Effects of Bunao-Fuyuan decoction serum on proliferation and migration of vascular smooth muscle cells in atherosclerotic

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[ABSTRACT] Atherosclerosis (AS) is a chronic inflammatory disease, the main causes of which include abnormal lipid metabolism, endothelial injury, physical and chemical injury, hemodynamic injury, genetic factors and so on. These causes can lead to inflammatory injury of blood vessels and local dysfunction. Bunao-Fuyuan decoction (BNFY) is a traditional Chinese medicine compound that can treat cardiovascular and cerebrovascular diseases, but its effect on AS is still unknown. The aim of this study was to investigate the effect and mechanism of BNFY in proliferation and migration of vascular smooth muscle cells (VSMCs) on AS. At first, the expression of α-SMA protein in ox-LDL-induced VSMCs, which was detected by immunofluorescence staining and western blot. CCK-8 technique and cloning technique were used to detect the cell proliferation of ox-LDL-induced VSMCs after adding BNFY. Meanwhile, the expression of proliferating protein Ki67 was detected by immunofluorescence staining. Western blot was also used to detect the expression of proliferation-related proteins CDK2, CyclinE1 and P27. Flow cytometry was used to detect the effect of BNFY on cell cycle. The effects of BNFY on proliferation and migration of cells were detected by cell scratch test and Transwell. Western blot was used to detect the expression of adhesion factors ICAM1, VCAM1, muc1, VE-cadherin and RHOA/ROCK-related proteins in cells. We found that the expression of AS marker α-SMA protein increased significantly and cells shriveled and a few floated on the medium after induction of ox-LDL on VSMCs. The proliferation rate of ox-LDL VSMCs decreased significantly after adding different doses of BNFY, and BNFY can inhibit cell cycle. Meanwhile, we also found that cell invasion and migration rate were significantly inhibited and related cell adhesion factors ICAM1, VCAM1, muc1 and VE-cadherin were inhibited too by BNFY. Finally, we found that BNFY inhibited the expression of RHOA, ROCK1, ROCK2, p-MLC proteins in the RHOA/ROCK signaling pathway. Therefore, we can summarize that BNFY may inhibit the proliferation and migration of atherosclerotic vascular smooth muscle cells by inhibiting the activity of RHOA/ROCK signaling pathway.

[KEY WORDS] Atherosclerosis; Bunao-Fuyuan decoction; Proliferation; Migration; RHOA/ROCK signaling pathway

[Introduction] Cardiovascular and cerebrovascular diseases are the leading causes of death in the world at present. Their key pathological basis is atherosclerosis (AS), which leads to memory myocardial infarction and stroke, etc., and the progress of atherosclerosis is insidious and sudden. Current diagnostic methods are insufficient to screen for early atherosclerosis, so how to effectively detect the atherosclerosis and how to intervene effectively has become the urgent need to solve the problem.

The main causes of AS include lipid metabolism abnormality, endothelial injury, physical and chemical injury, hemodynamic injury, genetic and other factors. When blood vessels are damaged by inflammation, the function of vascular endothelial cells is impaired, then the balance between vascular active substances which is synthesized and secreted by endothelial cells and cytokines is destroyed, causing white blood cells (especially the mononuclear cells and the vascular endothelial cells) adhesion. After that, phenotypic changes of the vascular smooth muscle cell (VSMCs) were induced...
and migrated to intracellular membranes. The activated VSMCs proliferated and ingested oxidized low density lipoprotein (ox-LDL) to form foam cells, and a large number of foam cells accumulated in the blood vessel walls to form lipid plaque, leading to the formation of the AS [1-3].

Nowadays, the therapeutic drugs for AS include statins, antioxidants, niacin and nitrates, etc. These drugs achieve certain therapeutic effects, while they cannot cure AS fundamentally and require long-term medication. Moreover, these western medicines have adverse effects that leave patients physically and mentally exhausted [4-5]. At present, more attention is paid to the traditional Chinese medicine in the treatment of cardiovascular and cerebrovascular diseases [6]. Greenway F et al. showed that eucommia leaf extract can improve the level of nitric oxide (NO) in plasma and effectively improve blood vessel function. Meanwhile, it also inhibits the production of intercellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) [7]. Polygonum multiflorum stilbene glycoside (PMS) is a water-soluble component of polygonum multiflorum, which has protective effect on cardiovascular and cerebrovascular diseases. Some studies have found that PMS reduces the production of ICAM-1 and VCAM-1 in foam cells by oxidative modification of high-density lipoprotein, proving that the PMS is a kind of effective drug resistant to atherosclerosis [8-9]. Wang et al. reported that tetramethylpyrazine (TMP) could inhibit the ox-LDL-induced activation of p-ERK, p-p38 lightening, and p-JNK mitogen-activated protein kinase (MAPK), proving that TMP protects endothelium and prevents atherosclerosis via inhibition of immunological responses [10].

Bunao-Fuyuan decoction (BNFY) is a traditional Chinese medicine compound consisting of astragalus root, angelica root, red peony root, szechwan lovage rhizome, safflower and peach kernel. Astragalus root has the effect of strengthening capillary resistance, and peach kernel has the effect of calming the spirit, promoting blood circulation and removing blood stasis, while szechwan lovage rhizome, safflower and angelica have the effect of enriching blood and nourishing blood, promoting blood circulation and removing blood stasis. So, the overall effects of BNFY are nourishing the brain, removing blood stasis, dredging blood vessels, improving blood flow and reducing blood viscosity. Therefore, we speculated whether BNFY had a good therapeutic effect on vascular diseases. Studies have found that when a variety of inflammatory stimulation signal molecules such as thrombin, vascular endothelial growth factor (VEGF) stimulate the capillaries of human brain, RHOA/ROCK pathway will be activated, which directly phosphorylates myosin light chain (MLC) or inhibits the activity of myosin light chain Phosphorylase (MLCP), increasing the phosphorylation level of MLC. Then it causes cell contraction and increases the permeability of vascular endothelial cells [11]. Endothelial cells are very important in the development of AS, as it can maintain the contraction of the VSMC phenotype through the intercellular gap junctions [12]. When vascular endothelial cell functions were damaged, VSMC phenotype changed from the contractility to synthetic, triggering AS [13]. Therefore, we speculated that RHOA/ROCK signaling pathway played a crucial role in the occurrence and development of AS. Meanwhile, the overall efficacy of BNFY mentioned above are removing blood stasis, dredging blood vessels, improving blood flow and reducing blood viscosity. Therefore, we speculate whether BNFY will treat AS by regulating the RHOA/ROCK signaling pathway.

In this study, we tested the effect of BNFY decoction on the migration and proliferation of VSMCs in AS, and explored its mechanism so as to provide a new idea for the treatment of AS.

Materials and Methods

Drug preparation

Bunao-Fuyuan decoction (BNFY, astragalus root 120 g, angelica root 6 g, red peony root 5 g, szechwan lovage rhizome 3 g, safflower 3 g and peach kernel 3 g) was provided by the first affiliated hospital of Wenzhou Medical University. Water with 10 times the weight of medicine was added so as to soak BNFY for 1 h, then 1 h of reflux extraction was performed. After that, the liquid was poured out, and 8 times the amount of distilled water was added to the residue. Then 1 h of reflux extraction was conducted. The two decoctions were combined together and concentrated by rotary evaporator to prepare BNFY decoction with the concentration of 1 g/mL. After cooling, BNFY was placed into the refrigerator at −4 °C.

Animal experiments

25 male Sprague-Dawley rats (200 ± 20 g) were purchased from Laboratory Animal Center of Central South University Xiangya Hospital (Changsha, China). They were divided into 5 groups at random and treated as follows: (1) NS group (NS): rats were given 0.9% NaCl by intragastric administration; (2) Positive control group (Atorvastatin): atorvastatin (10 mg·kg⁻¹) was given by intragastric administration; (3) BNFY-treated group: the experimental group was divided into low (3.72 g·kg⁻¹) and high (7.45 g·kg⁻¹), medium (14.9 g·kg⁻¹) doses, with n = 5 in each group. Each group was given gavage at 8:00 and 15:00 twice a day for 2 d, and sterile blood was collected within 1−2 h after the last gavage, which was separated by centrifugation at 3500 r·min⁻¹ for 10 min. The serum was then pumped and sterilized by 0.2 μm microporous filtration membrane and was inactivated at 56 °C for 30 min to remove the complement in the serum. At last the serum was stored at −20 °C for later use.

Cell culture

VSMC line was purchased from Shanghai Cell Collection (Shanghai, China). Cells were cultured in DMEM (Gibco; Thermo Fisher Scientific) added with 10% FBS (Gibco; Thermo Fisher Scientific), 100 U·mL⁻¹ penicillin, and 100 μg mL⁻¹ streptomycin at 37 °C with 5% CO₂. The medium was changed every 2–3 days.

Induction of cell models of AS

50 mg·mL⁻¹ oxidatively modified low density lipopro-
tein (ox-LDL) (cat. No. YB-002, Yiyuan Biotechnologies Co., Ltd.) induced VSMCs to be AS cells for 24 h.

**Cell administration and experimental grouping**

The following 500 μL drug containing serums were added to ox-LDL-induced VSMCs, and the experiments were divided into: normal saline control group (NS), low-dose group (L), medium-dose group (M), high-dose group (H), and atorvastatin group (Atorvastatin).

**Immunofluorescence assay**

Cells were washed with PBS, fixed with 4% paraformaldehyde for 30 min at room temperature, permeabilized with 0.2% Triton X-100 for 5 min and blocked with 5% BSA for 1 h at room temperature. Cells were then incubated with primary antibodies including anti-α-SMA (1 : 100, cat. No. ab32575, Abcam) and anti-Ki67 antibody (1 : 100, cat. No. ab197234, Abcam) for 1 h at 37 °C. After washing with PBS, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody (1 : 2000; cat. No. BM2004; Boster Biological Technology) for 1 h at room temperature in the dark. Images were acquired using a fluorescence microscope (Nikon Corporation) after staining the cell nuclei with DAPI (Boster Biological Technology).

**Western blot analysis**

Cells were seeded at 1 × 10^6 cells/well in six-well plates. Proteins were extracted from cells using a protein lysis buffer (RIPA; Beyotime Institute of Biotechnology). The concentration of protein was determined using a bichinchoninic acid assay protein assay kit (Beyotime Institute of Biotechnology). Proteins (25 μg/lane) were resolved using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane (EMD Millipore). These membranes were then incubated with primary antibodies and incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1 : 5000; cat. No. ab181658; Abcam) at room temperature for 1 h. Proteins were visualized using Image Quant™ LAS 4000 (GE Healthcare Life Sciences) and quantified using Image J (Version146; National Institutes of Health). Anti-α-SMA (1 : 100, cat. No. ab32575, Abcam), anti-CDK2 (1 : 1000; cat. No. ab5978), anti-CyclinE1 (1 : 1000; cat. No. ab5980), anti-P27 (1 : 1000; cat. No. ab205921), anti-ICAM1 (1 : 1000; cat. No. ab28472), anti-VCAM1 (1 : 1000; cat. No. 8480T), anti-muc1 (1 : 1000; cat. No. 3300T) and anti-VE-cadherin (1 : 1000; cat. No. 3300T) were purchased from Abcam, and anti-GAPDH (1 : 1000; cat. No. 5174S) antibodies were obtained from Cell Signaling Technology, Inc.

**Cell Counting Kit-8 (CCK-8) assay**

Cells were seeded into 96-well plates and incubated at 37 °C in a 5% CO₂ humidified incubator. Cell viability was determined using the CCK-8 reagent (Dojindo Molecular Technologies, Inc.), according to the manufacturer’s protocol. After transfection for 24, 48 and 72 h, 10 μL CCK-8 solution (Dojindo Molecular Technologies, Inc.) was added to each well for 4 h, then the optical density was measured at 450 nm using a microplate reader.

** Colony formation assay**

Cells were seeded at 500 cells/well into six-well plates and cultured for 7~12 days. Following this, the medium was removed, and the colonies were fixed with 4% paraformaldehyde for 20 min and stained with 0.2% crystal violet. Following being washed and air dried at room temperature, the colonies were visualized under a Nikon microscope. Magnification 100 ×.

**Cell invasion assay**

Transwell assay was used to investigate the invasive properties of AS cells. Cells in serum-free DMEM were seeded into the upper chamber which consisted of 8 μm-pore inserts coated with Matrigel (BD Biosciences) in culture plates. DMEM supplemented with 10% FBS was added to the lower chamber. Following a 48 h incubation, the Matrigel and the cells remaining in the upper chamber were removed with a cotton-tipped swab. Subsequently, the cells were fixed in 4% polyformaldehyde for 10 mins at room temperature and stained with 0.1% crystal violet for 15 min. The number of invasive cells in five random fields (magnification, ×200) was counted using a light microscope (Olympus Corporation).

**Scratch wound healing assay**

Cells were seeded into 12-well plates at a density of 1 × 10^5 cells/well for adherent culture. When cells reached 80% confluence, monolayer cells were scraped off using a 10 μL sterile pipette tip. A phase-contrast microscope (IX711; Olympus Corporation) was used to monitor cells at the borders of the scratches. The degree of scratch healing was observed and images were captured in each group at 0 and 24 h.

**Flow cytometry assay**

The cell cycle distribution was examined using the Cell Cycle Analysis kit (Beyotime Institute of Biotechnology), according to the manufacturer’s protocols. Ox-LDL-induced cells treated with different BNFY doses were treated with 70% cold ethanol at 4 °C overnight to increase cell membrane penetrability. Subsequently, 100 μg·mL⁻¹ RNase (Nanjing KeyGen Biotech Co., Ltd.) was used to treat cells at 37 °C for 20 min. Following staining with 30 μg·mL⁻¹ propidium iodide (Nanjing KeyGen Biotech Co., Ltd.) for 30 min at room temperature in the dark, the cell cycle was analyzed using a Gallios Flow Cytometer (Beckman Coulter, Inc.). The flow cytometry results were evaluated using a Cell Quest kit (BD CellQuest™ Pro software version 6.1; BD Biosciences), according to the manufacturer’s protocols.

**Cell cycle assay**

A cell cycle assay was conducted to determine the cell cycle distribution. In brief, cells were collected using 0.25% trypsin, washed and then fixed with 70% ethanol at 4 °C overnight. Subsequently, the cells were stained with RNAse A and PI at 4 °C for 30 min. Finally, flow cytometry with a flow cytometer (FACSCanto II; BD Biosciences) was performed to analyze the cell cycle distribution.

**Statistical analysis**

Data are expressed as the mean ± standard deviation.
(SD). SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses. Comparisons between groups were analyzed by Student’s t-test or one-way analysis of variance followed by Tukey’s test. \( P < 0.05 \) was considered to indicate a statistically significant difference.

**Results**

**VSMCs were induced by ox-LDL to become model cells of atherosclerosis**

To determine whether ox-LDL successfully induced the formation of atherosclerosis model in VSMCs, we examined the expression of \( \alpha \)-SMA protein, a marker protein for atherosclerosis in VSMCs. As shown in Fig. 1A, immunofluorescence staining showed a significant increase of \( \alpha \)-SMA in ox-LDL-induced VSMCs compared with VSMCs that adds nothing, while western blot showed the same result (Fig. 1B). This result showed that AS model was induced successfully.

**BNFY inhibited the proliferation of ox-LDL induced VSMCs**

We have shown that ox-LDL can induce the generation of AS model in VSMCs, so we next examined the effect of BNFY on the proliferation of model cells. Various methods were used to detect the proliferation rate of cells. As shown in

![Image](image.png)

**Fig. 1** VSMCs were induced by ox-LDL to become model cells of atherosclerosis. (A) Immunofluorescence staining (× 200) showed increased immunolabeling intensity of \( \alpha \)-SMA after ox-LDL treatment. (B) The expression of \( \alpha \)-SMA protein was detected by Western blot. Data are expressed as mean ± SD, \( n = 3 \), **\( P < 0.01 \) vs VSMC-ox-LDL.

![Image](image.png)

**Fig. 2** BNFY inhibited the proliferation of ox-LDL induced VSMCs. Proliferation rate was detected by cck-8 technique (A) and cell cloning technology (B)
Fig. 2A, CCK-8 results showed that the proliferation of ox-LDL VSMCs decreased gradually with the increasing dose of BNFY, compared with the NS group. Also, the effect of BNFY in medium dose was consistent with that of the atorvastatin group. Cell cloning technology showed the same results (Fig. 2B). Subsequently, we used immunofluorescence staining to stain Ki67, and found that the experimental results were consistent with the results of cck-8 and cloning technology (Fig. 2B). Then we detected the proliferation-related proteins CDK2, CyclinE1 and P27 and found that, compared with the NS group, the protein levels of CDK2 and CyclinE1 decreased and the protein levels of P27 increased as the dose of BNFY increased. What’s more, the effect of BNFY in medium dose was consistent with that of the Atorvastatin group (Fig. 4A). Finally, we tested the cell cycle, and found that with the increasing dose of BNFY, the cell cycle stagnated at G0 and G1 phase, while the S phase decreased compared with NS group (Figs. 4B–4C). All the above experimental results showed that BNFY could inhibit the proliferation of VSMCs in AS in a dose-dependent manner.

**BNFY inhibited proliferation, migration and invasion of ox-LDL induced VSMCs**

We tested the migration ability of ox-LDL VSMCs by cell scratch and invasion. The date showed that the migration (Figs. 5A and 5C) and invasion (Figs. 5B and 5D) ability of ox-LDL VSMCs decreased significantly with the increasing dose of BNFY, which was dose-dependent, compared with NS group. The effect of medium dose of BNFY on cell invasion and migration was similar to that of the Atorvastatin group. Meanwhile, we also detected the expression of adhesion factors ICAM1, VCAM1, muc1, VE-cadherin in cells. It was found that, compared with NS group, the expression of adhesion factors ICAM1, VCAM1, muc1, VE-cadherin in ox-LDL VSMCs was inhibited in a dose-dependent manner with the increasing dose of BNFY (Fig. 6). The results indicated that BNFY inhibited the migration ability of VSMCs in AS and inhibited the expression of related adhesion cytokines.

**BNFY inhibited proliferation, migration and invasion of ox-LDL induced VSMCs through the RHOA/ROCK signaling pathway**

Some studies have indicated that RHOA/ROCK signaling pathway plays an important role in the development of the AS [14-16]. We found that BNFY can inhibit the ox-LDL induced VSMCs proliferation and migration in early experiments, but the mechanism was not yet clear. Therefore, we detected the expression of RHOA/ROCK signaling pathway-related proteins RHOA, ROCK1, ROCK2, MLC and phosphorylated MCL. It was discovered that, compared with the NS group, the levels of RHOA, ROCK1 and ROCK2 decreased with the increasing dose of BNFY, while the total protein level of MLC remained unchanged, and the expression levels of p-MLC decreased with the increase of BNFY dose., and the effect of BNFY in medium dose was consistent with that of the Atorvastatin group (Fig. 7).

In order to further study the mechanism, RHOA was inhibited and overexpressed by cell transfection, and cells were divided into M, M + mimic-NC, M + RHOA mimic, M + RHOA mimic + BNFY, M + inhibitor-NC, M + RHOA inhibitor, M + RHOA inhibitor + BNFY. The results showed that the expression of RHOA, ROCK1, ROCK2 and p-MLC in the M + RHOA mimic group was significantly decreased compared with that in the M + RHOA mimic group. Moreover, compared with the M + RHOA inhibitor group, the expression of RHOA, ROCK1, ROCK2 and p-MLC in the M + RHOA inhibitor + BNFY group was further decreased (Fig. 8). These results suggested that BNFY inhibit the proliferation, migration and invasion of ox-LDL induced VSMCs through the RHOA/ROCK signaling pathway.
Discussion

AS is the main cause of acute cardiovascular diseases, such as coronary heart disease, cerebral infarction and peripheral vascular disease. Lipid metabolism disorder is the pathological basis of AS, which is characterized by the involvement of arterial lesions starting from the intima. General first lipid and compound carbohydrate accumulate. Then fibrous tissue hyperplasia and calcification, gradual disintegration and calcification of artery in the middle results in arterial wall thickening and hardening and the vascular cavity narrowing. This is called AS because of the yellow appearance of the lipid buildup in the lining of the arteries. AS seriously endangers people's life and health, so there are more and more research on its drug treatment.

VSMCs are one of the main cells that constitute the vascular wall tissue structure and maintain vascular tension, and their structural and functional changes are the cytopathological basis of AS. Phenotypic transformation, proliferation, invasion, and migration of VSMCs are closely related to the development and occurrence of AS [17-19]. Therefore, VSMCs were selected as the experimental cell in this study. Ox-LDL plays an important role in the initial stage of AS, and it can activate endothelial cells through inducing surface adhesion factors of multiple cells (VSMCs and macrophages, et al.) which eventually lead to the damage and dysfunction of endothelial cells, thereby contributing to the occurrence of AS [20-21]. Ox-LDL can not only induce cell inflammation [22], but also induce endothelial cell injury and apoptosis [23]. Chellan B et al. demonstrated that ox-LDL induced the formation of foam cell, mediated phenotypic transformation of VSMCs, and affected migration and calcification of VSMCs in AS [24]. α-SMA is an important marker of the contraction phenotype of VSMCs [25]. Therefore, in this study, ox-LDL was used to induce VSMCs to produce AS model, and the expression of α-SMA, the landmark protein in AS, was detected. We found that after the induction of ox-LDL to VSMCs, the expression of α-SMA in cells increased significantly, indicating that the experimental modeling was successful.

BNFY is a traditional Chinese medicine compound, consisting of 120 g astragalus root, 6 g angelica root, 5 g red perony root, 3 g szechwan lovage rhizome, 3 g safflower and 3 g peach kernel. The overall effects of BNFY is to nourish the brain, promote blood circulation, remove blood stasis, dredge blood vessels, improve blood flow and reduce blood viscosity. However, the research of BNFY on cardiovascular and cerebrovascular diseases has not been reported at present.
Fig. 5  BNFY inhibited the migration and invasion of ox-LDL induced VSMCs. (A) Cell migration was detected by cell scratch test. (B) cell invasion rate was detected by Transwell test (C) Statistical analysis of cell migration. (D) Statistical analysis of cell invasion rate.  * P < 0.05,  ** P < 0.01,  *** P < 0.001 vs NS;  # P < 0.05 vs atorvastatin

Fig. 6  BNFY inhibited the migration and invasion of ox-LDL induced VSMCs. The expression of adhesion factors was detected by western blot.  * P < 0.05,  ** P < 0.01,  *** P < 0.001 vs NS;  # P < 0.05,  ## P < 0.01,  ### P < 0.001 vs atorvastatin
Fig. 7  BNFY inhibited proliferation, migration and invasion of ox-LDL induced VSMCs through the RHOA/ROCK signaling pathway. RHOA/ROCK signaling pathway related proteins were detected by western blot. * P < 0.05, ** P < 0.01, *** P < 0.001 vs NS; # P < 0.05, ## P < 0.01 vs atorvastatin.

Fig. 8  BNFY inhibited proliferation, migration and invasion of ox-LDL induced VSMCs through the RHOA/ROCK signaling pathway. RHOA/ROCK signaling pathway related proteins were detected by western blot. * P < 0.05, ** P < 0.01, *** P < 0.001 vs NS; # P < 0.05, ## P < 0.01 vs atorvastatin.

AS important cells in the blood vessel walls, abnormal proliferation and migration will not occur in VSMCs under normal circumstances. But when the plaque of AS forms and blood vessel are damaged, VSMCs will proliferate and migrate abnormally, which exacerbate the condition of AS [14]. Moreover, the proliferation, migration and extracellular matrix synthesis of VSMCs are the pathological basis for the occurrence and development of vascular diseases such as AS. So inhibiting the proliferation and migration of VSMCs in the AS is treated as the main strategy [27]. Chen et al. found that MiR-155-5p can target AKT signaling pathway to inhibit the proliferation and migration of VSMCs in AS [28]. Chen Z et al. found that Baicalin can upregulate miR-126-5p by targeting HMGB1, thus inhibiting the proliferation and migration of ox-LDL-induced VSMCs in AS [29]. The effect of BNFY on proliferation and migration of ox-LDL-induced VSMCs remains unclear. Therefore, in this paper, we examined the effect of BNFY on proliferation, migration and migration in ox-LDL-induced VSMCs. We found that the inhibition rate of BNFY on the proliferation, invasion and migration of model cells increased with the increase of dose. Meanwhile, BNFY inhibited the expression of adhesion factors ICAM1, VCAM1, muc1 and VE-cadherin in cells in a dose-dependent manner. It shows that BNFY can relieve AS symptoms and thus achieve therapeutic effect.

Our study has confirmed that BNFY can inhibit the proliferation and migration of AS model cells. But the exact mechanism is not clear, so we discussed its mechanism next. Through the literature, we found that after injury of endothelial cells, endothelial nitric oxide synthase biological activity is decreased, which is the beginning stage of AS. Whereas ROCK signaling pathway could downregulate the expression of nitric oxide synthase by multiple ways [30]. Meanwhile, it was found that when various inflammatory stimulation signaling molecules such as thrombin and vascular endothelial growth factor (VEGF) stimulated capillaries in human brain, RHOA/ROCK signaling pathway was activated, and directly phosphorylated MLC or inhibited the activity of MLCP, resulting in increased phosphorylation level of MLC. It then causes contraction of cells and increases the permeability of VSMCs [31]. The increase of permeability of endothelial cells aggravates the degree of cell injury and further induces the occurrence of AS. Our experimental results also showed that RHOA/ROCK signaling pathway-related protein expression was inhibited when BNFY was added, and cell proliferation and mobility were also inhibited. At the same time, we found a significant increase in pathway-related proteins after overexpression of RHOA, indicating that the RHOA/ROCK pathway was activated, while a significant decrease in pathway-related proteins was found after addition of BNFY. In addition, when RHOA expression was inhibited, pathway-related proteins were significantly decreased, while when BNFY was added, the expression of pathway-related proteins was further decreased. Therefore, it was speculated that BNFY may inhibit the proliferation and migration of AS model cells through the RHOA/ROCK signaling pathway.

In conclusion, we found in this paper that ox-LDL can induce VSMCs to produce AS model. BNFY can inhibit the proliferation and migration of ox-LDL-induced VSMCs through the RHOA/ROCK signaling pathway, and reduce the expression of adhesion factors in cells. These results provide a new idea and method for the treatment of AS.

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