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Original Article

Intermittent low-level vagosympathetic nerve trunk stimulation inhibits ganglionated plexi activity to prevent atrial fibrillation

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Abstract: Background: The cardiac autonomic nervous system (CANS) plays a role in the occurrence and persistence of atrial fibrillation (AF). Low-level vagosympathetic nerve stimulation (LL-VNS) has been shown to inhibit the occurrence of AF. Objective: The novel objective of this study was to compare the effects of intermittent low- level vagosympathetic nerve stimulation (I-VNS) and continuous low-level vagosympathetic nerve stimulation (C-VNS). Methods: 19 beagles were randomly divided into 3 groups: Group A, rapid left atrial appendage pacing for 6 hours; Group B, rapid atrial pacing (RAP) for 6 hours and C-VNS (20 Hz, interval 0.1 ms, square wave) with 50% threshold voltage strength; Group C, RAP for 6 hours and I-VNS (continuously recurring cycles of 30-second ON, 30-second OFF). The atrial monophasic action potential (MAP) and the effective refractory periods (ERP) of the atrium and the pulmonary veins were measured at baseline, 1 hour, 3 hours and 6 hours after the experiment began. After the experiment, tyrosine hydroxylase (TH) and choline acetyl transferase (CHAT) expression levels in the anterior right ganglionated plexi (ARGP) from each group were measured. Results: Inter-group comparisons of MAP and ERP demonstrated that Group A was significantly different from Groups B and C ($P < 0.05$), while the difference between Groups B and C was not significant ($P > 0.05$). The MAP and ERP in Group A gradually decreased, reaching a minimum at 6 hours, but no significant changes were observed in Groups B and C. When compared to Group A, both Groups B and C had reduced TH and CHAT expression. Conclusions: During the occurrence and development of AF, I-VNS could protect the cardiovascular system, possibly replacing C-VNS. Additionally, both I-VNS and C-VNS inhibited ganglionated plexus (GP) activity during the AF prevention.

Keywords: Atrial fibrillation, vagosympathetic nerve, ganglionated plexus

Introduction

Atrial fibrillation (AF) is one of the most common arrhythmias and has severe, disabling and fatal complications. The current clinical drug treatments have unsatisfactory effects [1, 2]. In recent years, the focus of AF treatment and prevention has changed from radiofrequency ablation to vagosympathetic nerve stimulation (VNS), accompanied by various non-drug treatments.

In 1988, Jacob Zabarra first proposed the method of VNS in preventing and treating epilepsy [3]. Over the past two decades, VNS has become a relatively safe treatment for intractable epilepsy with few side effects and has been received by more than 100,000 patients [4, 5]. Additionally, because the cardiac autonomic nervous system (CANS) plays an essential role in the occurrence and persistence of AF, VNS is widely applied in basic research of AF prevention and treatment. Scherlag BJ, et al. has shown the effectiveness of Low-level vagosympathetic nerve stimulation (LL-VNS) in increasing the AF threshold of the atrium and the pulmonary vein. LL-VNS reduces the AF persistence period, lengthens AF circumference and reverses the “AF begets AF” phenomenon caused by rapid AF [6-8]. Yu, et al. further proposed that the role of LL-VNS in inhibiting AF was due to its inhibition effects on intrinsic CANS activity, analogous to ganglionated plexus (GP) damage by clinical ablation [9].

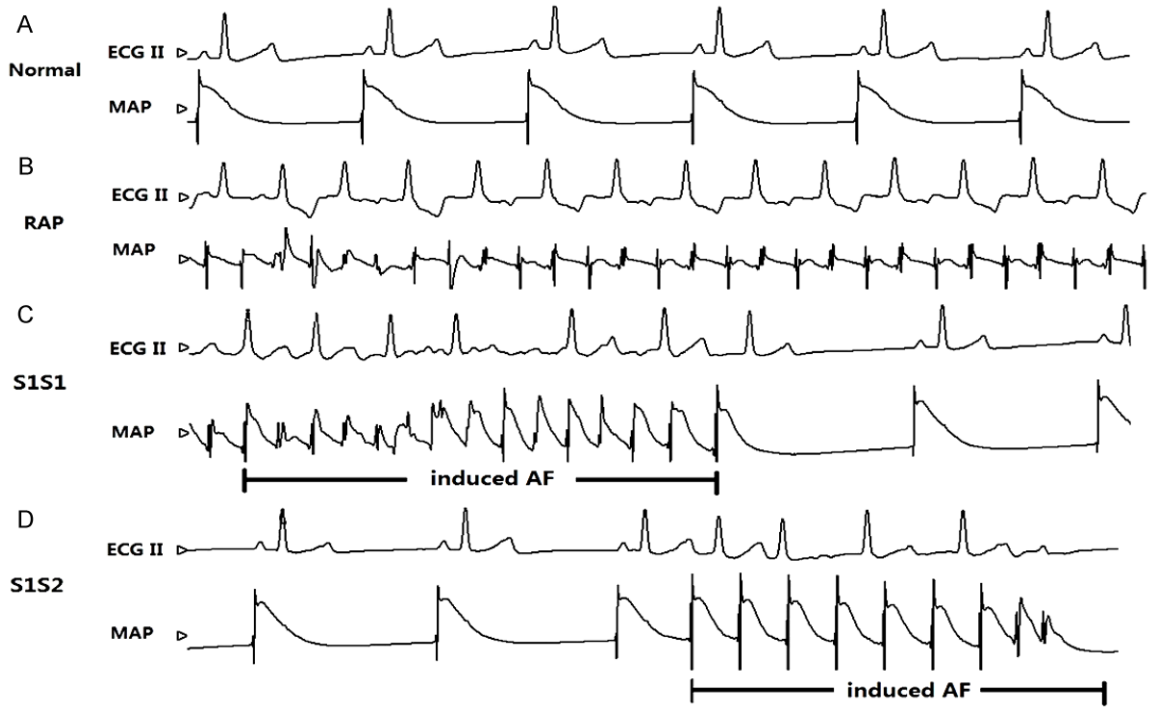

Figure 1. Atrial MAP record. A: Indicates atrial MAP recording the normal state; B: Means MAP of RAP (group A); C: Represents induced atrial fibrillation record of RAP (group A) by the S1S1 program; D: Represents induced atrial fibrillation record of RAP (group A) by S1S2 program.

Importantly, continuous low-level vagosympathetic nerve stimulation (C-VNS) has limited practical clinical application because continuous stimulation could induce rapid exhaustion of electrical energy. Even if C-VNS were used in clinics in the future, patients would require frequent surgeries for battery changes. Thus, the objective of this study was to compare the effectiveness of intermittent low-level vagosympathetic nerve stimulation (I-VNS) and C-VNS to find the optimal method and pathway for AF prevention and treatment.

Methods

Experimental animals

Our study was conducted with 19 healthy beagle dogs (mean body weight, 12.0 ± 1.5 kg; aged 1-2 years) provided by the First Affiliated Hospital of Xinjiang Medical University Animal Center. The study protocol was approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Xinjiang Medical University, China. All dogs were housed in an air conditioned room with good circulation and positive pressure ventilation. Each animal's body temperature was maintained at 36.5 ± 1.5°C using an insulation blanket. The dogs were anesthetized with sodium pentobarbital (30 mg/kg), after which, they received IV fluids and were catheterized. Each dog underwent a thoracotomy performed on both sides at the 4th intercostal space. Electrodes were sewn into the left atrial appendage to stimulate fast pacing, and multi-polar electrodes were sewn into the left and right atria, pulmonary veins for recording the effective refractory period (ERP) and monophasic action potential (MAP). The chest was then closed by suturing. After separating the vagus nerve on the right side of the neck, a needle was inserted parallel to the nerve to provide vagus nerve stimulation. Standard ECG leads were used to continuously record heart rate and rhythm. Parameters of oxygen saturation, arterial blood pressure, and other physiologic indices were routinely monitored.

Establishing the acute AF model and recording of electrophysiological data

The left atrial appendage (LAA) of each animal was sutured with electrodes to provide pacing (1200 HZ frequency, 0.5 ms pulse width, and 2-fold threshold voltage) for 6 hours, as recorded by a multi-conductive physiological recorder

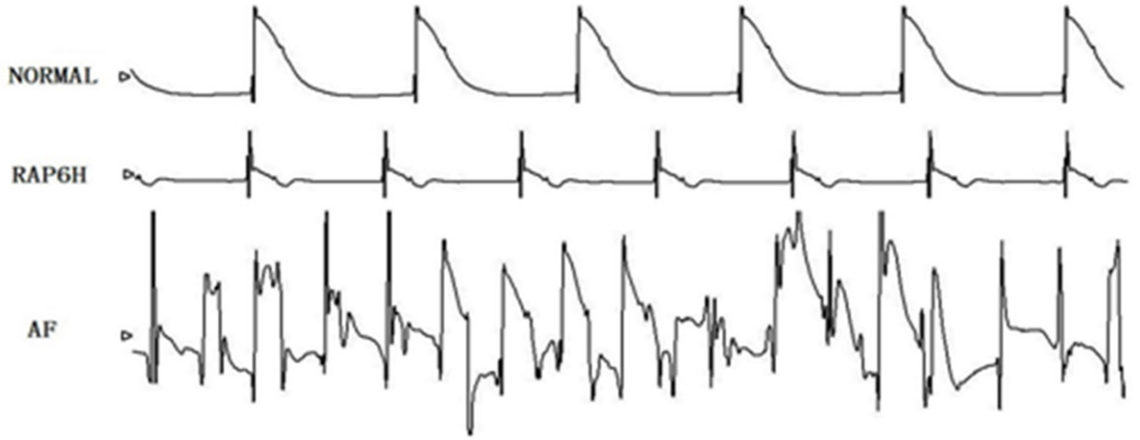

Figure 2. Atrial MAP record. While inducing AF, the cardiac cycle became irregular, the MAP was polymorphic and the diastolic interval between continuous MAPs disappeared.

(Lead-7000 EP Control, Sichuan Jinjiang, China). AF was defined as having more than 5 s of irregular 500 beats/min heart rate with an irregular atrioventricular conduction. The values for ERP were measured both before and after pacing. During ERP measurements, the pacing was paused, and ERP was measured using the S1S2 method (S1-S1 = 300 ms, S1: S2 = 8:1, V = 2 × Threshold), where the longest S1S2 interval failed to produce a response. The anterior walls of the left and right atrium were sutured with a multi-electrode for MAP recording under different conditions (**Figure 1**), and the multi-electrode was connected to a conductive physiological cardiac multi-metre (Lead-7000 EP amp, Sichuan Jinjiang, China). A 90% repolarisation of action potential duration (APD_{90}) based on MAP was used for data comparison.

Electrical nerve stimulation

Two silver wires (0.1-mm diameter) were inserted parallel to the trunk of the right sympathetic vagus nerve. The stem of the vagus nerve received high frequency electrical stimulation (20 Hz, interval 0.1 ms, square wave) from a Grass stimulator (S88X, Astro-Med Inc., Warwick, RI, USA). The lowest voltage at which VNS delayed sinus rhythm or promoted a second-degree atrioventricular block was considered the threshold voltage. The voltage used for LL-VNS was ~50% below the threshold. The effects of C-VNS and I-VNS (continuously recurring cycles of 30-second ON, 30-second OFF) was compared.

Immunohistochemistry studies

After 6 h of LL-VNS, samples of the anterior right ganglionated plexi (ARGP) were removed from each dog, fixed in 4% paraformaldehyde, and stored in 70% ethanol. The samples were then desiccated, embedded in paraffin, and cut into 5 um sections. Immunohistochemical staining was performed with antibodies against tyrosine hydroxylase (TH) and choline acetyltransferase (CHAT), using Rabbit polyclonal anti-TH (ab93291, Abcam, UK) and Cat polyclonal anti-CHAT (AB144P, Millipore, USA); Image-Pro Plus 6.0 image analysis software was used to determine positive area density, defined as the entire detection area divided by the area showing positive staining (um^2/mm^2). Each section was observed with a ×40 eyepiece, and three visual fields which displayed the highest levels of positive density were selected for analysis. The mean density of any given section was reported as the positive area density of that section.

Experimental protocol

Adult beagle dogs were randomly assigned to 3 groups: a rapid atrial appendage pacing (RAP) group (Group A, n = 6), a RAP + C-VNS group (Group B, n = 6), or a RAP + I-VNS group (Group C, n = 7). All three groups were tested for ERP in the atrium and pulmonary veins, and MAP values in the right and left atrium were recorded at baseline, and again after 1, 3, and 6 hours of atrial pacing. The animals were then sacrificed. The anterior right ganglionated plexi (ARGP) of each dog was removed and examined to deter-

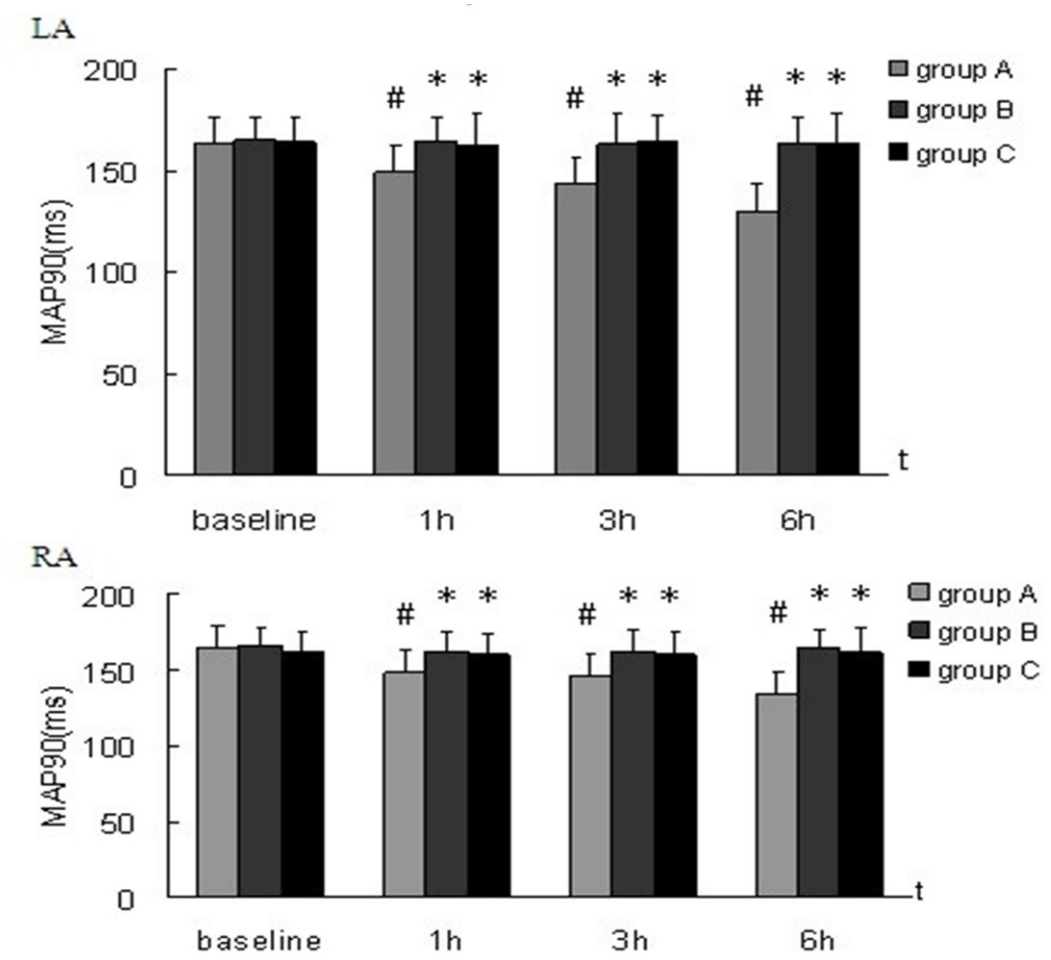

Figure 3. MAP in the three groups at different time points. *$P < 0.05$ comparing with group A; #$P < 0.05$ comparing with baseline. LA: left atrium; RA: right atrium.

mine expression of TH and CHAT by immunohistochemistry.

Statistical analysis

Statistical analyses were performed using SPSS for Windows, Version 16.0. Chicago, IL: SPSS Inc. All values are expressed as the mean ± standard deviation of the mean (SD). The mean ERP and MAP values at different time points were analyzed using analysis of variance for repeated measurements. Comparisons of TH and CHAT protein levels were performed by one-way ANOVA, while pair-wise comparisons between multiple groups were analyzed using the LSD method. *P*-values < 0.05 were considered statistically significant.

Results

Throughout the experiment, the blood pressure of the animals remained stable without any sign of heart failure caused by rapid pacing. The vagus nerve stem was kept moist without discoloration and changes from the stimulating effect, thereby confirming that the neural stem was uninjured.

The effect of LL-VNS on MAP variation

With an increased pacing period, the morphology of the control group changed significantly. The amplitude of vibration was decreased and the time limit was shortened. While inducing AF, the cardiac cycle became irregular, the MAP was polymorphic and the diastolic interval between continuous MAPs disappeared (**Figure 2**).

Inter-group comparisons showed significant differences in APD_{90} among the three groups. Specifically, Group A was significantly different from Groups B and C, but there was no significant difference between Groups B and C.

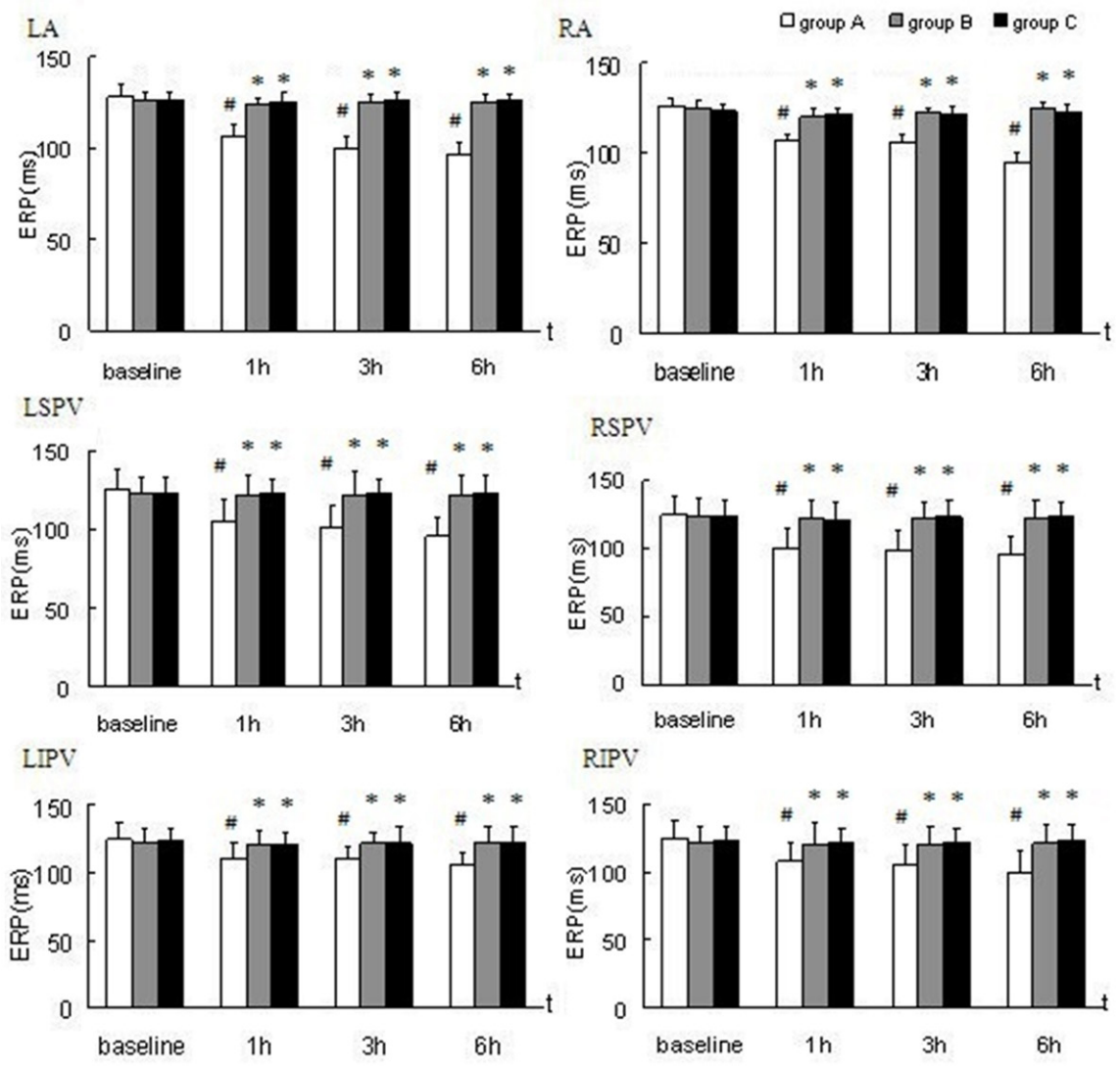

Figure 4. ERP in the three groups at different time points. ERP: effective refractory period; LA: left atrium; RA: right atrium; LSPV: left superior pulmonary vein; RSPV: right superior pulmonary vein; LIPV: left inferior pulmonary vein; RIPV: right inferior pulmonary vein. *$P < 0.05$ comparing with group A; #$P < 0.05$ comparing with baseline.

Intra-group comparisons also showed that the APD_{90} values of Group A at 1, 3 and 6 hours were significantly different from the baseline value, with the minimum APD_{90} value occurring at 6 hours. The APD_{90} values for Groups B and C were not significantly different when compared with the baseline value (**Figure 3**).

The effect of LL-VNS on ERP variation

Inter-group comparisons showed significant differences in ERP among the three groups. Specifically, Group A was significantly different from Groups B and C, but there was no significant difference between Groups B and C.

Intra-group comparisons also showed that the ERP values of Group A at 1, 3 and 6 hours were significantly different from the baseline value, with the minimum APD_{90} value occurring at 6 hours. The APD_{90} values for Groups B and C were not significantly different when compared with the baseline value (**Figure 4**).

TH and CHAT expression in the ARGP

Ganglionated plexi are an important component of the epicardial neural network, which plays an essential role in AF occurrence and persistence [10]. Under various conditions in this experiment, TH and CHAT protein expres-

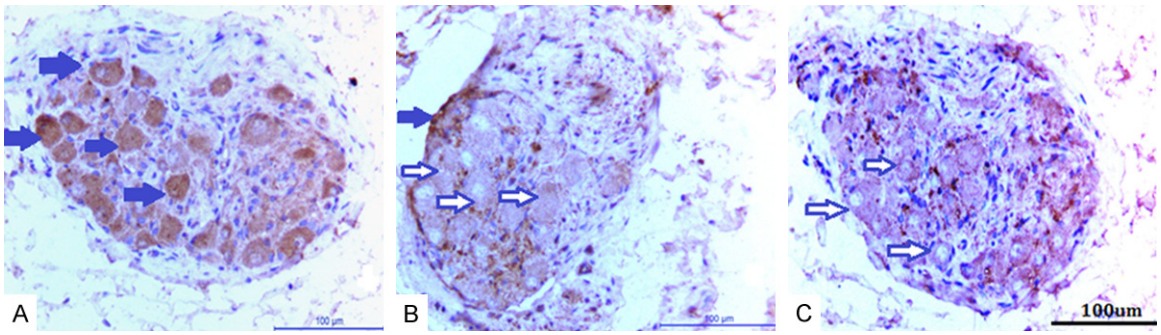

Figure 5. ARGP CHAT levels as shown by immunohistochemistry. A: ARGP CHAT staining in RAP (group A). B: ARGP CHAT staining in C-VNS (group B). C: ARGP CHAT staining in I-VNS (group C). The staining revealed several nigger-brown positive ganglion cells (blue arrow) and unpigmented negative ganglion cells (white arrow) of various shapes with blue cores.

sion levels in ARGP were measured by immunohistochemical staining. The staining revealed several nigger-brown positive ganglion cells of various shapes with blue cores (**Figure 5**).

The ARGP TH expression levels in Groups A, B and C were (25.63 ± 1.20) × 1000 $\mu m^2/mm^2$, (10.85 ± 3.24) × 1000 $\mu m^2/mm^2$ and (9.56 ± 3.12) × 1000 $\mu m^2/mm^2$, respectively. Group A was significantly different from Groups B and C ($P < 0.05$), while Groups B and C were not significantly different.

The ARGP CHAT expression levels in Groups A, B and C were (72.35 ± 4.65) × 1000 $\mu m^2/mm^2$, (30.47 ± 3.62) × 1000 $\mu m^2/mm^2$ and (33.36 ± 5.24) × 1000 $\mu m^2/mm^2$ respectively. Group A was significantly different from Groups B and C ($P < 0.05$), while Groups B and C were not significantly different (**Figure 6**).

Conclusions

Key findings

In this study, the effects of I-VNS on induced AF were proven. We showed that I-VNS could also prevent and block APD_{90} and ERP decreases. Additionally, both I-VNS and C-VNS reduced TH and CHAT protein expression in the ARGP. It was previously speculated that LL-VNS inhibited GP activity during AF prevention. Now, we have shown that I-VNS has similar effects as C-VNS on AF prevention.

LL-VNS effectively prevents AF

Surgical treatments for AF, such as isolation and ablation of the anterior pulmonary vein, have low success rates. Even with ablation of ganglionated plexus, the effectiveness of clinical surgery remains controversial. In addition to individual differences in AF patients, the accurate positioning of all ganglionated plexus is a formidable barrier [11, 12]. Therefore, discovery of other AF treatment approaches is an important research goal of scholars. As a result, non-drug and non-ablation methods like LL-VNS have gradually drawn public attention due to their effects on reversing and preventing AF [13, 14].

In this study, we found that with a prolonged pacing period, Group A had shortened ERP and APD_{90}. These results validated that the typical RAP-induced AF "electrical remodeling" model was successfully established [15]. Groups B (C-VNS) and C (I-VNS) had no significant differences during the different time intervals compared to the initial value, indicating that LL-VNS prevented AF caused by RAP-induced "electrical remodeling". This is contrary to the theory that AF is prone to occur and persist under VNS and demonstrates that VNS does not always induce AF. VNS of different strengths ultimately resulted in different cardiac effects. Our findings are consistent with the results from Sunny Po's team that LL-VNS could be used to inhibit high excitation of the local atrium to inhibit focal AF. The anti-arrhythmia function is independent from the increased activity of the vagus nerve afferent fiber. Consequently, LL-VNS is an important physiological regulation strategy for AF prevention and treatment [16].

Hemi-I-VNS effectively prevents AF

C-VNS is therapeutically effective in the treatment of epilepsy and heart failure [17, 18].

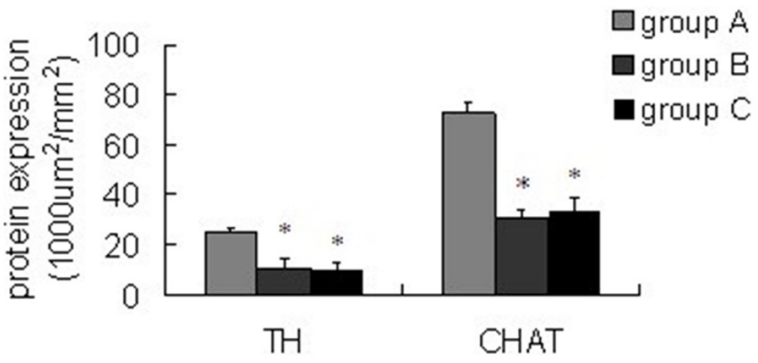

Figure 6. TH and CHAT expression levels in ARGP. ARGP: anterior right ganglionated plexi; TH: tyrosine hydroxylase; CHAT: choline acetyl transferase. *$P < 0.05$ comparing with group A.

Based on these data, Krekwit et al. reported that I-VNS could reduce myocardial infarction area and improve ventricular function [19]. Through intermittent stimulation of the vagus nerve rami auricularis of dogs suffering from myocardial infarction, Wang et al. found that non-specific inflammatory factor in plasma was decreased, and that left ventricular reconstruction was alleviated after myocardial infarction [20]. Although intermittent stimulation has beneficial effects in many diseases and is easy to use in clinics, there are no studies on the effectiveness of intermittent stimulation in AF prevention.

In this study, I-VNS was administered to a common AF model to determine if it had similar effects as C-VNS. We found that C-VNS and I-VNS caused insignificant differences in APD_{90} and ERP values and insignificant differences in TH and CHAT expression in ARGP. These data suggest that, similar to C-VNS, I-VNS also inhibits its variation in atrial electro physiologic indexes and TH and CHAT expressions in GP. Consequently, I-VNS inhibited the occurrence of AF. Since I-VNS reduced the stimulation time, it is a more practical treatment than C-VNS.

Nerve inhibition effects are a potential mechanism for AF electrical remodeling intervention

The mechanism by which LL-VNS prevents AF has been extensively discussed in various experiments. Sheng X, et al. proposed that LL-VNS could inhibit the activity of the cardiac intrinsic autonomic nervous system. This produces an obvious anticholinergic effect, preventing and reversing the RAP-induced acute atrial electrical remodeling [21]. Sha, et al. found that low-level unilateral VNS has dual anticholinergic and antiadrenergic functions, producing chronotropic effects on the vagus and autonomic nerves [22]. Shen MJ, et al. observed that chronic unilateral neck VNS could significantly reduce the sympathetic activity of stellate ganglion [23, 24]. All of these experiments confirm that LL-VNS, no matter whether it is unilateral or bilateral, intermittent or continuous, could inhibit atrial electrical remodeling and autonomic neural remodeling by inhibiting cholinergic and adrenergic nerve activities. After our experiment, the ARGP was selected to investigate the TH and CHAT expression in GP tissues. We found that under LL-VNS both TH and CHAT expression was decreased, further validating that LL-VNS affected plexus cardiac activity, which may be the mechanism of AF prevention.

Clinical significance

This study shows that the non-continuous VNS method can reverse AF that is induced by electrical remodeling. As a physiological regulatory approach to treating arrhythmia, I-VNS is advantageous over radiofrequency ablation because it poses no risk of damaging the cardiac conduction system. Furthermore, I-VNS avoids nervous fatigue due to continuous stimulation and also extends the VNS instrument's battery life. The effectiveness and safety of this method, however, needs to be further investigated in basic and clinical studies.

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Disclosure of conflict of interest

None.

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