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Prevention of Atrial Fibrillation by Using Sarcoplasmic Reticulum Calcium ATPase Pump Overexpression in a Rabbit Model of Rapid Atrial Pacing

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Background: Recent research suggests that abnormal Ca^{2+} handling plays a role in the occurrence and maintenance of atrial fibrillation (AF). Therefore, Ca^{2+} release and ingestion depend on properties of the ryanodine receptor (RyR) and sarcoplasmic reticulum Ca^{2+} ATPase2a (SERCA2a). This study aimed to detect whether SERCA2a gene overexpression has a preventive effect on atrial fibrillation caused by rapid pacing right atrium.

Material/Methods: Forty-eight New Zealand white rabbits were randomly divided into a control group, AF group, AAV9/GFP group, and AAV9/SERCA2a group. The right atrium was rapidly paced at 600 beats/min for 30 days after an intraperitoneal injection of an adeno-associated virus expressing the SERCA2a gene and GFP. The AF induction rate and the effective refraction period (ERP) were measured after 0, 4, 8, 12, and 24 h of pacing. Western blot analysis was used to test for the expression of SERCA2a. Changes in atrial tissue structure were observed by H&E staining and electron microscopy.

Results: The AF induction rate was higher in the AF groups than in the AAV9/SERCA2a group at different time points of pacing. After 12 h of pacing, ERP was significantly prolonged in the AAV9/SERCA2a group compared to the AF and AAV9/GFP groups ($p < 0.05$). SERCA2a protein expression was significantly lower in the AF and AAV9/GFP groups compared to the control group ($p < 0.05$), while expression was significantly higher in the AAV9/SERCA2a group than in the AF and AAV9/GFP groups ($p < 0.05$). The myocardial structure of the AAV9/SERCA2a group was significantly improved compared with the AF group, indicating that SERCA2a overexpression relieved the structural remodeling of atrial fibrillation.

Conclusions: SERCA2a overexpression is capable of suppressing ERP shortening and AF induced by rapid pacing atrium. SERCA2a gene therapy is expected to be a new anti-atrial fibrillation strategy.

MeSH Keywords: **Atrial Fibrillation • Atrial Remodeling • Sarcoplasmic Reticulum Calcium-Transporting ATPases**

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Background

Atrial fibrillation (AF) is a common cause of clinical arrhythmias, and has become a major epidemic disease in the cardiovascular field, resulting in high morbidity, mortality, and disability [1]. At present, the main treatment for AF is drug and interventional therapy, and while great progress has been achieved, many issues remain, such as recurrence of AF and complications after treatment, which can seriously endanger human health [2,3]. In-depth studies of the molecular mechanisms underlying cardiovascular disease have led to progress in gene therapy, and are providing new therapeutic strategies for the clinical treatment of cardiovascular diseases [4,5]. Currently, AF is classified based on its clinical presentation: paroxysmal atrial fibrillation (usually within 48 h can be self-terminating, the longest lasting no more than 7 d), persistent atrial fibrillation (duration of more than 7 d, or need drugs or electric cardio trembling), long-term persistent atrial fibrillation (duration >12 months), and permanent atrial fibrillation (patients and physicians jointly decide to give up further attempts to restore and/or maintain sinus rhythm) [6].

Most fatal arrhythmias are caused by intracellular calcium abnormalities [7,8], in which sarcoplasmic reticulum Ca^{2+} -ATPase (SERCA2a) plays an important role [9,10]. Sarcoplasmic reticulum calcium ATPase is a major protein involved in calcium regulation. It functions to promote myocardial diastole by discharging calcium ions from the pulp into the sarcoplasmic reticulum via an ATP-dependent inverse concentration gradient [11]. The results from a large number of animal experiments and clinical trials of patients with heart failure have shown that a significant decrease in SERCA2a expression causes intracellular calcium abnormalities and decreased myocardial contractility [12,13]. Thus, transgenic therapy using SERCA2a as the target gene has led to significant achievements in the treatment of patients with heart failure [14–17]. A study by Dobre et al. found that Ca^{2+} pump dysfunction leads to decreased myocardial contractility, thereby increasing the occurrence of atrial arrhythmias [18,19]. SERCA2a is involved in the circulation of calcium to atrial myocytes. We hypothesized that SERCA2a overexpression affects atrial electromyography and structural remodeling in rabbits with AF. We assessed the atrial effective refraction period (ERP) of these rabbits, as well as changes in atrial morphology and histology of the atrial muscle following pericardial injection of an adeno-associated virus expressing the SERCA2a gene. We also evaluated the effect on electrical and structural remodeling.

Material and Methods

Animal preparation

A total of 48 healthy adult New Zealand white rabbits, regardless of sex, with weight 3.0 ± 0.5 kg and age of 1 year to 2 years, were provided by the First Affiliated Hospital of Xinjiang Medical University Animal Center. Surface electrocardiography was used to confirm heart rate, and echocardiography was used to exclude other heart disease. All rabbits were given an insulation blanket to maintain body temperature at $35.5 \pm 1.5^\circ\text{C}$ in the experimental operation. This study was performed according to the Guide for the Care and Use of Laboratory Animals issued by the National Institutes of Health. The experimental design and processes were approved by the Animal Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University (IACUC). Animals were randomly divided into 4 groups, each group including 12 rabbits: control group (implanted pacing electrode but no pacing), AF group (implanted pacing electrode pacing), AAV9/GFP group (post-empty pacing), and AAV9/SERCA2a group (transferred to SERCA2a pacing), after transgenic therapy for 30 days, then rapid right atrial pacing at a frequency of 600 beats/min.

Gene transduction

The rabbits were anesthetized by injecting 3% pentobarbital sodium at 30 mg/kg through the ear vein. A chest incision was made through the third or fourth rib to expose the heart. Using microscopic surgery, the outer membrane of the pericardium was raised using ophthalmic tweezers, and the space between the raised part and the pericardium was used for the injection. During the systolic period, a needle was placed into the pericardial cavity, and 500 μL AAV9/SERCA2a and 500 μL AAV9/GFP were slowly injected into the pericardial cavity. Pacing was initiated 30 days after injection.

Rapid atrial pacing (RAP) model preparation

The rabbits were anesthetized through ear vein injection of 30 mg/kg sodium pentobarbital, according to previously described methods [20]. The neck of the rabbit was cut, then the right internal jugular vein was separated. We inserted a puncture needle and 4F sheath into the 0.05-cm pitch 4-pole electrode in the right atrium and used Lead-7000 electrophysiological record of cardiac electrocardiogram and body limb lead ECG. The right external jugular vein was cannulated and used for catheter insertion into the right atrium to perform RAP. The electrophysiology instrument was used to give continuous single stimulus for 24 h of RAP, pacing at a frequency of 600 beats/min. The sham group was also implanted with pacing electrodes, but without RAP.

Data collection for cardiac electrophysiology

Rate of AF: Based on our previous model, the lead tube was fixed when a big A wave and a small V wave were observed on the heart ECG, and remained steady. AF was defined as irregular atrial rates >500 beats/min and lasting >5 s associated with irregular atrioventricular conduction [21]. Burst mode S1S1 circumference was 100 ms, a stimulation voltage of twice the threshold value was the output voltage, and the right atrium was rapidly paced in order to induce AF at 0, 4, 8, 12, and 24 h, repeated 3 times, for 30 s each time, and we recorded the number of AFs.

ERP of right atrium: Programmed stimulation (S1S2) was used for the right auricles at 4, 8, 12, and 24 h after the pacing to measure the ERP. The program-controlled reverse scanning was performed with the initial pacing-length of 250 ms and shortened by 5 ms until no response was observed (S1: S2=8: 1, twice-threshold current). The longest S1S2 interval was recorded as the ERP.

Transmission electron microscopy

With the animals under anesthesia, an incision was made in the chest and tissues from the right atrium were removed for analysis. Tissues were fixed with 3% glutaraldehyde and cut into 1-mm³ blocks. Tissue blocks were dehydrated through an ethanol gradient, and then embedded with epoxy resin to prepare ultrathin tissue slices. Tissue slices were stained with a uranium acetate solution and lemon lead solution. Ultrastructural changes in the right atrial myocardium were observed using a JEM-1230 lens electron microscope (Japan).

Histopathology

Atrial myocardia were fixed in a 4% paraformaldehyde for 24 h and embedded. For cellular structure visualization, a 5- μ m-thick slice was stained with haematoxylin and eosin (H&E).

Atrial myocardia were examined by immunohistochemistry to evaluate the expression of SERCA2a. The sections were incubated with SERCA2a monoclonal antibody overnight at 4°C, followed by a 1-h incubation with goat anti-mouse polyclonal antibody, and was colored and photographed using Leica Qwin software under magnification.

Western blots

Rapidly frozen rabbit right atrium tissue was homogenized in RIPA lysis and extraction buffer which contained protease and phosphatase inhibitors. Protein content was determined by the Bradford method. After denaturation at 95°C for 5 min, proteins were separated by SDS-polyacrylamide gel. Electrophoresis

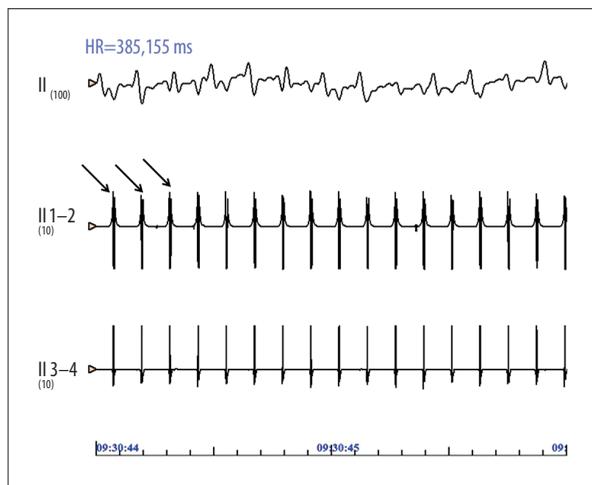


Figure 1. Rabbit rapid pacing induced atrial fibrillation II: Body lead. HRA – high right atrium. The arrows refer to the stimulus signal.

proteins were transferred onto PVDF membranes and blocked with 5% milk. For immunoreaction, the blot was incubated with 1: 1000 diluted SERCA2a and GAPDH (Abcam, 1: 1000) primary antibodies overnight at 4°C. On the second day, the membrane was incubated with secondary fluorescent anti-mouse and anti-rabbit antibodies (Cell Signalling, 1: 3000 dilution) for 1 h. The membrane was then scanned with a gel imaging system and bands were analyzed with imaging software. The values obtained were normalized to GAPDH to correct for variations in protein loading.

Statistical analyses

We used SPSS 17.0 statistical software for data analysis. All data are reported as mean values \pm standard deviation ($\bar{x} \pm s$), and compared using the variance analysis of repeated measurements. A value of $p < 0.05$ was deemed statistically significant.

Results

Preparation of AF model

During the course of the experiment, 2 rabbits died as the result of an anesthesia accident, and were substituted with animals of the same characteristics to the appropriate groups. There was no typical P wave in the electrocardiogram of the body surface (Figure 1).

AF induction rate

The AF model was constructed after 24 h of rapid pacing, and the results demonstrated no obvious changes in AF induction rates in the control group at different time points. At 4 h of

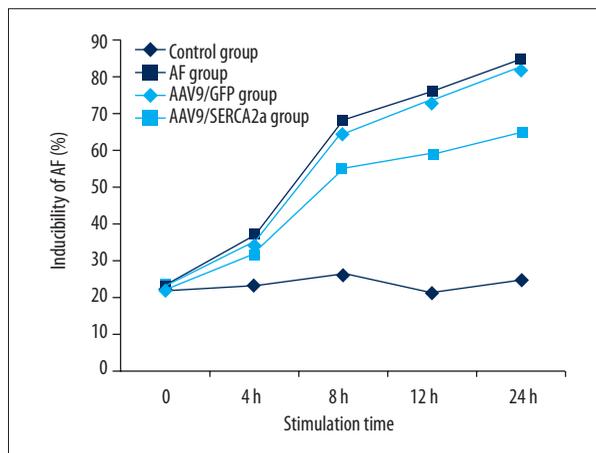


Figure 2. Induction rate of atrial fibrillation at different time points in each group.

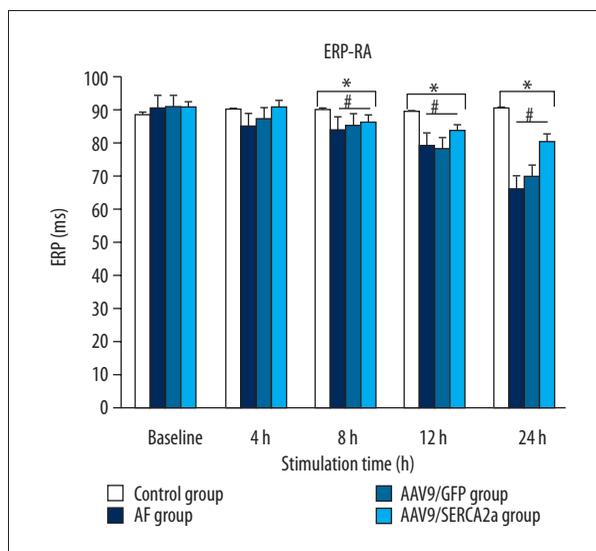


Figure 3. Four sets of different time points ERP comparison. * Compared with the control group ($p < 0.05$); # compared with AF group ($p < 0.05$).

spacing, the AF induction rate in the AF and AAV9-GFP groups were significantly higher than in the control group ($p < 0.05$), whereas there were no obvious difference between the AAV9/SERCA2a and control groups ($p > 0.05$). At 8 h of pacing, the AF induction rate in the AF and AAV9-GFP groups was significantly higher than in the SERCA2a transduction group ($p < 0.05$) (Figure 2). These results suggest that SERCA2A overexpression reduces the occurrence of AF.

ERP is prolonged after SERCA2a overexpression

A comparison of ERP at different time points was performed for all experimental groups. After 4 h of pacing, ERP in the AF and AAV9/GFP groups was significantly shortened compared to the control group ($p < 0.05$). This shortening became more

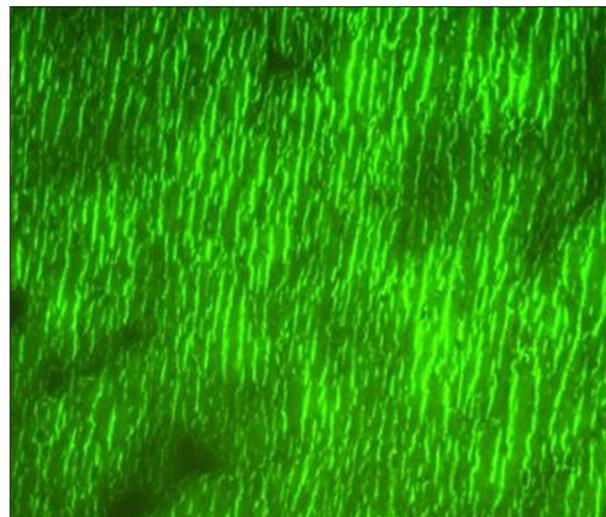


Figure 4. Fluorescence detection of myocardial sites. The AAV9/GFP group showed a strong green fluorescent protein expression under a fluorescence microscope.

obvious with a more prolonged pacing time. After 12 h of pacing, ERP was prolonged significantly in the AAV9/SERCA2a group compared to the AF and AAV9/GFP groups ($p < 0.05$) (Figure 3).

Immunofluorescence

Thirty days after injection of the virus vectors, frozen slices of the right atrial myocardium of rabbits from the AAV9/GFP group were analyzed. Results revealed strong green fluorescence in the atrial myocardium (Figure 4). Green fluorescent protein (GFP) in the cardiomyocytes showed a small patchy or scattered distribution, suggesting that the adeno-associated virus had infection efficiency in the atrial myocardium, which would then mediate efficient expression of the targeted gene in the atrial myocardium.

Pathological morphology of the atrial tissue

H&E staining showed that the myocardial fibers in the control group were neatly arranged with regular morphology and clear stripes (Figure 5A). However, the myocardial fibers in the AF and AAV9/GFP groups were not neatly arranged, and instead displayed unclear stripes and small regions of vacuolar degeneration. Moreover, the muscle gap was larger in animals from the AF and AAV9/GFP groups, and there were areas of scattered inflammatory cell infiltration (Figure 5B, 5C). In the AAV9/SERCA2a group, the structure of the myocardial cells was intact, the nucleus was clear with no obvious abnormalities, there was a moderate number of interstitial fibroblasts that showed regular formation, and there was a slight increase in the number of muscle fiber cells compared to the control group (Figure 5D). Compared with the AF and AAV9/GFP groups, the irregular arrangement of atrial myocardium

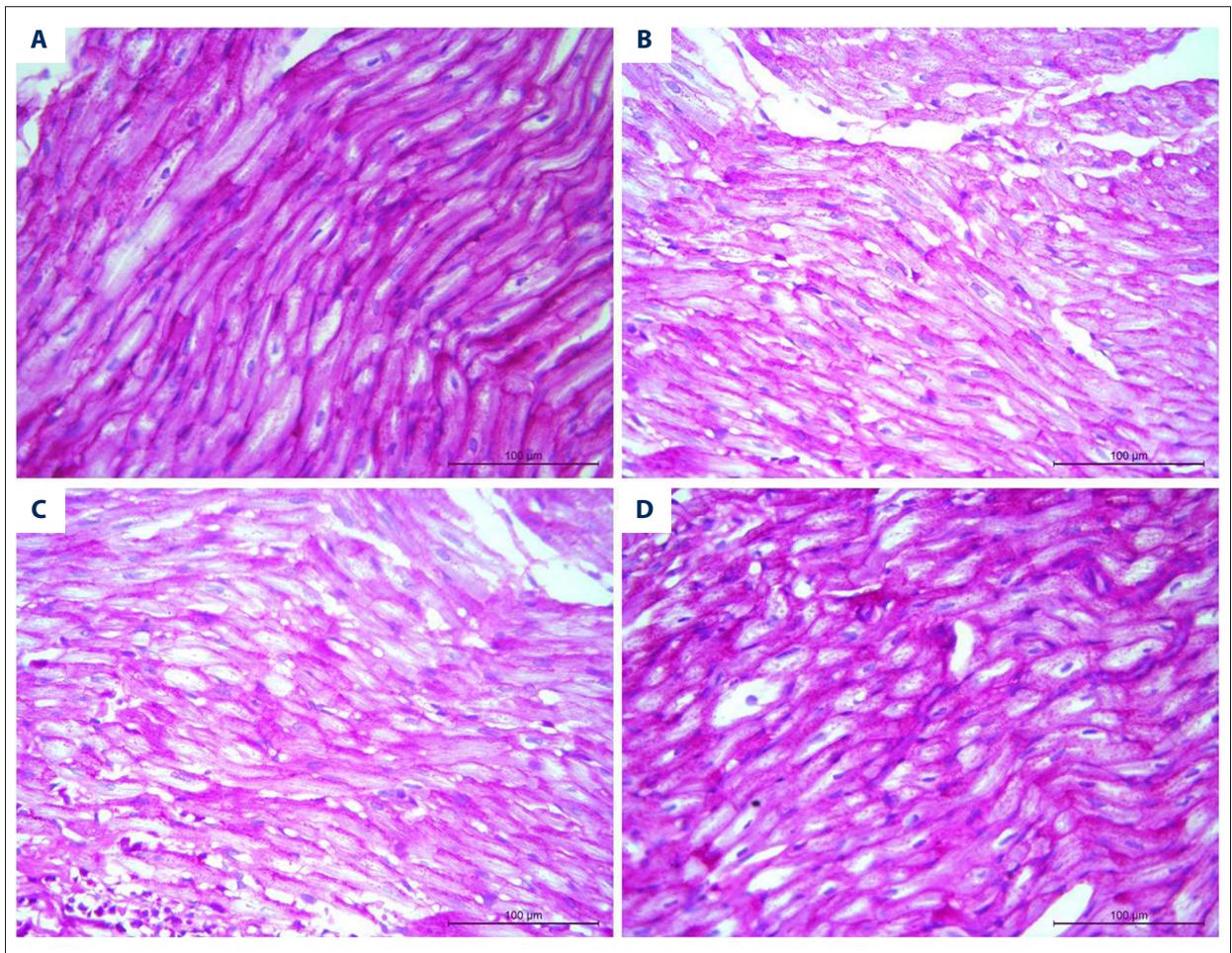


Figure 5. Right atrial H&E staining (20×10). (A) Control group, (B) AF group, (C) AAV9/GFP group, (D) AAV9/SERCA2a group.

cells in the transgenic groups had improved significantly in the AAV9/SERCA2a animals.

Ultrastructural analysis

In the control group, atrial muscle fibers were regularly arranged, mitochondrial morphology was normal, and the nuclear membrane was smooth and complete. However, in the AF and AAV9/GFP groups, the mitochondria were deformed, part of the ridge membrane was ruptured, a large number of vacuoles had formed, and aggregation of glycogen granules was apparent. In the AAV9/SERCA2a group, sarcomere arrangement in the atrial myocytes was slightly irregular. Mitochondrial edema and deformation were still visible in these animals, but the vacuoles and disorderly ridge arrangement were all significantly decreased compared to the AF group (Figure 6A–6D). These results suggest that SERCA2a overexpression improved muscle fiber damage due to AF.

Immunohistochemistry

SERCA2a protein was expressed mainly in the cytoplasm of atrial myocytes. SERCA2a protein expression was significantly higher in the AAV9/SERCA2a group than in the AF group ($p < 0.05$) and AAV9/GFP ($p < 0.05$) group, but was not significantly different from the control group (Figure 7A–7D). This suggests that transduction of the SERCA2a gene into rabbits with AF can significantly improve the downregulation of SERCA2a gene expression.

Western blot analysis

The expression of SERCA2a protein in myocardial tissue was assayed using the ratio SERCA2a: GAPDH. The results are summarized in Figure 8. The expression of SERCA2a protein in the SERCA2a group was much higher than that in the AF group ($P < 0.05$); even though the expression was higher than in control group, the difference was not significant.

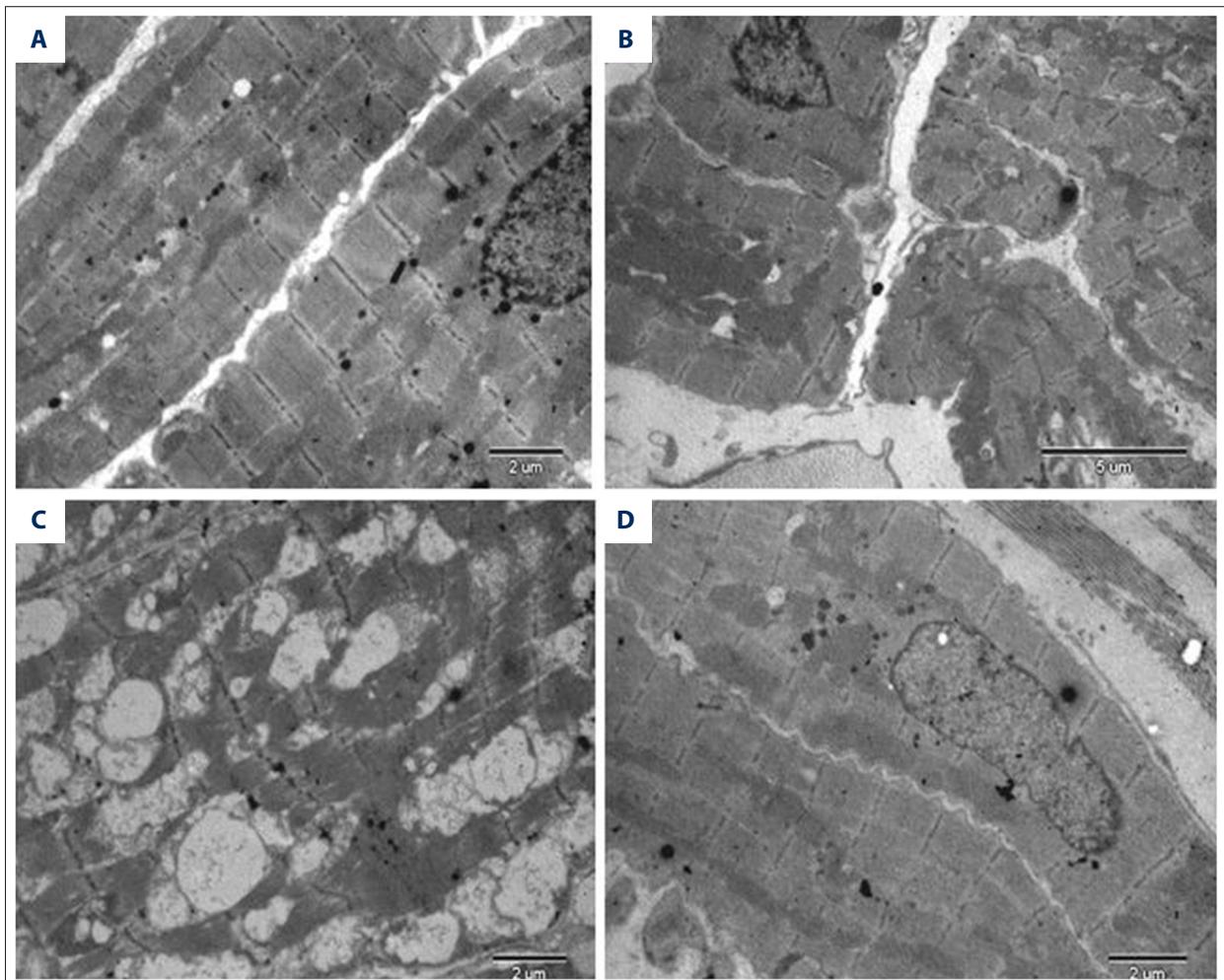


Figure 6. Changes of right atrial ultrastructure (40×10). (A) Control group, (B) AF group, (C) AAV9/GFP group, (D) AAV9/SERCA2a group.

Discussion

Cytoplasmic Ca^{2+} arises mainly from calcium release from the sarcoplasmic reticulum. Abnormal expression of the sarcoplasmic reticulum Ca^{2+} -ATPase (SERCA2a) gene leads to an imbalance in the concentration of cytoplasmic free Ca^{2+} , and results in cardiac systolic and diastolic dysfunction [16,22]. The present study found that transduction of exogenous SERCA2a reduced the AF induction rate in rabbits with AF, prolonged ERP, induced pathological changes in the atrial muscle, and increased SERCA2a protein expression. Thus, AAV9 vector-mediated SERCA2a gene transduction effectively reversed the electrical and structural remodeling of the atrial myocardium following rapid atrial pacing, and therefore could be a potential method for the treatment of AF.

Impact of SERCA2a overexpression on cardiovascular disease

A study by Prunier et al. suggested that SERCA2a overexpression effectively prevented the occurrence of ventricular arrhythmias

after cardiac ischemia [23], indicating that SERCA2a plays an important role in the development of reperfusion arrhythmias. SERCA2a is an important enzyme for the circulatory regulation of intracellular Ca^{2+} , accounting for 40% of total protein in the sarcoplasmic reticulum. SERCA2a is responsible for the uptake of cytoplasmic Ca^{2+} into the sarcoplasmic reticulum, and the reduction of intracellular Ca^{2+} concentration. Studies have reported that transduction of the SERCA2a gene into the ventricular myocardium in an animal heart failure model can increase SERCA2a protein expression, reduce cardiomyocyte death, shorten the time course of ventricular motion potential, and accelerate the repolarization of cardiomyocytes [24,25]. A study by Hajjar et al. revealed that SERCA2a gene treatment of heart failure is at the second stage of clinical trials; however, the occurrence of heart failure can lead to an increase in AF [26]. A large number of studies have shown that calcium overload is an important factor leading to AF [27,28]. Therefore, the effect of SERCA2a gene transduction on the atrial myocardium using atrial electromyography and structural remodeling in an AF model requires further studies.

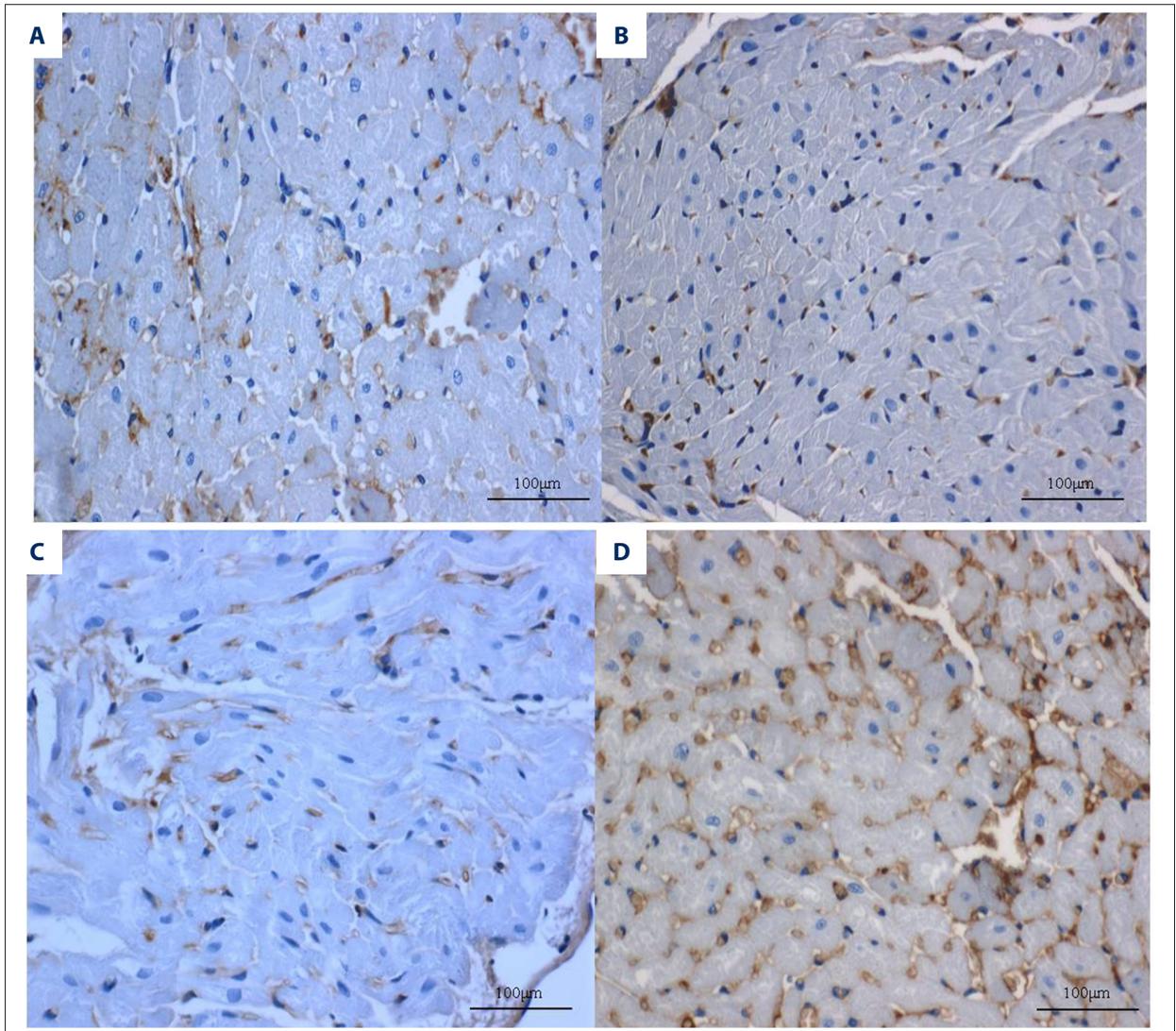


Figure 7. Right atrial SERCA2a protein positive expression (20×10). (A) Control group, (B) AF group, (C) AAV9/GFP group, (D) AAV9/SERCA2a group. Brown fine particles for the positive expression, in the cytoplasmic changes of right atrial ultrastructure had a dotted distribution.

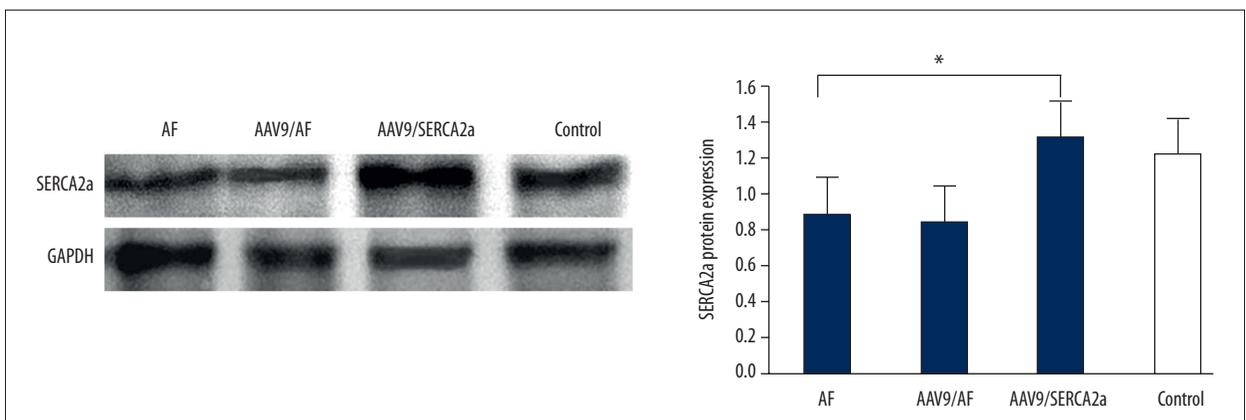


Figure 8. SERCA2a protein positive expression in the right atrium. SERCA2a protein expression in the AAV9/SERCA2a group was higher than that in the AF group ($P<0.05$), but there was no significant difference compared with the control group. * $P<0.05$.

Effect of rapid pacing on atrial fibrillation on ventricular arrhythmia

Atrial fibrillation patients show a serious rhythm and rate disorder in every chamber of the heart, resulting in changes in hemodynamics and related complications, prompting cardiovascular physicians to perform related research and seek breakthroughs [29]. Atrial fibrillation treatment, rhythm, and rate, especially atrial fibrillation ventricular rate control, has always been an important part of the related complications and prognosis. There is no doubt that there are serious, life-threatening symptoms of atrial fibrillation, and patients with aortic stenosis should do everything possible to restore and maintain sinus heart rate. Some studies suggest that rapid atrial activation can cause changes in the electrophysiological characteristics of atrioventricular nodules, mainly in the action potential shortening and frequency adaptation to reduce the reactivity, suggesting that this change in electrophysiological properties determines the atrioventricular node "filtration characteristics". This conduction controls the atrial fibrillation ventricular rate, reducing the incidence of ventricular arrhythmias and premature beats. We used intracardiac electrocardiography to observe the positioning of the electrode. The electrode was only in the right atrium, with atrioventricular node conduction with independent protection, reaching the ventricular pacing frequency to reduce the impact on the ventricle, but this was not obvious.

SERCA2a gene transduction method

Gene therapy as a therapeutic strategy that has broad prospects for the treatment of cardiovascular disease, and requires the establishment of cardiac-specific and potent gene transduction. A variety of gene transduction methods have been used to date, all of which can achieve efficient cardiac-specific transduction [30]. Commonly used cardiac gene transduction methods include intravenous injection, myocardial injection, and pericardial injection, each of which has advantages and disadvantages [31]. AAV9 is currently the most secure viral vector used in the transgenic treatment of cardiovascular disease [32]. It is safe, with low immunogenicity, and can be used to drive long-term exogenous gene expression in the body. Aoki et al. used an intra-pericardial injection of HVJ-liposome as a carrier to target myocardial tissue inferior to the pericardium, as well as transgene expression in several layers of cardiomyocytes and fibroblasts [33]. Fromes et al. performed a cardiac transgenic study using pericardial injection, and successfully achieved target gene expression in the myocardium [34]. In accordance with our previous gene transduction study [35], we used pericardial injection in the present study. We were able to successfully transduce SERCA2a gene expression in the atria, reducing the risk of major trauma and infection that can result from pericardial incision, and increasing clinical operability.

Improved AF atrial structure following SERCA2a overexpression

Studies have shown that intracellular Ca^{2+} overload can cause structural changes to the atrial myocardium. In particular, mitochondrial swelling and sarcoplasmic reticulum rupture in atrial myocytes are commonly associated with calcium overload during AF. Ausma et al. found that short-term calcium overload was consistent with myocardial ultrastructural changes in goats with AF, and suggested that the calcium overload activated the proteolytic enzyme pathway, which in turn led to morphological remodeling [36]. Overexpression of the SERCA2a gene can reduce intracellular calcium overload, inhibit the proteolytic enzyme pathway, and improve the mitochondrial swelling and sarcoplasmic reticular rupture associated with Ca^{2+} overload. Morillo et al. used a dog model of AF to show that muscle fibers of the atrial muscle tissue were thickened and irregularly arranged after rapid atrial pacing [37]. Moreover, mitochondria were larger, the sarcoplasmic reticulum was swollen, and nuclei were enlarged. In the present study, we observed the same effect using a rabbit model after a sustained stimulation of the right atrium at 600 beats/min frequency for 8 h. However, this was significantly improved following transduction of the SERCA2a gene group: myocardial tissues were regularly arranged, and vacuolar degeneration as well as inflammatory cell infiltration were decreased, as determined by pathological examination and electron microscopy. A number of possible mechanisms could explain the effect of SERCA2a overexpression on AF. For example, a direct increase in the ability of the sarcoplasmic reticulum to uptake calcium could reduce calcium overload. Alternatively, SERCA2a overexpression could reduce calcium overload directly by reducing the amount of Ca^{2+} in L-type calcium channels. Reducing intracellular calcium overload is a possible mechanism by which SERCA2a overexpression functions in the treatment of AF, providing a basis for the clinical application of SERCA2a gene therapy.

Limitations

We investigated the effect of SERCA2a overexpression on acute AF in rabbits, but did not study the effect on chronic AF. Our study did not use patch clamp technique, which changes the calcium ion channel current. This study also lacked clinical drug research. Therefore, this study provides some evidence but cannot be directly used in clinical practice. SERCA2a overexpression has an improved effect on AF and requires further study of the mechanism. Our study did not observe the electrophysiological and structural changes of the left atrium after rapid pacing, but the electrical and structural changes in the right atrium were consistent. The study did not use a variety of transduction gene methods to compare the effect of gene transduction, and did not observe the pericardium injection of foreign genes; the gene in the heart outside the liver and kidney tissue transgenic distribution and liver and kidney toxicity

effect. The experiments also lacked data on pro-collagen and rough endoplasmic reticulum. The mechanisms of calcium overload of AF involved in expression of SERCA2a could be better explained if the rough endoplasmic reticulum was recorded.

Conclusions

Gene transfer effectively increased SERCA2a protein levels, attenuated atrial effective refractory period prolongation,

improved electrical remodeling, and reduced atrial pathological changes in the rabbit AF model. Therefore, SERCA2a gene therapy has potential prevention effects against AF.

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