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Effect of miR-467b on atherosclerosis of rats

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ABSTRACT

Objective: To observe the effect of miR-467b on the atherosclerosis (AS) of rats with apolipoprotein E (ApoE) gene knockout ($ApoE^{-/-}$).
Methods: $ApoE^{-/-}$ rats were fed with high fat and high cholesterol diet and were randomly divided into group A, group B and group C, with 10 rats in each group. Group A: rats were injected with ApoE agonist through the caudal vein; group B: rats were injected with ApoE antagonist through the caudal vein; group C: as negative control group. Enzyme oxidation method was used to detect the blood lipid levels of rats. Western blotting method was used to detect the aortic lipoprotein lipase (LPL) expression levels of rats. HE staining and oil red O staining were performed to observe the AS lesions and lipid accumulation state.
Results: Compared with group C, blood lipid level, aortic intima and aortic sinus lipid accumulation area ratio, aortic sinus lesion area and LPL expression level in group A significantly reduced; while blood lipid level, aortic intima and aortic sinus lipid accumulation area ratio, aortic sinus lesion area, and LPL expression level in group B significantly increased, with the statistical difference ($P < 0.05$).
Conclusions: miR-467b can alleviate the AS lesions of $ApoE^{-/-}$ rats, and its inhibiting effect on AS may be related to LPL expression.

1. Introduction

Atherosclerosis (AS) refers to the complex pathological process induced by many factors. Cardiovascnlar and cerebro-vascular disease, which are induced by AS, would bring great harm to health, especially for middle-aged and aged people [1]. The lipid metabolism disorders, excessive cholesterol accumulation and vascular inflammatory responses for AS patients are the key factors resulting in AS [2,3]. Apolipoprotein E (ApoE) plays an important role during the process of lipid transmission and metabolism [4]. In this study, $ApoE$ gene knockout ($ApoE^{-/-}$) rats fed with high fat and high cholesterol diet were selected for the building of AS model.

miRNA is a kind of endogenous single mature non-coding RNA, which could induce the miRNA degradation and inhibit its transcription translation process through incomplete pairing with target mRNA. In addition, miRNA is involved in many physiological process, like regulating cell viability, embryonic development, lipid metabolism, inflammatory response and autoimmunity [5,6]. Recent researches found that miR-467b expression was increased in $ApoE^{-/-}$ mice, which might play an important role in the occurrence and development of AS and its action mechanism maybe achieved through target regulating of lipoprotein lipase (LPL) [7]. LPL in vascular cells could promote the formation of foam cells, increase the inflammatory factor expression, cause the lipid accumulation and then result in the occurrence of AS [8]. Some researches showed that the AS lesions of rats with high fat and high cholesterol diet feeding and macrophage LPL gene deletion were obviously alleviated [9]. In this study, intravenous injection of miR-467 agonist and antagonist for $ApoE^{-/-}$ rats was performed to observe the LPL, lipid levels and AS lesions, which could provide theoretical basis for the prevention and targeted treatment of AS.

2. Materials and methods

2.1. Animals

A total of 30 healthy male SPF $ApoE^{-/-}$ rats were provided by the Institute for Experimental Animals of Chinese Academy

of Medical Sciences, with weight range (250–300) g, and were fed at room temperature range (18–20) °C, with illumination for at least 12 h and humidity (45%–55%).

2.2. Instruments and reagents

Real-time quantification PCR Amplifier Lightcycler 480 was purchased from Roche Life Science; the high speed micro-centrifuge G508009 from Sangon; the precise one-way adjust-able-Volume Pipettor from Sangon; the geldocument system GOS7500 from UVP; the electronic balance from Sartorius; the ultra cold storage freezer (−86 °C DW-86W100) from Haier; Real-time quantification PCR kit from Roche Group; miRNA agomir/antagomir from RiboBio; Bovine Serum Albumin (A7030) from Sigma; the oil red O staining reagents from Sigma; BCA protein quantification kit from Pulitzer gene technology Co., LTD; the LPL primary antibody from Abcam; Goat Anti-rabbit second antibody from Abcam; and GoScript™ Reverse Transcription System from Promega.

2.3. Experimental methods

2.3.1. Animal grouping
All experimental $ApoE^{-/-}$ rats were fed with high fat and high cholesterol diet. After 1 week, these rats were randomly divided into group A, group B and group C ($n = 10$). Group A: rats were injected with ApoE agonist through the caudal vein; group B: rats were injected with ApoE antagonist through the caudal vein; group C: as negative control group. Rats in group A were given ApoE agonist for once, and rats in group B were given ApoE antagonist for once respectively, with continuous treatment for 10 d. In addition, rats in A and group B were fed with high fat and high cholesterol diet, with diet formula (large oil, unprocessed food grains and 0.35% of cholesterol).

2.3.2. Tissues sampling and trimming
Fasting for 12 h before rats were put to death and rats were anesthetized with intraperitoneal injection of 10% of chloral hydrate, with dosage of 300 mg/kg. After adequate anesthesia, blood was collected from eyeballs for high speed centrifugation. Supernate was obtained and enzyme oxidation method was used to detect the venous blood lipid indexes of rats, with indexes including triglyceride (TG), total cholesterol (TC), high-density lipoprotein (HDL) and low density lipoprotein (LDL). The aorta of rats was obtained and Western blotting method was used to detect the LPL expression levels of aortic tissues of rats. 4% of neutral formalin was used for 30 min perfusion for rats. Heart and partial aorta were obtained from rats: one part was applied to the quick frozen sections for aortic sinus tissue, with section thickness (10 μm), then oil red O staining was performed; the other part was applied to quick slicing for heart tissue, which was fixed with 10% of paraformaldehyde, embedded with paraffin, with serial section and section thickness (10 μm), then HE staining was performed.

2.3.3. Method statement
ELISA Method: Serum of rats with a certain dilution multiple was added into the purified inflammatory factor antibody coating microplates successively. After washing and coloration, spectrophotometer was used to detect the homologous OD value and homologous inflammatory factor concentration was calculated.

Oil red O staining: aorta was obtained and fixed on the paraffin plate, with oil red O staining. After sealing piece, observation and statistics were performed under light microscope. Image Analysis Software was used to detect the percentage of fatty streak/total aortic area. HE staining: paraffin section was prepared with HE staining for 10 min, 1% of hydrochloric acid differentiation and PBS solution for washing, and then HE staining for 2 min once more. After dehydration, transparency and sealing piece were finished, observations were performed under microscope. Western blotting method: aortic tissues of rats were obtained and separated using polyacrylamide gel electrophoresis for extracting the protein. After coloration, gel image analysis system was performed for scanning and the gray area ratio of object of study/β-action was calculated.

2.4. Statistical method

SPSS 20.0 software was used for experimental data treatment. Blood lipid levels and lesion locations were expressed by Mean ± SD deviation. One-way ANOVA analysis or t test was used for comparison among groups, where $P < 0.05$ showed the statistical significance.

3. Results

3.1. Blood lipid level comparison of rats

Fully automatic biochemical analyzer was used to detect the TG, TC, HDL levels of experimental rats in three groups. Compared with group C (control group), blood lipid levels in group A obviously reduced, while blood lipid levels in group B obviously increased, where difference had statistical significance ($P < 0.05$). It could be proved that the usage of ApoE agonist could reduce the blood lipid levels, while the usage of antagonist could increase the blood lipid levels (Table 1).

3.2. Aortic AS lesions of rats

The oil red O staining was employed to detect the aortic intima lipid accumulation and aortic sinus lipid accumulation of rats. The proportion of lipid accumulation area, intima area and aortic lumen area were calculated by software. Compared with group C, the proportion of aortic intima lipid accumulation and aortic sinus lipid accumulation of rats in group A significantly reduced; while the proportion of aortic intima lipid accumulation and aortic sinus lipid accumulation of rats in group B significantly increased. Comparative difference among groups had statistical significance ($P < 0.05$). HE staining was performed to detect the aortic sinus AS lesion area of rats. Group C: obvious inflammatory cell infiltration and the formation of foam cells were visible. Group A: inflammatory cell infiltration was reduced and foam cells were little and even invisible. Group B: a large number of inflammatory cell infiltration and the formation

Table 1
Blood lipid level comparison of rats (mmol/L).

Group	n	TG	TC	HDL	LDL
A	10	1.27 ± 0.29	12.06 ± 3.37	2.02 ± 0.67	10.08 ± 1.65
B	10	3.21 ± 0.58	19.01 ± 4.14	5.26 ± 1.13	15.03 ± 3.04
C	10	2.28 ± 0.35	15.95 ± 3.68	3.64 ± 0.96	12.63 ± 2.42

of foam cells were visible. The proportion of AS lesion area accounted for the total lumen area was calculated by software. Compared with group C, the aortic sinus AS lesion area proportion of rats in group A significantly reduced; while the aortic sinus AS lesion area proportion of rats in group B significantly increased. Comparative difference among groups had statistical significance (Table 2) ($P < 0.05$).

3.3. Aortic LPL protein expression levels of rats

Western blot method was used to detect the aortic LPL protein expression levels of rats (Figure 1). Compared with group C, aortic LPL protein expression levels in group A significantly reduced, while aortic LPL protein expression levels in group B significantly increased, and comparative difference among groups had statistical significance ($P < 0.05$).

4. Discussion

AS often occurred in the large and medium-sized artery. The appearance of lipid accumulation (cholesterol, glucolipid *etc*.) in vessel wall intima was the key factor of the occurrence and development of AS. Cholesterol mainly came from plasma lipoprotein. Plasma lipoprotein could increase the cholesterol internal flow and deposition within arterial walls, like LDL could promote the AS occurrence. In addition, plasma lipoprotein could promote the cholesterol outward transport from blood vessels, like HDL had inhibition effects on AS [10–13]. ApoE was an important component of plasma lipoprotein and played an important role in regulating the cholesterol *etc* [14]. Related report [15] showed that pathological symptoms of $ApoE^{-/-}$ rats were in accordance with all stages of AS and were similar to human beings. Therefore, in this study, $ApoE^{-/-}$ rats were selected and were fed with high fat and high cholesterol diet for successful building of AS model of rats to discuss the effect mechanism of miR-467b on AS.

Not only miRNA could play effects in the non-coding region of targeting miRNA but also could miRNA play effects in coding region of targeting miRNA, which could affect the transcription translation process, increase the degradation speed of mRNA, involve and regulate the endothelial cell growth, inflammatory response, lipid regulation *etc*, which were related to AS, and finally affected the occurrence and development of AS [16,17]. Recent researches indicated that numerous miRNA therapeutic targets involved in the process of AS, in addition, miR-33, miR-122 were closely related to AS [18,19]. Some report [7] showed that miR-467b also involved in the occurrence and development of AS, but its specific mechanism was not clear. In this study, we also found that miR-467b could obviously reduce the blood lipid levels of $ApoE^{-/-}$ rats. In addition, LDL was obviously reduced, in the meantime, HDL was also reduced, which indicated that miR-467b acted on the ATP binding cassette transponer 1 A1 and acyl-coenzyme A:
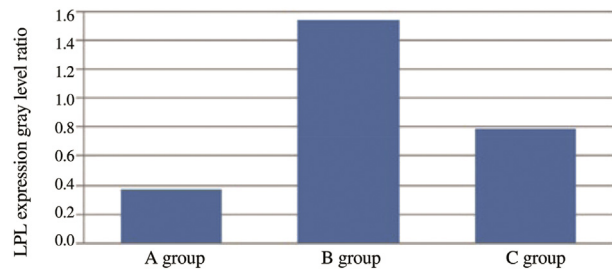

Figure 1. Aortic LPL protein expression levels of rats in three groups.

Acyl Cholesterol Acyl Transferase 1, which indirectly inhibited the reverse transport process of cholesterol, and then resulted in the blocking of HDL formation.

Through the observations of lipid accumulation area and lesion area, we found that miR-467b could obviously improve the aortic intima and aortic sinus lipid accumulation area, meanwhile, obviously reduce the atherosclerotic lesion area. The worsening of lipid accumulation area and lesion area appeared in miR-467b antagonist group. AS lipid infiltration theory was the common accepted explanation for AS pathogenesis. Lesion limitation began from aortic intima, then lipid, carbohydrate compound accumulation and endothelial injuries resulted in the metamorphosis, calcification and decreased flexibility of arterial intima; in addition, the deposition of cholesterol, glucolipid materials also accelerated the formation of atheromatous plaque. Monocytes in blood were attracted to the subcutaneous tissues after lipid particles were modified. Monocytes were translated into macrophages, which could phagocytose the lipid particles largely. When the outward transport capacity of which was exceeded, foam cells, which were formed by macrophages, began to die, and the accumulation of dead foam cells formed the lipid pool, which was covered by collagen and elastic fibers, which were released by smooth muscle cells, finally lesions came into being [20,21]. miR-467b involved in multiple steps of the forming process of lesion, which indicated that miR-467b might alleviate the aortic lipid accumulation and AS lesion range.

There were researches showed that miR-467b could targetedly regulate the LPL expression of RAW 264.7 macrophagocytes of rats and affect the lipid accumulation and inflammatory factor release. This study also indicated that miR-467b could obviously reduce the LPL expression levels of aorta of rats. LPL was a kind of TG degradation rate-limiting enzyme and a kind of glycoprotein which was secreted by adipocytes, myocardial cells and macrophagocytes. LPL in macrophagocytes could mediate the lipid retention and modification, accelerate the formation of foam cells, promote the inflammatory factor expression and then promote the AS occurrence [22]. On the other hand, LPL also had a certain anti-AS effect through reducing plasma TG and increasing HDL levels [23]. Therefore, LPL could not explain the inhibiting effect of miR-467b on AS. The relationship between miR-467b and LPL *in vivo* should be analyzed in the further research.

Conflict of interest statement

We declare that we have no conflict of interest.

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Table 2
Aortic AS lesions of rats (%).

Group	n	Aortic intima lipid accumulation	Aortic sinus lipid accumulation	Aortic sinus lesion area
A	10	3.71 ± 0.52	5.45 ± 1.34	8.92 ± 1.76
B	10	7.44 ± 1.27	22.65 ± 6.78	29.24 ± 7.53
C	10	6.23 ± 0.93	13.07 ± 3.58	16.85 ± 4.39

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