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An extract from medical leech improve the function of endothelial cells in vitro and attenuates atherosclerosis in ApoE null mice by reducing macrophages in the lesions

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ABSTRACT

Aim: Medicinal leech has been widely used as a traditional Chinese medicine in cardiovascular diseases. However, its pharmaceutical effect is not fully revealed. The goal of this study was to determine whether a leech extract has the effect of anti-atherosclerosis in ApoE −/− mice and the mechanism of this effect.
Methods and results: In vivo experiments: ApoE −/− mice fed on high-cholesterol diet were separated into 5 groups. Control group was administrated with normal water; leech extract of low dose treatment group was given a leech extract of 0.02 g/kg/d; leech extract of medium dose treatment group was given a leech extract of 0.1 g/kg/d; leech extract of high dose treatment group was given a leech extract of 0.5 g/kg/d; simvastatin group was given simvastatin of 10 mg/kg/d. Leech extract significantly reduced atherosclerotic lesions in aortic root compared with control group. And the number of macrophage in or around the atherosclerosis plaque is significantly reduced in the leech extract groups compared with control group.
In vitro experiments: human endothelial cell line, EA.hy926, was induced with TNF-α to perform endothelial dysfunction. Control group: EA.hy926 cells with no special treatment; TNF-α group: EA.hy926 cells were induced by 10 ng/ml TNF-α for 6 h; leech extract only group: EA.hy926 cells were treated with 200 mg/ml leech extract only; leech extract and TNF-α group: 200 mg/ml leech extract was applied before TNF-α induction. Protein and mRNA level were detected in each group, leech extract can decrease the expression of intercellular adhesion factor (ICAM-1) and monocyte chemotactic protein (MCP-1) compared with TNF-α group. Furthermore, it showed less adhesion and migration of THP-1 cells to EA.hy926 cells in the adhesion assay and transwell assay. The NF-κB translation to nucleus was blocked by leech extract in the NF-κB translocation assay.
Conclusions: Leech extract could obviously attenuate the area of atherosclerosis lesion in ApoE −/− mice. And this effect is dose dependent. The effect is mainly a result of reduced invasion of monocyte in artery walls by blocking NF-κB translocation.

1. Introduction

Medicinal leech has been used as a traditional Chinese medicine in cardiovascular diseases and other chronic diseases for more than 2000 years. Some researches had been carried out and proved that anticoagulation is the main pharmaceutical effect of medicinal leech [1–3], but this cannot fully explain the effects in the treatment of cardiovascular diseases.

Atherosclerosis is a main event and fundament of many cardiovascular diseases [4]. It is the second cause of death in elder people. Atherosclerosis has now been regarded as a chronic inflammatory disease. Endothelial dysfunction is one of the first key steps. Risk factors could lead to endothelial dysfunction [4,5]. Afterward, monocytes in the circulation adhere to the artery wall and move to the tunica intima. In the tunica intima monocytes differentiate to macrophages and then engulf modified LDL to form early atherosclerosis lesions. The adhesion of monocytes to endothelium is dependent on integrins, such as VCAM-1 and ICAM-1, and the migration is driven by chemokines such as MCP-1.

Here we investigated the anti-atherosclerosis effect of an extract from medicinal leech using ApoE −/− mice, and found the treatment of leech extract could obviously attenuate the area of atherosclerosis lesion. And this effect is dose dependent. Furthermore, we proved the effect is mainly a result of reduced

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invasion of monocyte in artery walls by blocking NF-κB translocation. This study proves that leech extract could be promising in the treatment of cardiovascular diseases.

2. Materials and methods

2.1. Chemicals and antibodies

The leech extract was provided by Shandong University Weihai International Biotechnology Research Centre (Weihai, China). Antibodies used includes anti-monocyte and macrophage antibody (MOMA-2) for IHC, anti-ICAM-1, anti-MCP-1 and anti-actin for Western blot.

2.2. Animals

ApoE −/− mice, male, 7-week-old, were purchased from Vital River (Beijing, China) and housed under specific pathogen-free conditions in micro-isolator cages. All animals were used in accordance with institutional guidelines and the current experiments were approved by the Use Committee for Animal Care. All the mice were fed a high-cholesterol diet for 20 weeks. And at the first day of the 10th week, the mice were separated into 5 groups and 15 in each group. Control group was administrated with normal water; leech extract of low dose treatment group was given a leech extract of 0.02 g/kg/d; leech extract of medium dose treatment group was given a leech extract of 0.1 g/kg/d; leech extract of high dose treatment group was given a leech extract of 0.5 g/kg/d; simvastatin group was given simvastatin of 10 mg/kg/d. All medicines were given p.o.. The treatments lasted for 10 weeks.

2.3. Oil red O staining of the root of aorta

Serial cryostat sections of 10 μm were cut from aortic sinus to aortic arch. Slides were soak in 60% isopropanol for 30 s then stain with oil red O for 10 min and followed by soaking in 60% isopropanol for 10 s to remove redundant dye.

2.4. Immunohistochemical analyses

Serial cryostat sections of 10 μm were cut from aortic sinus to aortic arch. Serial frozen sections were air-dried, fixed in acetone for 10 min at −20 °C, air-dried and rehydrated with PBS before being incubated in 3% H_2O_2/PBS to block endogenous peroxidase. For macrophage detection, sections were blocked in 5% normal goat serum in PBS followed by staining using MOMA-2 antibody overnight. Slides were colorated by DAB, counterstained in haematoxylin, dehydrated in ethanol, cleared in xylene and mounted in gum.

2.5. Ultrasound biomicroscopic imaging

Anesthetized mice were laid supine on a platform with four limbs taped to electrocardiographic electrodes for heart rate monitoring. Body temperature was monitored via a rectal thermometer and maintained to 36–38 °C. The neck hair was shaved, and preheated ultrasound gel was used as an acoustic coupling medium. The aorta was imaged with the use of the UBM system (Vevo 2000) and a 30-MHz transducer with B-scan imaging and Doppler flow measurement capabilities. The maximum lumen diameters were measured at root of the aorta. Care was taken to place the mice in similar postures to ensure similar orientation of vessels during ultrasonic imaging, and measurements were averaged from three consecutive cardiac cycles.

2.6. Cell culture

The human endothelial cell line, EA.hy926 and human monocyte cell line, THP-1 were purchased from Shanghai institute for biological sciences, Chinese academy of sciences institute of cell resource center (Shanghai, China). Cell culture was according to manufacturer's protocol. All the cell lines were grown at 37 °C in a 5% CO_2/95% air atmosphere and were revived every 3–4 months.

2.7. Cell viability assay

Cell viability was tested with a MTT assay. Briefly, EA.hy926 cells were raised in 96-well plate and incubated overnight before treating with or without test agents for the indicated time. 15 μL MTT solution (5 mg/mL) was added into each well 4 h before the end of treatments. The culture medium was aspirated and replaced with 100 μL DMSO. The absorbance at 570 nm (A570) for each well was determined by an ELISA reader (SUNRISE). The percentage of living cells was calculated as $A570_{test}/A570_{control}$.

2.8. Adhesion assay

EA.hy926 cells were plated in 24-well plate and allowed to grow to confluent monolayers. The EA.hy926 cells were treated with leech extract for 24 h. Then they were treated with TNF-α for 6 h. THP-1 cells were suspended overnight in RPMI-1640 medium containing 0.1% fetal bovine serum. After 24 h, 1×10^6 THP-1 cells were added to the endothelial monolayers and incubated at 37 °C for 30 min. The nonadherent cells were collected carefully and the wells were washed with media and collected, then merged with aforementioned THP-1 and counted under a microscope with a counting plate. The adhesion rate equals ((total cell number of THP-1 added) − (nonadherent cell number of THP-1))/(total cell number of THP-1 added).

2.9. Migration assay

EA.hy926 cells were plated in the lower chambers of a transwell plate and allowed to grow to confluent monolayers. The EA.hy926 cells were treated with leech extract for 24 h. Then they were treated with TNF-α for 6 h. THP-1 cells were suspended overnight in RPMI-1640 medium containing 0.1% fetal bovine serum. After 24 h, 1×10^6 THP-1 cells were added to the upper chambers and incubated at 37 °C for 30 min. After 30 min, the membrane of each well was cut off carefully and fixed with resin on a slide and observed under a microscope.

2.10. RT-PCR

Total RNA isolation and RT-PCR were performed according to manufacturer's protocol. In brief, total RNA was isolated from EA.hy926 cells with Trizol reagent. Then it was quantified spectrophotometrically at a ratio of 260–280 nm. Total RNA was used in first-strand cDNA synthesis performed by a reverse transcriptase enzyme kit. The PCR reactions were performed by a PCR kit. The primer sequences were synthesized by Nanjing GenScript Biotech Company as follows: GAPDH (forward: 5′-CACCAGGGCTGCTTTTAACTCTGGTA-3′, reverse: 5′-CCTTGACGGTGCCATGGAATTGC-3′, T_m 50 °C), eNOS (forward: 5′-TTCCGGGGATTCTGGCAGGAG-3′, reverse: 5′-GCCATGGTAACATCGCCGCAG-3′, T_m 50 °C), ICAM-1 (forward: 5′-CAGTCACCTATGGCAACGAC-3′, reverse: 5′-CAGTCACCTATGGCAACGAC-3′, T_m 50 °C), MCP-1 (forward: 5′-AACTGAAGCTCGCACTCTCG-3′, reverse: 5′-TCAGCACAGATCTCCTTGGC-3′, T_m 57 °C). The PCR amplification were performed with denaturation at 95 °C for 30 s, annealing at T_m for 30 s and DNA elongation at 72 °C for 60 s. A total of 35 cycles were carried out. The results were analyzed

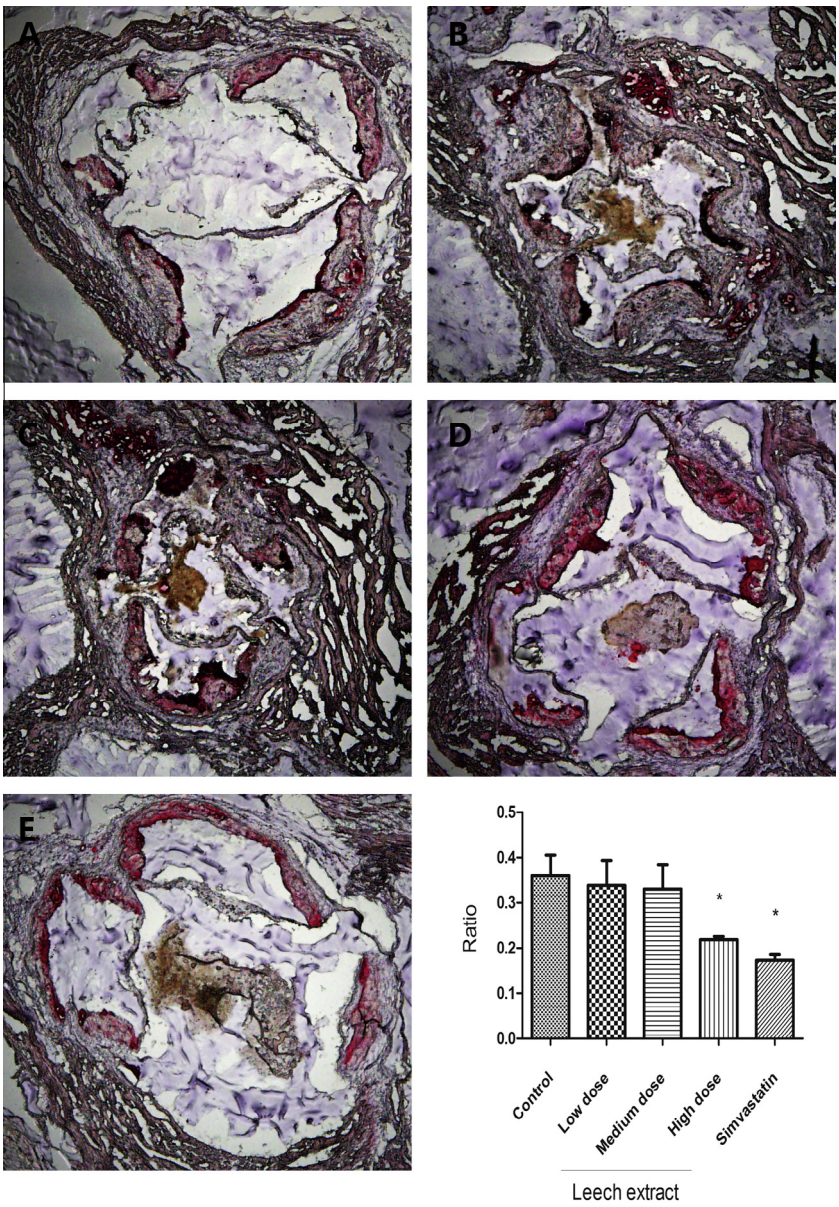

Fig. 1. Effect of leech extract on atherosclerosis lesion of ApoE null mice (40×). (A) control group, (B) leech extract of low dose group (0.02 g/kg/d), (C) leech extract of medium dose group (0.1 g/kg/d), (D) leech extract of high dose group (0.5 g/kg/d), (E) simvastatin group (10 m g/kg/d). Ratio of AS lesion to aorta root section in group D and E is obviously reduced compared to control group ($n = 5$, $^{*}p < 0.05$).

by ImageJ 1.47. The intensities of specific PCR products were quantified relative to the GAPDH product from the same sample.

2.11. Western blot

Protein extraction and Western blot analysis was performed with ICAM-1 and MCP-1. Briefly, 20 μg proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking, the filters were incubated with ICAM-1, MCP-1 antibodies at 4 °C overnight. After washing and incubation with anti-mouse secondary antibodies IgG for 1 h, membranes were visualized with Simon imaging system.

2.12. NF-κB translocation assay

NF-κB translocation assay was performed with a test kit following the instruction [6]. Briefly, EA.hy926 cells raised in the 6-well plate were incubated with or without various test treatments in HBSS for 1 h at 37 °C, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 for 15 min, and blocked with 5% BSA for 2 h at room temperature. The cells were then incubated overnight with the primary antibody against the p65 subunit of NF-κB at 4 °C, washed in PBS, and incubated with Cy3-labeled secondary antibody for 2 h at room temperature. Finally, the cells were stained with 2 μM DAPI for 5 min, and then observed using a fluorescence microscope.

2.13. Statistical analysis

Data was described as the mean ± SD. Comparisons between different groups were undertaken using the Student's two-tailed *t*-test. The criteria of statistical significance was $p < 0.05$. Statistical analysis was done with SPSS 22.0 software.

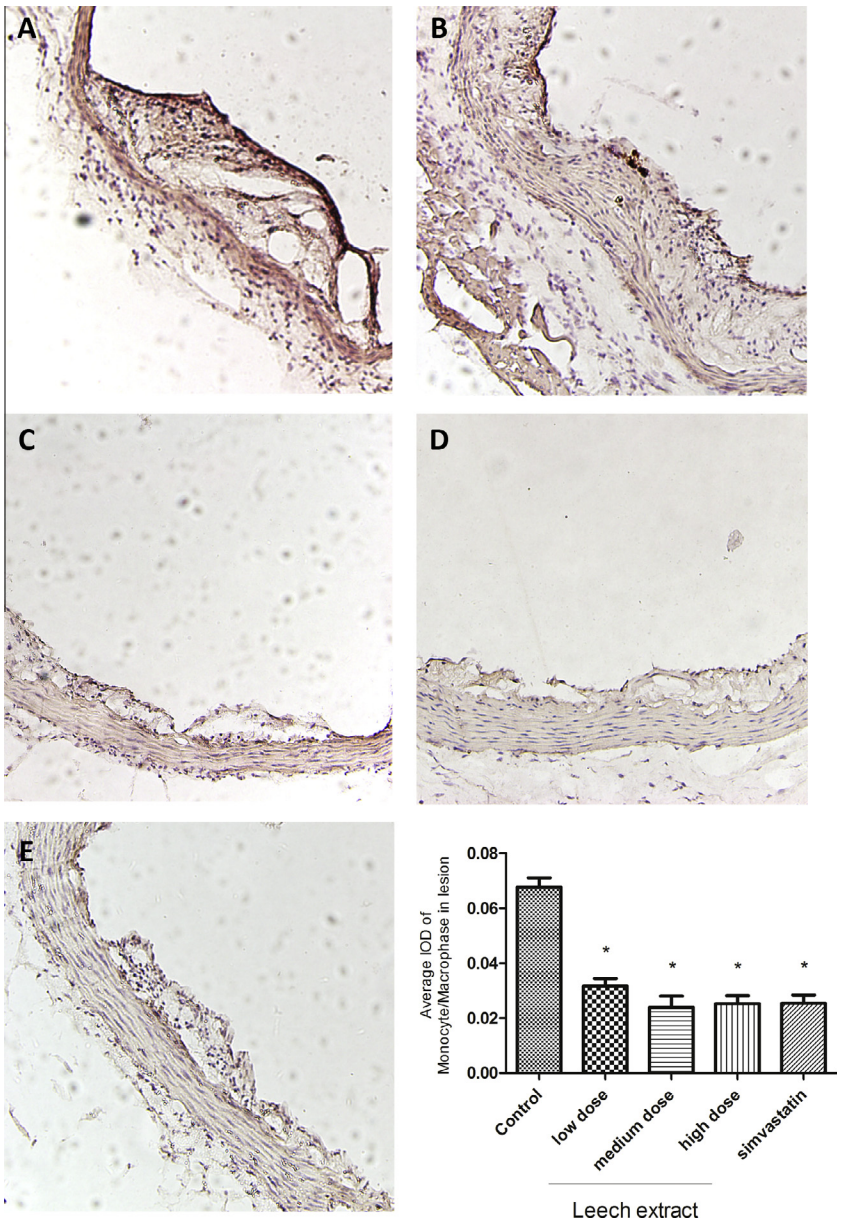

Fig. 2. Effect of leech extract on monocyte/macrophage in atherosclerosis lesion of ApoE null mice (100×). (A) Control group, (B) leech extract of low dose group (0.02 g/kg/d), (C) leech extract of medium dose group (0.1 g/kg/d), (D) leech extract of high dose group (0.5 g/kg/d), (E) simvastatin group (10 m g/kg/d). Ratio of AS lesion to aorta root section in group B, C, D and E is obviously reduced compared to control group ($n = 5$, $^{*}p < 0.05$).

3. Results

3.1. Isolation of leech extract

The process of leech extract was according to the patent [7] and described briefly as below. Dry leech was weighted and smashed, and then dd-H_2O was added. After 48 h stilled at 4 °C in a refrigerator, the mix was homogenized with a homogenizer for 15 min at 3000 rpm in an ice-bath. After homogenization, Tris (pH 8.5) was added to adjust the pH to 8.5. Then trypsin and crude enzymes were added. The mix was moved to a 50 °C water-bath, stirred for 8 h. in this process the pH should be adjusted every 30 min to keep stable at 8.5. After this process this liquor was cooled down to the room temperature and absolute alcohol was added then stilled at 4 °C for 12 h. The supernatant was separated and acetone of 2 times of the volume was added, then stilled at 4 °C for 12 h. The supernatant was separated and spin steamed to evaporate all organic solvents. Then the liquor was ultrafiltration with a 6 k Dalton molecular sieve. The part lower than 6 kD was collected and freeze-dried. The leech extract powder was finally collected and stored at −20 °C.

3.2. Lumen diameters of root of the aorta are wider in leech extract treatment groups

To evaluate the effect of this leech extract, lumen out diameter (OD) and outer diameter (ID) of root of the aorta were measured by Ultrasound biomicroscopic (UBM) imaging method before the mice in each group were sacrificed. The results showed that the ratio of ID to OD is higher in leech extract treatment groups (17.3% in high dose group) and simvastatin group (19.4%) (Supplementary material, Fig. S1).

3.3. Atherosclerosis lesion area is obviously reduced in leech extract treatment group

To evaluate the effect of this leech extract, atherosclerosis lesions in the aortic root of each group were measured and the ratio of lesion area to whole aortic root section was calculated. ApoE −/− mice of 17-week-old developed obvious atherosclerosis before drug treatment. Atherosclerosis lesion can be observed in the anatomy. After 10 weeks treatment, the mice in each group were sacrificed. The results showed that the ratio of area stained with oil red O to area of aorta cross section was reduced in the high dose leech extract group (9.2%, $^{*}p < 0.05$) and simvastatin group (11.6%, $^{*}p < 0.05$) significantly (Fig. 1).

3.4. Less macrophage is observed in the lesion in leech extract treatment group

The number of macrophage in or around the atherosclerosis plaque is stained with MOMA-2, and the result showed that macrophages were reduced in all leech extract group and simvastatin group significantly (B: 3.50%, C: 4.27%, D: 4.48%, E: 4.64%, $^{*}p < 0.05$, Fig. 2).

The results in vivo suggest that leech extract could attenuate atherosclerosis lesion development and this might related to the macrophages migration. Monocyte in the circulation is one of the major resources of macrophage in atherosclerosis lesions. The changes of expression of ICAM-1 and MCP-1 affect the adhesion and migration process greatly. To testify this hypothesis we ran tests in vitro to study whether leech extract affects the processes of THP-1 cells adhesion and migration to EA.hy926 cells.

First we screened the proper dose of leech extract by MTT to eliminate the effect of cytotoxicity. It showed no cytotoxicity from 50 to 800 mg/ml (Supplementary material, Fig. S2). And we used the upregulating of mRNA level of eNOS to judge the effect of leech extract in promoting endothelial function. At the dose of 200 mg/ml, the effect of upregulating mRNA level of eNOS is most significant. And the dose of leech extract was determined to 200 mg/ml (Fig. 3A).

Experiments were carried out in four groups. Control group: EA.hy926 cells were given no special treatments; TNF-α group: EA.hy926 cells were induced by 10 ng/ml TNF-α for 6 h; leech

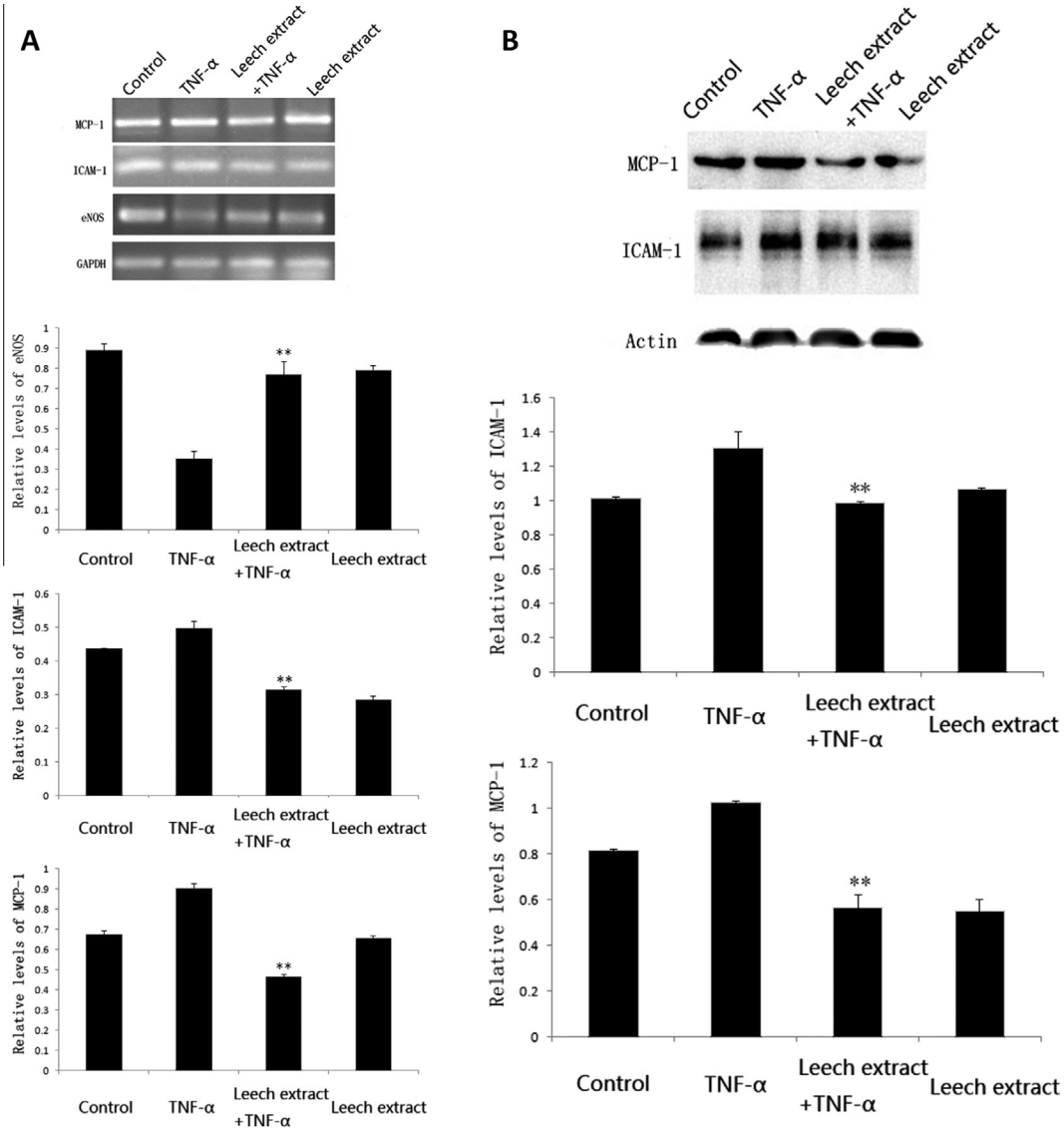

Fig. 3. Effect of leech extract on eNOS, ICAM-1 and MCP-1 mRNA level and ICAM-1 and MCP-1 protein expression of EA.hy926 cells. EA.hy926 cells were treated with leech extract (200 mg/mL) for 24 h and followed with TNF-α (10 ng/mL) for 6 h. mRNA levels of eNOS, ICAM-1 and MCP-1 were measured by RT-PCR, and protein expression of ICAM-1 and MCP-1 were measured by Western blot ($n = 3$, $^{*}p < 0.05$).

extract only group: EA.hy926 cells were treated with 200 mg/ml leech extract only; leech extract and TNF-α group: 200 mg/ml leech extract was applied before TNF-α induction.

3.5. Effect of leech extract on mRNA and protein expression of ICAM-1 and MCP-1 in EA.hy926 cells

First we verified whether leech extract altered the expression of ICAM-1 and MCP-1 in EA.hy926 cells. The results showed that mRNA level of ICAM-1 and MCP-1 in leech extract and TNF-α group reduced 36.9% and 48.5% compared with TNF-α group (Fig. 3A). And protein expression reduced 31.97% and 45.03% in leech extract and TNF-α group compared with TNF-α group (Fig. 3B).

3.6. Leech extract attenuates THP-1 adhesion and migration to EA.hy926 cells

We have already observed the differences of mRNA expression of ICAM-1 and MCP-1 between the leech extract treated group and TNF-α group. Both mRNA and protein expression of ICAM-1 and MCP-1 in EA.hy926 cells were lower in leech extract treated group compared with TNF-α group. Furthermore, adhesion assay and migration assay were employed to verify the effect of leech extract on cell adhesion and migration. The results showed that the adhesion rate of THP-1 was lower in the leech extract group (Supplementary material, Fig. S3). And less THP-1 cells were observed in the transwell assay in the leech extract group (Supplementary material, Fig. S4).

3.7. Leech extract block the NF-κB translocation to nucleus

To further study the mechanism of leech extract, NF-κB translocation assay were carried out. NF-κB plays an important role in controlling the expressing of ICAM-1 and MCP-1. This experiment showed that leech extract can block the translocation of p65, the subunit of NF-κB, from cytoplasm to nucleus (Fig. 4).

4. Discussion

In this study we extracted a leech extract from *Whitmania pigra* Whitman. This is the first study fully explaining the pharmacological effect of medical leech in atherosclerosis. Our studies show that the area of atherosclerosis lesion in artery root from mice given leech extract is significantly reduced compared with control group (Figs. 1 and S1). And this reduction is dose-dependent. Also the number of macrophages in the advanced plaques is reduced. In

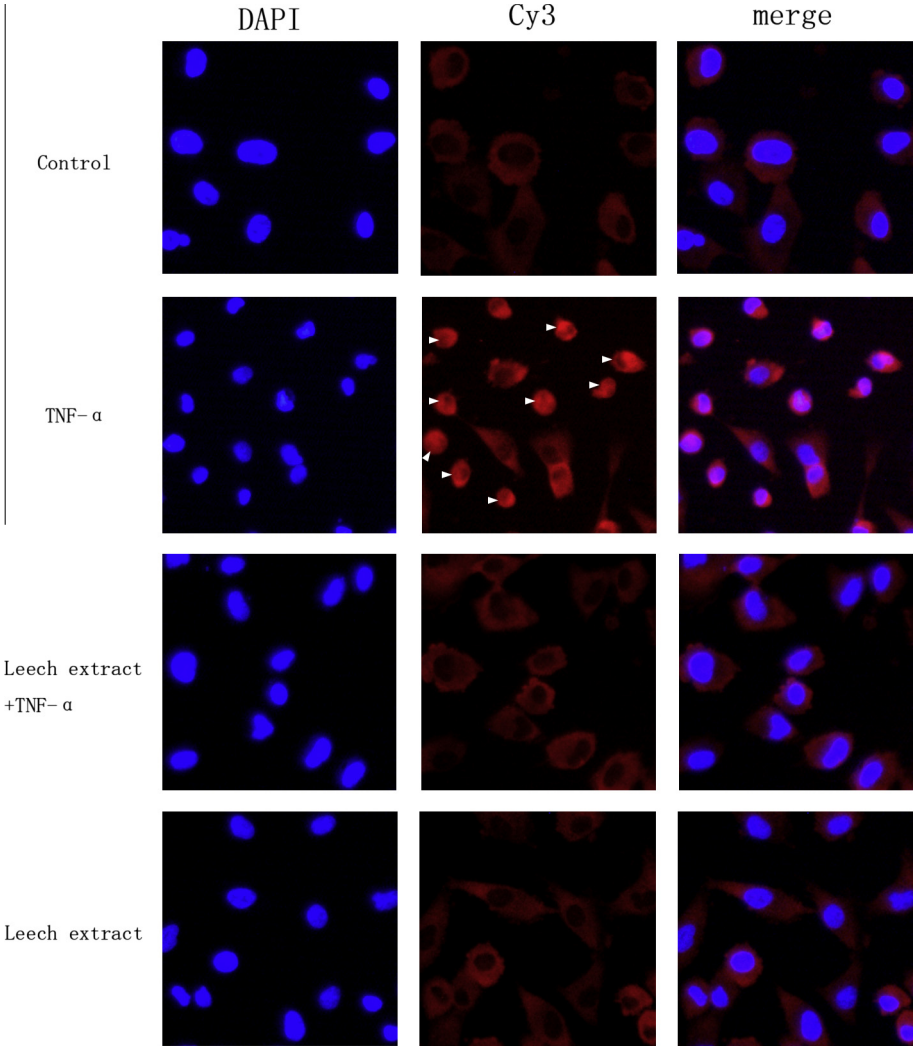

Fig. 4. Effect of leech extract on NF-κB activation of EA.hy926 cell. EA.hy926 cells were treated with leech extract (200 mg/mL) for 24 h and followed with TNF-α (10 ng/mL) for 6 h. p65 subunit of NF-κB was indicated with red color under fluorescence microscope ($n = 3$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

vitro studies show that leech extract could downregulate the expression of ICAM-1 and MCP-1 in TNF-α induced EA.hy926 cells by blocking p65, a subunit of NF-κB, translocation to nucleus.

Leech is a traditional Chinese medicine and the researches previously focus on the anti- coagulation effects in vitro. But the effects and clinical administration could not be fully explained by the anti-coagulation effect [8,9] and antithrombins [10]. In this study, the results suggest that there are other substances in leech extract that response to the anti-atherosclerosis effect.

Atherosclerosis is now regarded as a chronic inflammatory disease [11]. Its initiation and development is a complex process and affected by many elements. The essential steps of atherosclerosis development are that monocytes adhere to endothelium, migrate into the subendothelial arterial space and differentiate into macrophages, which lead to rapid uptake of modified LDL and subsequent foam-cell formation [7]. Thus lipid is accumulated and atherosclerosis lesion is formed. In this process monocytes adhesion and migration are the key steps. In the ApoE −/− mice, atherosclerosis lesion develops first in aorta root then the aorta artery. In this study, high dose of leech extract obviously attenuated the area of atherosclerosis lesion and shows an equivalent effect compared to simvastatin group. And MOMA-2 staining results suggest that this effect is related to macrophages in the lesions.

Endothelium is the most inner site that contact directly with blood and cells and chemistry agents. The function of endothelium is important for the body to maintain homeostatic. In general, it protects the vessel by expression substances function as anti-coagulation and anti-atherosclerosis against the risk factors as compensation. But with long time consistent risk factor stimulation, endothelium shifted to dysfunction. Endothelial dysfunction is the function shift of endothelium. Monocytes adhesion and migration are driven by the adhesion factors (such as VCAM-1 and ICAM-1) and chemokines (such as MCP-1) expressed by the activated endothelium. The changes are closely related to atherosclerosis and other cardiovascular diseases. We proposed the hypothesis that leech extract could affect endothelial cell functions to lower the expression of adhesion factors and chemokines which are upregulated in the process of atherosclerosis. TNF-α is a common inflammatory factor and found in cardiovascular diseases [12]. Endothelial cells induced with proper dose of TNF-α would perform dysfunction which is similar to the process in the atherosclerosis [12,13]. We established an endothelial cell dysfunction model with EA.hy926 cells induced by TNF-α in vitro. In vitro experiments treatment of leech extract previous to TNF-α induction could obviously downregulate the mRNA level and protein expression of ICAM-1 and MCP-1. And also it could affect the process of adhesion and migration of THP-1 to EA.hy926 cells. This might reflect the process of macrophages infiltration in atherosclerosis.

Other researches showed that ICAM-1 and MCP-1 are usually controlled by NF-κB [14,15]. NF-κB translocation assay showed that leech extract can significantly block the process. This could be response for the effect of leech extract.

In conclusion, the experiments have proven that leech extract has obviously decreased the development of atherosclerosis in the ApoE −/− mice by inhibiting macrophage infiltration. Leech extract downregulated the expression of ICAM-1 and MCP-1. It inhibits the adhesion and migration of monocytes by blocking NF-κB translocation. These results indicate that leech extract is a promising drug in atherosclerosis treatment. And the effect of leech extract on endothelial cells could explain its wide applications in clinical.

Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.10.135.

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