Long-Term Effects of Ganglionated Plexi Ablation on Electrophysiological Characteristics and Neuron Remodeling in Target Atrial Tissues in a Canine Model

Running title: Wang et al.; Long-term effects of ganglionated plexi ablation

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Abstract:

Background - The long-term effects of ganglionated plexi (GP) ablation on electrophysiological characteristics and neuron remodeling in target atrial tissues remain unclear.

Methods and Results - Dogs in group 1 (control, n=8) were not subjected to GP ablation and observed for 1 month, and dogs in groups 2-4 (ablation groups, n=8 each) underwent ablation of the right-sided GP and observed for 1, 6, and 12 months, respectively. Atrial electrophysiological characteristics were examined before ablation, immediately and continuously after ablation. Target atrial tissues were subjected to immunohistochemical staining and Western blot analysis. Atrial effective refractory period (AERP) was significantly prolonged immediately after ablation (P<0.001), and persisted for 1 month (P<0.05). Nerve densities decreased 1 month after ablation (P<0.001). These parameters reverted to pre-ablation levels after 6 and 12 months. In the ablation groups, atrial fibrillation (AF) was induced in five of eight dogs after 1 month and in all animals after 6 and 12 months. AF was not observed in the control group and in the experimental groups immediately after ablation. Moreover, the expression of the growth-associated protein 43 was upregulated after ablation.

Conclusions - GP ablation effectively prolonged AERP for a short period, but the long-term effects on AERP and the suppression of AF induction were not persistent. Targeted atrial neuron remodeling may be an important mechanism underlying the observed electrophysiological changes.

Key words: electrophysiology, atrial fibrillation, catheter ablation, ganglionated plexi, neuron remodeling, atrial effective refractory period,
Introduction

Since the first report that paroxysmal atrial fibrillation (AF) is primarily initiated by ectopic foci originating from the pulmonary veins\(^1\), radiofrequency ablation that targets these veins, has become the most popular strategy to treat AF. Nevertheless, application of this process, also known as pulmonary vein isolation, is constrained by its associated risks and high recurrence\(^2-4\).

Many basic and clinical studies on AF\(^5,6\) have suggested that rapid firing is possibly initiated by activation of the intrinsic cardiac autonomic nervous system, this ectopic impulse may be effectively eliminated through ablation of the main ganglionated plexi (GP), which are embedded in fat pads. Although many recent studies utilize catheter ablation of GPs to eliminate AF\(^7,8\), the efficacy of this procedure remains controversial\(^9-11\), and the long-term effect on AF has not been elucidated. For example, Oh et al\(^12\) reported that radiofrequency ablation of fat pads may not achieve long-term suppression of AF induction in a canine model. By contrast, Pokushalov\(^13\) and Calo\(^14\) confirmed that GP ablation in humans could be an efficient treatment for AF.

Many researchers have investigated the role of cardiac autonomic nerve remodeling in AF recurrence. Sakamoto\(^15\) observed that vagal effects were restored at 4- weeks after GP ablation, which indicates the occurrence of early atrial reinnervation. Zhao\(^16\) also demonstrated that atrial autonomic innervation remodeling easily induced AF after GP ablation. However, a study\(^17\) on GP ablation of paroxysmal AF did not manifest neuron remodeling after a 12-month follow-up. Similarly, Po\(^18\), found that destroyed autonomic neurons in the GP do not regenerate. Given the contrasting results of previous studies, in the present study, we ablated the anterior and inferior right GP in a canine model to explore long-term effects on electrophysiological...
characteristics and neuron remodeling in target atrial tissues. We also explored the role of cardiac automatic neuron remodeling in induced AF.

Methods

Animal Preparation

Thirty-two conditioned dogs of both sexes weighing 14-16 kg were divided into four groups (n=8 in each group). Dogs in group 1 (control group) underwent right thoracotomy at the fourth intercostal space without GP ablation. Groups 2-4 (ablation groups) underwent right thoracotomy, followed by GP ablation. Groups 1 and 2 were observed for 1 month, group 3 for 6 months, and group 4 for 12 months. This experiment was approved by the Institutional Animal Care and Use committee.

The animals were intravenously anesthetized with 3% sodium pentobarbital (30 mg/kg), and ventilated with room air. Supplemental doses were administered to maintain stable anesthesia. A catheter introducer was inserted into the right femoral artery to monitor arterial blood pressure. Continuous surface electrocardiograph leads (I, II, and III) and intracardiac tracings were recorded. Under sterile surgical conditions, the chest was opened transversely at the right fourth intercostal space to expose the right atrium and a pericardial cradle was created.

GP ablation

In the ablation groups (group 2-4), radiofrequency current was delivered to the two exposed major right-sided GPs (anterior and inferior right GPs, Figure 1), by using a 7Fr deflectable quadripolar catheter with a 4-mm tip electrode. The catheter tips were positioned manually on
the epicardial surface of the GP under direct visual control to ensure optimal tissue contact and energy delivery. Radiofrequency was delivered at a power of 20-30 W for 30-60s at each ablation point. The catheter tip was flushed with small amounts of saline during epicardial delivery of radiofrequency current to avoid increased impedance and tissue charring. The end-point of ablation was defined as the disappearance of vagal response induced by high-frequency stimulation in the GP area.

**Electrophysiological study**

Three bipolar electrodes were placed at the right atrial free wall (FRA) close to the anterior right GP, high right atrium (HRA) away from the anterior right GP, and right atrial appendage (RAA) (Figure 1). All pacing electrodes were in close contact with atrial tissues during the experiment. The pacing electrodes were removed after the initial study (baseline) and replaced during the final experiment (on months 1, 6, and 12). Programmed electrical stimulation was performed using the LEAD-7000 electrophysiology management system (Sichuan Jinjiang Electronic Science and Technology Co., Ltd., Chengdu, China). The drive train contained eight stimuli at a basic cycle length of 300 ms, followed by premature extra stimulus (S2) at a coupling interval of 200 ms with 5 ms step reductions. Atrial effective refractory period (AERP) was defined as the longest S1-S2 interval that failed to produce a propagated response. Data before ablation, immediately after ablation and follow-up after ablation were collected from each animal. AF inducibility was defined as the percentage of the total attempts, as evaluated by S1S1 (120 ms cycle length) programmed stimulus method. The duration of S1S1 stimulation was 10 s. AF was defined as the fast atrial rate (>500 bpm) that persisted for at least 10 s after the end of burst
stimulation. The S1S1 stimulus program was performed twice on the FRA, close to the anterior right GP of the right atrium in each dog. AERP and AF inducibility were determined pre-ablation, immediately after ablation and continuously after ablation for each animal.

**Tissue preparation**

Yuan reported that in the canine intrinsic cardiac nervous system, atrial plexuses which are interconnecting nerves between the ganglia in GP, form complete loops that are -5 mm to 2 cm in diameter. Thus, we defined ~1 cm of tissue away from the GP as the target atrial tissue. After electrophysiological studies were conducted, the target atrial tissues were obtained from all animals. Some tissues were fixed in 4% formalin for at least 24 h, and the remaining tissues were stored in liquid nitrogen for Western blot analysis.

**Immunohistochemistry studies**

Immunohistochemical staining was performed as described previously for tyrosine hydroxylase (TH, a sympathetic nerve index) and choline acetyltransferase (CHAT, a parasympathetic nerve index) in all the groups. Nerve density was determined using a computer-assisted image analysis system (Image-Pro Plus 6.0, Media Cybernetics, Inc., Rockville, MD, USA). Three fields with the highest nerve densities were selected under a microscope and averaged to represent the nerve density of the slide. A computer calculated the number and area occupied by the nerves in the field.

**Western blot analysis**

The expression of TH, CHAT, and growth-associated protein 43 (GAP43) was assessed using Western blot analysis as described previously. The density of the bands on the Western blots...
was quantified using a Tanon-4500SF image analysis system (Tanon Science & Technology Co., Ltd., Shanghai, China).

**Statistical analysis**

All values are presented as medians with first and third quartiles. AERPs between pre-ablation, and immediately after ablation, 1 month, 6 months and 12 months in ablation groups (group 2-4) were compared using a generalized estimated equation method. ANOVA with Bonferroni test was used for multiple comparisons of nerves densities positive for TH and CHAT and protein expression of GAP43, TH and CHAT between the control group and ablation groups. All analyses were performed with SPSS v17.0 software (SPSS, Inc, Chicago, IL, USA). Two-sided probability value $P<0.05$ was considered statistically significant.

**Results**

1. **Status of animals after surgery**

Twenty-nine dogs were stable throughout the postoperative period and the statistics were based on these dogs. One dog died of over-anesthesia 4 h after GP ablation, one dog died of severe infection 2 weeks after GP ablation, and one dog died of diarrhea 6 weeks after ablation. Overall, seven dogs completed the experimental protocol in group 1, six dogs in group 4 and eight dogs in groups 2 and 3.

2. **Effect of GP ablation on AERP**

Figure 2 shows AERP variations at pre-ablation and at different time points after ablation at three sites. AERP was significantly longer immediately after GP ablation than that at pre-ablation at all
sites. Specifically, the ERP at the HRA prolonged from 105 ms [96.25, 108.75] to 120.0 ms [110.0, 125.0] (P<0.001). The ERP at the FRA prolonged from 100.0 ms [95.0, 110.0] to 120.0 ms [110.3, 120.0] (P<0.001). The ERP at the RAA prolonged from 110.0 ms [100.0, 115.0] to 125.0 ms [120.0, 130.0] (P<0.001). These AERP variations persisted at all sites within 1 month after GP ablation (P=0.005, P=0.003, and P=0.022; Figure 2, pre-ablation vs. 1 month after ablation). At 6 months after GP ablation, the ERP in the right atrium reverted to pre-ablation levels and was not significant (Figure 2; 6 months vs pre-ablation): 105.0 ms [103.8, 110.0] versus 105 ms [96.25, 108.75] on the HRA (P=0.286), 110.0 ms [100.00, 110.00] versus 100.0 ms [95.0, 110.0] on the FRA (P=0.084), 115.0 ms [113.8, 121.3] versus 110.0 ms [100.0, 115.0] on the RAA (P=0.838). These AERP recoveries persisted for 12 months after ablation, and were not significantly different from those observed at pre-ablation, with data as follows (Figure 2, 12 months vs. pre-ablation): 97.5 ms [87.5, 100.0] versus 105.0 ms [96.25, 108.75] on the HRA (P=0.154), 97.5 ms [95.00, 100.00] versus 100.0 [95.0, 110.0] on the FRA (P=0.073), 102.5 ms [92.5, 105.0] versus 110.0 [100.0, 115.0] on the RAA (P=0.051).

3. Effect of GP ablation on AF inducibility

In the control group, AF was not induced after right thoracotomy and after 1 month. Similarly, the premature extra stimuli did not induce AF before or immediately after GP ablation in all ablation groups. However, AF inducibility significantly increased 1 month after GP ablation. In particular, AF was induced in five of eight dogs after 1 month in group 2, and in all animals after 6 months in group 3 and after 12 months in group 4.
4. Nerve density

Immunohistochemical staining was conducted to determine the nerves positive for TH and CHAT in group 1 and at different time points after ablation (groups 2-4). At 1 month after GP ablation, TH- and CHAT-positive nerves in the right atrium were significant reduced compared to the control group (figure 3-4, 1 month vs. control group), with data as follows: for TH, 803.5 um²/mm² [695.25, 916.5] versus 1764.0 um²/mm² [1652.25, 1843.75] (P<0.001), and for CHAT, 805.0 um²/mm² [700.0, 905] versus 1793 um²/mm² [1671.5, 1843.5] (P<0.001). However, the densities of TH- and CHAT-positive nerves between 1 and 6- months were comparable (TH, P<0.001; CHAT: P<0.001). The expression of TH and CHAT was higher at the end of follow-up in groups 3 and 4 than that in group 2. The densities of the two types of nerves were not significantly different between 6 months after ablation and control group and between 12 months after ablation and control group (TH-positive nerve density at 6 months vs. control group, P = 0.066, 12 months vs. control group, P= 0.055; CHAT-positive nerve density at 6 months vs. control group, P =0.057, 12 months vs. control group, P = 0.054). Figures 3- and 4 demonstrate the diffuse and heterogeneous CHAT- and TH-positive nerves after GP ablation, respectively.

5. Protein expression of GAP43, TH and CHAT

The protein expression levels of GAP43 was upregulated 6 and 12 months after ablation compared with that in the control group, whereas no significant difference was found between the control and ablation groups after 1 month (6 months vs. control group, P<0.001; 12 months vs. control group, P<0.001, 1 month vs. control group, P= 0.083, Figure 5C). GAP43 expression was significantly higher 6 months after ablation than that at 1 month after ablation (6 months vs.
1 month, \( P < 0.001 \), Figure 5C). Compared with the control group, the expression of TH and CHAT in the ablation groups were downregulated 1 month after GP ablation (TH, 0.702 [0.649, 0.807] vs. 1.874 [1.654, 1.935], \( P < 0.001 \); CHAT, 0.773 [0.735, 0.817] vs. 1.795 [1.737, 1.838], \( P < 0.001 \)), the lowest expression was observed 1 month after ablation among the different time points (Figure 5). The protein expression levels of TH and CHAT were upregulated at the end of follow-up in groups 3 and 4 compared with that in group 2. No significant difference in the densities of the two types of nerves was observed between the control group and the ablation group after 6 months and between the control group and the ablation group after 12 months (TH protein expression at 6 months vs. control group, \( P = 0.141 \), 12 months vs. control group, \( P = 0.065 \); CHAT protein expression at 6 months vs. control group, \( P = 0.114 \), 12 months vs. control group, \( P = 0.09 \)).

Discussion

Major findings

The main findings in this study are as follows: (1) All ERPs in the right atria significantly prolonged immediately after GP ablation and persisted in all sites in the right atrium at 1 month after GP ablation. However, these AERP variations disappeared and the values reverted to the pre-ablation levels after 6 and 12 months. AF was not induced before or immediately after GP ablation in all groups. AF inducibility increased 1 month after GP ablation, and it was much more inducible after 6 and 12 months compared with that in the control group. (2) The densities of cholinergic and adrenergic nerves decreased in the right atrium at 1 month after GP ablation,
but reverted to baseline after 6 and 12 months. Nerve fiber regeneration was observed after 6 months. GAP43 protein expression was upregulated 1 month after GP ablation, whereas it was much higher at 6 and 12 months after ablation than that in the control group.

**Changes in the electrophysiological characteristics in the target atrial tissues after GP ablation**

GP consists of ganglia and fibers that are distributed throughout the heart\(^{21}\). Numerous studies have observed multiple major and small GPs in the atria of mammalian hearts \(^{19,22-24}\). These GPs modulate the interaction and balance between the extrinsic and intrinsic autonomic nervous system and contain efferent cholinergic and adrenergic neurons which influence the atrial myocardium\(^{25}\), in terms of action potential duration, AERP, and conduction velocity. In many animal studies, autonomic denervation exhibited short-term efficacy\(^{26-28}\). The effectiveness of denervation on vagal stimulation of the sinus node, atrioventricular node, and AERP was significantly eliminated immediately after GP ablation. These denervation effects disappeared after 4 weeks\(^{12}\). In the present study, AERP was significantly prolonged immediately after GP ablation, which could be due to the effect of GP ablation on the autonomic nerve tone. Changes in the AERP also persisted for 1 month after GP ablation but reverted to baseline after 6 and 12 months. This result suggests that GP ablation does not exhibit long-term effect of prolonging AERP.

Ablation targeting the intrinsic cardiac autonomic nervous system has been shown to improve the success rate of AF ablation\(^{9,14}\). However, an animal study\(^{27}\) reported ablation of the right pulmonary vein-atrial junction fat pads facilitated rather than prevented the initiation of AF.
Another clinical study assessed the long-term outcome of patients who underwent anatomic GP ablation for paroxysmal AF\textsuperscript{29}, results demonstrated that anatomic GP ablation could only achieve short-term suppression of AF inducibility; however, this result was not maintained over the long-term. In the present study, AF was not induced before and immediately after GP ablation. Nevertheless, AF inducibility significantly increased over a long-term period after GP ablation indicating that the long-term effects of GP ablation on AF inducibility must be further evaluated.

**Autonomic neuron remodeling in target atrial tissues after GP ablation**

An animal study\textsuperscript{15} has demonstrated that GP ablation significantly reduced atrial vagal innervation immediately after ablation, but innervation reverted to baseline after 4- weeks. A clinical report by Pappone et al\textsuperscript{7} indicated that the use of vagal denervation with radiofrequency current to prevent recurrent AF could alter heart rate variability parameters, but these parameters returned to pre-ablation levels after 6 months. These findings suggest that the atrial autonomic neuron remodeling occurs after GP ablation or autonomic denervation. Although the potential mechanisms of neuron remodeling remain unclear, they may be explained by the following factors: (1) incomplete ablation as GP ablation only eliminates major GPs, does not affect other GPs, or only injures bypassing nerve axons without affecting the body of nerve cells; (2) neuroreceptor hypersensitivity, which has been documented in a canine model with parasympathetic denervation\textsuperscript{30} and in heart failure models with damaged ganglionic neurotransmission\textsuperscript{31}; and (3) autonomic regeneration. Another animal study\textsuperscript{16} observed the occurrence of atrial autonomic neuron remodeling 8 weeks after GP ablation. Pokushalov et al\textsuperscript{32} conducted radiofrequency ablation in anatomic GP sites in 56 patients with paroxysmal AF and
found that heart rhythm/min, heart rhythm/mean, and the low frequency: high frequency ratio increased immediately after ablation; nevertheless, these values returned to baseline 6 months after the procedure. In the present study, the protein expression of TH and CHAT in target atrial tissues was significantly downregulated 1 month after GP ablation, indicating that the denervation effects were completed within a short period after GP ablation. Moreover, the sympathetic and parasympathetic nerves were significantly regenerated for a long period. This nerve sprouting and regeneration phenomena were also detected in infarcted hearts\textsuperscript{33} and heart transplant cases\textsuperscript{34, 35}.

GAP43, a protein expressed in the growth cones of sprouting axons, is a marker for nerve sprouting. Zhou\textsuperscript{33} observed the upregulated GAP43 expression in infarcted hearts and they confirmed that GAP43 was retrogradely transported to the left stellate ganglion, thereby inducing nerve sprouting in the noninfarcted free wall of left ventricle and, to a lesser degree, at the infarcted site. We also observed a robust upregulation of GAP43, accompanied by nerve regeneration in the target atrial tissues at 6 and 12- months after GP ablation. This phenomenon suggests that upregulated GAP43 expression is responsible for atrial autonomic reinnervation in canine models. The potential mechanism of GAP43 inducing autonomic reinnervation must be further investigated.

**Long-term effects of autonomic neuron remodeling on electrophysiological characteristics**

The initiation and maintenance of AF greatly depend on variations in the autonomic tone. Vagal nerve activation or stimulation may result in formation of reentrant circuits. In this study, the densities of TH- and CHAT-positive nerves significantly decreased in target atrial tissues at 1...
month after GP ablation, which indicates that the balance between the cholinergic and adrenergic nerves was destroyed. This imbalance led to an increased dispersion of atrial refractoriness and repolarization, resulting in short-term AF induction. We considered that regeneration of TH and CHAT-positive nerves after 6 and 12 months contributed to the recovery of AERP, which may play an important role in AF initiation. These findings suggest that GP ablation prolonged AERP for a short period, but failed to maintain long-term effects on AERP and on suppression of AF induction. Targeted atrial autonomic neuron remodeling may be an important mechanism underlying the electrophysiological changes observed.

Clinical implications

This study demonstrated that GP ablation did not cause the long term suppression of AF. The efficacy of GP ablation in preventing AF must be reevaluated.

Study limitations

In this study, we did not ablate the two left-side GPs located adjacent to the base of the left superior and left inferior pulmonary veins because of the long-term survival rate of the animals. Nevertheless, our findings are conclusive because we focused on atrial tissues targeted by GP ablation.

Conclusion

Right-sided GP ablation produced short-term effects on prolonging AERP. Nevertheless, long-term AERP prolongation and suppression of AF induction was not be achieved by GP ablation in the present canine model, which could be due to targeted atrial autonomic neuron
remodeling. Importantly, these findings raise concern regarding the long-term effect of right-sided GP ablation for treating AF.

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**Conflict of Interest Disclosures:** None.

**References:**


Figure Legends:

Figure 1: Ganglionated plexi location and recording sites. A: Yellow areas indicate ARGP and IRGP. Asterisks indicate the three sites at which the recording/pacing electrodes were placed: right atrial free wall, high right atrial and right atrial appendage. Right thoracotomy approach: B: ARGP located at the RSPV–atrial junction; C: IRGP located at the junction of the IVC and both atria. ARGP= anterior right ganglionated plexi; RSPV= right superior pulmonary vein; IRGP= inferior right ganglionated plexi; IVC= inferior vena cava; SVC = superior vena cava; RIPV= right inferior pulmonary vein.

Figure 2: ERP in the right atrium of dogs at three sites (A: HRA; B: FRA; C: RAA) determined at pre-ablation and immediately after ablation, as well as 1, 6, and 12 months after ablation. Data are expressed as medians with first and third quartiles. ERP of right atrium was prolonged immediately after ganglionated plexi ablation at all sites and maintained within 1 month of ablation. However, these changes disappeared and recovered to the pre-ablation levels after 6 and 12 months. ERP = effective refractory period; HRA = high right atrium; FRA = right atrial free wall; RAA = right atrial appendage.

Figure 3: Examples of TH immunostaining. A: group 1, B: group 2, C: group 3, D: group 4. E: Density of TH-positive nerves in the target atrial tissues at different time points. Data are expressed as medians with first and third quartiles. TH-positive nerves were significantly lower
at 1-month after ablation compared with that in the control group. TH positive nerves were higher 6 and 12 months after ablation compared with that at 1 month. Magnification of objective lens: ×20. TH = tyrosine hydroxylase.

**Figure 4:** Examples of CHAT immunostaining. A: group 1, B: group 2, C: group 3, D: group 4. E: Density of CHAT-positive nerves in the target atrial tissues at different time points. Data are expressed as medians with first and third quartiles. CHAT-positive nerves were significantly lower 1 month after ablation compared with that in the control group, CHAT positive nerves were higher 6 and 12 months after ablation compared with that at 1 month. Magnification of objective lens: ×20. CHAT= choline acetyltransferase.

**Figure 5:** Expression levels of (A) TH, (B) CHAT and (C) GAP43 in the target atrial tissues at different time points in the control and ablation groups. Data are expressed as medians with first and third quartiles. The expressions levels in the cholinergic and adrenergic nerves were significantly lower 1 month after ablation, which reverted to baseline after 6 and 12 months. The GAP43 protein expression had no significant difference between 1 month after ablation and control group, whereas it was much higher at 6 and 12 months after ablation than that in the control group. GAP43 = growth-associated protein 43.
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