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Correlation between endothelial cell apoptosis and SIRT3 gene expression in atherosclerosis rats

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Abstract. – OBJECTIVE: To investigate the correlation between the endothelial cell apoptosis and sirtuin-3 (SIRT3) gene expression in atherosclerosis (AS) rats.

MATERIALS AND METHODS: The AS model in rats was established through the high-fat diet. A total of 12 rats fed normally were enrolled as the control group, while 12 rats fed with high-fat diet were enrolled as the experimental group. After the experiment, the aortic tissues of rats were collected, and the relative area of the arterial plaque (total area of plaque/total area of the vessel) was measured *via* oil red O staining. The serum was collected to detect the levels of blood lipid, including total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C). Moreover, the expression levels of SIRT3 and apoptotic genes were determined *via* Reverse Transcription-Polymerase Chain Reaction (RT-PCR), Western blotting and immunohistochemistry (IHC), respectively. The apoptosis was detected *via* terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining.

RESULTS: The area of aortic plaque in the experimental group [(36.15±9.52)%] was significantly larger than that in the control group [(11.62±3.25)%] ($p<0.01$). Compared with those in the control group, the serum TC, TG and LDL-C levels were significantly increased in the experimental group, while the HDL-C level was significantly decreased ($p<0.05$). Compared with those in the control group, the mRNA and protein expression levels of SIRT3 in the aorta of rats markedly declined in the experimental group ($p<0.05$), while Caspase-3 and Caspase-9 expressions were significantly increased ($p<0.05$), respectively. The results of TUNEL staining revealed that the apoptosis in the aorta of rats in the experimental group was remarkably higher than that in the control group ($p<0.05$).

CONCLUSIONS: The expression of SIRT3 is deleted in the aorta of AS rats and closely related to the apoptosis. SIRT3 may serve as a potential target for the treatment of AS.

Key Words:
Atherosclerosis, Apoptosis, SIRT3.

Introduction

Atherosclerosis (AS), as a kind of chronic disease of the arterial wall, is a major cause of shortened lifespan and death of patients around the world[1]. Studies have demonstrated that AS is also affected by some factors, such as the gender difference (more common in male), hypertension, family history and expansion for decades between subcapsular smooth muscle cells (SMCs) and endothelial cells[2]. The structural and functional integrity of endothelium plays a key role in maintaining cardiovascular balance and preventing AS. It has been confirmed that endothelial dysfunction is a primary cause of atherosclerotic cardiovascular diseases and the occurrence and development of AS[3].

Sirtuin (SIRT) is a nicotinamide adenine dinucleotide (NAD^+)-dependent histone deacetylase family, which plays an important role in regulating cellular energy metabolism and senescence[4,5]. SIRT3, as a member of the SIRT family, features in energy balance, cardiac remodeling and heart failure. Increasingly more evidence has shown that SIRT3 exerts key effects in protecting the heart from cardiac hypertrophy and heart failure-related cardiac dysfunction and protecting the heart cells from stress-mediated death[6,7]. SIRT3-mediated mitochondrial homeostasis and

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regulation on cardiac function have been studied deeply, but the influence of SIRT3 on endothelial cells has not been well studied.

The primary purpose of this work was to investigate the correlation between the endothelial cell apoptosis and sirtuin-3 (SIRT3) gene expression in atherosclerosis (AS) rats.

Materials and Methods

Animal Modeling

24 male Sprague-Dawley (SD) rats weighing (200±20) g were purchased and raised in the Laboratory Animal Center of Third Military Medical University. They were fed with different feed formulas in a specific pathogen-free environment at the room temperature of (25±2)°C. In the control group, rats were fed with basal diet and had free access to food and water. In the experimental group (high-fat diet group), rats were fed with high-fat diet according to a Jiang et al[8]. The feed formula is as follows: 100 g basal food, 10 g milk powder, 10 g lard oil, 1 egg, 10 drops of concentrated cod-liver oil (including 17000 U vitamin A and 1700 U vitamin D), 250 g of fresh soybean sprout, 10 g of casein (protein ≥85%), and 20 g of sucrose. This study was approved by the Animal Ethics Committee of the Third Military Medical University Animal Center.

Oil Red O Staining of Aorta

The rats were dissected under a stereomicroscope. The peripheral adipose tissues of the whole aorta were removed, the aorta was longitudinally cut and peeled off along the medial aortic arch, and immediately fixed in 10% paraformaldehyde solution overnight. The aorta was taken and washed with Phosphate-Buffered Saline (PBS) on a shaking table at 4°C overnight. Then, the oil red O dye solution was prepared: 0.1 g of oil red O powder was weighed, dissolved in 10 mL of isopropanol, shaken evenly and placed in an incubator at 60°C for 40 min. An equal volume (10 mL) of double-distilled water (ddH_2O) was added, and the mixture was filtered to obtain the oil red O dye solution. The aorta was placed into an Eppendorf (EP) tube containing red oil O dye solution at room temperature for staining in a dark place for 2-4 h (the staining time should not be too long). Then, the aorta was taken, washed with 85% isopropanol 3-5 times (3 min/time) (the washing times and duration were adjusted according to the aortic background color), and washed again with PBS for several times. Finally, the stained aorta was unfolded, fixed, observed and photographed under a microscope.

Detection of Biochemical Indexes

The baseline blood lipid and blood lipid levels after intervention (TC, TG, HDL-C and LDL-C) in each group were measured according to the instructions of the blood lipid assay kit (Solarbio, Beijing, China). Total cholesterol (TC) and triglyceride (TG) were detected using the glycerol-3-phosphate oxidase-peroxidase anti-peroxidase (GPO-PAP) method, low-density lipoprotein cholesterol (LDL-C) was detected using the polyvinyl sulfuric acid precipitation method, and high-density lipoprotein cholesterol (HDL-C) was detected using the phosphotungstic acid-magnesium precipitation method. The fasting serum blood glucose level in each group was measured after intervention using a fast blood glucose meter.

RNA extraction and qRT-PCR (Quantitative Real-Time Polymerase Chain Reaction)

Total RNA was extracted from the aortic tissues using the TRIzol kit (Invitrogen, Carlsbad, CA, USA), respectively, followed by measurement of RNA concentration using an ultraviolet spectrophotometer (Hitachi, Tokyo, Japan). The complementary Deoxyribose Nucleic Acid (cDNA) was synthesized according to the instructions of the PrimeScriptTM RT MasterMix kit (Invitrogen, Carlsbad, CA, USA). QRT-PCR reaction conditions were as follows: 94°C for 30 s, 55°C for 30 s and72°C for 90 s, for a total of 40 cycles. The relative expression level of the target gene was expressed by $2^{-\Delta\Delta Ct}$. Primer sequences used in this study were as follows: SIRT3, F: 5'-CTTGACTCCTCCTTACTC-3', R: 5'-CTAATGTGCCCGAAGGA-3'; Caspase-3, F: 5'-GATGCACCTGTACGATCATG-3', R: 5'-ACTTTCAACACGCAGTCG-3'; Caspase-9, F: 5'-AGCATAAAGACATACAA-3', R: 5'-CCTTCTCCACAACC CTCTGTCT-3'; β-actin: F: 5›-CCTGGCACCCAGCACAAT-3›, R: 5›-GCTGATCCACATCTGCTGGAA-3›.

Detection of Protein Expression Via Western Blotting

The aortic tissues of rats were cut into pieces, placed into the 2.5 mL EP tube and added with 150 μL mixture of radioimmunoprecipitation

assay buffer (RIPA) and protease phosphorylation inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO, USA), followed by homogenization in a tissue homogenizer for 10 min. Then, the EP tube was taken and ultrasonic dispersion (40 A, 3 s/time) was performed 3 times, followed by centrifugation at 12000 g and 4°C for 15 min. The supernatant liquid was taken as the tissue protein. The protein concentration was determined using a bicinchoninic acid (BCA) kit (Beyotime, Shanghai, China). After denaturation, the total protein was separated using the 10% acrylamide gel, transferred onto a 0.22 μm-thick nitrocellulose membrane (Millipore, Billerica, MA, USA) for 1.5 h, sealed with 5% skim milk for 1 h and incubated with the SIRT3 (1:1000), Caspase-3 (1:2000), Caspase-9 (1:1000), and GAPDH (1:2000) monoclonal antibodies overnight. Afterward, the band was incubated with the anti-mouse or anti-rabbit IgG secondary antibody for 1 h, and the target protein band was developed using the ECL system (Bio-Rad, Hercules, CA, USA). The relative content of target protein was expressed as the ratio of the gray value of target protein to that of internal reference band.

Measurement of SIRT3 Protein Level Via Immunohistochemistry (IHC)

The paraffin-embedded tissues were sliced into 5 μm-thick sections, baked in an oven at 65°C for 3 h and deparaffinized according to the standard procedure. After the antigen retrieval of tissue sections, the MTSS1 antibody (Cell Signaling Technology Inc., Danvers, MA, USA) was dropped onto the glass slide, followed by incubation with the horseradish peroxidase-conjugated secondary antibody. The color was developed using the Histostain Plus kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and 3,3-diaminobenzidine (DAB) (Sigma-Aldrich, St. Louis, MO, USA) was used as the color developing agent. The sections were counterstained with hematoxylin, and PBS took the place of antibody in incubation as a negative control.

Determination of Apoptosis Level Via TUNEL Staining

After deparaffinization at 60°C for 20 min and transparentization with xylene twice for 5-10 min, the sections were hydrated with gradient ethanol from high to low, so that the subsequent binding reaction could be sufficient and uniform. The cells were incubated with proteinase K and transparentized for 10-30 min, followed by incubation with the TUNEL solution (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 1 h and color development with DAB for about 10 min (the background color was controlled under the microscope, 30 min at most). After sections were washed with PBS, they were sealed and observed under a fluorescence microscope.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 software (IBM, Armonk, NY, USA) was used for the statistical analysis. The data were expressed as mean ± standard deviation, and the *t*-test was adopted for the comparison between the two groups. $p<0.05$ suggested that the difference was statistically significant.

Results

Oil Red O Staining of Aorta

The aorta of rats was peeled off for oil red O staining, and the relative area of the red-stained lipid plaque on the inner vascular wall (total area of plaque/total area of the vessel) was observed. The results revealed that after the high-fat diet for 12 weeks, there were lipid plaques formed in the aorta in both groups. In addition, the area of lipid plaque was (11.62±3.25)% in the control group and (36.15±9.52)% in the experimental group, displaying a statistically significant difference ($p<0.01$) (Figure 1).

Measurement of Blood Lipid Levels

After the high-fat diet for 12 weeks, the blood lipid levels (TC, TG, LDL-C, and HDL-C) in blood samples of each group were determined. The results showed that, compared with those in the control group, the serum TC, TG and LDL-C levels were significantly increased in the experimental group, while the HDL-C level was significantly decreased ($p<0.05$) (Table I).

Changes in Blood Glucose Level

After the experiment, the fasting serum blood glucose level in each group was measured using a fast blood glucose meter. The blood glucose level in the experimental group was significantly increased compared with that in the control group after the high-fat diet for 12 weeks ($p<0.001$) (Figure 2).

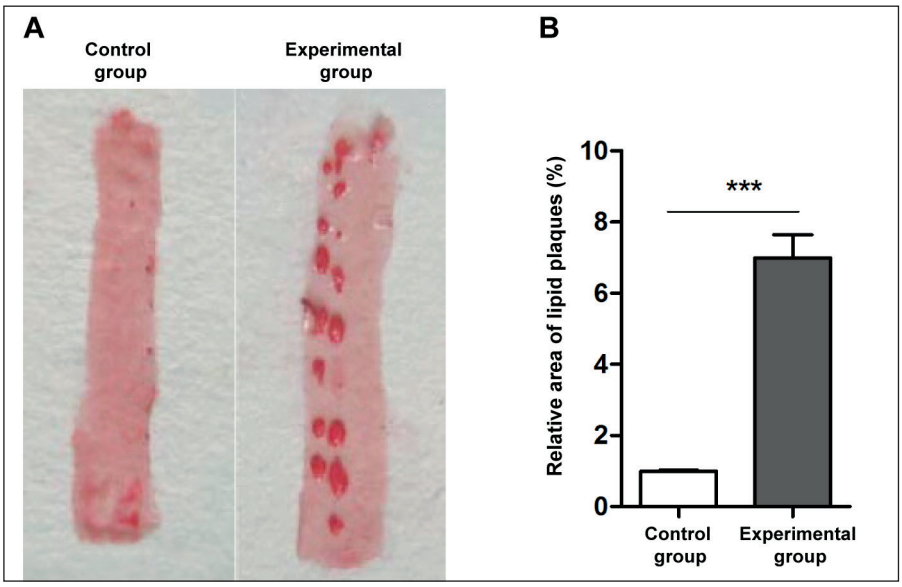

Figure 1. Oil red O staining of aorta. **A,** Relative area of aortic lipid plaques detected *via* oil red O staining. **B,** Statistical graph of relative area of lipid plaques in both groups.

SIRT3 Expression Level Determined Via Western Blotting and Quantitative PCR (qPCR)

The aortic endothelial cells were extracted from rats in both groups to measure the mRNA and protein expression levels of SIRT3 and apoptotic indexes *via* Western blotting and qPCR, respectively. As shown in Figure 3, compared with the control group, the experimental group had significantly declined SIRT3 mRNA and protein levels in endothelial cells ($p<0.05$). Then, Caspase-3 and Caspase-9 expressions were further detected, and it was found that they were substantially increased in the experimental group ($p<0.05$). All those results suggested that the down-regulation of SIRT3 may be closely related to endothelial cell apoptosis.

SIRT3 Expression Deletion Promoted Endothelial Cell Apoptosis

To further clarify the correlation between SIRT3 expression in endothelial cells and endothelial cell apoptosis, the SIRT3 protein expression and apoptosis were detected *via* IHC and TUNEL, respectively. The results of IHC showed that the experimental group had a negative expression of SIRT3 while the control group had

Table I. Comparisons of blood lipid levels between the two groups after modeling ($\bar{x} \pm s$, mmol/L).

	Control group	Experimental group
TC	12.3 ± 0.2	32.6 ± 1.2***
TG	1.56 ± 0.1	2.36 ± 0.1*
LDL-C	7.65 ± 0.6	18.62 ± 0.3**
HDL-C	2.02 ± 0.4	0.36 ± 0.1**

Note: *$p<0.1$, **$p<0.05$ and ***$p<0.01$ *vs.* control group.

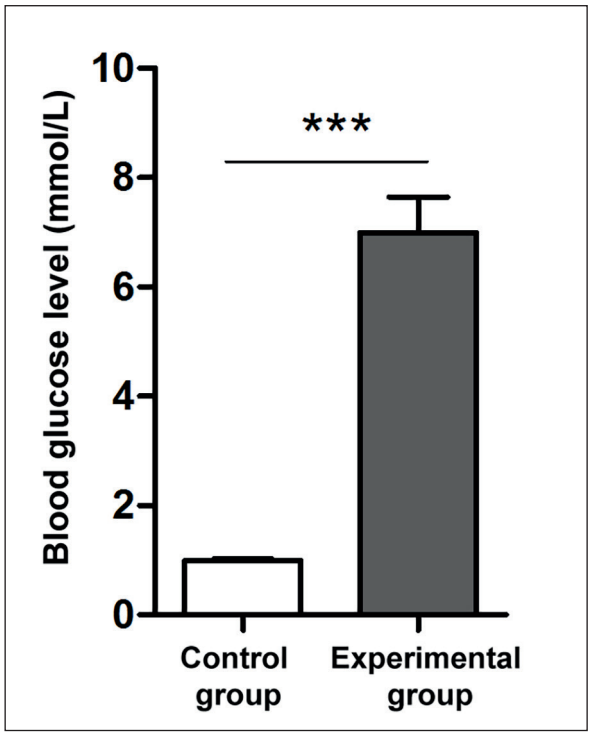

Figure 2. Comparison of blood glucose level between the two groups after intervention. Comparison of blood glucose level between the control group and the experimental group. Note: ***$p<0.001$ *vs.* control group.

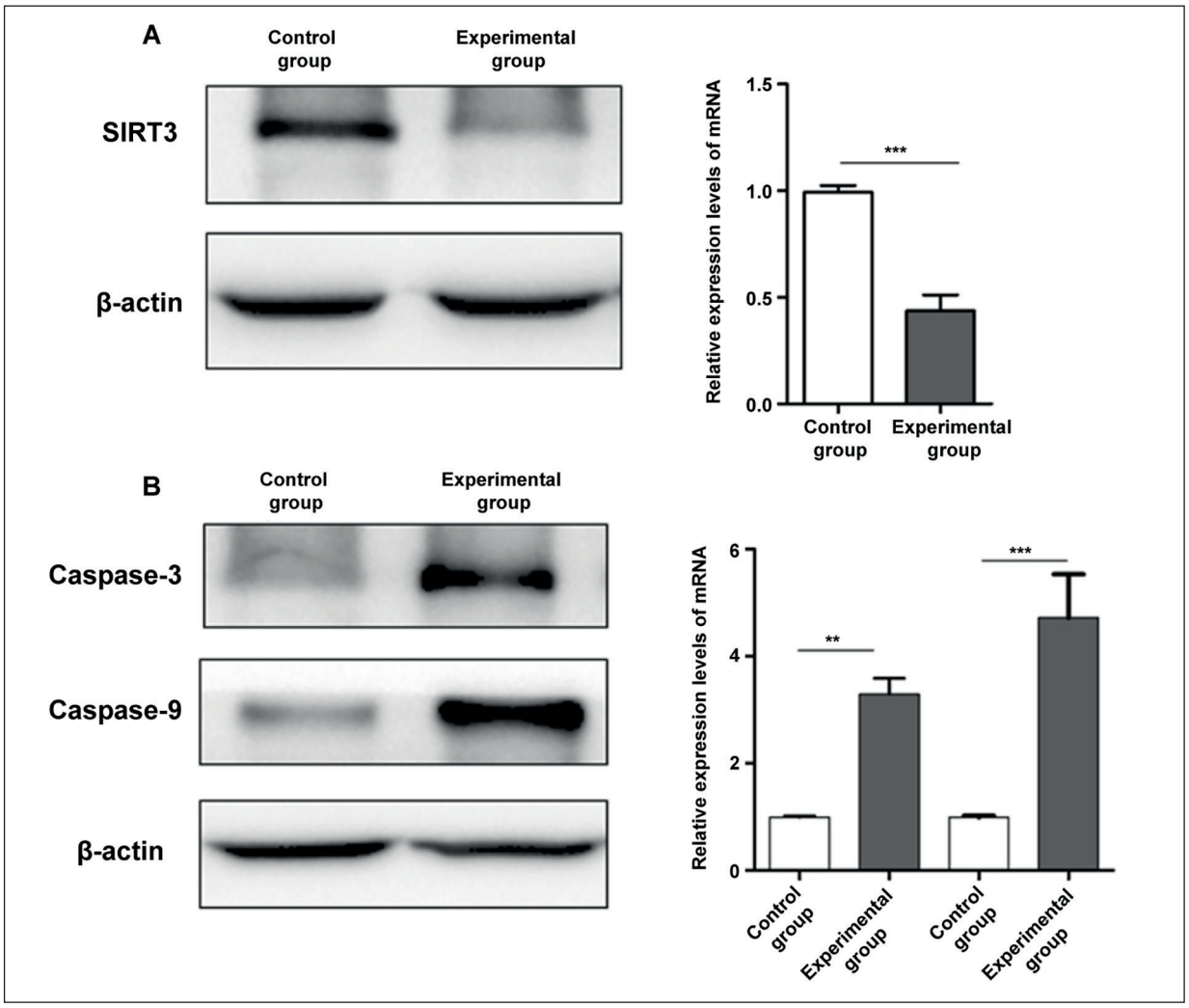

Figure 3. Expression levels of SIRT3 and apoptotic genes in both groups. **A,** Comparisons of SIRT3 mRNA and protein expression levels in endothelial cells in both groups. **B,** Expressions of Caspase-3 and Caspase-9. Note: **p<0.001; ***p<0.001.

a positive expression of SIRT3 in aortic cells. TUNEL results showed that the aortic cell apoptosis in the experimental group was significantly higher than that in the control group (p<0.05) (Figure 4).

Discussion

The formation of AS is a slow and complex pathological process. Some studies[9,10] have found that AS plaques exhibit the apoptosis and necrosis of a variety of cells, such as vascular endothelial cells, vascular SMCs and macrophages. In addition, the endothelial cell injury is a key factor in the occurrence of AS, and endothelial cell apoptosis can lead to necrosis and shedding of AS plaques, thereby inducing thrombosis[11]. Furthermore, after atherosclerotic coronary heart disease occurs, the vascular stenosis-induced myocardial ischemia and hypoxia and ischemia-reperfusion injury will also initiate the myocardial apoptosis, indicating that apoptosis plays an important role in AS.

SIRT3 exerts a certain regulatory effect on cell survival, proliferation, metabolism, death and senescence, as well as the longevity of organs[12]. Additionally, the expression of SIRT3 declines with the increase of age and cardiovascular and metabolic diseases[13]. According to Bell et al[14], SIRT3 can initiate the tumor cell apoptosis by inhibiting reactive oxygen species and regulating the stability of hypoxia-inducible factor-1. Moreover, SIRT3 plays a key role in protecting the heart

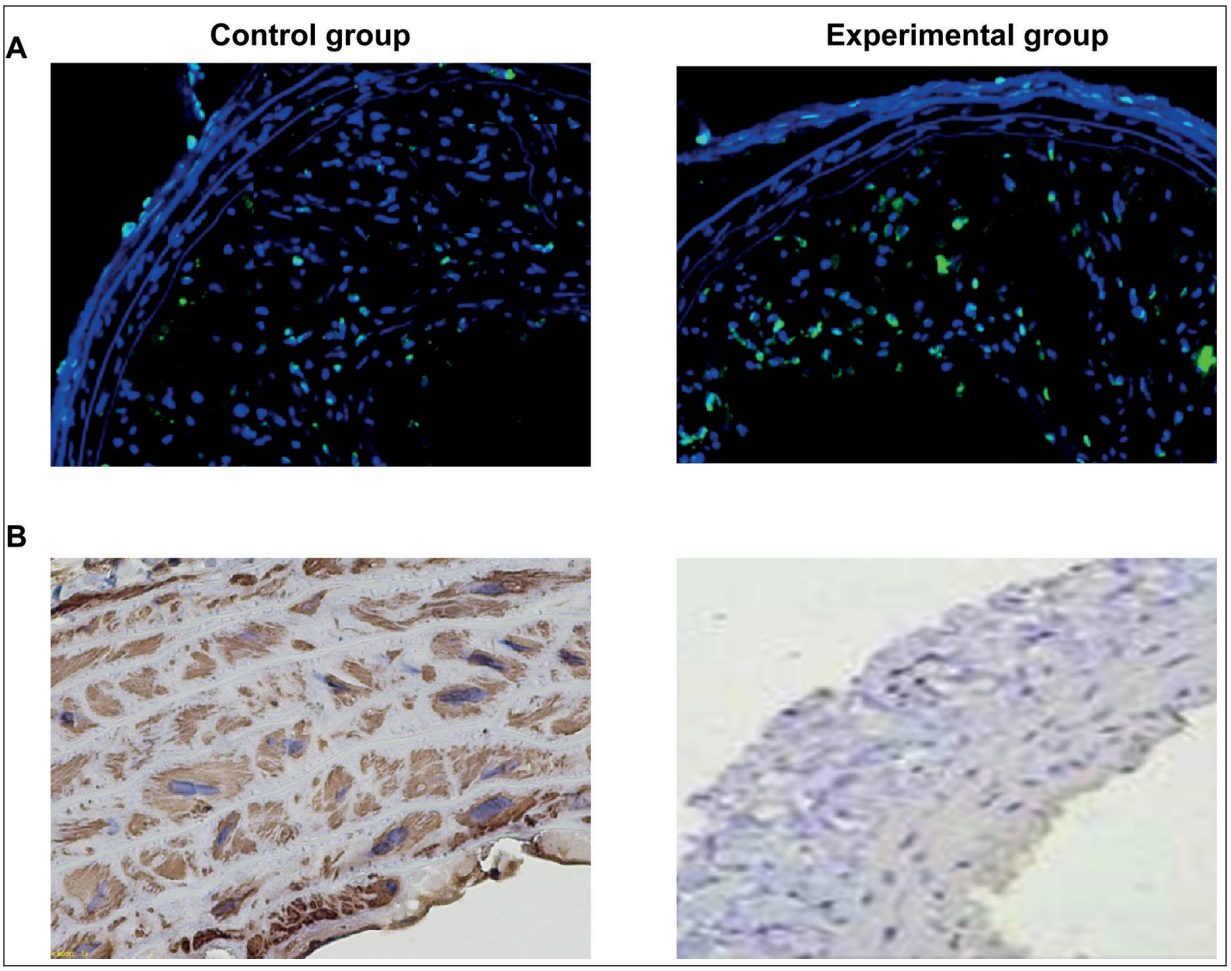

Figure 4. SIRT3 expression and apoptotic levels in the aorta in both groups (×200 in TUNEL, ×400 in IHC). **A,** Apoptosis of aortic endothelial cells detected *via* TUNEL. **B,** SIRT3 protein expression detected *via* IHC.

from cardiac hypertrophy and heart failure-related cardiac dysfunction and protecting the heart cells from stress-mediated death[15]. Chang et al[16] have shown that heart failure with preserved ejection fraction in patients with abnormal coronary microcirculation is correlated with endothelial dysfunction and coronary microvascular stenosis. SIRT3-mediated mitochondrial homeostasis and regulation on cardiac function have been studied deeply, but the influence of SIRT3 on AS cell apoptosis has not been well studied.

In this work, the AS model in rats was established *via* high-fat diet. After high-fat diet for 12 weeks, the aorta of rats was peeled off, and the relative area of the red stained lipid plaque on the inner vascular wall was observed. It was found that the area of aortic plaque in the experimental group [(36.15±9.52)%] was significantly larger than that in the control group [(11.62±3.25)%]. Furthermore, the mRNA and total protein were extracted from aortic tissues in both groups to determine the expression levels of SIRT3 and apoptotic indexes (Caspase-3 and Caspase-9), respectively. Our results showed that, compared with those in the control group, the SIRT3 mRNA and protein levels in the aorta markedly declined in the experimental group ($p<0.05$), while Caspase-3 and Caspase-9 expressions were significantly increased ($p<0.05$). TUNEL results also manifested that the apoptosis in the aorta of rats in the experimental group was also significantly higher than that in the control group ($p<0.05$). The above results suggested that the down-regulation of SIRT3 is closely related to endothelial cell apoptosis in AS. Sun et al[17] found that SIRT activators, such as metformin and resveratrol, can significantly reduce the risk of AS, which is consistent with this study. All those

results indicated that SIRT3 as a therapeutic target of AS may provide a new direction for the treatment of AS.

Lipid metabolism disorder is closely related to the formation of AS. In lipid metabolism disorder, TC, TG, and β-lipoprotein are abnormally increased, LDL and VLDL are increased and HDL declines[18]. New evidence proved that SIRT3 is involved in regulating endothelial metabolism and angiogenesis, thus affecting the development of aging-associated cardiovascular diseases[19]. Therefore, the influence of SIRT3 on blood lipid indexes was also studied, and it was found that, compared with those in the control group, the serum TC, TG and LDL-C levels were significantly increased in the experimental group, while the HDL-C level was significantly decreased. According to Yu et al[20], the activation of SIRT3 is conducive to the lipid metabolism and antioxidation. The latest study revealed that SIRT3 increases the expression and release of endothelial nitric oxide by promoting LDL-induced apoptosis[21]. Therefore, SIRT3 may also play a protective role in the formation of AS *via* regulating lipid metabolism.

Conclusions

In this work, AS model in rats was established. The expression of SIRT3 is deleted in the aorta of AS rats and closely related to the apoptosis. We found that SIRT3 may serve as a potential target for the treatment of AS.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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