Effects of spironolactone towards rabbit atrial remodeling with rapid pacing

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Abstract: This study aimed to observe the effects of spironolactone towards the rabbit atrial remodeling with rapid atrial pacing (RAP). 30 rabbits were randomly divided into control group, RAP group and spironolactone group, with 10 rabbits in each group. RAP was performed at the speed of 800 beats/min for 8 h, atrial effective refractory period (AERP) was determined before and at the 1st, 2nd, 4th, 6th and 8th of the pacing, the expressions of atrial muscular calcium channel α1C subunit and β1 subunit mRNA were performed the RT-PCR detection, and ultrastructural changes of atrial myocytes were observed. AERP of RAP group shortened, with poor frequency adaptability; the expressions of calcium channel α1C subunit and β1 subunit mRNA decreased 22% and 26%, respectively, when compared with the control group; ultrastructure of atrial myocytes changed significantly. AERP of spironotlactone group shortened less that RAP group, and the frequency adaptability was maintained, the decreased expressions of calcium channel α1C subunit and β1 subunit mRNA significantly reduced. RAP could cause atrial remodeling, while spironolactone could inhibit RAP-induced atrial remodeling.

Keywords: Fibrillation, rapid atrial pacing, electrical remodeling, structural remodeling, spironolactone.

INTRODUCTION

Atrial fibrillation (AF) is one of the most common clinical arrhythmias, and one of the biggest challenges of cardiology in the 21st century, which incidence would increase with the increasing age. The mechanism of AF is complex, a lot of studies have shown that the atrial remodeling plays an extremely important role in AF incidence and continuous existance. Renin-angiotensin-aldosterone system (RAAS) is an important human nervous-hormone system, playing an important role in regulating the body blood pressure, fluid and electrolyte balance (Ma *et al.*, 2010). Recent studies have confirmed that RAAS also plays an important role in atrial remodeling, participating the pathophysiology of AF formation and maintenance (De Maria *et al.*, 2012). Currently, the treatment of AF is still the weakest link in the treatment of arrhythmia, although non-drug treatments, such as surgical maze procedure, radio frequency ablation and pacing prevention, have aroused widespread concern about AF occurrence in recent years, the drug therapy is still the cornerstone of AF treatment. Based on in-depth research on atrial remodeling, prevention of AF occurrence and recurrence through inhibiting RAAS has become a new hot spot.

With the in depth studies of AF mechanism, angiotensin-converting enzyme inhibitors (ACEI) and angiotensin receptor blocker (ARB) have a formally positive effect towards the AF prevention and AF-recurrence (Burchill *et al.*, 2012), providing a new way for the clinical AF treatment. Previously it is believed that, ACEI and ARB drugs can effectively inhibit the production of aldosterone. However, recent studies have found that, due to aldosterone escape, ACE and ARB drugs cannot completely prevent the aldosterone production. Aldosterone is the last link of RAAS, reaching more and more attention in the atrial remodeling, a series of studies have shown that aldosterone is closely related with AF. Compared with the sinus rhythm, aldosterone levels of AF patients significantly increased (Dixen *et al.*, 2007). The specific mechanism of aldosterone affecting AF is not clear presently, while it's normally thought that the strong aldosterone-induced myocardial fibrosis is the central influence on AF. In this study, the rabbit atrial remodeling model with rapid atrial pacing was established, the effects of spironolactone towards rabbit atrial electrical remodeling and structural remodeling with rapid atrial pacing for 8 h were observed. The objective is to explore the possibility of AF treatment with spironolactone, and provide a theoretical basis of clinical application of spironolactone in AF treatment.

MATERIALS AND METHODS

Experimental animals and grouping

30 healthy normal rabbits, male or female, weight 2.0-3.2 kg, were provided by the Experimental Animal Center of Nanchang University. The rabbits were randomly divided into 3 groups: Control group (n=10), pacing group (n=10) and spironolactone group (n=10). Spironolactone group (Hangzhou Minsheng Pharmaceutical, Hangzhou, China)

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was orally administered spironolactone 20mg/(kg·d) for two weeks, while the other 2 groups were administered with the equal volume of saline for 1 week. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of People's Liberation Army No. 105 Hospital.

Establishment of AF model

3% sodium pentobarbital were intravenously injected at 30mg/kg into rabbit ear vein for anesthesia, and then 1000 U heparin was also intravenously injected. Then fixed the rabbit on the operation table, recorded the surface ECG; performed the neck incision, separated the right internal jugular vein and cut open, ligated the proximal segment, then inserted into a 4 F 4-electrode catheter (5 mm electrode spacing) to the right atrium, and the insertion depth was about 6 cm; connected the distal electrode pair with the ECG left and right electrodes, tuned the ECG to mode I and recorded the intracardiac bipolar ECG, the large A and small V indicated that the electrode was in the right atrium, then fixed the electrode catheters. Along the ear vein, atropine and metoprolol were injected to block the effect of autonomic nerve on heart rate, the first dose of atropine was 0.04mg/kg, intravenous injection, after that 0.007mg/(kg h) intravenous infusion was performed for the maintenance, the first dose of metoprolol was 0.2mg/kg, after that the 0.02mg/(kg·h) was performed for the maintenance.

AERP determination

DF-5A electrophysiology instrument (Suzhou Dongfang Electronic Instrument Factory, Suzhou, China) was used, with the starting frequency 10%-20% higher than the sinus pacing, and the pulse width was 0.5ms. The pacing threshold was measured for the set up of the output voltage, which was twice of the pacing threshold. The atrial effective refraction period (AERP) were measured at 200 ms and 150 ms S1S1, respectively, and labeled as AERP200 and AERP150. Measuring method: the programmable pre-period stimulation (S1S2) increment scanning method was applied, the S1S2 stimulation frequency was 8:1, with step as 5ms, r the longest S1S2 interval which could not be passed down to the atrium was labeled as AERP. Pacing group and spironolactone group was performed the rapid atrial pacing with 800 beats/min, and the AERP was measured before and 1 h, 2 h, 4h, 6h and 8h after the pacing, respectively, and the above values were denoted by P_0, P_1, P_2, P_4, P_6 and P_8. The control group would not be performed rapid pacing in the corresponding period, only measured AERP. AERP200-AERP150 was set as the AERP frequency adaptability judgment index.

mRNA expression of L-type calcium channel α1c and β1 subunits by semi-quantitative RT-PCR

According to the design requirements, Primer5.0 software was applied to design, and the synthesis was performed by Shanghai Boya Biotechnology Co. Ltd., the primer length and reaction conditions were shown in table 1.

According to the experimental grouping, after the pacing, the rabbit was performed thoracotomy with aseptic technique under the anesthesia status, the heart was rapidly removed and rinsed with calcium-free Tyrode's solution, the right atrial tissue was then cut and cryopreserved in liquid nitrogen. The atrial tissue was finely grounded and fully homogenized in Trizol RNA extraction kit (Life Technologies Company, California, USA) according to instructions for the extraction of total RNA. The extracted RNA was dissolved in 30μl enzyme-free water and quantitatively determined the concentration and purity with nucleic acids quantitative determination instrument, the RNA was then performed electrophoresis in 0.8% agarose gel for the RNA integrity observation.

The atrial α1c and β1mRNA changes were detected by RT-PCR. 1μg total RNA was performed the reverse transcription reaction in 20μl reverse transcription reaction system according to the kit instructions, and the resulted cDNA was used as the template for the subsequent 25μl PCR assay. PCR reaction system: 5μl cDNA, 12.5μl PCR Master Mix, 5.5μl no-ribozyme water and 1μl upstream and downstream primer (10 μmol/l). The PCR product was then electrophoresed in 2% agarose gel, the gel imaging system was used for the imaging and

Table 1 Primer sequences and PCR reaction conditions

Gene	Primer sequence	Renaturation temp	Cycles	Length of amplified segment
α1c	Upstream 5---GACTCCACTTTCACCCCC---3 Downstream 5---TCTCCCCCTTGATTCTTCTGCC---3	60	35	490
β1	Upstream 5---AGCTTGCGCTGAGTTCTTGC----3 Downstream 5---TCCCTTGCTTTGCTCTCTG---3	52	35	494
GAPDH	Upstream 5---GCTTTTAACTCTGGCAAAGTG---3 Downstream 5---GATGATGACCCTTTTGGCTC---3	58	35	190

Table 3: Comparison of AERP with basic pacing perimeter as 150

Group	P_0	P_1	P_2	P_4	P_6	P_8
Control	111.1±6.3	110.9±5.3	111.3±5.4	111.2±5.7	111.0±7.0	111.5±6.4
Pacing	110.3±8.6	104.2±6.4*	103.6±7.6*	103.1±7.7*	103.3±7.3*	103.0±6.9*
Spironolactone	112.0±7.9	111.0±7.8$^{\#}$	111.1±8.4$^{\#}$	110.8±8.1$^{\#}$	111.1±7.3$^{\#}$	110.9±8.3$^{\#}$

Table 4: Changes of AERP frequency adaptability

Group	AERP200-AERP150					
	P_0	P_1	P_2	P_4	P_6	P_8
Control	11.7±5.0	12.2±3.0	13.6±4.9	11.9±6.3	12.5±5.1	11.0±4.8
Pacing	12.1±5.3	5.9±3.1*	5.3±4.9*	4.8±5.5**	3.3±6.4*	4.7±5.2**
Spironolactone	11.1±5.0	10.2±5.3$^{\#}$	10.4±5.2$^{\#}$	10.7±5.2$^{\#}$	10.0±5.3$^{\#}$	10.0±4.6$^{\#}$

Compared with the relative time point of the control group $^{*}p<0.01$, $^{**}p<0.05$, compared with the relative time point of the pacing group $^{\#}p<0.05$.

Table 5: Comparison of the subunits in the three groups

Group	Subunit	
	α1c	β1
Control	0.68±0.04	0.83±0.05
Pacing	0.53±0.05*	0.61±0.05*
Spironolactone	0.60±0.04$^{*,\#}$	0.69±0.05$^{*,\#}$

Compared with the control group $^{*}p<0.01$, compared with the pacing group $^{\#}p<0.05$.

measuring the absorbance (A value), the GAPDH was used as the internal reference, the ratios of Aα1c/AGAPDH and Aβ1/AGAPDH were used for the statistical analysis.

Observation of the ultrastructure

After the execution of rabbit, right atrial tissue was isolated, a small piece of atrial tissue (1mm × 1mm) was immediately fixed in 3% glutaraldehyde at 4^{o}C, the specimen was performed gradual ethanol dehydration, soaked with acetone-embedding medium mixture (1:1) for 1 h at room temperature, then embedded and fixed with pure embedding medium and prepared the semi-thin slices and then ultrathin slice; uranyl acetate and lead citrate were used for the double staining, then rinsed the specimen for the differentiation, when it was dry, observed the specimen with transmission electron microscopy (Hitachi H-600, Tokyo, Japan).

STATISTICAL ANALYSIS

All data were expressed as $\bar{x} \pm s$, SPSS13.0 statistical software was used, the repeated measurement ANOVA was performed for the statistical analysis, and $p<0.05$ was considered as statistical significance.

RESULTS

Changes of atrial effective refractory period

The control group showed no significant changes of AERP200 and AERP150 throughout the whole period, while after the rapid atrial pacing, the AERP200 and AERP150 of the pacing group shortened when compared with the data before pacing, 8 h after the pacing, the data shortened 16.7ms ($p<0.01$) and 7.3ms ($p<0.05$), respectively. The shortening degrees of AERP200 and AERP150 of the spironolactone group were less than the pacing group, 8h after the pacing, the AERP200 and AERP150 of the spironolactone group were 2.2 ms ($p>0.05$) and 1.1ms ($p>0.05$) shorter than P_0 (tables 2 and 3).

Changes of atrial frequency adaptability

During the experiment, the shorter the S1S1 perimeter of the control group, the shorter the AERP, namely the AERP frequency adaptability was normal. The AERP frequency adaptability of the rapid pacing group was normal before the pacing, AERP150 was shorter than AERP200 (AERP200-AERP150) 12.1±5.3ms, but 8 h after the pacing, the ratio became 4.7±5.2ms ($p<0.05$), while the AERP200-AERP150 of the spironolactone group before

the pacing was 11.1±5.0ms, and 10.0±4.6 ms 8h after the rapid pacing, which had no significant difference when compared with the control group ($p>0.05$) (table 4).

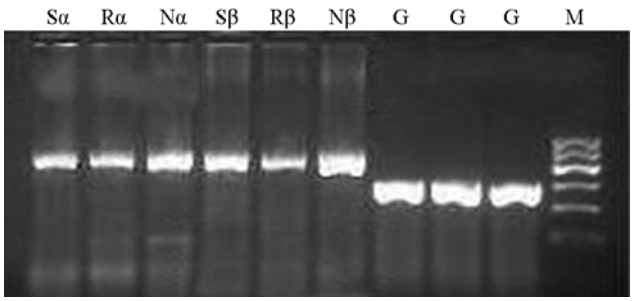

Fig. 1: Electrophoresis of L calcium channel subunits mRNA. S, spironolactone group; R, pacing group; N, control group; G, internal standard APDH; M, Marker.

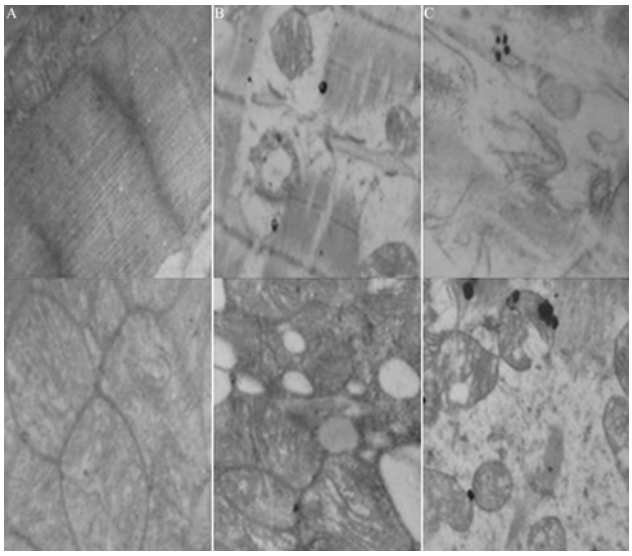

Fig. 2: TEM of atrial tissue. (A) control group. (B) spironolactone group. (C) Pacing group.

Semi-quantitative RT-PCR electrophoresis results of L calcium channel subunits mRNA

The positions of the amplified bands of target genes and the internal standard GAPDH were consistent with the theoretical values (fig. 1). 8 h after the rapid atrial pacing, the expressions of atrial L calcium channel α1c and β1 subunit mRNA decreased by 22% and 26%, respectively, when compared with the control group, with $p<0.01$, while the descent degrees of spironolactone group significantly reduced to 13% and 17%, when compared with the pacing group, with $p<0.05$, indicating that spironolactone had a protective effect on rapid atrial pacing-induced expression of L calcium channels (table 5).

Results of electron microscopy

There were abundant intracellular myofilaments in the control group, arranged regularly, with regular sarcomeres, cell morphology and mitochondrial morphology (fig. 2A). In the spironolactone group, the myofibrils arranged in a disordered rule, the sarcomeres were irregular, some myofilaments dissolved and ruptured, cells and mitochondria were swelled, with reducing number and different sizes (fig. 2B). TEM images of the pacing group revealed that the myofibrils dissolved and ruptured, the sarcomeres were unclear, the myofilaments significantly reduced, the density of the cytoplasmic electron decreased, the mitochondria was swelled and some were ruptured, and the number reduced (fig. 2C).

DISCUSSION

Atrial remodeling includes atrial electrical remodeling and atrial structural remodeling, atrial electrical remodeling refers to the progressive shortening of atrial effective refractory period (AERP) and action potential duration (APD) when AF happens, at the same time, the action potential conduction velocity and the frequency adaptation would also diminish. Wijffels *et al* (1995) firstly proposed the concept of atrial electrical remodeling and the theory of "AF-induced AF", which established the theory of atrial electrical remodeling and AF animal model, and confirmed by a subsequent animal experiments and clinical researches. The mechanism of atrial remodeling is still unclear, it is currently considered that the changes of atrial ion channels (Heijman *et al.*, 2013), calcium overload (Yamashita *et al.*, 2011), inflammation and oxidative stress (Naji and Sabovic, 2013), renin-angiotensin-aldosterone system and many other factors all involve in the occurrence of atrial remodeling. Although atrial remodeling involves a variety of mechanisms in AF, the atrial muscle calcium channel changes and RAAS activation are thought to be the most closely related to atrial remodeling. Ion current of calcium channel is the main procedure of myocardial action potential and its exciting function. There are varieties of Ca^{2+} channel protein on myocardial cells, among which L-type calcium channel plays an important role in cardiac repolarization. L-type calcium channel would be activated and generate the inward current in the initial stage of action potential, and would continue through the whole plateau stage, so the changes of L-type calcium channel function could lead to arrhythmias. $I_{ca\text{-}L}$ channel has an important role in atrial remodeling, which has been confirmed by a number of animal tests and clinical trials (Voigt *et al.*, 2012). Tsai *et al* (2007) observed that the expression of α1c mRNA significantly reduced in persistent atrial fibrillation, when compared with sinus rhythm, which was also found in this study that the expression of L Ca^{2+} subunit mRNA of the pacing group decreased. In this study, the rabbit atrial remodeling model with rapid atrial pacing was established, the effects of aldosterone receptor antagonist spironolactone towards rabbit atrial electrical remodeling and structural

remodeling with rapid atrial pacing for 8 h were observed. Results found that, rapid atrial pacing could shorten AERP and decrease AERP frequency adaptability, which was similar to the previous studies, indicating that rapid atrial pacing could induce acute atrial electrical remodeling, and cause changes similar to AF-induced electrical remodeling. However, the electrical remodeling could be fully restored a few weeks after the AF patients restored the sinus rhythm, while it would still be easy to induce AF (Lee, 2013, Melduni and Cullen, 2012), indicating in addition to electrical remodeling, there were other factors involved in the occurrence and maintenance of AF. Studies have shown that AF could induce significant electrophysiological changes of atrium, and progressively induce the structural changes of the heart, leading to atrial enlargement and myocardial fibrosis (Fukui *et al.*, 2013), namely atrial structural remodeling, which might play the more important role in persistent AF. The main phenomena of atrial structural remodeling included: atrial myocytes hypertrophy, glycogen accumulation around the nucleus, sarcomere disappearing (myolysis), connexin expression changing, mitochondria shape changing, sarcoplasmic reticulum fragmentation, homogeneous distribution of nuclear staining, quantity and location changing of cell structure protein, interstitial fibrosis (Hatem, 2012). The atrial structural changes existed in levels of atrial myocytes and extracellular matrix (Pellman *et al.*, 2010). In this study, the pacing group exhibited obvious changes in atrial structural remodeling, such as broken myofilaments, swelled cell, changes of mitochondria and different nucleus sizes, *etc*. Atrial electrical remodeling could cause atrial structural remodeling, and atrial structural remodeling is also beneficial to the maintenance of atrial electrical remodeling, the above two normally combine with each other, jointly promoting the occurrence and maintenance of AF.

Aldosterone, as the last link of RAAS, plays an important role in the maintenance of fluid balance. Recent studies have found that aldosterone also was important in atrial remodeling. In 1990s, Berglund *et al* (1990) had found that the serum aldosterone levels of AF patients were significantly higher than the patients with sinus rhythm. Goette *et al* (2001) found that the serum aldosterone level significantly increased when AF happened, while the level would significantly decrease after 48 hours of electrical cardio version, and would increase again when AF reoccurred. These studies revealed the close link between aldosterone and AF, though the specific mechanism was still not fully clear. At present, it's considered that the strong myocardial fibrosis effects of aldosterone is the central link which influences AF, in addition, it could also influence the occurrence and maintenance of AF through inducing muscle dissolving, myocardial apoptosis, inflammation activation, oxidative stress and direct electrophysiological effects.

Limitations of this study are mainly as follows: Firstly, the rapid atrial pacing time is only 8 h, which relatively short. So the effect of spironolactone on chronic AF can not be observed. Secondly, the effect of spironolactone on atrial remodeling is only observed from a macro point, but not from the ion level or channel protein level. In future research, based on enlarged sample size, the effects of spironolactone on chronic AF and atrial remodeling should be observed from the molecular level.

The important role of aldosterone in atrial remodeling has increasingly attracted the attention. Currently, the impact of aldosterone receptor antagonist towards AF is still only in the stage of animal experiments. Yang *et al* (2008) studied chronic heart failure, and found that spironolactone, aldosterone antagonist, could not change the atrial effective refractory period, but could shorten the interauricular conduction time, increase atrial conduction velocity, reduce atrial fibrosis, and reduce the incidence of atrial fibrillation and atrial fibrillation duration. Milliez *et al* (2005) conducted a study, spironolactone, lisinopril and atenolol was administrated alone or in combination to mice three months after myocardial, the results showed that three months after the myocardial infarction, the atrial diameter of the mice increased, the atrial fibrosis and P-wave duration also increased, 24 h dynamic electrocardiogram revealed that atrial premature happened frequently. While a month after the administration of drugs, the atrial over-excitement reduced, and only spironolactone could reduce the atrial fibrosis and shorten P wave duration. In the present study, it was found that spironolactone could inhibit the AERP shortening and AERP frequency adaptability decreasing induced by rapid pacing, the decreasing amplitude of atrial myocytes L-subunit mRNA expression decreased, indicating that spironolactone might affect the atrial electrical remodeling through inhibiting the changes of calcium channel. It was also observed that spironolactone could effectively reduce the changes of myocardial structural remodeling, providing a theoretical support for the application of spironolactone in the treatment of AF.

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