percutaneous coronary intervention or coronary artery bypass grafting, has been widely performed in the management of acute myocardial infarction over the past few years [2]. However, reperfusion itself can ironically lead to life-threatening Vas [1]. Both experimental and clinical studies have shown that a marked autonomic imbalance characterized by sympathetic overactivity and vagal attenuation is significantly associated with adverse cardiac events [3, 4]. Vagal nerve stimulation (VNS), however, can activate the parasympathetic nervous system and reduce ischemia-related VAs in hearts with myocardial infarction, post-infarction heart failure and ischemia/reperfusion (I/R) [5, 6]. The atrial ganglionated plexus, as an intrinsic cardiac nervous system, is mainly innervated by parasympathetic nerve [7]. Our previous study showed that ganglionated plexus stimulation (GPS) could exert a VNS-like effect on ventricular electrophysiology [8]. Furthermore, our studies also showed that GP ablation might facilitate, whereas GPS might

inhibit, the incidence of VAs after acute myocardial infarction [9, 10]. However, the mechanism underlying the antiarrhythmic effect of GPS has remained unknown.

Connexin43 (Cx43), as the main gap junction component of the heart ventricle, is mainly located at the intercalated discs. It contributes to the mechanical stability, electrical coupling and chemical coupling of cardiomyocytes [11]. Previous studies have shown that Cx43 was highly remodeled in the diseased heart, an effect that was mainly characterized by down-regulation and heterogeneous redistribution to the lateral sides of cardiomyocytes [12]. Furthermore, remodeling the impaired Cx43 expression could restore normal cardiac function and normalize electrical stability [12]. Ando et al showed that VNS might reduce ischemia-related VAs through preserving the loss of Cx43 [13]. Therefore, we hypothesized that GPS might also provide protective effects against I/R-induced VAs through preserving Cx43.

Materials and methods

Animal preparation

The study was reviewed and approved by Wuhan University (Permit Number: 2014-0561) and conformed to the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. A total of 20 adult mongrel dogs weighing 17-24 kg were anesthetized with 3% Na-pentobarbital at a basal dose of 30 mg/kg and a maintenance dose of 2 mg/kg*h. All dogs were ventilated with a positive pressure ventilator, and the core body temperature was kept at 36.5 ± 1.5°C with a heating pad. A body surface electrocardiogram and the arterial blood pressure were recorded throughout the whole procedure using a computer-based Lab System (Lead 2000B, Jingjiang Inc., Wuhan, China). In addition, all efforts were made to minimize suffering, and euthanasia was administered at the end of the experiment.

Measurement of the ventricular effective refractory period

Both left and right thoracotomies were performed at the fourth intercostal space to expose the heart. A multi-electrode catheter was sutured to the left and right ventricular free walls. The ventricular effective refractory period (ERP) was recorded from the following six sites (**Figure 1**): left ventricular apex (LVA), left ventricular base (LVB), median area of the left ventricle (LVM), right ventricular apex (RVA), right ventricular base (RVB) and median area of the right ventricle (RVM). The ERP at each site was measured by programmed pacing comprising eight consecutive stimuli (S1-S1, 350 ms cycle length) followed by a premature stimulus (S2). The S1-S2 interval was progressively reduced until refractoriness was achieved. The ERP was defined as the longest S1-S2 interval that failed to capture the ventricles [8, 10].

Measurements of heart rate variability

The spectral power of the heart rate variability was analyzed on 5-minute electrocardiogram recording segments obtained at baseline and 1 h after GPS, and an autoregressive algorithm was used to analyze digitized signals from the electrocardiographic recordings. The following power spectral variables were assessed: high frequency component (HF, 0.15-0.4 Hz), low frequency component (LF, 0.04-0.15 Hz) and ratio between the LF and HF powers (LF/HF, index of the interaction between the sympathetic and vagal activities) [14].

GPS

A left thoracotomy at the fourth intercostal space was performed to expose the superior left ganglionated plexi located adjacent to the left superior pulmonary vein/atrial junction within the left atrial appendage. A custom-made bipolar plaque electrode was sutured on top of the fat pad containing the superior left ganglionated plexi for stimulation (**Figure 1A** and **1B**). High frequency electrical stimulation (20 Hz, 0.1 ms duration) at a voltage able to induce a 10% decrease in the sinus rate was applied to the superior left ganglionated plexi via an electronic stimulator (Grass-S88; Astro-Med, West Warwick, RI, USA) [8]. Continuous GPS was performed from 1 minute before LAD occlusion until the end of the experiment.

Measurement of the reperfusion-induced ventricular arrhythmias

Electrocardiograms were continuously monitored for 1 hour to record the incidence and duration of reperfusion-induced ventricular

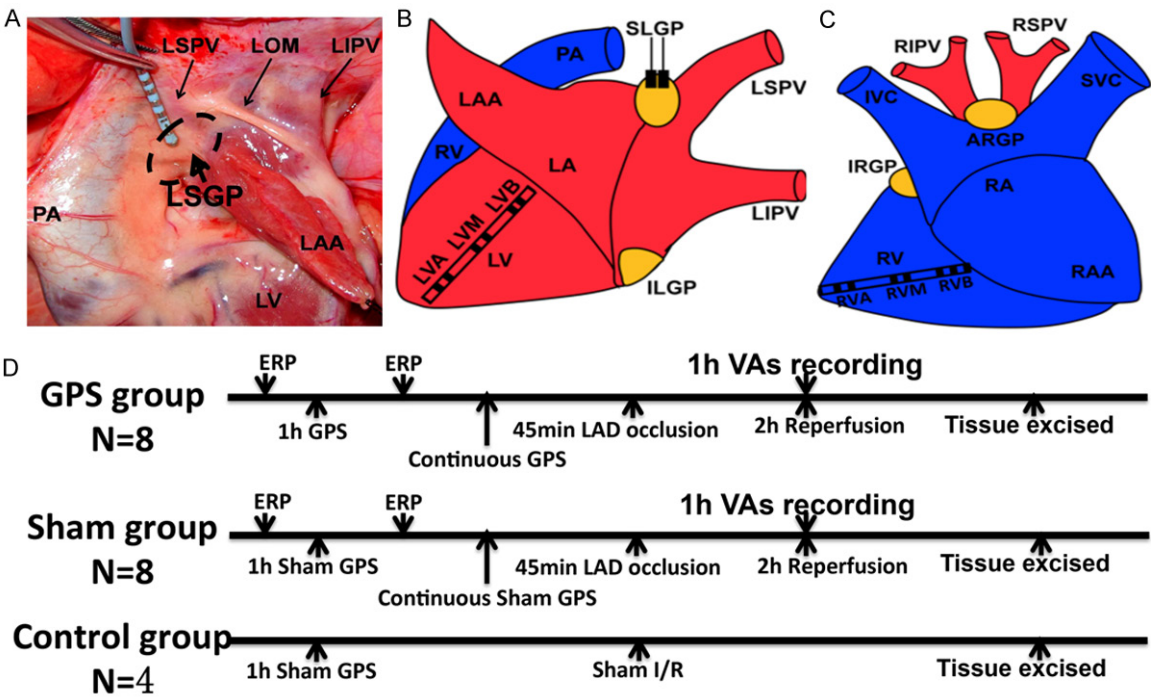

Figure 1. Schematic representation of the catheter position and a flow chart. LV and RV: left ventricle and right ventricle; LAA and RAA, left and right atrial appendage; LA and RA, left and right atrium; LOM, ligament of Marshall; LSPV, LIPV, RSPV and RIPV: left superior, left inferior, right superior, right inferior pulmonary vein; PA, pulmonary artery; SVC and IVC: superior and inferior vena cava; LVA and LVB, left ventricular apex and base; LVM, the median area between the LVA and LVB; RVA and RVB: right ventricular apex and base; RVM, the median area between the RVA and RVB; SLGP, ILGP, ARGP and IRGP: superior left, inferior left, anterior right and inferior right ganglionated plexus. GPS, ganglionated plexus stimulation; GPS group, receiving GPS and ischemia/reperfusion; Sham group, receiving sham GPS and ischemia/reperfusion; Control group, receiving sham GPS and sham ischemia/reperfusion; ERP, effective refractory period; LAD, left anterior descending coronary artery; VA, ventricular arrhythmia.

arrhythmias. The following definitions were used for reperfusion-induced VAs recorded on the ECG [15]: ventricular premature beats (VPBs), identifiable premature QRS complexes; ventricular tachycardia (VT), three or more consecutive VPBs; non-sustained VT, VT terminating spontaneously within 30 s; sustained VT, VT persisting for more than 30 s; and ventricular fibrillation (VF), a tachycardia with random ECG morphology and an associated loss of arterial blood pressure that degenerates into ventricular asystole.

Measurement of the infarct size

The infarct size was assessed with 0.5% Evans Blue and 1.0% triphenyltetrazolium chloride staining [16]. Briefly, Evans Blue was infused into the left atrial appendage to define the ischemic area at risk of necrosis (AAR, uncolored by the blue dye) as previously described. The deeply anesthetized dog was then sacrificed via an injection of potassium chloride. The heart was quickly removed, frozen at -20°C for 3 hours and then sliced into 2- to 5-mm-thick sections perpendicular to the left anterior descending artery occlusion site. Five continuous slices from the occlusion site were incubated in triphenyltetrazolium chloride at 37°C for 15 minutes to discriminate the infarcted tissues from the viable myocardium. After overnight fixation with 4% paraformaldehyde, each slice was photographed with a digital camera. The area measurement was performed using Image Tool software, version 3.0. The ratio of the AAR to the total ventricular mass and the infarct size normalized to the AAR were calculated.

Western blot analysis

Peri-infarct cardiac tissues were excised. Equal amounts of homogenated proteins were loaded for sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane. Phosphorylated Cx43

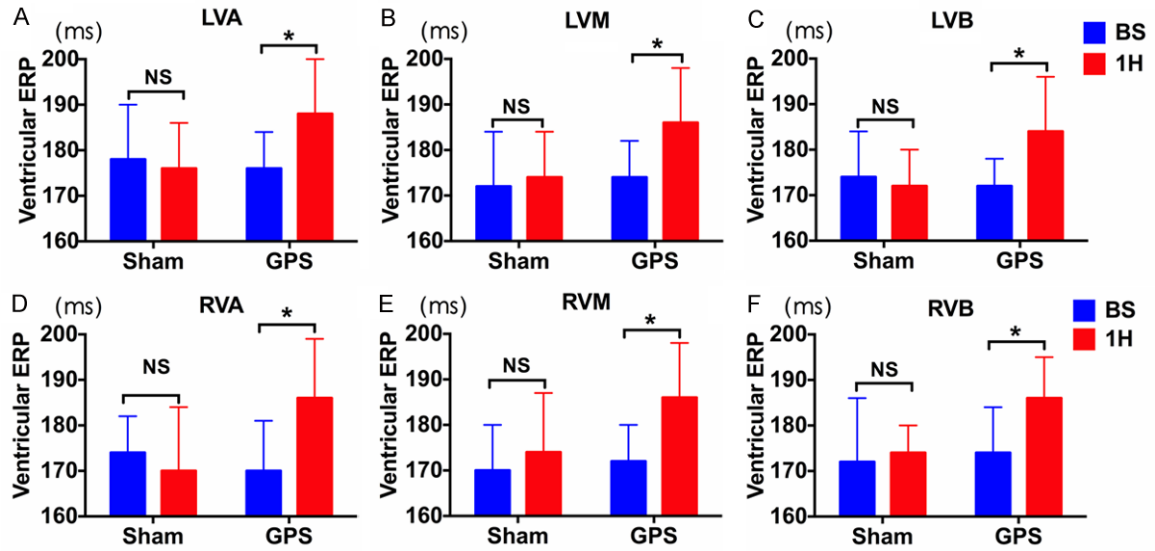

Figure 2. Effect of GPS on the ventricular ERP at six sites. BS, baseline; NS, $P > 0.05$, $^{*}P < 0.05$ versus baseline, other abbreviations are identical to those in **Figure 1**.

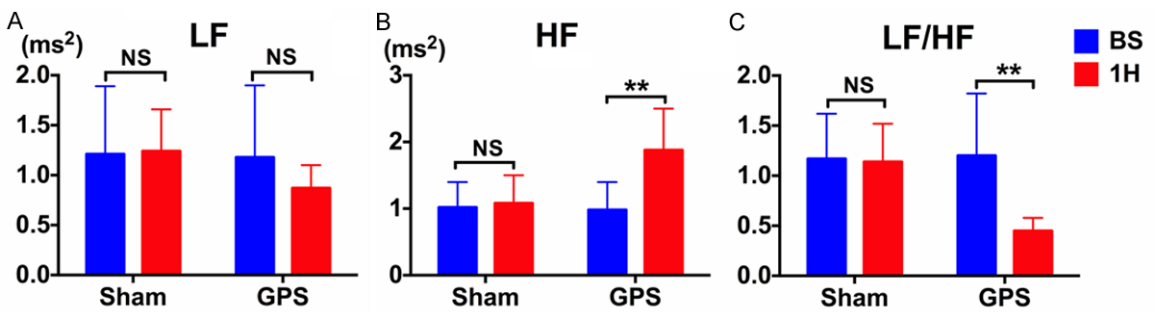

Figure 3. Effect of GPS on the heart rate variability. LF, low frequency; HF, high frequency; LF/HF, the ratio between LF and HF; BS, baseline; 1H, a hour after GPS or sham GPS; NS, $P > 0.05$, $^{**}P < 0.01$ versus baseline; other abbreviations are identical to those in **Figure 1**.

and total Cx43 were probed with a mouse monoclonal antipan-Cx43 antibody (71-0700, Zymed), diluted 1:1000 [13]. Antibody-binding protein bands were visualized by 125I-protein and quantified with a Bio-Rad Personal FX PhosphorImager, not by densitometry of the film. We adjusted the exposure to ensure that the protein bands were not saturated. The same Western blots were reprobed with anti-GAPDH (1:5000, Research Diagnostics Inc.) to normalize the gel loadings. Every blot was performed in triplicate, and the mean intensity value was presented as the value for the tissue.

Immunofluorescence analysis

The infarct size was measured as shown above. Multiple tissue paraffin blocks were sampled from peri-infarct myocardium using sharp skin biopsy punches (Acu-Punch, Acuderm, Inc.) and a scalpel and were routinely processed as described in a previous study [13]. Serial 4-μm-thick sections were mounted on slides. Immunofluorescence was assayed with an anti-Cx43 antibody (71-0700, Zymed), diluted 1:100. Labeled sections were visualized with a fluorescence microscope, and digital manipulations were restricted to conventional techniques. All immunoreactivity was quantified by a commercial software (ImagePro, Media Cybernetics, Inc, Rockville, MD). The nerve density based on the immunoreactivity of each slide was determined by the average of 3 fields with the highest nerve density. The nerve density was expressed as the total area of positive staining per square millimeter ($\mu m^2/mm^2$).

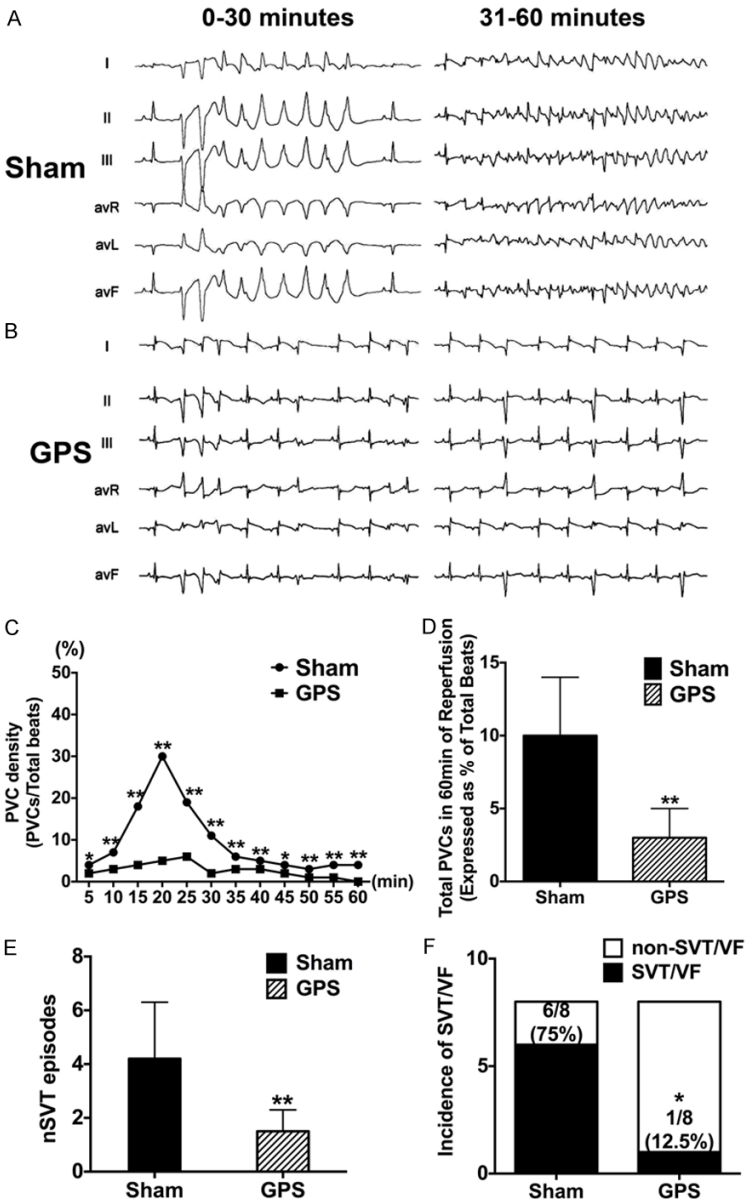

Figure 4. Effect of GPS on ischemia/reperfusion-induced VAs. (A and B) show the representative incidence of VAs during 0-30 and 31-60 minutes after reperfusion, respectively. (C-F) show the PVC density (C), total PVCs (D), nSVT episodes (E) and incidence of SVT/VF (F). VA, ventricular arrhythmia; PVC, premature ventricular contraction; nSVT, non-sustained ventricular tachycardia; SVT, sustained ventricular tachycardia; VF, ventricular fibrillation; NS, $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the Sham group; other abbreviations are identical to those in **Figure 1**.

Experimental protocol

A total of 16 adult mongrel dogs were randomly divided into two groups, a GPS group (n = 8, with GPS) and a Sham group (n = 8, with sham GPS). ERP and HRV were measured at baseline and 1 hour after GPS in the normal hearts. The left anterior descending artery (LAD) proximal to the first diagonal branch was then occluded with a reversible silk ligature for 45 minutes, and reperfusion was performed by releasing the ligature for 2 h. ST segment elevation that exceeded 2 mV was regarded as the criterion for ischemia. VA was measured at the first hour after reperfusion, and the hearts were finally excised at the peri-infarct zone. In another 4 dogs (Control group, sham GPS combined with sham I/R), the hearts were also excised at the corresponding site.

Statistical analysis

Continuous variables are presented as the mean ± SD and were analyzed by t test, one-way ANOVA, or two-way repeated-measures ANOVA with a Bonferroni posthoc test. To compare the incidence of incidence of sustained VT/VF between groups, Fisher's exact test was used. All data was analyzed by GraphPad Prism version 5.0 software (GraphPad Software, Inc., San Diego, CA), and two-tailed $P \leq 0.05$ was considered significant.

Results

Effect of GPS on ventricular ERP

Figure 2 summarizes ventricular ERP at 6 epicardial sites in the GPS and Sham groups. Compared to baseline, both left and right ventricular ERPs were significantly prolonged by GPS (LVA, 176 ± 8 ms vs. 188 ± 12 ms; LVM, 174 ± 8 ms vs. 186 ± 12 ms; LVB, 172 ± 6 ms vs. 184 ± 12 ms; RVA, 170 ± 11 ms vs. 186 ± 13 ms; RNM, 172 ± 8 ms vs. 186 ± 12 ms; RVB, 174 ± 10 ms vs. 186 ± 9 ms; $P < 0.05$ for all), whereas no significant change was caused by 1 h sham GPS.

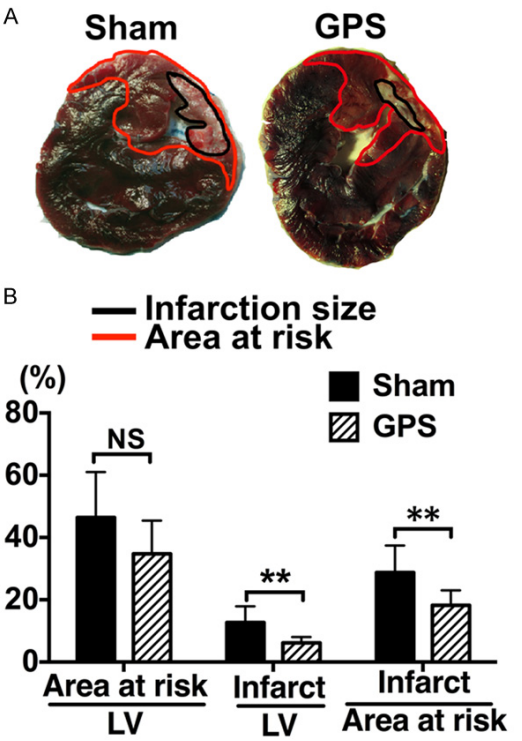

Figure 5. Effect of GPS on ischemia/reperfusion-induced infarct size and area at risk. A. Shows the representative examples of infarction size and area at risk. Blue indicates the viable myocardium; red indicates the non-infarct area; white indicates myocardial infarction. B. Shows the quantification analysis of infarct size and area at risk. NS, $P > 0.05$, $^{**}P < 0.01$ compared with the Sham group; other abbreviations are identical to those in **Figure 1**.

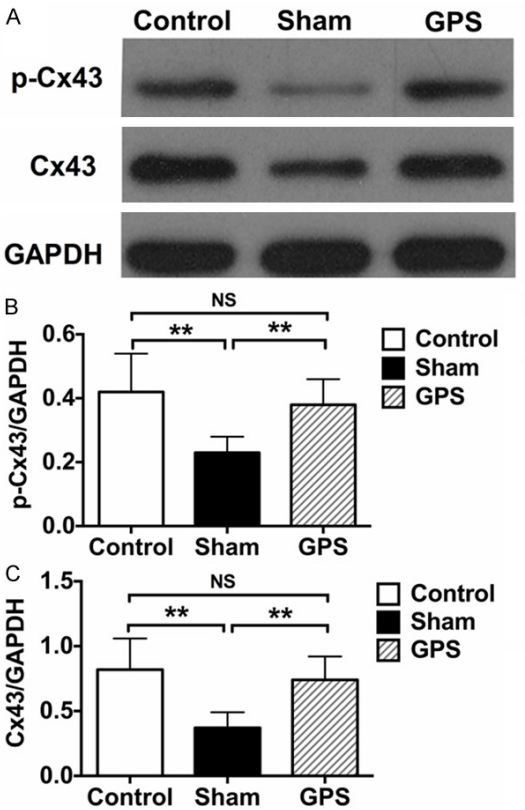

Figure 6. Representative Western blot examples (A) and relative levels (B and C) of p-Cx43 and Cx43 protein in the peri-infarct myocardium. Cx43, connexin43; p-Cx43, phosphorylated Cx43; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NS, $P > 0.05$ versus the Control group; $^{**}P < 0.01$ versus the Sham group; other abbreviations are identical to those in **Figure 1**.

Effect of GPS on HRV

As shown in **Figure 3**, 1 hour of GPS induced a significant increase in HF (1.88 ± 0.62 ms^2 vs. 0.98 ± 0.42 ms^2, $P < 0.01$ versus baseline) and a significant decrease in LF/HF (0.45 ± 0.13 vs. 1.20 ± 0.62, $P < 0.01$ versus baseline). Furthermore, no significant change was caused by GPS (0.87 ± 0.23 ms^2 vs. 1.18 ± 0.72 ms^2, $P > 0.05$ as compared to group baseline). Sham GPS, however, did not induce a significant change in LF, HF or LF/HF.

Effect of GPS on spontaneous VAs induced by I/R

Spontaneous VAs were induced by I/R injury (**Figure 4A** and **4B**). During the first hour after reperfusion, the number of VPBs was significantly reduced by GPS compared with sham GPS (**Figure 4C**). The total percentages of VPBs in the GPS and Sham groups were 3 ± 2% and 10 ± 4% ($P < 0.01$), respectively (**Figure 4D**). As shown in **Figure 4E**, the runs of non-sustained VT (1.5 ± 0.8 vs. 4.2 ± 2.1, $P < 0.01$) and the incidence of sustained VT/VF (12.5% vs. 75%, $P < 0.05$) were significantly reduced in the GPS group compared with the Sham group (**Figure 4F**).

Effect of GPS on myocardial infarct size

Figure 5 shows typical examples of the myocardial infarct size and AAR. Both the AAR and the infarct size were expressed as percentages of the total ventricular mass. In comparison with Sham group, a significant decrease was shown in infarct size (12.7 ± 5.2 vs. 6.2 ± 1.8, $P < 0.05$) but not in AAR (46.4 ± 14.6% vs. 34.8 ± 10.6, $P > 0.05$) in GPS group. The infarct size in the GPS group was, on average, 26 ± 6% of the

AAR, compared with 44 ± 12% of the AAR in the Sham group ($P < 0.01$), and a reduction of approximately 40% was found in the GPS group (**Figure 4B**).

Western blot analysis

Figure 6 shows representative blots and quantitative results in which the Cx43 and phosphorylated Cx43 band intensities were normalized to the measurements for GAPDH. Densitometric analysis of the immunoblots revealed significant reductions in the total Cx43 (0.37 ± 0.12 vs. 0.82 ± 0.24, $P < 0.01$) and phosphorylated Cx43 (0.23 ± 0.05 vs. 0.42 ± 0.12, $P < 0.01$) in the Sham group compared with the Control group. However, the Cx43 and phosphorylated Cx43 signals in the GPS group were almost comparable to those in the Control group (total Cx43, 0.74 ± 0.18; phosphorylated Cx43, 0.38 ± 0.08, both $P > 0.05$ compared with the Control group).

Immunohistochemistry

Figure 7A and **7C** shows the distribution and expression of ventricular phosphorylated Cx43 in the Control, Sham and GPS groups. In comparison with Control group (1328 ± 29 $\mu m^2/mm^2$), a significant decrease in phosphorylated Cx43 was found in Sham group (673 ± 149 $\mu m^2/mm^2$, $P < 0.01$), whereas the phosphorylated Cx43 remained at a nearly normal level in GPS group (1217 ± 235 $\mu m^2/mm^2$, $P > 0.05$).

Discussion

In the present study, we observed that GPS significantly (1) prolonged ventricular ERP, decreased LF/HF and increased HF in the normal heart and (2) decreased VAs, reduced myocardial infarct size/AAR and prevented the loss of Cx43 in the I/R model. GPS may protect against I/R induced ventricular arrhythmias, accompanied by preserving Cx43.

The atrial ganglionated plexi, which is mainly innervated by parasympathetic postganglionic neurons, has been shown to serve not only as a relay station that transmits central parasympathetic impulses to the heart but also function as the "integration center" of the extrinsic and intrinsic cardiac autonomic nervous system to modulate atrial and ventricular electrophysiology [7, 17]. Both human and animal studies have shown that VNS is able to prolong ventricular ERP and reduce VAs induced by ischemia or I/R [5, 18, 19]. Brack et al showed that the prolongation effect of VNS on ventricular ERP was mediated by ganglionated plexi and that this effect could be eliminated by ganglionic blockade [20]. Our previous study showed that ganglionated plexi ablation might facilitate the occurrence of VAs in acute myocardial ischemic hearts [4]. GPS, however, significantly reduced VAs during acute myocardial ischemia [21]. Our present study also showed that GPS was able to significantly prolong ventricular ERP and reduce reperfusion-induced VAs. Furthermore, 1 hour of GPS was able to cause a significant increase in HF and an evident decrease in LF/HF. All of these observations indicate that GPS might exert a VNS-like effect on hearts after I/R.

Gap-junction channels, which comprise highly homologous proteins known as connexins in the heart, have been implicated in electrical coupling. It has been widely accepted that alterations in gap junction organization and connexin expression might contribute to abnormal impulse propagation and arrhythmia during acquired heart disease [22]. Cx43, the principal component of ventricular gap-junction proteins, plays an important role in the mechanical stability, as well as the electrical and chemical coupling, of cardiomyocytes [23]. The reduction, dephosphorylation and lateralization of Cx43 reported in hearts with ischemia or heart failure were shown to be potential arrhythmogenic substrates [24]. Previous studies demonstrated that reductions in Cx43 might result in slowed conduction velocity and accelerated reperfusion-induced VA onset [25]. Furthermore, Cx43 was partially phosphorylated under physiological conditions and at the onset of ischemia [4]. Following prolonged ischemia, however, Cx43 dephosphorylation [26], a process associated with the opening of Cx43-formed hemichannels [4] and the translocation of Cx43 from the gap junctions toward the free plasma membrane [26] or an intracellular pool [13], was shown. Preventing the loss and dephosphorylation of Cx43 by VNS or gap junction blockers, however, was shown to be antiarrhythmic in previous studies [13, 27]. In the present study, the reduction and dephosphorylation of Cx43 combined with increased VAs were shown in the I/R group, and all of these

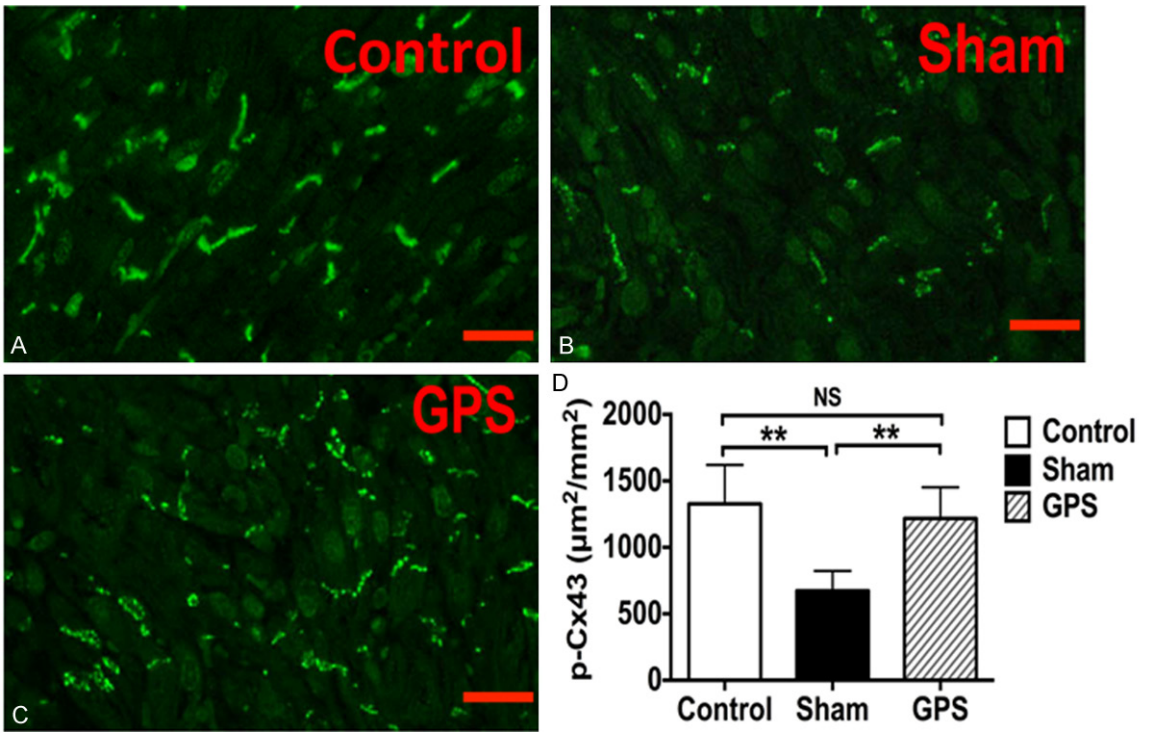

Figure 7. Immunofluorescence of p-Cx43 in the peri-infarct myocardium. NS, $P > 0.05$ versus the Control group; $^{**}P < 0.01$ versus the Sham group; other abbreviations are identical to those in **Figure 1**.

effects were significantly attenuated by GPS. All of these observations indicated that GPS might exert its antiarrhythmic effect by preventing the loss of Cx43 and a further study is needed to corroborate this.

The mechanism underlying the preservation of Cx43 and the antiarrhythmic effect of GPS remains unknown. Several potential mechanisms might be involved. First, the altered release of multiple neuromodulators may lead to anti-arrhythmic effects. For example, nitric oxide release, which is involved in higher connexin gene expression and the anti-ventricular fibrillation effects of VNS, could be induced by GPS [28, 29]. Second, Ando et al showed that VNS might block the degradation pathway and the dephosphorylation of Cx43 [13]. In the present study, the reduced expression and dephosphorylation of Cx43 induced by reperfusion were significantly attenuated by GPS. Therefore, it is possible that GPS can similarly prevent the degradation and dephosphorylation of Cx43 to maintain Cx43 at a normal level. Third, GPS is also postulated to suppress excessive inflammation. Rossi et al showed that 6 hours of GPS could reduce the postoperative inflammatory response, with significant reductions in interleukin-6, tumor necrosis factor-a, vascular endothelial growth factor and epidermal growth factor [30]. Our recent study also showed that GPS could decrease serum inflammatory factors [9].

We showed that GPS protected against VAs resulting from brief myocardial I/R as well as preserving Cx43. GPS induced by implanting a standard pacing lead in a suitable position for short-term neuromodulation may serve as a simple and feasible clinical approach to protect against VAs during coronary bypass surgery.

Limitations of this study included: First, fibrosis, inflammation and the degeneration of cardiomyocytes may also contribute to reperfusion-induced VAs in the ischemic heart [31]. The present study exclusively focused on the antiarrhythmic mechanism of GPS. We found that the underlying mechanism of GPS might be mediated by preserving the loss of Cx43, but this finding needs a further study to corroborate it and does not exclude other mechanisms. Second, functional changes in the gap junctions of the myocardial tissue were not measured in this study. Third, we only investi-

gated the acute impact of GPS on VAs. Whether these effects can be translated into a long-term benefit should be verified by further studies.

In conclusion, GPS significantly reduced VAs and the myocardial infarct size/AAR in hearts undergoing I/R. The salutary effect was accompanied by prevention of the loss and the dephosphorylation of Cx43.

Acknowledgements

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Disclosure of conflict of interest

None.

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