Experimental Animals
Exp. Anim. 70(3), 293–301, 2021

Original

Effects of Angiotensin II type I receptor shRNA on blood pressure and left ventricular remodeling in spontaneously hypertensive rats

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Abstract: This study was designed to investigate the effects of the Angiotensin II type I receptor (*AT1R*) shRNA on blood pressure and left ventricular remodeling in spontaneously hypertensive rats. Ten Wistar Kyoto (WKY) rats were used as a normal blood pressure control group, and 20 spontaneously hypertensive rats (SHR) were randomly divided into the experimental and hypertension control groups. The rats in the experimental group were injected with *AT1R* shRNA recombinant adenovirus (Ad5-*AT1R*-shRNA) via a tail vein, and the rats in the other two groups were injected with recombinant adenovirus (Ad5-EGFP). The systolic blood pressure (SBP) at rat arteria caudalis was measured before and after the injection, and the heart, kidney, aorta, and adrenal tissues were obtained two days after repeated injection to observe the distribution of Ad5-*AT1R*-shRNA under a fluorescence microscope. Before the injection of Ad5-*AT1R*-shRNA, the blood pressure of the experimental group and the hypertension control group was significantly higher than that of the normal blood pressure control group (P<0.01). After two injections, the blood pressure in the experimental group decreased significantly, and the duration of blood pressure reduction reached 19 days. In the experimental group, the kidney, heart, aorta, and adrenal gland tissues showed vigorous fluorescence expression under the fluorescence microscope. Repeated administration of Ad5-*AT1R*-shRNA has a long-lasting hypotensive effect on SHR and can significantly improve ventricular remodeling.
Key words: hypertension, RNAi, spontaneously hypertensive rats, type 1 Angiotensin II receptor, ventricular remodeling

Introduction

Ventricular (or myocardial) remodeling is a concept proposed in the study of ischemic cardiomyopathy, hypertensive heart disease, dilated cardiomyopathy, and other forms of heart damage, which are mainly characterized by the changes after myocardial injury, such as cardiomyocyte hypertrophy, non-myocyte proliferation, and myocardial fibrosis, and the heart cavity expansion and heart weight increase on this basis [1]. Cardiac hypertrophy is the most common pathological change in hypertension, where a series of changes of subcellular morphology and structure often appears in hypertrophic myocardium.

A previous study has confirmed that the over activation of the RAS system (renin-Angiotensin system) plays a crucial role in the pathological process of hypertensive ventricular remodeling, due to vasopressor function, cell growth and proliferation promoting effect [2]. Ang II is the main active substance of RAS, and can exert biological effect by specifically activating *AT1R*, which has high affinity on the surface of cardiac fibroblasts [3]. In addition, Ang II also has a certain growth factor-like effect and can directly promote the synthesis of RNA

(Received 13 October 2020 / Accepted 18 January 2021 / Published online in J-STAGE 11 February 2021)
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and protein in cardiomyocytes to promote the proliferation of fiber cells, causing ventricular remodeling [4].

Furthermore, RNA interference technology refers to using shRNA to produce active RNA short interfering RNA (siRNA) in cells and specifically binds and degrades the mRNA of complementary target genes, so as to inhibit the expression of the target gene [5]. Recombinant adenovirus vector (Ad5-*AT1R*-shRNA), designed based on the principle of RNA interference, specifically blocked the expression of *AT1R* and has exerted significant hypotensive effect in previous antihypertensive studies conducted by the investigators [6–8]. However, as a relatively new form of gene therapy for hypertension, it is unknown whether it has an effect on ventricular remodeling caused by hypertension and the effects and side effects of repeated intravenous injection have not been reported.

In the present study, spontaneously hypertensive rats (SHR) were selected as the experimental subjects, the blood pressure and ventricular remodeling of SHR were observed after repeated injection of Ad5-*AT1R*-shRNA to investigate the effect of gene blocking of *AT1R* induced by Ad5-*AT1R*-shRNA on the remodeling in hypertension.

Materials and Methods

Drugs, reagents, and instruments

The Ad5-*AT1R*-shRNA and the control recombinant adenovirus vector (Ad5-EGFP) carrying the Angiotensin II type 1 receptor (*AT1R*) shRNA were constructed and synthesized by Benyuan-Zhengyang Gene Technology Co., Ltd. (Beijing, China). The hydroxyproline (HYA) detection kit was purchased from the Nanjing Jiancheng Biological Engineering Institute. The non-invasive blood pressure instrument for the rat tail artery (PowerLab, ADInstruments, New South Wales, Australia) was provided by the Laboratory of Cardiology in our college. The 722 grating spectrophotometer was made by Shanghai Third Analytical Instrument Factory (Shanghai, China). The transmission electron microscope (Fei Tecnai G2 12 type transmission electron microscopy, FEI Company, Hillsboro, OR, USA) was provided by the Electron Microscope Room of Tongji Medical College, Huazhong University of Science and Technology. The current study has been approved by the Ethics Committee of Tian Jin Chest Hospital.

Animal grouping and administration

Adult male SHR and Wistar Kyoto (WKY) rats were all 12 weeks old and weighed 280–320 g. There were 3–5 rats per cage, the room temperature was kept at 24 ± 2°C, and the relative humidity was maintained at 60–65%. A 12-h artificial day and night cycle was provided in which 7:00 AM–7:00 PM indicated the lighted portion of the cycle, and 7:00 PM–7:00 AM indicated the dark portion of the cycle. The rats could freely eat and drink. The groups of rats included a normal blood pressure control group (WKY group, n=10), a hypertension control group (SHR group, n=10), and a hypertension experimental group (experimental group, n=10).

The rats in the experimental group were intravenously injected with 1 ml of Ad5-*AT1R*-shRNA with a titer of TCID50 1.7×10^9 via a tail vein, and the rats in the SHR and WKY groups were intravenously injected with 1 ml of Ad5-EGFP with a titer of TCID50 1.7×10^9 via a tail vein. The injection time took place at the beginning of the experiment, and when the blood pressure had basically returned to the initial state after the first injection.

For all rats in each group, about 80–90 g of myocardial tissue was taken, and the operation was carried out strictly according to the instructions of hydroxyco3ine detection kit.

Measurement of the blood pressure and heart rate and observation of experimental animals

The systolic blood pressure (SBP) of the tail artery and the heart rate were measured by the tail-cuff method in a quiet environment at 0, 1, 2, 3, 4, 5, 6, 7, 9, 11, 13, 15, 17, 19, and 21 days of injection. The tail of that rat was preheated to about 40°C and fixed in a comfortable position. The compression cuff was placed at the root of the tail of the rat, inflated, and pressurized. When the pressure reached 270 mm Hg (1 mm Hg = 0.133 kPa), the pressure reduced automatically and slowly, and the blood pressure and heart rate were recorded by the Medlab biological function experiment system connected to a pressure transducer and an amplifier. All rats were measured five times continuously, and the means were calculated and used as the SBP and heart rate. The appetite, activity, body temperature, body weight (BW), and other reactions of the animals were observed daily until the end of the experiment.

Detection of distribution of the recombinant adenovirus vector in the main tissues

Animals in the experimental group were injected with Ad5-*AT1R*-shRNA labeled with fluorescent protein. Two days after repeated injection of Ad5-*AT1R*-shRNA, the heart, kidney, aorta, and adrenal tissues of the experimental group were obtained and then immediately prepared into frozen sections. The fluorescence expression was observed under a fluorescence microscope. When blood pressure clearly reduced, some animals were sac-

rificed and weighed. The animals were injected peritoneally with 0.4 ml/100 g of pentobarbital for anesthesia, and the heart, kidney, aorta, and adrenal tissues were rapidly removed. With a sharp blade, these organs were cut into 1 × 1 × 1 cm small tissue pieces and were sent to the Department of Pathology, where they were prepared into frozen sections. Their thickness measured between 5–8 μm, and the expression of fluorescence protein was observed under a fluorescence microscope to evaluate the absorption of Ad5-*AT1R*-shRNA by major organs.

Preparation of myocardial samples

At the end of the experiment, the BW of rats was measured. The rats were injected peritoneally with 0.4 ml/100 g of pentobarbital for anesthesia. After opening the chest, the heart was quickly removed, the surrounding blood vessels were cut off, the heart blood was dried with filter paper, the whole heart wet weight (HW) was weighed, and the ratio of heart weight to BW (HW/BW) was calculated [9, 10]. Some 1-mm^3 myocardial tissue pieces were taken from the apical region, rinsed with 0.1 mol/l phosphate-buffered saline (PBS), then initially fixed with 2.5% glutaraldehyde (pH 7.2). They were then fixed with a 1% osmium tetroxide solution (pH 7.2), dehydrated by ascending gradient alcohol and acetones, soaked and embedded with epoxy resin, and prepared into ultrathin sections. Another 60–100 g of left ventricular myocardium was taken for the measurement of HYA.

Observation indexes

The SBP of the tail artery in a quiet waking state was recorded before injection and at all time points after injection. About 80–90 g of myocardial tissue was taken, and the content of HYA in the myocardial tissue was detected using a biological method, according to the instructions of the HYA detection kit, and then converted to collagen content, according to the formula: collagen content = HYA content × 7.46 [11]. The BW and HW of the rats were recorded, and the HW/BW ratio was calculated. The ultrathin sections were stained with both uranyl acetate and lead citrate and observed and photographed under a transmission electron microscopy.

Detection of the expression of *AT1R* mRNA by real-time fluorescent quantitative polymerase chain reaction (RT-qPCR)

At the end of the experiment, 0.5 g of left ventricle tissue was taken from all groups to detect the expression of *AT1R* mRNA. The rat *AT1R* gene sequence was selected on GenBank (serial number NM_030685), and the primers and probes were designed using Primer Premier 5.0 and synthesized by Sangon Biotech (Shanghai, China). The *AT1R* upstream primer was 5'-ACG ATG CTG GTG GCC AAA GT -3', and the downstream primer was 5'-ATG ATA AGG AAA GGG AAC AAG AAG C -3'. The probe sequence was 5'-ATT ATG AGT CTC GGA ATT CGA CGC TCC CCA-3', and the probe modification was 5'-FAM, 3'-TAMRA, 3'-PO4. The β-actin upstream primer was 5'-AGG GAA ATC GTG CGT GAC AT -3', and the downstream primer was 5'-CCA TAC CCA GGA AGG AAG GCT-3'. The probe sequence was 5'-CAC TGC CGC ATC CTC TTC CTC CCT GG-3', and the probe modification was 5'-FAM, 3'-TAMRA, 3'-PO4. The total RNA was extracted from the tissues using the Trizol one-step method and reversely transcribed into cDNA by the Moloney Murine Leukemia Virus (MMLV) reverse transcription system. The preparation of standard curve samples consisted of the target gene and the housekeeping gene (β-actin) of the sample needed to be amplified by PCR (polymerase chain reaction). The product was diluted by gradient to make a standard curve. The PCR reaction system was 2 μl of 10 × Buffer, 2 μl of $MgCl_2$, and 0.5 μl of dNTPs. There was 0.5 μl of the upstream primer, 0.5 μl of the downstream primer, 1 μl of the probe, 0.4 μl of the Taq enzyme, and 2 μl of reaction template, and sterile deionized water was added until the volume reached 20 μl. The PCR system was placed in a fluorescent PCR tube, amplification was carried out, and fluorescence signals were collected on the Rotor-gene fluorescence quantitative PCR detector. The reaction conditions were as follows: 95°C, 5 min; 94°C, 5 s; 60°C, 30 s for a total of 45 performed cycles. The fluorescent signals were then collected, and the results were analyzed. In order to further eliminate the impacts of mRNA quantification and reaction efficiency of PCR on the results in the experiment, the quantitative detection of the absolute copy number of internal control β-actin was carried out simultaneously, the ratio of the absolute copy number of *AT1R* to β-actin was used as the relative expression of the *AT1R* gene.

Statistical analysis

The statistical analysis of data was conducted using statistical software SPSS 12.0. Repeated measurement data among the three groups were analyzed using analysis of covariance. All data sets fulfilled assumptions for carrying out ANCOVA. The Student-Newman-Keuls (SNK)-q test was used for multiple comparisons among means of multiple groups. Data in all groups were expressed as mean ± SD. $P<0.05$ was considered statistically significant.

Results

Ad5-*AT1R*-shRNA significantly reduced SBP in SHR

Before injection, SBP was significantly higher in the experimental group, and that of the SHR group was higher than in the WKY group ($P<0.05$). Twenty-four hours after the first injection, SBP was significantly lower in the experimental group than in the SHR group ($P<0.01$), and SBP did not decrease significantly in the SHR group and WKY group ($P>0.05$). After the first injection of Ad5-*AT1R*-shRNA, the hypotensive effect lasted for five days in the experimental group, and the blood pressure was basically recovered on the 7th day. After the second injection, the blood pressure significantly decreased again, and the duration was longer than that after the first injection, lasting for 14 days (Fig. 1). The local and systemic reactions of animals were observed every day, and no abnormalities, such as anorexia, weight loss, and increased temperature, were found. There were no significant changes in heart rate in the three groups, and the differences among the three groups were not statistically significant.

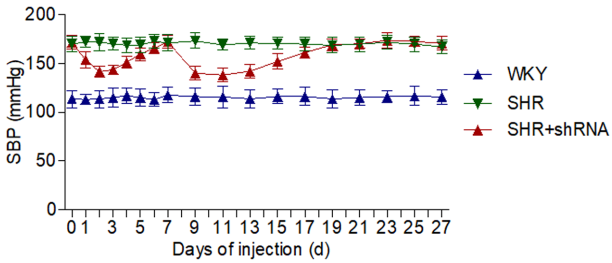

Fig. 1. Effect of Ad5-*AT1R*-shRNA on SBP in SHR .WKY group: Ad5-EGFP was injected at 0th and 7th days. SHR group: Ad5-EGFP was injected at 0th and 7th days. Experimental group: Ad5-*AT1R*-shRNA was injected at 0th and 7th days. The SBP of tail artery in rats was monitored every day after the first injection and every two days after the second injection.

Ad5-*AT1R*-shRNA significantly reduces the *AT1R* mRNA expression in heart tissue

In order to verify whether Ad5-*AT1R*-shRNA has the effect of RNA interference on the expression of *AT1R* mRNA in tissues, on the 21st day after the Ad5-*AT1R*-shRNA injection, the total RNA of the heart tissue was extracted in all groups. After reverse transcription into cDNA, the copy number of genes was detected by real-time fluorescence quantitative PCR. The results of the PCR amplification curve revealed that the expression of *AT1R* mRNA in the experimental group was significantly lower than that in the hypertension control group. Moreover, the difference was statistically significant ($P<0.05$, Fig. 2).

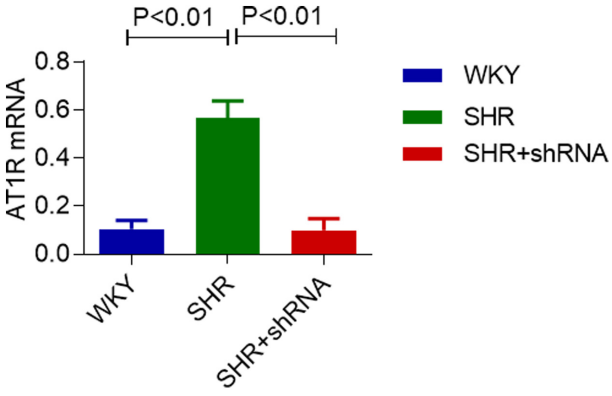

Fig. 2. Effect of injection of Ad5-*AT1R*-shRNA on *AT1R* mRNA in heart tissue: On the 21st day of injection, the heart tissue of the animal was obtained, the change of *AT1R* mRNA was detected by RT-PCR.

Distribution of Ad5-*AT1R*-shRNA in tissues

The fluorescence-labeled recombinant adenovirus was distributed in in the heart, kidney, aorta, and adrenal tissues after 2 days of repeated injection of Ad5-*AT1R*-shRNA (Fig. 3). Since *AT1R* was widely distributed in the tissue, the results of this study were consistent with the *in vivo* distribution of *AT1R*.

Ad5-*AT1R*-shRNA significantly reduces myocardial hypertrophy in SHR

The condition of myocardial hypertrophy of rats can be reflected by measuring the HW and the HW/BW ratio. By measuring the heart weight and body weight of rats, this study revealed that HW and HW/BW were significantly higher in the SHR group than those in the WKY group ($P<0.01$). The result suggests that there was obvious myocardial hypertrophy in untreated SHR. On the 21st day after Ad5-*AT1R*-shRNA injection, HW and HW/BW were lower in the experimental group than in the SHR group ($P<0.05$) (Fig. 4).

Ad5-*AT1R*-shRNA can reduce the degree of myocardial fibrosis in SHR

The contents of HYA and collagen were significantly higher in the SHR group than in the WKY group ($P<0.01$). These results suggest that obvious myocardial fibrosis has been found in the cardiac ventricle in the SHR group. The contents of HYA and collagen were significantly lower in the SHR group than in the SHR group ($P<0.01$) (Fig. 5).

Ad5-*AT1R*-shRNA significantly improves the myocardial ultrastructure of SHR

The ultrastructural observation of the myocardium by

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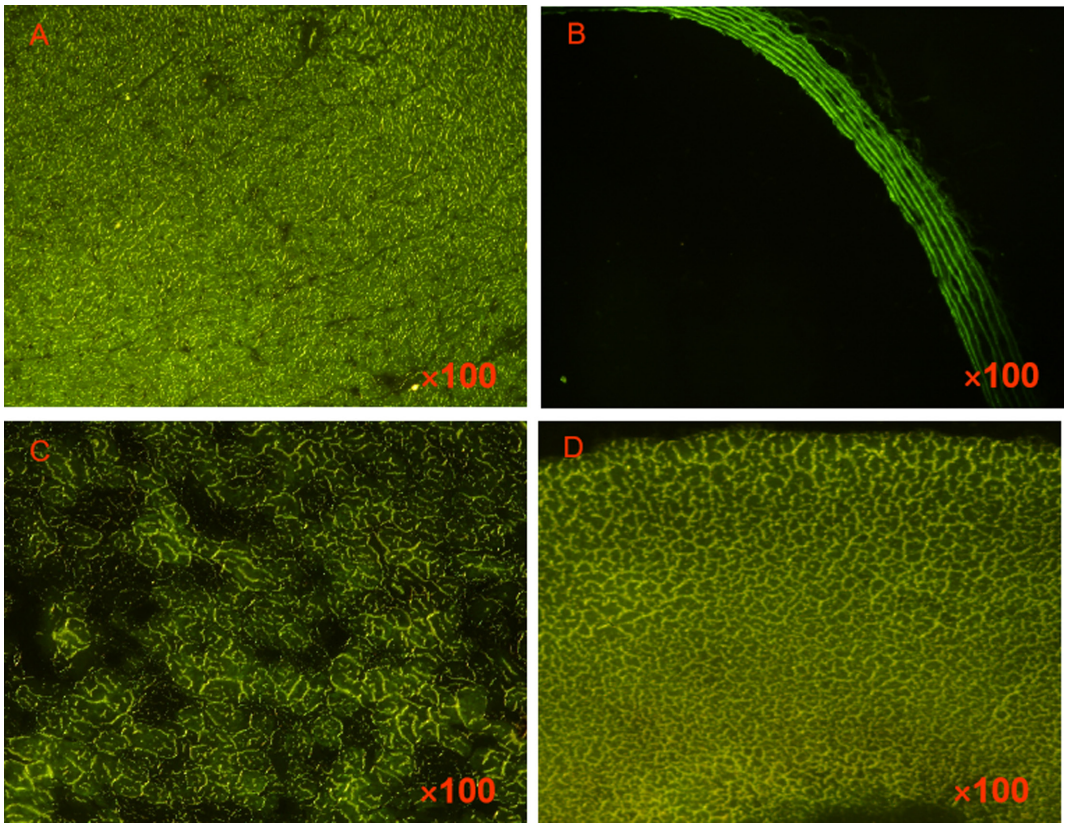

Fig. 3. Partial tissue distribution of injection of Ad5-*AT1R*-shRNA. The heart, kidney, aorta and adrenal tissues of all groups were obtained, then prepared into frozen sections immediately, and the fluorescence expression was observed under a fluorescence microscope, to observe the fluorescence expression of myocardial tissue (A) aortic tissue (B) renal tissue (C) and adrenal tissue (D).

transmission electron microscope showed that in the WKY group, the nuclear membrane of myocardial cells was intact, the myofibrils were clear, the arrangement was neat, the transverse lines were clear, the mitochondria were clear, and there was no collagen fiber proliferation in the myocardial interstitium (Figs. 6A and B). In the SHR group, the nuclear membrane of myocardial cells was not intact, the myofibrils were fuzzy, and the arrangement was irregular, some of the myofilaments disappeared. It could be seen that the transverse lines were not clear, and mitochondria were swollen and accumulated. Moreover, vacuoles were formed in it, a large number of endoplasmic reticulum hyperplasia and collagen fiber proliferation were observed in the myocardial interstitium (Figs. 6C and D). Compared with the WKY and SHR groups, in the experimental group, the nuclear membrane of myocardial cells was relatively intact, the myofibrils were clear, the arrangement was relatively neat, the transverse lines were clear, and the mitochondria were not swollen. However, the number of mitochondria increased in local regions, and there was no obvious collagen fiber proliferation in the myocardial interstitium (Figs. 6E and F).

Discussion

In the present study, we found that Ad5-*AT1R*-shRNA could significantly reduce the blood pressure of SHR, reduce the *AT1R* mRNA expression in heart tissue. The recombinant adenovirus vector has a certain targeting effect, which can be absorbed by the tissue containing *AT1R* after tail vein injection. Further, Ad5-*AT1R*-shRNA could reduce myocardial hypertrophy in SHR, improve myocardial fibrosis in SHR by inhibiting *AT1R* expression and improve the ultrastructural changes of the myocardium in SHR.

In normal rats, SBP is 110–120 mmHg, and in SHR, SBP can be as high as more than 200 mmHg. The incidence of hypertension was 100%, and these rats also indicated hypertensive cardiovascular disease. Therefore, this type of rat is suitable for the study of human hypertension. Spontaneously hypertensive rats usually begin to exhibit left ventricular hypertrophy at the age of 10 weeks, which aggravates progressively with age, collagen content in myocardium increases significantly, and myocardial fibrosis and myocardial interstitial remodeling occurs [12, 13]. The present study revealed

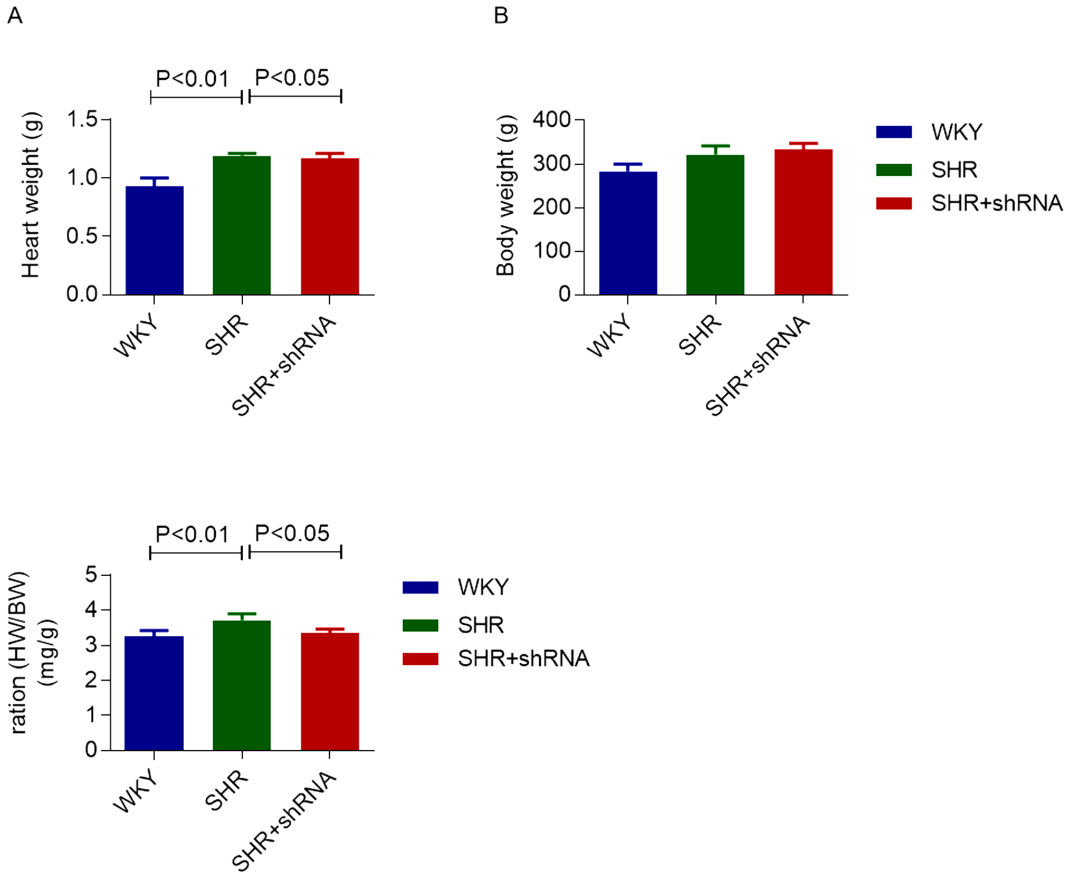

Fig. 4. Effect of Ad5-*AT1R*-shRNA injection on the ratio of heart weight to body weight in SHR. On the 21st day after injection, the rats' weight and the whole heart weight were measured and the ratio was calculated.

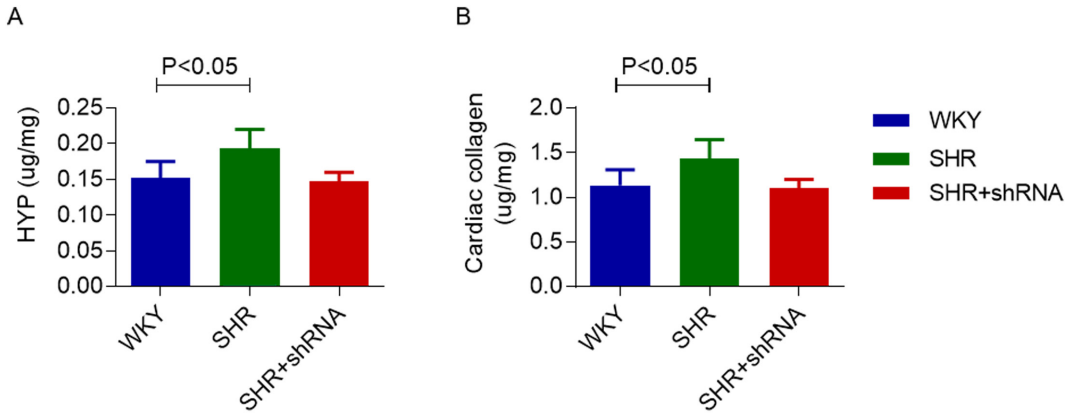

Fig. 5. Effect of Ad5-*AT1R*-shRNA injection on the content of HYA and collagen in myocardium in SHR: On the 21st day of injection, the heart tissue of the animal was obtained, and the content of HYA and collagen in myocardium was measured by biological method.

that compared with 16-week-old WKY rats, HW, HW/BW, the contents of HPA, and collagen in the myocardium significantly increased in 16-week-old SHR. The increase in the HW and HW/BW reflects the proliferation and hypertrophy of cardiomyocytes and the increase of heart weight. Furthermore, HYA is unique to collagen fibers, and its content can reflect collagen content and fibrosis degree. Furthermore, the results of electron microscopic examination revealed that in the SHR group, the nuclear membrane of myocardial cells was not intact, the myofibrils were fuzzy, the arrangement was irregular, some of the myofilaments disappeared, the transverse lines were unclear, mitochondria were swollen and accumulated, and vacuoles were formed in it. A large number of endoplasmic reticulum hyperplasia and collagen fiber proliferation were observed in the myocardial in-

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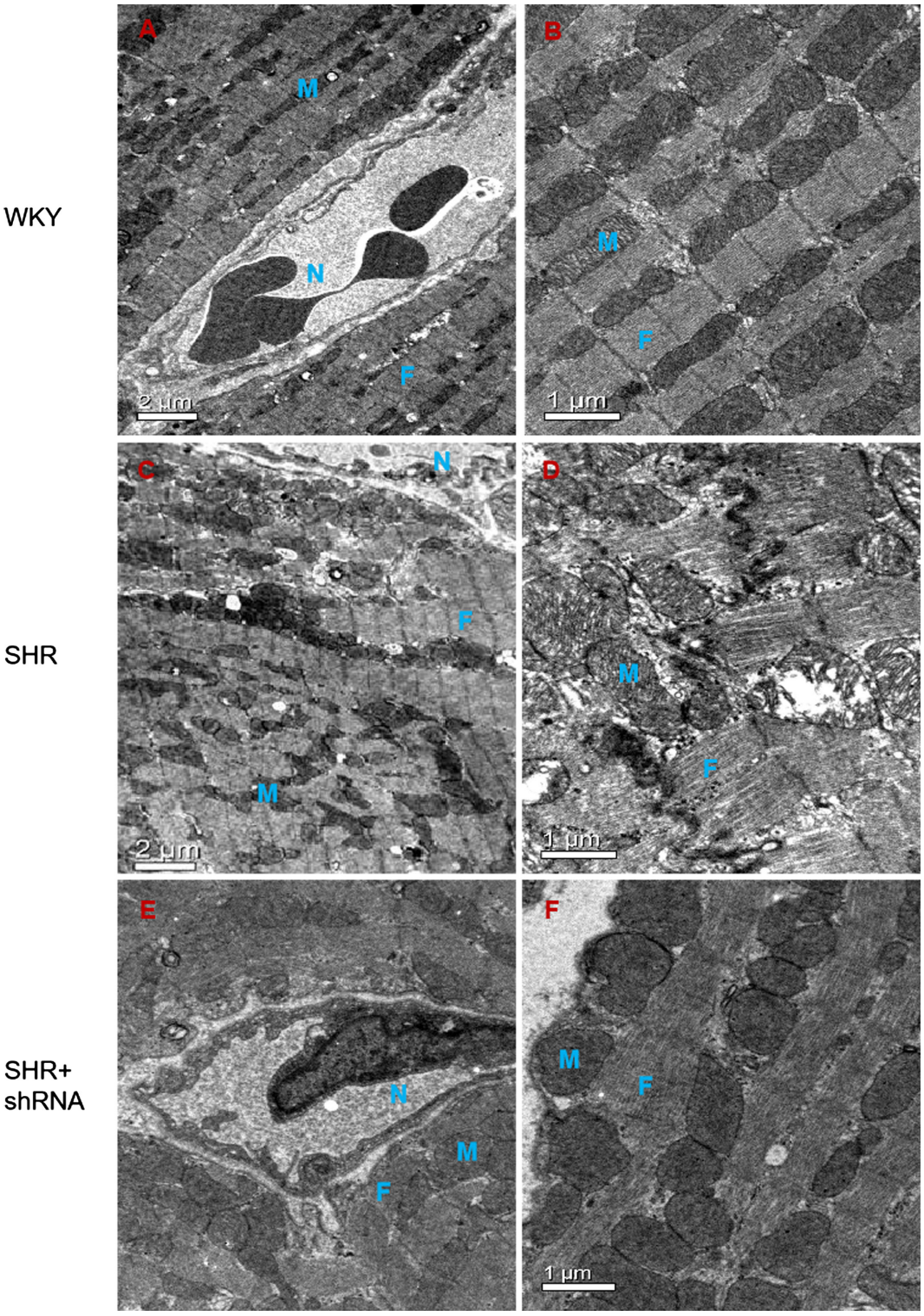

Fig. 6. Effect of injection of Ad5-*AT1R*-shRNA on myocardial ultrastructure: A–B: Myocardial ultrastructure in the WKY group; C–D: Myocardial ultrastructure in the SHR group; E–F: Myocardial ultrastructure in the experimental group.

terstitium. The results revealed that the ventricular remodeling was obvious in 16-week-old SHR.

Studies of gene therapy for hypertension revealed that antisense inhibition of the expressions of *AT1R*, and other members of the RAS system had reduced blood pressure and improved the pathological changes of the heart in SHR [14, 15]. The results of previous studies revealed that shRNA was highly expressed in the kidney, heart, liver, aorta, and adrenal tissues. This is similar to the results of this study. In addition, Ad5-ACE-*AT1R*-

shRNA can improve the ultrastructure of cardiomyocytes and reduce blood pressure by down-regulating the expressions of ACE and *AT1R*. Similarly, RNA interference technology was used in previous experiments conducted by other researchers [16], where the gene expression of *AT1R* was inhibited by tail vein infusion of Ad5-*AT1R*-shRNA, which has reduced blood pressure. We attempted to reverse left ventricular hypertrophy and myocardial fibrosis through gene therapy. The present study revealed that after repeated injection of Ad5-*AT1R*-shRNA, the blood pressure in SHR not only demonstrated an obvious hypotensive effect but also had the effect of superposition, that is, the amplitude and duration of hypotensive effect after the second injection were significantly higher than those after the first injection. The results suggest that the effect of repeated injection of Ad5-*AT1R*-shRNA is better. The HW, HW/BW, and the contents of HYA and collagen in the heart were significantly lower in the experimental group than in the SHR group. The results of electron microscopy examination also revealed that the myocardial damage was significantly lighter in the experimental group than in the SHR group, and the expression of *AT1R* mRNA was significantly lower in the experimental group than in the SHR group. All of these results revealed that Ad5-*AT1R*-shRNA produced the effect of RNA interference, which blocked a series of biological effects of the combination of Ang II and *AT1R* induced by hypertension and ventricular remodeling, suggesting that this gene therapy can reduce not only blood pressure but also improve the ventricular remodeling in SHR. Plasma Angiotensin II level would increase by blocking gene of Angiotensin II type 1 receptor. The classical effects of Angiotensin II on its target organs are mostly mediated by two membrane receptors, type 1 receptors (*AT1R*s) and type 2 receptors (AT2Rs), which mediate different functions. *AT1R* is proved to promote growth and proliferation, such as vasoconstriction, increased BP, cardiac contractility, and mediate detrimental effects, including endothelial dysfunction, cardiovascular diseases and inflammation. By contrast, AT2R is considered to induce opposing effects, inhibit growth, promote apoptosis, vasodilatation, hypotension, and prevent cardiovascular remodeling [17]. After repeated injection, the adenovirus vector did not produce obvious adverse reactions, and the hypotensive effect was more long-lasting. At the end of the experiment, in the experimental group, and the blood pressure essentially returned to the initial state. However, the expression of *AT1R* in the myocardium was lower than that in the SHR group. This does not suggest that the change of blood pressure is directly related to the expression of *AT1R* in the myocardium. It is speculated that the RAS system of local tissue is mainly involved in the role of tissue fibrosis.

One limitation of this study was that blood pressure should be measured invasively with radio-telemetry, or at least by a tethered system, but due to technical problems, we could only use Tail-cuff measurements of blood pressure, which was not very reliable.

Conclusion

Gene therapy using RNAi technology to inhibit *AT1R* expression can not only be used as a new method to reduce blood pressure but is also expected to be a new method to improve myocardial remodeling and heart failure. Nevertheless, at present, this new technology is limited to animal research. The safety and effectiveness of its application in human treatment must be further studied. For example, the safety and efficiency of the transduction vector, and whether this gene therapy strategy can be applied to other types of myocardial changes, requires further study.

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