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Original Article

MiR-34a ameliorates arterial blood flow in rats with lower limb arteriosclerosis obliterans via Sirt1 signaling pathway

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Abstract

In this study, we investigated the impact of microRNA-34a (miR-34a) on lower limb arteriosclerosis obliterans in rats through the Sirtuin 1 (Sirt1) signaling pathway. Thirty-six Sprague-Dawley rats were divided into normal, model, and miR-34a mimics groups. Rats in the normal group were raised normally, while the model group underwent lower limb arteriosclerosis obliterans induction and received saline injections. The miR-34a mimics group also underwent arteriosclerosis obliterans modeling but received miR-34a mimics injections. Immunohistochemistry revealed significantly increased vascular endothelial growth factor (VEGF) expression in both model and miR-34a mimics groups compared to the normal group, with the miR-34a mimics group showing higher levels. Western blotting indicated elevated Sirt1 protein expression in both non-normal groups, with the miR-34a mimics group exhibiting significantly higher levels. Quantitative polymerase chain reaction (qPCR) demonstrated higher levels of miR-34a, VEGF mRNA, and Sirt1 mRNA in the model group compared to the normal group, but significantly lower levels than the miR-34a mimics group. Enzyme-linked immunosorbent assay (ELISA) showed increased VEGF content in the model group compared to the normal group but decreased compared to the miR-34a mimics group. Hemorrheological detection revealed a reduced PU index in both non-normal groups compared to the normal group, with a significant increase in the miR-34a mimics group compared to the model group. Overall, miR-34a upregulation enhanced VEGF expression in rat blood vessels, ameliorating arterial blood flow in lower limb arteriosclerosis obliterans through the Sirt1 signaling pathway.

Keywords: Lower limb arteriosclerosis obliterans, miR-34a, Sirt1 signaling pathway, VEGF.

1. Introduction

Lower limb arteriosclerosis obliterans, a prevalent peripheral vascular disease in clinical practice, stands as a widespread manifestation of peripheral atherosclerosis in vascular surgery. The incidence of this condition, predominantly affecting the elderly population, has demonstrated a steady increase in recent years [1-3]. Marked by degenerative and proliferative alterations in the intima and media of lower limb arteries, lower limb arteriosclerosis obliterans lead to luminal narrowing and potential occlusion. Consequently, patients often experience intermittent pain, limb soreness, and, in severe cases, limb necrosis. Given the escalating prevalence of this ailment, there is a pressing need for comprehensive studies into its etiology and associated pathogenesis.

Current understanding attributes lower limb arteriosclerosis obliterans to vascular lesions arising from various factors, perpetuating ischemia and hypoxia in the affected limb. The intricate interplay between degenerative changes in the arterial layers results in adverse outcomes for patients, necessitating a deeper comprehension of the underlying mechanisms [4-6]. Amid the molecular orchestrations, the sirtuin 1 (Sirt1) signaling pathway emerges as a pivotal player in the regulation of vascular regeneration and repair [7, 8]. Its involvement spans the modulation of diverse substances, protein expressions, and cellular processes, including proliferation, differentiation, and apoptosis.

Micro ribonucleic acid (miR)-34a, a prominent member of the miRNA family, assumes a crucial role in the regulatory landscape of downstream signaling pathways. Notably, it is recognized as a key regulatory factor intricately linked with vascular regeneration and repair [9, 10]. Against this backdrop, our research endeavors to delve into the intricate interplay between miR-34a and lower limb arteriosclerosis obliterans in rats, elucidating the regulatory influence of miR-34a through the Sirt1 signaling pathway. As current therapeutic interventions for lower limb arteriosclerosis obliterans remain a clinical challenge, our exploration aims to contribute valuable insights that could pave the way for novel treatment modalities and a deeper understanding of this debilitating vascular condition.

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2. Materials and methods

2.1. Laboratory animals

A total of 36 experimental SPF Sprague-Dawley rats [License No.: SCXK (Shanghai) 2014-0003] were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). All the rats were fed with normal diet and sterile filtered water every day in the Laboratory Animal Center with a 12/12 h light/dark cycle and conventional room temperature and humidity. This study was approved by the Animal Ethics Committee of the Affiliated Hospital of Guangdong Medical University.

2.2. Experimental reagents and instruments

MiR-34a mimics (MCE, Monmouth Junction, NJ, USA), anti-vascular endothelial growth factor (VEGF) and anti-Sirt1 primary antibodies and secondary antibodies (Abcam, Cambridge, MA, USA), and immunohistochemistry kit and quantitative polymerase chain reaction (qPCR) kit (Vazyme, Nanjing, China).

2.3. Animal grouping and treatment

The 36 rats were divided into normal group (n=12), model group (n=12) and miR-34a mimics group (n=12) using a random number table. All of them were fed adaptively in the Laboratory Animal Center for 7 d before experiments.

The rats in normal group were raised normally, without any treatment. The model of lower limb arteriosclerosis obliterans was established in model group, and normal saline was injected intraperitoneally every day. The rats miR-34a in mimics group were utilized to prepare the model of lower limb arteriosclerosis obliterans and then daily administered with miR-34a mimics (0.5 μM/day) via intraperitoneal injection. Subsequently, the specimens were obtained after 7 consecutive days of intervention in each group.

2.4. Modeling

First, ketamine (50 mg/kg) was intraperitoneally injected into the rats. After successful anesthesia, the rats were disinfected, and the inguinal regions on both sides were exposed, on which an incision (about 1 cm) was made to expose the femoral artery. Next, the lower 1/3 of the femoral artery was ligated using an aseptic silk suture, and the branches were obstructed by vascular clamps. 5 min later, the ligation was released, the incision was sutured, and the modeling was completed.

2.5. Detection of blood flow via color Doppler ultrasound

After the rats were anesthetized successfully, the blood flow of the femoral artery at the medial thighs was examined using a Doppler flowmetry. The results were presented as perfusion unit (PU) value, and the PU index = PU value $_{(\text{affected side})}$/PU value $_{(\text{healthy side})}$ was applied to evaluate the blood flow in the microcirculation on the affected side.

2.6. Specimen acquisition

The specimens were directly obtained from 6 rats in each group after successful anesthesia. Specifically, the vascular tissues were taken out directly, flushed in normal saline and stored in Eppendorf (EP) tubes at -80°C for subsequent Western blotting (WB) assay and enzyme-linked immunosorbent assay (ELISA). As for the remaining rats in each group, the specimens were collected by means of perfusion-fixation. In detail, the thoracic cavity was cut open to expose the heart, and 400 mL of 4% paraformaldehyde was perfused from the left atrial appendage. Then the tissues were dissected and fixed in 4% paraformaldehyde solution, which was used for immunohistochemistry and TUNEL assay.

2.7. Immunohistochemistry

The tissues embedded in paraffin in advance were sliced into 5 μm-thick sections, spread in warm water at 42°C, collected and baked. Therefore, the paraffin-embedded sections were prepared. Then the sections obtained were soaked in xylene solution and gradient alcohol for routine deparaffinization until rehydration. Subsequently, those sections were immersed in citric acid buffer solution and heated in a microwave oven repeatedly for 3 min and braised for 5 min for 3 times, so as to achieve adequate antigen retrieval. Then endogenous peroxidase blocker was added dropwise onto the specimens and reacted for 10 min after rinsing. Next, the specimens were rinsed and added with goat serum in drops for sealing for 20 min, and anti-VEGF primary antibody (1:200) was added after the goat serum blocking buffer was shaken off, followed by incubation in a refrigerator at 4°C overnight. The next day, the specimens were rinsed and added dropwise with the secondary antibody solution for reaction for 10 min. After rinsing adequately, streptavidin-peroxidase solution was added for reaction for 10 min, followed by color development with diaminobenzidine (DAB) added in drops, counterstaining of the nucleus with hematoxylin, mounting and observation.

2.8. Western blotting (WB) assay

The lysis buffer was added into the cryopreserved cardiac tissues for ice bath for 1 h, then the tissues were put into a centrifuge for centrifugation at 14,000 g for 10 min, and the protein was quantified using bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). Next, the absorbance and standard curve of the protein were obtained through a microplate reader, which were applied to calculate the protein concentration in tissues. Subsequently, the proteins in tissue specimens were subjected to denaturation and separated via sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The position of the Marker proteins was observed, and the electrophoresis was stopped when the Marker proteins reached the bottom of glass plate in a straight line. Then the proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA) and reacted with blocking buffer for 1.5 h. After that, anti-Sirt1 primary antibody (1:1000) and secondary antibody (1:1000) were added in sequence. Finally, the image was fully developed with chemiluminescent reagent in the dark for 1 min after rinsing.

2.9. Detection via qPCR

The total RNA was extracted from the tissue specimens and then reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using the reverse transcription kit, with a reaction system of 20 μL. Reaction conditions: reaction at 51°C for 2 min, pre-denaturation at 96°C for 10 min, denaturation at 96°C for 10 s and annealing at 60°C for 30 s, 40 cycles in total. The relative expression of

Table 1. Primer sequences.

Organization	Primer sequence
VEGF	Forward primer: 5' TCCACTTCCAAGCTGAGCGAG 3' Reverse primer: 5 'GTCCAGGGCATGATGGTTCT 3'
SIRT1	Forward primer: 5' TCCACTTCCAAGCTGAGCGAG 3' Reverse primer: 5' GTCCAGGGCATGATGGTTCT 3'
MiR-34a	Forward primer: 5' TCCACTTCCAAGCTGAGCGAG 3' Reverse primer: 5' GTCCAGGGCATGATGGTTCT 3'
GADPH	Forward primer: 5' ACGGCAAGTTCAACGGCAGTGGCA 3' Reverse primer: 5' GAAGACGCCAGTAGACTCCACGAC 3'

relevant messenger RNAs (mRNAs) was calculated with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal reference. The detailed primer sequences are shown in Table 1.

2.10. ELISA

The blood of abdominal aorta was centrifuged in a high-speed centrifuge at 14,000 g for 10 min, and the supernatant was collected. According to the instructions of the ELISA kit, the samples were loaded, standard substance, biotinylated antibody working solution and enzyme conjugate working solution were added, and the plate was washed successively. Finally, the tissues were detected using the microplate reader at 450 nm.

2.11. Statistical analysis

In this research, Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) was adopted for statistical analysis. The enumeration data were expressed as mean ± standard deviation. t-test was performed for data meeting normal distribution and homogeneity of variance, corrected t-test for those meeting normal distribution and heterogeneity of variance, and non-parametric test for those not meeting normal distribution and homogeneity of variance. The rank sum test was utilized for the ranked data and chi-square test for the enumeration data.

3. Results

3.1. Immunohistochemistry results

As shown in Figure 1, the tissues with positive expression were sepia. There were few positive expressions of VEGF in normal group and relatively more positive expressions in the other two groups. According to the statistical results (Figure 1), the average optical density of the positive expression of VEGF was increased remarkably in model group and miR-34a mimics group compared with that in normal group, displaying statistically significant differences (P<0.05). However, it was raised notably in miR-34a mimics group compared with that in model group, with a statistically significant difference (P<0.05).

3.2. Relative expressions of proteins detected via WB assay

The protein expression of Sirt1 was lower in normal group but higher in the remaining groups (Figure 2A). It was shown in the statistical results (Figure 2B) that the relative protein expression level of Sirt1 was elevated clearly in the other two groups in comparison with that in normal group, and it was markedly raised in miR-34a mimics group in contrast with that in model group. All the differences were statistically significant (P<0.05).

3.3. QPCR results

Model group and miR-34a mimics group exhibited distinctly higher relative expression levels of miR-34a, VEGF mRNA and Sirt1 mRNA than normal group, and there were statistically significant differences (P<0.05). Besides, miR-34a mimics group had prominently higher relative expression levels of miR-34a, VEGF mRNA and

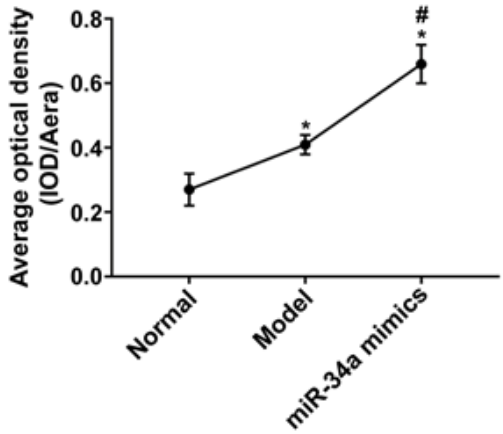

Fig. 1. Immunohistochemistry results determined by average optical density of positive expressions in each group. *P <0.05 vs. normal group, #P<0.05 vs. model group.

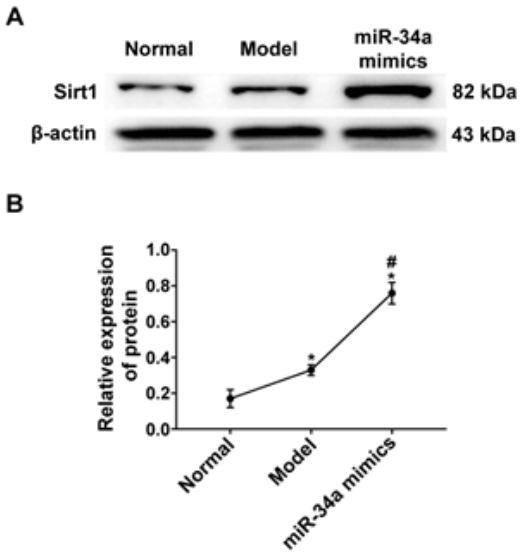

Fig. 2. Protein expressions detected via WB assay. Note: A, WB assay, B, Relative expression levels of proteins in each group. *P<0.05 vs. normal group, #P<0.05 vs. model group.

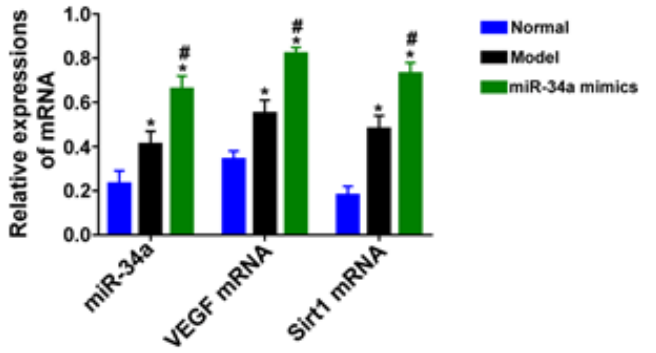

Fig. 3. Relative expressions of related mRNAs in each group. Note: *P<0.05 *vs.* normal group, #P<0.05 *vs.* model group.

Sirt1 mRNA than model group, with statistically significant differences (P<0.05) (Figure 3).

3.4. ELISA results

Compared with that in normal group, the content of VEGF was increased obviously in the other two groups, showing statistically significant differences (P<0.05). Moreover, in comparison with model group, miR-34a mimics group model group had clearly increased content of VEGF, and the difference was statistically significant (P<0.05) (Figure 4).

3.5. Hemorrheological detection results

Based on Figure 5, the PU index was the highest in normal group and the lowest in model group. the PU index was reduced remarkably in model group and miR-34a mimics group compared with that in normal group, while it was raised significantly in miR-34a mimics group in contrast with that in model group, and all the differences were statistically significant (P<0.05).

4. Discussion

The incidence rate of lower limb arteriosclerosis obliterans becomes higher and higher with the aggravation of population aging and changes in people's lifestyles, such as sedentariness, long-standing and changes in eating habits. Related studies have demonstrated that the incidence rate of lower limb arteriosclerosis obliterans is as high as 10% around the world and shows an upward trend year by year [11, 12]. As a fairly common peripheral vascular disease in the clinic, lower limb arteriosclerosis obliterans may occur in every artery of the lower limbs and induce arterial intima-media thickening and fibrosis due to various reasons, ultimately resulting in luminal narrowing, ischaemia and hypoxia [13, 14]. However, the specific mechanism of lower limb arteriosclerosis obliterans remains unclear at present. According to studies [15, 16], vascular injury is one of the leading causes of lower limb arteriosclerosis obliterans, and the repair and reconstruction after artery injury are closely correlated with the prognosis of the disease. Currently, the primary therapeutic objectives of lower limb arteriosclerosis obliterans are to restore the blood supply, preserve the limb function and prevent amputation. However, there have been no ideal therapeutic methods for the disease yet, so it is essential to deeply investigate the related pathogenesis to lower limb arteriosclerosis obliterans. Sirt1 is a crucial NAD-dependent histone deacetylase in the body, which

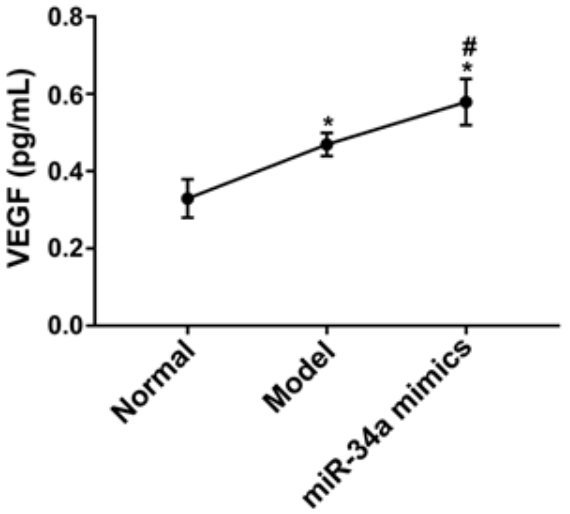

Fig. 4. ELISA. Note: *P<0.05 *vs.* normal group, #P<0.05 *vs.* model group.

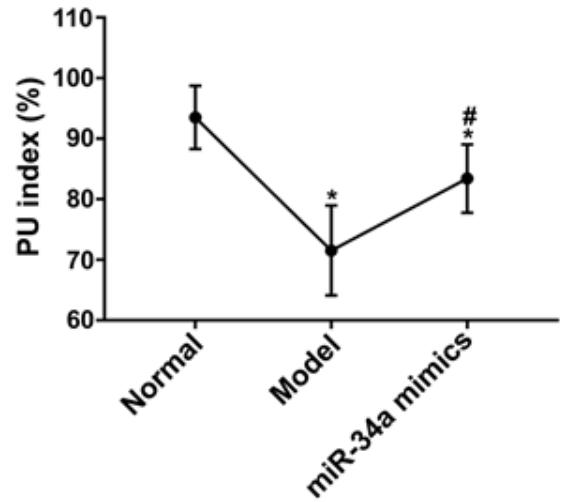

Fig. 5. Blood flow each group. Note: *P<0.05 *vs.* normal group, #P<0.05 *vs.* model group.

plays very important roles in regulating vascular endothelial injury, repair of vascular injury and regeneration. It has been revealed by studies [17-19] that Sirt1 has vital vascular protective effects after injury, and it can exert critical regulatory effects through modulating a variety of physiological and pathological responses. In addition, Sirt1 in the Sirt1 signaling pathway can activate multiple downstream signaling pathways via high expression and phosphorylation under the stimulation of injury factors, thus controlling diverse protein expressions and participating in the regulation of physiological and pathological responses [20]. The results of this research indicated that Sirt1 was highly expressed in the vascular tissues of rats with lower limb arteriosclerosis obliterans, suggesting that the Sirt1 signaling pathway is activated. Meanwhile, high expression of VEGF, a vital effector promoting vascular repair and regeneration, was also observed, implying that the activation of the Sirt1 signaling pathway may facilitate vascular repair by regulating VEGF expression. As an essential signaling pathway in organisms, the Sirt1 signaling pathway is also regulated by upstream regulatory factors. Furthermore, miR-34a is one of the crucial members of the miRNA family that exerts important regulatory effects on

a number of downstream signaling pathways. It was manifested in this research that the intervention with miR-34a mimics could remarkably increase the expression of Sirt1 in the blood vessels, activate the Sirt1 signaling pathway and up-regulate VEGF expression of the rats with lower limb arteriosclerosis obliterans at the same time, which may be an important reason of ameliorated arterial blood flow in those rats after the intervention with miR-34a mimics.

In conclusion, it can be seen that miR-34a enhances the expression of VEGF in the blood vessels of rats with lower limb arteriosclerosis obliterans by up-regulating the Sirt1 signaling pathway, thus ameliorating the arterial blood flow.

Conflict of interests
The author has no conflicts with any step of the article preparation.

Consent for publications
The author read and approved the final manuscript for publication.

Ethics approval and consent to participate
This study was approved by the Animal Ethics Committee of the Affiliated Hospital of Guangdong Medical University Animal Center.

Informed Consent
The authors declare that no patients were used in this study.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request

Authors' contributions
Tuo Xu and Huilai Miao designed the study and performed the experiments, Changwei Zheng and Yongkang Wu collected the data, Changwei Zheng, Yongkang Wu and Zhengde Chen analyzed the data, Tuo Xu and Huilai Miao prepared the manuscript. All authors read and approved the final manuscript.

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