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Original Article

Reduce of atrial remodeling of rapid atrial pacing pig by inhibition of renin-angiotensin system with telmisartan

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Abstract: Objective: To investigate the effects of telmisartan on rapid atrial pacing pig left atrial remodeling and renin angiotensin aldosterone system. Methods: 18 healthy pigs were randomly divided into sham operation group (sham group), rapid atrial pacing group (rap group), angiotensin angiotensin II receptor inhibitor group (pacing + telmisartan, ARB group), and each group had 6 heads. Rapid pacing atrial (500 beats/min) of rap group and ARB group for 2 weeks, while install pacemaker (AOO) of sham group without pacing stimuli. 2 weeks later, left atrial myocardium was collected, and collagen was detected by Masson staining. The protein expression of TGF-β1, ACE2, p-ERK1/ERK2, ERK1/ERK2 and collagen-I was detected by Western-blot test. The Ang1-7 expression was detected by ELISA test. The mRNA expression of TGF-β1, ACE2 and ERK2 was detected by RT-PCR test. Results: 1 Compared with Sham group, expression of collagen positive staining and collagen-I increased obviously of RAP group, while compared with RAP group, expression of collagen positive staining and collagen-I decreased obviously of ARB group. 2. Compared with Sham group, protein expression of TGF-β1, p-ERK1/ERK2, ERK1/ERK2 and mRNA expression of TGF-β1, ERK2 increased ($P<0.05$), protein and mRNA expression of ACE2 decreased ($P<0.05$), and Ang1-7 expression decreased of RAP group. While compared with RAP group, protein expression of TGF-β1, p-ERK1/ERK2, ERK1/ERK2 and mRNA expression of TGF-β1, ERK2 decreased ($P<0.05$), protein and mRNA expression of ACE2 increased ($P<0.05$), and Ang1-7 expression increased of ARB group. Conclusion: Telmisartan can reduce atrial fibrosis of rapid pacing pig, and the mechanism may by increase of ACE2 to promote Ang1-7 synthesis, inhibition of TGF-β1-ERK pathway activation, to effectively inhibit matrix formation and maintenance of atrial fibrillation (AF).

Keywords: Atrial remodeling, renin-angiotensin system, telmisartan, rapid atrial pacing pig

Introduction

Atrial fibrosis (atrial fibrillation, AF) is one of the most common persistent arrhythmia in clinic. Atrial fibrosis is one of the most important pathological changes in atrial fibrillation, and related research has confirmed that fibrosis plays an important role in the development and progression [1, 2]. At present, there is sufficient evidence to suggest that atrial remodeling in AF is associated with activation of Rennin-Angiotensin system (RAS). In 2000, Donoghue found a homologue of ACE, named ACE2, the main biological effect was Ang1-7 promotion by degradation of Ang II. In contrast to the effect of Ang II, Ang1-7 had the effect of relaxing blood vessels, anti proliferation, anti fibrosis, can inhibit and reverse ventricular remodeling, and had protective effect on heart [4]. However, the ACE2, Ang1-7 expression in AF atrial tissue and the possible mechanism of ACE2, Ang1-7 expression in AF atrial structural remodeling and the effect of ARB drugs on the expression of ACE2, Ang1-7 were not reported. In this experiment, the pathological changes of atrial fibrillation were simulated by using a long time rapid right atrial pacing model in pigs, and Telmisartan was applied to observe left atrial myocardial fibrosis and expression changes of ACE2, Ang1-7, transformation growth factor β1 (transformation growth factor-Beta 1, TGF-β1), phosphorylated extracellular signal regulating kinase 1/2 (Phosphorylation extracellular signal regulating kinase1/2, p-ERK1/ERK2) and ERK1/ERK2. And investigate the effects of Telmisartan on atrial fibrillation atrial fibrosis and its possible mechanism.

Table 1. Primer sequences

Gene	Gene No.	Upstream or downstream sequences (5'→3')	Product size (bp)
ACE2	NM_001123070	GCCTCATTCCTGTAACTGCTGCTC	376
		GGGTCCAAAACTTTCCCACTACTGT	
TGF-β1	NM_214015.1	GCAGTGGCTGACCCGCAGAG	156
		AAGGGCCGGTTCATGCCGTG	
ERK2	XM_001929509.1	GAGCAGGCTCTTGCCCACCC	207
		GCACGTCCCGTCCTTGGAGC	
β-actin	DQ452569.1	GCTGCCGTAAAGGGGCCTCG	412
		GTAGCCCCGCTCCGTCAGGA	

Materials and methods

Healthy young pigs were collected, male and female, weight (37.4 + 2.5) kg, aged from 2 to 3 months. (Xuzhou Medical College Animal Experimental Center, Experimental Animal, License Number: SYXK200220038).

Main reagents

ACE2 antibody (Santa Cruz America, No.: SC-17719, dilution 1:1000), TGF-β1 antibody (Abcom America, No.: ab27969, dilution 1:1000), Collagen-I antibody (Abcom America), horseradish peroxidase marked mouse anti goat secondary antibody and horseradish peroxidase marked Rabbit anti mouse second antibody (Sigma, America), reverse transcription polymerase chain reaction (RT-PCR) kit (Promega, America), angiotensin 1-7 enzyme-linked immunosorbent adsorption assay (ELISA) kit (West of Shanghai XiTang Biotechnology Co., Ltd.).

Main instruments

Cardiac stimulation (Jinjiang Electronic instrument), external defibrillator (American Philips), fixed rate pacemaker (Nanjing Medical University), atrial "J" pacing electrodes (American Medtronic), C arm X-ray digital subtraction angiography (American Philips).

Investigated objects and divided groups

18 healthy pigs were randomly divided into three groups according to the random number table, namely sham operation group (sham group), rapid pacing group (RAP group) and Telmisartan group (ARB), with 6 pigs in each group.

Telmisartan TC was provided by Xuzhou Wanbang Pharmaceutical Co., Ltd., experiments were fed with 1.5 mg·kg-1·d-1, the ARB group was fed from three days before operation to two weeks after operation. Telmisartan was mixed with small amounts of ordinary feed, mixed feeding, and then fed ordinary feed, to ensure that the medicine was fully served.

The method to make rapid pacing model is as follows

3% pentobarbital 1 ml/kg intramuscular injection of pig after anesthesia, and the right jugular vein was punctured by Seldinger puncture technique, put into "J" shaped passive atrial electrode under X-ray, electrode head fixed in the right auricle, after measuring the electrical parameters (voltage threshold <1 V, 500~1000 Ω) with pacing analyzer, link the end of electrode and the experimental high frequency cardiac pacemaker controlled by embedded in vitro, and placed the pacemaker under the skin of neck, with pacemaker pacing mode AOO and pacing frequency 500 beats per minute. Intramuscular injected Sodium sodium 3.0 daily for 4 days to anti infection. Conducted ECG examination every other day during the pacing to verify whether the pacing was good.

Animals sampling

Animals were sacrificed after 2 weeks, intramuscular injected 3% pentobarbital 1 ml/kg, fixed pigs on the operating table after fully anesthetized, tracheal intubation, ventilator mechanical ventilation, pressure triggered synchronous mode, tidal volume 12-15 ml/kg, respiratory rate 25 breaths per minute, oxygen concentration 40%, injected 500 IU/kg heparin sodium heparin, opened the chest from the left margin of the pig, removed the heart and lung body tissue quickly, cut left atrium and left atrial tissue, flushed with physiological saline and removed blood fat tissue, put into liquid nitrogen tank quickly after packed and marked with aluminum foil package, put into -80°C box frozen storage for use after 48 hours. At the same time, fixed with 10% formalin, conducted paraffin block pathological detection.

Animal experiment scheme was in accordance with the National Institutes of Health (National Institutes of Health, the NIH) related experi-

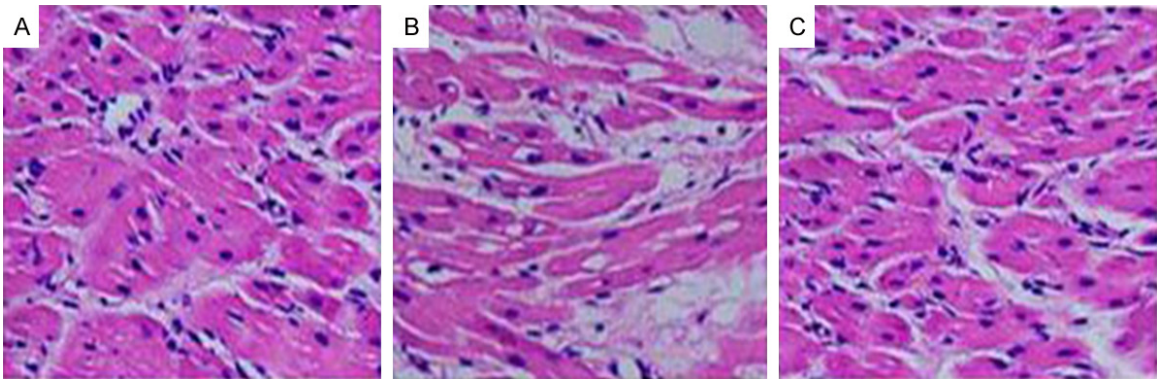

Figure 1. Groups of atrial tissue with Masson staining (×400). A. Sham group showed a small amount of collagen fibers in atrial muscle interstitial. B. RAP group showed significant fibrous tissue proliferation in atrial muscle interstitial, there was a large amount of collagen fibers around atrial muscle cells. C. ARB group showed a small amount of collagen fibers in atrial muscle interstitial, decreased compared with RAP group.

mental animal guide [5], raised by the Xuzhou Medical Animal Experiment Center, and the researchers had Experimental Animals Qualification Certificate of Jiangsu province.

Testing indexes

Tested the changes of collagen with mason (Masson) three color dyeing: collagen fibers were blue and muscle fibers were red. (2) Tested the expression of angiotensin 1-7 in left atrial with Enzyme-linked immunosorbent method: in strict accordance with the method of enzyme-linked immunosorbent kit manual operation. (3) Tested protein expression of TGF-β1, ACE2, p-ERK1/ERK2, ERK1/ERK2 and collagen-I with protein imprinting (Western blot) determination: took about 0.1 g of fresh left atrial myocardial tissue at the end of test, extractied cytoplasm protein in strict accordance with the organization extraction kit steps. Lowry-phenol reagent method was applied to protein quantitative. Conducted SDS polyacrylamide gel electrophoresis with total protein of 100 μg of each sample, transferred to nitrocellulose membrane, closed antibody for one night under 4°C, second antibody incubated 1 h under 37°C, infrared fluorescent color. J Image analyzer was used to conduct stripe gray analysis and calculation of target protein and inside protein β-action or internal GADPH bands ratio, which was used as a semi-quantitative analysis. Tested the expression of messenger RNA (mRNA) of TGF-β1, ACE2 in the left atrial with reverse transcription polymerase chain reaction (RT-PCR) determination: took about 0.1 g of fresh left atrial myocardial tissue at the end of test, extracted left atrial myocardial tissue ribonucleic acid (RNA) of pig with Trizol reagent (Shanghai Invitrogen Biotechnology Company) one-step method. One-step method was used to reverse transcription polymerase chain reaction. Primers were synthesized from Shanghai Invitrogen Biotechnology Company, and primer sequences were shown in **Table 1**. Reaction conditions were as follows: ACE2: 42°C 45 min 1 cycle, 94°C 2 min 1 cycle, 94°C 30 s, 55°C 45 s, 68°C 1 min 30 cycle, 68°C 7 min 1 cycle, TGF-β1: 42°C 45 min 1 cycle, 94°C 2 min 1 cycle, 94°C 30 s, 54°C 45 s, 68°C 1 min 30 cycle, 68°C 7 min 1 cycle; ERK2: 42°C 45 min 1 cycle, 94°C 2 min 1 cycle, 94°C 30 s, 54°C 45 s, 68°C 30 s 30 cycle, 68°C 7 min 1 cycle; β-actin: 42°C 45 min 1 cycle, 94°C 2 min 1 cycle, 94°C 30 s, 54°C 45 s, 68°C 1 min 30 cycle, 68°C 7 min 1 cycle.

Took reverse transcription polymerase chain reaction products 5 μl, put in 1.5% agarose gel and conducted electrophoresis, tested integral absorbance ratio of purpose gene and inside gene with gel imaging scan and analysis system.

Statistical processing

Measuring data were expressed as mean ± standard deviation, using SPSS13.00 software for statistical analysis, using single factor analysis of variance (ANOVA) (data accord with normal distribution, variance) and rank and inspection (data do not accord with normal distribution) analysis data, with $P<0.05$ for the difference which was statistically significant.

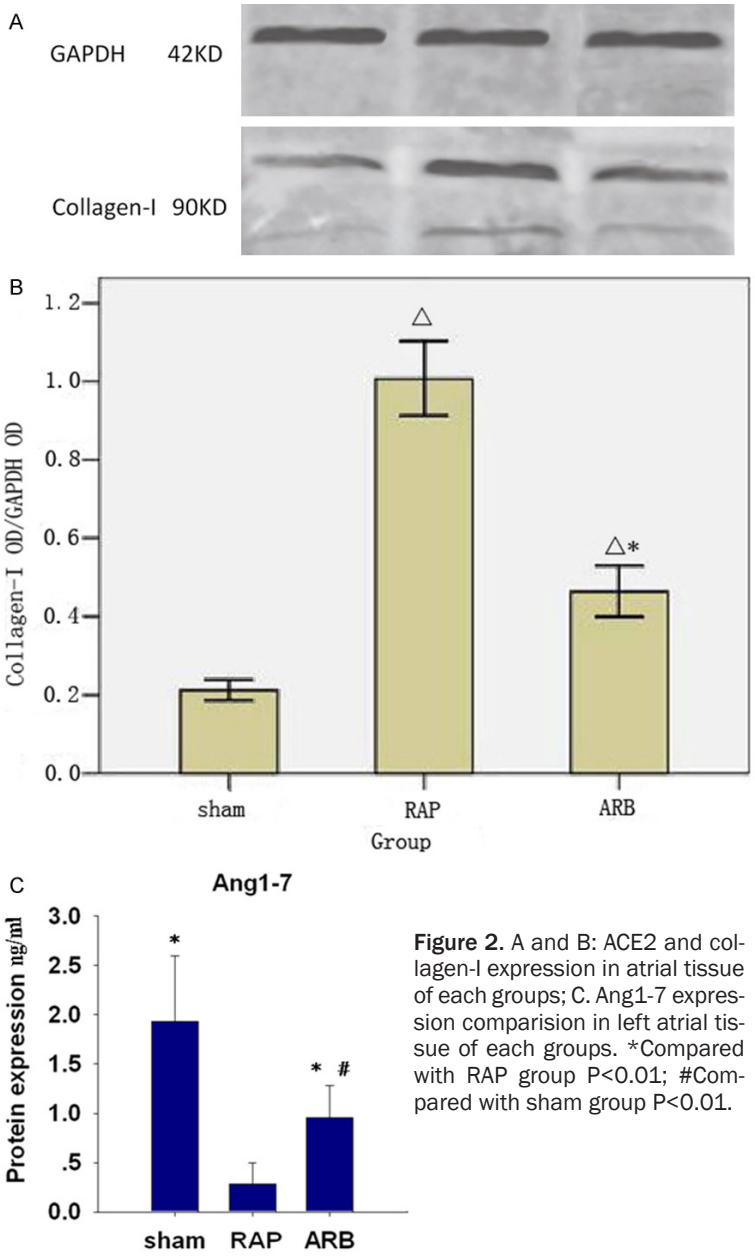

Figure 2. A and B: ACE2 and collagen-I expression in atrial tissue of each groups; C. Ang1-7 expression comparision in left atrial tissue of each groups. *Compared with RAP group P<0.01; #Compared with sham group P<0.01.

Results

Atrial fibrosis of three groups of animals

Sham group: as shown in **Figure 1A**, there was little atrial muscle tissue collagen positive staining, mainly situated around blood vessels, and there was no collagen deposition in myocardial interstitial cells. RAP group: as shown in **Figure 1B**, AF atrial muscle tissue collagen positive staining increased, not only situated around blood vessels, collagen deposition in myocardial interstitial cells increased as well. ARB group: as shown in **Figure 1C**, compared with RAP group, there was less atrial muscle tissue collagen positive staining, mainly situated around blood vessels and myocardial interstitial cells.

Content of collagen-I of RAP group was obviously higher than that of normal control group, ARB group (P<0.05), while content of collagen-I of ARB group was obviously lower than that of RAP group (P<0.05), but still higher than normal control group (P<0.05) (**Figure 2**).

Ang1-7 expression changes of each group with ELISA test

Compared with sham group, Ang1-7 express in left atrial myocardial decreased of RAP group [(1.93±0.67) ng/ml than (0.28±0.22) ng/ml, P<0.01]; while compared with RAP group, Ang1-7 express increased of ARB group [(0.28±0.22) ng/ml than (0.96±0.32) ng/ml, P<0.01] (**Figure 2C**).

Western-blot result

Tested protein expression of TGF-β1, ACE2, p-ERK1/ERK2 and ERK1/ERK2 of each group with Western-blot method. Compared with sham group, protein expression of TGF-β1, p-ERK1/ERK2 and ERK1/ERK2 in left atrial myocardial increased (P<0.05), and protein expression of ACE2 decreased (P<0.05) of RAP group; While compared with RAP group, protein expression of TGF-β1, p-ERK1/ERK2 and ERK1/ERK2 decreased (P<0.05), and protein expression of ACE2 increased (P<0.05) of ARB group (**Figure 3**).

RT-PCR result

Tested mRNA expression of TGF-β1, ACE2 and ERK2 in left atrial myocardial with RT-PCR. Compared with sham group, mRNA expression of TGF-β1 and ERK2 in left atrial myocardial

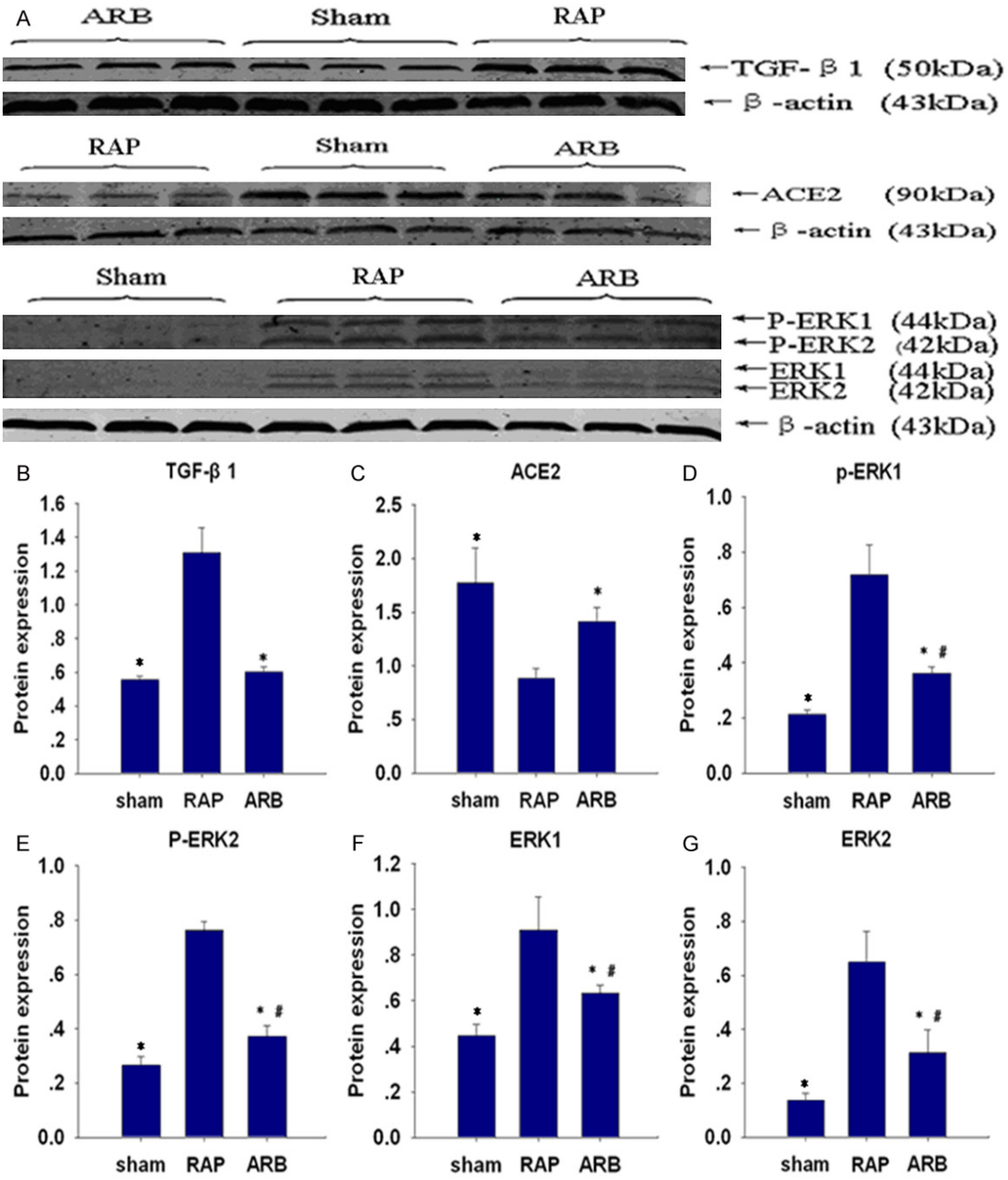

Figure 3. Protein expression of TGF-β1, ACE2, p-ERK1/ERK2 and ERK1/ERK2 in left atrial tissue of each groups. *Compared with RAP group P<0.05; #Compared with sham group P<0.05. A: Protein expression electrophoresis map of TGF-β1, ACE2, p-ERK1/ERK2 and ERK1/ERK2 in left atrial tissue of each groups; B-G: Protein expression semi-quantitative analysis of TGF-β1, ACE2, p-ERK1/ERK2 and ERK1/ERK2 in left atrial tissue of each groups.

increased (P<0.05), and mRNA expression of ACE2 decreased (P<0.05) of RAP group; While compared with RAP group, mRNA expression of TGF-β1 and ERK2 decreased (P<0.05), and mRNA expression of ACE2 increased (P<0.05) of ARB group (**Figure 4A-D**).

Correlation of the Ang1-7 and TGF-β1 protein expression

Protein expression of Ang1-7 and protein expression of TGF-β1 in atrial tissue had a negative correlation (r=0.813, P<0.01) (**Figure 4E**).

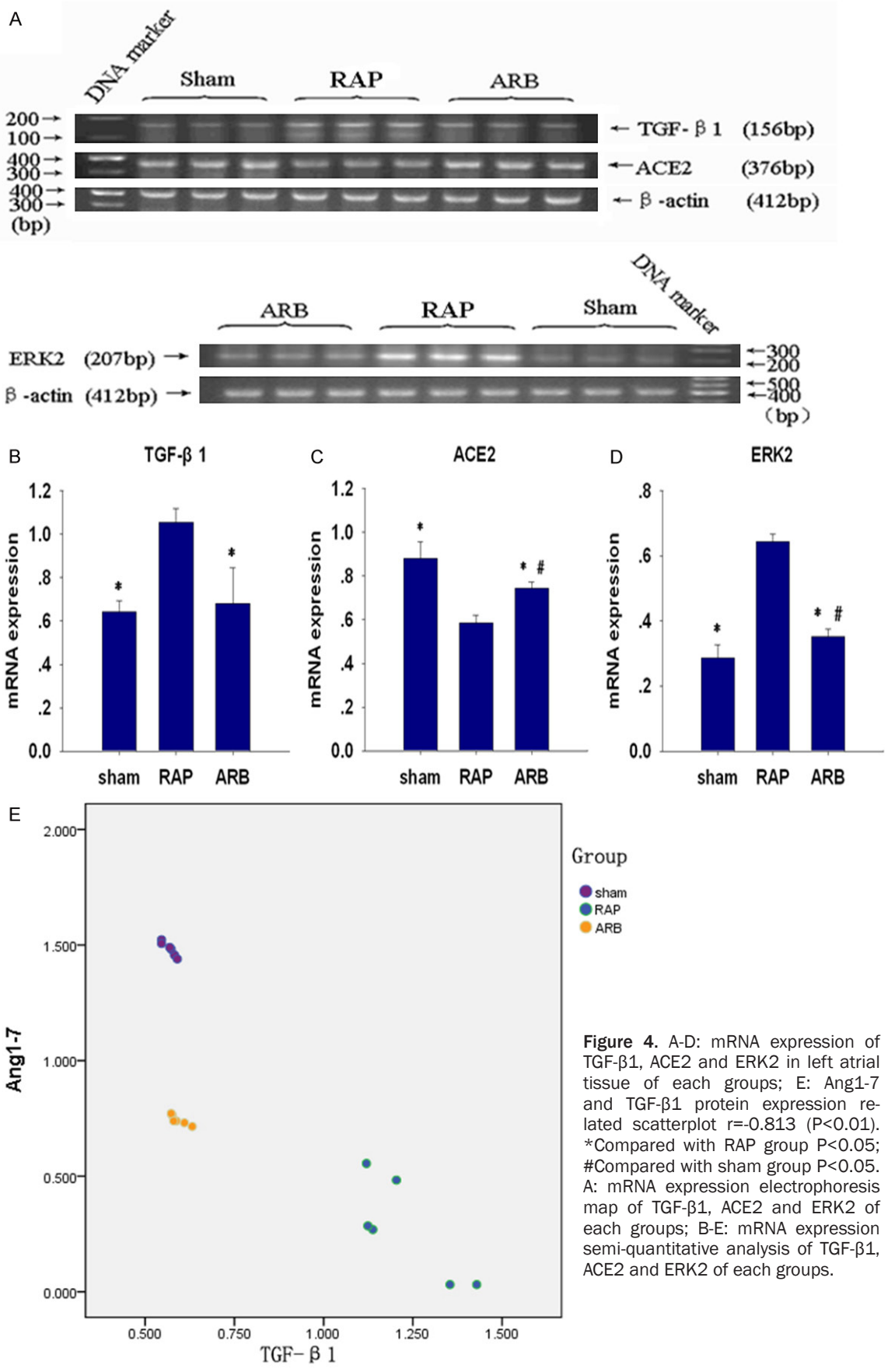

Figure 4. A-D: mRNA expression of TGF-β1, ACE2 and ERK2 in left atrial tissue of each groups; E: Ang1-7 and TGF-β1 protein expression related scatterplot r=-0.813 (P<0.01). *Compared with RAP group P<0.05; #Compared with sham group P<0.05. A: mRNA expression electrophoresis map of TGF-β1, ACE2 and ERK2 of each groups; B-E: mRNA expression semi-quantitative analysis of TGF-β1, ACE2 and ERK2 of each groups.

Discussion

There has been more than one hundred years for atrial fibrillation mechanism research so far, but there is no hypothesis which can fully explain all phenomenon. Atrial fibrillation may be a complex arrhythmia produced by a variety of mechanisms. In the early 1990s, Allessie and wijffel and other researchers adopted epicardial pacemaker method to establish the chronic persistent AF animal model, which laid a good foundation for atrial fibrillation mechanism research. But this method had disadvantages such as large damage and complex operation [6, 7]. In 1995, Morillo pioneered in implanted pacemaker method for rapid pacing to establish animal models of chronic AF dogs [8]. This research succeeds in long-time rapid pacing through pacemaker implantation in pigs, to a certain extent, which can imitate atrial fibrillation pathological process. We punctured jugular vein directly with neck Selding blind puncture method, avoided vein incision, and chose the right jugular vein, for the reason that we had known from dissection that pig's right external carotid venous confluence with former vena cava is flat with left external carotid venous confluence with former vena cava, so that pacemaker electrode can go through successfully. The right auricle of pig is bigger, and J type passive electrode retrace slightly after flowing into right atrium through superior vena cava, which can be fixed at the tip of right atrium. This model was simple with high success rate, and it has not found complications such as electrode dislocation and pacemaker dysfunction in a long process of pacemaker.

Currently recognized that atrial remodeling plays an important role in generation and maintain of AF [9]. Fibrosis is the most important structure reconstruction, and there is significant atrial fibrosis in patients with persistent AF and AF animal models with organic cardiopathy [10, 11]. Our study found that: left and right atrium amplified after 2 weeks' rapid atrial pacing; collagen deposition increased around blood vessels and in myocardial interstitial cells, adipose tissue infiltrated, cell arrangement and organization structure broken, and there was significant interstitial fibrosis, which indicated that 2 weeks' quick pacemaker can induce atrial clear refactoring.

Partial RAS exists in myocardial tissue, which has huge effects on cardiac remodeling. RAS are involved in myocardial fibrosis process in diseases such as hypertensive heart disease, congestive heart failure and others [12, 13]. Previous studies show that ACE concentration in atrial tissue increased of AF patients. Ang II and related transmitters are the key link to promote atrial fibrosis [14, 15]. Classic RAS is mainly composed of Renin, Ang II and ACE, and ACE is the core enzyme of RAS, which produce the most important effect factor-Ang II of RAS. In 2000, Donoghue and Tipnis cloned homologue of ACE from left ventricular tissue cDNA library of human lymphoma and enlarged heart CHF patients respectively, named ACE2 [4, 16]. The expression parts of ACE2 a re more limited than that of ACE, which is mainly expressed in the heart, kidney and testis. ACE2 can degrade Ang II to produce Ang1-7 [17]. Grobe found that long-term application of Ang1 can prevent myocardial hypertrophy and fibrosis caused by long-time Ang II infusion, and found that application of Ang1-7 can make TGF-β levels fell by 40% of long-term Ang II infusion group through measuring fibrosis promoting signaling molecules TGF-β level in plasma in experiments, which indicated that anti fibrosis and antiproliferative effect of Ang1-7 may be achieved by inhibition of TGF-β [18]. Compared with wild-type mice, myocardial fibrosis level and degree of myocardial hypertrophy were both lower of ACE2 transgenic mice after injection of Ang II, which indicated that excessive expression of ACE2 can partly resist myocardial hypertrophy and fibrosis induced by Ang II [19]. This study find that after continued rapid atrial pacing, ACE2 expression level in left atrial myocardial tissue decreased, and Ang1-7 expression level decreased, which indicates that decline of Ang1-7 expression may play an important role in atrial fibrillation atrial remodeling. However, when Ang1-7 expression declined, expression of TGF-β1, p-ERK1/ERK2, and ERK1/ERK2 increased. Correlation analysis has showed that protein expression of Ang1-7 and protein expression of TGF-β1 in atrial tissue has a negative correlation, therefore suggests that Ang1-7 may play the role of inhibition of myocardial fibrosis through decreasing expression of TGF-β1, p-ERK1/ERK2 and ERK1/ERK2, which indicates that decline of ACE2 expression may play an important role in atrial remodeling when AF.

A large number of studies have confirmed the ARB can prevent AF episodes [20-22], and can significantly reduce the chance of AF recurrence after ring of pulmonary vein linear radiofrequency ablation surgery [23]. Losartan can effectively prevent atrial fibrillation of hypertension with atrial fibrillation patients [24]. Olmesartan can reverse moderate myocardial hypertrophy, reduce structure reconstruction caused by atrial fibrillation, and prevent recurrence of atrial fibrillation [25]. Candesartan also can reduce sensitive index of atrial fibrillation through inhibition of atrial remodeling to reduce occurrence of atrial fibrillation [26]. And plasma Angl-7 increases significantly of normal blood pressure volunteers after using irbesartan [27]. ACE2 expression and activity increase in hearts of myocardial infarction (MI) rats with losartan treatment, and contributs to the increases of Ang-(1-7) activity to reverse myocardial remodeling [28]. This study finds that ACE2 and Ang1-7 expression levels in left atrial myocardial tissue of Telmisartan group are higher than that of RAP group, while expression levels of TGF-β1, p-ERK1/ERK2 and ERK1/ERK2 are lower than that of RAP group, which indicates that Telmisartan can prevent expression of ACE2, which leads to increase of Ang1-7, and can inhibit myocardial fibrosis through declining expression of TGF-β1, p-ERK1/ERK2 and ERK1/ERK2.

Lack of research

Because the time of pacemaker is short, and in the process of pacemaker, it has not been conducted atrioventricular node ablation and implantation of ventricular pacing at the same time, which may not be able to simulate persistent AF pathological process completely.

Disclosure of conflict of interest

None.

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