Original article

Differential densities of cholinergic nerves in canine supraventricular regions of hearts

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A R T I C L E   I N F O

Article history:
Received 5 August 2012
Received in revised form 13 November 2012
Accepted 4 December 2012
Available online 9 February 2013

Keywords:
Cholinergic nerve
Histology
Atrial fibrillation
Innervation

A B S T R A C T

Purpose: Cholinergic nerve plays an important role in the induction and maintenance of atrial fibrillation (AF). Cholinergic innervation at supraventricular tissues is considered to be the histiological basis and intervention-associated target site for the arrhythmia; however, the distribution of cholinergic nerve in supraventricular tissues has not been clearly studied. In this study, we investigated the cholinergic nerve innervation in canine supraventricular regions of hearts.

Methods: We performed histological and immunohistochemical staining on canine tissues of left atrial appendage (LAA), right atrial appendage (RAA), left atrium (LA), right atrium (RA), atrial septum (AS), crista terminalis (CT), pulmonary vein (PV), and super vena cava (SVC) using hematoxylin and eosin (H&E) and antibodies to choline acetyltransferase.

Results: Normal canine cardiovascular histological structures were shown from H&E staining. Cholinergic nerve densities at LAA and RAA were significantly higher than LA, which was higher than RA, but no significant difference was observed between LAA and RAA. Furthermore, RA was significantly higher than AS, CT, PV, and SVC and there were no significant differences among the latter four.

Conclusion: The heterogeneity of different densities of cholinergic nerve innervation of canine supraventricular regions establishes the histological basis of cholinergic nerve-mediated pathological conditions.

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Introduction

The cholinergic nerve system in mammals is a crucial determinant in producing and maintaining atrial fibrillation (AF) [1–9]. Vagal stimulation (VS) is able to induce and maintain the processes of AF; on the contrary, catheter ablation of the cardiac parasympathetic nerves obviously contributes to eliminate vagally mediated AF. Furthermore, adjunctive complete vagal denervation during circumferential pulmonary vein (PV) ablation for rescuing AF significantly reduces the recurrence rate of AF [10]. On the other side, the perfusion of the cholinergic neurotransmitter, acetylcholine (ACh), is capable of initiating atrial tachyarrhythmias while the environment that lacks ACh prevents AF [11–13]. ACh binds to M2 receptors and then triggers G protein to activate the ACh-activated potassium channels (Kir3) which induce ACh-induced inward rectifier potassium current (I\textsubscript{K,ACh}) [14,15].

However, the mechanisms of cholinergic nerves working on AF have not been studied in great detail. An increasing number of researchers insist that the heterogeneous nature of supraventricular regions contributes to the mechanisms: for example, heterogeneous vagal innervation enables VS to initiate re-entrant atrial tachyarrhythmias by enhancing the dispersion of refractoriness which is attributed to increased spatial dispersion of refractoriness [16]; a greater quantity of Kir3 channels and higher density of I\textsubscript{K,ACh} in left atrium (LA) than right atrium (RA) generate more ACh-induced speeding-up of rotors in LA than that in RA [17]; and differential densities of M2 receptor and I\textsubscript{K,ACh} are different among atria, atrial appendages, PV, and super vena cava (SVC) and reduced I\textsubscript{K,ACh} dispersion is the mechanism for amiodarone to treat AF [18].

The cholinergic innervations at different regions of heart supraventricular structures have not been reported yet. We have measured the cholinergic nerve distribution densities at atrial appendages, atria, atrial septum (AS), crista terminalis (CT), PV, and SVC which comprise the supraventricular structures of hearts using...
hematoxylin and eosin (H&E) and antibodies to choline acetyltransferase (ChAT) staining techniques.

Materials and methods

Experimental animals

Six adult health male dogs, weighing 12–15 kg, were used for experimental research. This study was performed according to the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication no. 85-23, revised 1996). The protocols of the study were approved by the Ethical Committee of Wuhan University in China. Animal handling was carried out according to the Wuhan Directive for Animal Research.

Atrial preparations

Dogs were abdominally anesthetized with pentobarbital sodium (30 mg/kg). Using median sternotomies, hearts with normal size were quickly excised and removed. Then we placed the hearts in Tyrode’s solution equilibrated at 36 ± 0.5 °C with 95% O2 and 5% CO2 to clean the remaining blood. The ingredients of Tyrode’s solution are (mM): NaCl 137, NaHCO3 15, KCl 4, CaCl2 2.7, MgCl2 0.5, NaH2PO4 0.5, and glucose 11 and adjusted the pH value to 7.4.

Tissue processing

Hearts were fixed after the procedure at room temperature in 4% paraformaldehyde phosphate-buffered saline (PBS) solution overnight. Each supraventricular part of the hearts was sectioned into eight pieces according to the anatomical boundaries: LAA, RAA, LA, RA, AS, CT, PV, and SVC.

Histology

Each piece of tissue was dehydrated through a series of graded ethanol, cleared in xylene, and processed for embedding into paraflin block, following routine protocols. Blocks were cut by microtome into 5-μm-thick sections and mounted on glass slides. For each paraffin block, one slide was stained with H&E at an original magnification of 200× to show the histopathological structure of the heart.

Immunohistochemistry

For immunohistochemistry, mounted tissue sections were deparaffinized in xylene, and rehydrated through a series of graded ethanol. In order to retrieve antigen activity in the tissues, sections were incubated with citric acid buffer (pH 6.0) for 30 min at 95 °C in a water bath. Sections were incubated for 30 min using 3% H2O2 in order to block the endogenous peroxidase. Non-specific binding sites were blocked by incubation with normal sera. Rat monoclonal antibody to ChAT (Calbiochem, Merck KGaA, Darmstadt, Germany) diluted in PBS for immunocytochemical staining was used to label cholinergic nerves [19]. The tissues were stained in the same session.

Structural analysis

We determined nerve densities with a computer-assisted image analysis system (Image-Pro Plus 3.0, Media Cybernetics, Carlsbad, CA, USA). Each slide was examined under a microscope to select five fields with the highest density of nerves. The computer then automatically calculated the area occupied by the nerves in the field. We divided the each ChAT positive area by the total area on each microscope field and set the value as the nerve density (μm²/mm²). The mean density of nerves in these five selected fields was used to represent the nerve density of that slide.

Statistical analysis

Values are shown as mean ± SEM. Data analysis was performed using Origin 7 (Originlab, Northampton, MA, USA) software and statistical comparisons were made by ANOVA. Statistical significance was assumed if p-values were less than 0.05. Statistically significant results (p < 0.05) are indicated in the figures by an asterisk (*).

Results

Histopathologic structures of supraventricular regions are normal

As expected, normal histopathologic structures were exhibited in the supraventricular regions of experimental animals. A longitudinal section of H&E-stained cardiac tissue is presented in Fig. 1. H&E staining demonstrated the branching nature of cardiac muscle fibers from different supraventricular regions. Cytoplasm and large central nuclei are evenly stained and clearly displayed. Striations within myocytes and intercalated discs between myocytes are visible and there are numerous blood vessels between the myocytes.

Cholinergic nerves are distributed at different densities among supraventricular regions

Fig. 2 shows that examples of nerves in each supraventricular region immunoreactive to ChAT were stained as dark brown twigs. ChAT immunohistochemical staining showed that cholinergic nerve densities of LAA and RAA are the highest among all eight supraventricular regions, in addition, there is no difference between that of LAA and RAA (Fig. 2A and B). The next highest was LA (Fig. 2C) and then RA (Fig. 2D). Furthermore, the cholinergic nerve density of RA is significantly higher than the remaining four regions of supraventricular structures, which are AS, CT, PV, and SVC (Fig. 2E–H). However, the cholinergic nerve innervations located at AS, CT, PV, and SVC were comparable. Fig. 3 shows the mean cholinergic nerve densities compared with the eight regions of heart supraventricular structures, respectively.

Fig. 1. Histopathologic structure of supraventricular tissues is normal (hematoxylin and eosin staining).
Discussion

We used histochemical and immunohistochemical staining on canine tissues of atrial appendages, atria, AS, CT, PV, and SVC by H&E and antibodies to ChAT staining. As expected, normal histopathologic structures were illustrated in the supraventricular tissues of experimental animals. However, cholinergic nerve densities were different in different regions. LAA and RAA were higher than LA, which was higher than RA. But RA was higher than AS, CT, PV, and SVC. Furthermore, there were no significant differences...
Statistically, cholinergic conduction is observed to attenuate pro-arrhythmic responses. This is especially true for atrial appendages, which are more susceptible to vagal stimuli. This is further supported by research showing that vagal nerve innervation is highest in the atria compared to other regions of the heart. The higher density of cholinergic nerves in the atria suggests a significant role in promoting and maintaining atrial fibrillation (AF).

Cholinergic nerve plays a significant role in promoting and sustaining AF

The relationships between cholinergic nerves and AF have long been investigated, with a focus on the mechanisms of cholinergic nerves working on AF. To compare sympathetic and cholinergic effects on atrial effective refractory period (ERP) and reentrant wavelength, Liu and Nattel [5] discovered that sympathetic stimulation is much less effective than vagal stimulation in promoting AF and the heterogeneity of atrial ERP is important in sustaining AF. In addition, Huang et al. [6] reported that high vagal tone is associated with a high dispersion of atrial ERP during recovery from atrial electrical remodeling, indicating that the vagus and sympathetic have a synergistic effect on the refractory period. Chiou et al. [7] found that long-term vagal denervation of the atria and sinus and AV nodes can be produced by radiofrequency current catheter ablation of the fat pads and results in vagal denervation supersensitivity. Vagal denervation prevents induction of AF in this model too. In contrast, when recording the activation sequence in VS-induced AF, with increasing frequency of VS, the occurrence of vagally induced atrial premature depolarizations (APDs) and AF increases [1]. Similar results have been observed that high frequency electrical stimulation evokes rapid ectopic beats from PV and SVC, which show variable degrees of conduction block to the atria and induce AF, resembling findings in patients with focal idiopathic paroxysmal AF [3]. Other research [20,21] found that posterior left atrium (PLA) and the ligament of Marshall (LOM) contain a predominance of cholinergic nerve fibers. Targeted cholinergic blockade in the PLA and ablation of LOM attenuate vagal responses respectively and, consequently, beneficially change AF substrate for the genesis and maintenance of the arrhythmia.

Heterogeneity of supraventricular regions contributes to the basic mechanisms for AF

An increasing amount of research on the heterogeneity of supraventricular regions focused on cholinergic electrophysiological evidence and neurotransmitter receptors. The heterogeneous distribution of cholinergic innervation of heart contributes to the pro-arrhythmic ability of vagal nerve stimulation. Huang et al. [18] showed that inherent $I_{KACH}$ differences between atrial appendages and atria play an important role in initiation and maintenance of cholinergic AF. Another study inspected the onset of vagally mediated AF and found the cycle length of the re-entry around LAA was slow, stepwise, and initially shortened. A focal activity also appeared in RAA and LAA, and occurred more for intense VS frequencies than other types of AF. These results imply that atrial appendages dominate the effects on the vagally mediated AF [1]. The result in our study that the cholinergic innervation at atrial appendage is the densest among different regions of supraventricular parts of the heart goes along with these conclusions.

Other research [22–25] considered that LA acts as a driving and superior region compared to RA for the contributions to AF. The ERP differences between canine LA and RA contribute to the differences in APD which is shorter in LA myocytes than that in RA myocytes. LA acts as a drive region compared to RA for AF on the basis of these differences [25]. In isolated sheep hearts, ACh-mediated AF is maintained by the faster re-entrant sources in LA with fibrillation conduction toward the slower RA [24]. In a chronic rapid atrial pacing model, the mean AF cycle length was also significantly shorter in the LA than in the RA [22]. In isolated sheep hearts, an LA to RA frequency gradient and an overwhelming predominance of left to right impulses transmitted across Bachmann’s bundle and the interposterior pathway during AF were verified by Mansour et al. [23]. Our result that the density of cholinergic nerves in LA is higher than RA is consistent with the above researches.

Furthermore, PV isolation has a lower efficacy in patients with vagotonic paroxysmal AF than with adrenergic or random episodes of paroxysmal AF. The results suggest that PV plays a less important role in vagotonic paroxysmal AF [26]. Another study showed that VS increased the frequency of premature atrial depolarizations wherever from the atria. The result indicates that PV isolation is less effective for eliminating vagotonic paroxysmal AF than other types of paroxysmal AF [27]. Razavi et al. [28] revealed that ablation around the PV ostia decreased left atrial response to VS and the atrial vulnerability window. They thought that diminished vagal response after ablation contributes to the restraint of AF. Thus, their results also support that PV plays a subsidiary role in vagally mediated AF. Our result that cholinergic innervation at PV with one of the lowest densities of all the regions resembles the conclusions of these reports.

$M_2$ receptors mediate the cholinergic effects on cardiac functions with one of the mechanisms for activating $I_{KACH}$ [14]. Differential densities of $M_2$ receptor and $I_{KACH}$ assist in atrial remodeling in AF induced by congestive heart failure [29]. More specifically, previous works [18,30] demonstrated that the densities of $M_2$ receptors and $I_{KACH}$ are higher in myocytes obtained from the RAA and LAA than from atria, PV, and SVC. Additionally the densities of $M_2$ receptors and $I_{KACH}$ are higher in myocytes from LA than in that from RA, PV, and SVC. These results correlate well with the distribution of cholinergic nerve innervation at these regions in our study showing that atrial appendages have the highest density, LA less but higher than RA which is still higher than PV and SVC.

Zhao et al. [31] measured the autonomic innervation and density of $I_{KACH}$ in CT and concluded that the different densities of adrenergic nerves in different regions of CT determine the genesis of atrial arrhythmias; however, cholinergic innervation did not show this heterogeneity on CT. Our study characterizes cholinergic nerves that exhibit heterogeneity over different supraventricular regions and the distribution on CT is low which supports the conclusion of their research. Sharifov et al. [1] indicated that AS can play an important role in both the initiation and the maintenance of VS-induced AF. However, the cholinergic innervation at AS in our research is as low as CT, PV, and SVC which according to their results were observed in epicardium but the cholinergic innervation was measured in the myocytes between epicardium and endocardium.
Study limitations

Our study was performed in canines; therefore, the results may not be able to be directly extrapolated to human hearts. Further studies are needed to assess cholinergic innervation in AF model using more specific nerve density determination measurements to further demonstrate the relationships between the heterogeneity of cholinergic innervation and the pathological processes of AF. Furthermore, other tissues crucial to initiate and promote AF such as ganglion plexus will be studied in our future research.

Acknowledgments

We are grateful for the financial support of Health Vocation Proprietary Grant of Ministry of Health to Huang Cong-Xin (No. 200802157) and Special Grant for Basic Scientific Research Expenses of the Central Universities to Zhe Li (No. 302274994).

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