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ORIGINAL ARTICLE

Ruanmailing Oral Liquid Inhibits Atherosclerosis in ApoE$^{-/-}$ Mice via Regulation of TGF-β1/SMAD4 Signaling Pathway

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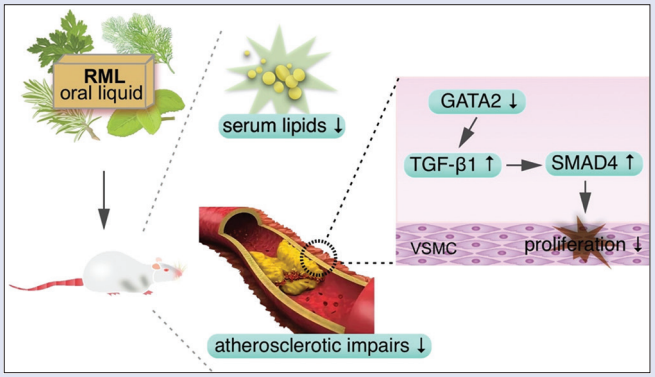
ABSTRACT

Background: In this study, we aimed to investigate the effect of Ruanmailing oral liquid on atherosclerosis and transforming growth factor (TGF)-β1/SMAD4 signaling pathway in apolipoprotein E-knockout (ApoE$^{-/-}$) mice induced by a high-fat diet. **Materials and Methods:** A total of 40 ApoE$^{-/-}$ mice were randomly divided into five groups: control group, model group, low-dose group, high-dose group, and Lipitor group. Mice fed with standard diet formed the control group. ApoE$^{-/-}$ mice exhibited high-fat diet-induced atherosclerotic phenotype. The other four groups were high-fat diet model groups, low- and high-dose Ruanmailing groups (1.75 and 4.55 mL/kg/day, respectively), and Lipitor group (3.0 mg/kg/day). After 12 weeks of administration, the levels of total cholesterol (TC), triglyceride (TG), low-density lipoprotein-cholesterol (LDL-C), and high-density lipoprotein-cholesterol (HDL-C) were measured by blood sampling from the orbital vein of the mice, and the pathological changes in thoracic aorta due to atherosclerosis were observed by hematoxylin and eosin (H and E) staining. Enzyme-linked immunosorbent assay (ELISA) was performed to detect the concentration of serum TGF-β1, and reverse transcriptase polymerase chain reaction (RT-PCR) and western blot analysis were performed to detect the expression of *SMAD4* and *GATA2* in the thoracic aorta of mice in each group. **Results:** Compared with the high-fat model group, the level of serum lipids in the test group were reduced ($P < 0.01$ or $P < 0.05$) and the ratio of plaque area to luminal area (W/L) was significantly reduced ($P < 0.05$). The pathological examination indicated that the atherosclerotic lesions in the thoracic aorta of ApoE$^{-/-}$ mice were alleviated, and the high-dose Ruanmailing group had the most significant anti-atherosclerotic effect. **Conclusion:** Ruanmailing oral liquid exhibited an anti-atherosclerotic effect, and its mechanism may be related to the intervention of GATA2 in the TGF-β1/SMAD4 signaling pathway to reduce the differentiation and proliferation of arterial smooth muscle cells.

Key words: Atherosclerosis, GATA2, Ruanmailing oral liquid, SMAD4, TGF-β1

SUMMARY

- RML regulates the serum lipids in ApoE$^{-/-}$ mice.
- RML reduces atherosclerotic impairs in the thoracic aorta of ApoE$^{-/-}$ mice.
- The mechanism may be related to inhibiting the expression of *GATA2*, promoting the TGF-β1/SMAD4 signaling pathway, and reducing the differentiation and proliferation of arterial smooth muscle cells.
- RML can be used to treat atherosclerosis.



Abbreviations used: AS: atherosclerosis; TGF-β: transforming growth factor β; ELISA: enzyme-linked immunosorbent assay; TC: total cholesterol; TG: triglyceride; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; VSMCs: vascular smooth muscle cells; W/L: the ratio of vascular wall (mesomembrane) area to vascular lumen area.

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INTRODUCTION

Atherosclerosis (AS) is an important pathological basis of cardiovascular and cerebrovascular diseases and also an important cause of death in patients with cardiovascular and cerebrovascular diseases.[1] The development of atherosclerotic plaque involves pathological changes such as abnormal recruitment of inflammatory cells, formation of foam cells, proliferation of smooth muscle cells, synthesis of extracellular matrix proteins, production of reactive oxygen species (ROS), and arterial remodeling.[2,3] Among these changes, abnormal proliferation of smooth muscle cells is responsible for the occurrence and development of AS. A previous study has shown that atherosclerotic lesions are caused by an excessive inflammatory–fibroproliferative response caused by vascular endothelial and smooth muscle cells.[4] Therefore, inhibiting this response is the key to reducing the development of AS and delaying the stenosis of the arterial lumen.

Transforming growth factor β (TGF-β) is a potent cytokine with multiple biological functions. It regulates various mechanisms such as

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cell growth, proliferation, differentiation, and migration. It stimulates the synthesis and secretion of active substances such as various cytokines and inflammatory mediators, and participates in the synthesis and degradation of extracellular matrix. Therefore, it can exhibit the dual effects of inhibition and promotion of various signaling pathways. TGF-β1 is related to the proliferation of vascular smooth muscle cells (VSMCs) and vascular remodeling. It is the primary regulator of fibrogenesis, and its role in preventing the formation of AS has gradually attracted attention.[5] SMADs are a group of proteins transducing extracellular signals directly to the nucleus. SMAD4, a member of the SMAD family, is the only signaling molecule that mediates the TGF-β1 signal from the cell membrane to the nucleus.[6] Previous studies have shown that the TGF-β1/SMAD4 signaling pathway plays an important role in cardiovascular and cerebrovascular diseases, especially in the development of AS.[7,8]

The GATA family of transcription factors is an evolutionary conserved zinc-finger protein transcription factor. The factors function by binding to the DNA sequence (G/A) GATA (G/A). They are widely expressed in different species and play a very important role in proliferation, differentiation, and gene regulation.[9] Among them, GATA2 is necessary for the expansion and maintenance of hematopoietic stem cells and pluripotent progenitor cells. It controls the proliferation and differentiation of stem cells. A previous study has reported that GATA2 negatively regulates the TGF-β signaling pathway in a SMAD4-dependent manner, and that it specifically interacts with the N-terminal of SMAD4.[10] With the overexpression of *GATA2*, the DNA-binding activity of SMAD4 is significantly reduced, indicating that GATA2 is a new negative regulator of the TGF-β/SMAD4 signaling pathway.

Ruanmailing oral liquid (RML) is a Chinese medicinal preparation. It nourishes the liver and kidneys, replenishes Qi, and activates the blood. It is widely used in the clinical treatment of AS and related cardiovascular and cerebrovascular diseases with remarkable curative effects.[11] Numerous experimental studies have revealed the anti-atherosclerotic effects of RML, such as reducing the migration of platelet-derived growth factor (PDGF)-induced VSMCs and formation of stress fibers,[12] enhancing the expression and redistribution of twinfilin-1 in VSMCs, and promoting the reorganization of the cytoskeleton,[13] reducing the expression of CD105 protein to inhibit angiogenesis and stabilize atherosclerotic plaque.[14] In this study, RML was used to interfere with the occurrence and development of AS in the thoracic aorta of apolipoprotein E-knockout ($ApoE^{-/-}$) mice, to analyze the regulation of GATA2 on the TGF-β1/SMAD4 signaling pathway during AS, and to explore the anti-atherosclerotic effect and its mechanism of action.

MATERIALS AND METHODS

Animals and Regents

In this study, $ApoE^{-/-}$ mice were used to study hyperlipidemia and its complications. A total of 40 male, 10-week-old $ApoE^{-/-}$ mice weighing about 20–22 g were used. To the standard diet of mice, 2.0% cholesterol, 2.5% bile salt, and 10% lard were added to prepare high-fat diet (HFD). The feed was fully mixed and processed into granules by the experimental animal center of Fujian Medical University. The experimental animals were purchased from the Department of Medicine, Peking University, license no. SCXK (Beijing) 2014-0004. RML (Z35020207) was provided by Fujian Xinwuyi Pharmaceutical Co., Ltd., and Lipitor (atorvastatin calcium, J20030047) was purchased from Pfizer Inc. (New York, NY, USA). All animal experiments were performed by following the National Animal Experimentation Guidelines after obtaining approval for the experimental protocols from the Institutional Animal Experimentation Committee of Fujian Medical University. The experiment has been approved by the Ethics Committee of Fujian Medical University, and the month of approval is August 2017.

Instruments

In this study, the following instruments were used: vortex shaker (ql-902 haimenqi Linbei Instrument Manufacturing Co., Ltd.); biophotometer (Eppendorf); fluorescence quantitative CFXTM Real-Time PCR Detection System (Bio Rad Laboratories); deionized water instrument (Purelab Plus; Pall, Port Washington, NY, USA); high-speed centrifuge (Eppendorf, Germany); and low-temperature centrifuge (5418r Eppendorf, Hamburg, Germany).

Animal grouping, model preparation, and administration method

A total of 40 $ApoE^{-/-}$ mice were randomly divided into normal control group (control group), model group, low- and high-dose Ruanmailing groups, and Lipitor group, with eight mice in each group. The mice were raised in a net cage with constant temperature (22°C ± 2°C) and humidity (55% ± 5%). The mice were provided with 12 h/12 h light/dark cycles through artificial lighting. They were provided with drinking water *ad libitum*. $ApoE^{-/-}$ mice were fed with an HFD to induce AS. According to the body surface area between humans and animals, the equivalent dose administered to mice was calculated. Based on the group, the mice were fed with the following regimen: (1) control group mice were fed with the standard diet; (2) model group mice were fed with HFD and 0.2 mL/day distilled water; (3) high-dose group mice were fed with HFD, 4.55 mL/kg/day Ruanmailing, and 0.2 mL/day distilled water; (4) low-dose group mice were fed with HFD, 1.75 mL/kg/day Ruanmailing, and 0.2 mL/day distilled water; (5) Lipitor group mice were fed with an HFD and Lipitor 3.0 mg/kg/day that was dissolved in 0.2 mL of distilled water. Every week, the animals were weighed and the dosage of Lipitor was adjusted accordingly. After 12 weeks of drug intervention, the mice were fasted for 12 h, and subsequently, 0.04 mL/10 g of 10% chloral hydrate was injected into the abdominal cavity of the mice.[15] After complete anesthesia, 0.5 mL of orbital venous blood of mice was collected, centrifuged at 13,000 rpm for 10 min, and the supernatant was stored in a refrigerator at −80°C for later use.

Determination of Serum Lipids

In this study, total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) of $ApoE^{-/-}$ mice were measured using Beckman automatic biochemical analyzer.

Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) was performed to determine the concentration of TGF-β1 in the serum samples collected from $ApoE^{-/-}$ mice. The test was performed by following the instructions provided in the kit (CSB-E04726m; CUSABIO, Wuhan, China).

Histopathology

After blood was collected from the orbital vein of the mice, the thoracic cavity was opened and the aorta was removed from the root of the aorta. Some part of the tissue was fixed with 10% paraformaldehyde, embedded in paraffin, sectioned, stained with hematoxylin and eosin (H&E), and then examined under a light microscope. The ratio of the area of the vascular wall (middle membrane) to the area of the vascular lumen (W/L) was measured concerning the imaging quadrature method of the image analyzer.

RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction Assay

Total RNA from the thoracic aorta tissue of ApoE$^{-/-}$ mice was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and the concentration and purity of the extracted RNA were measured. TIANScript RT kit (TIANGEN Biotech Ltd., Beijing, China) was used to reverse transcribe RNAs into cDNA. In accordance with the manufacturer's instructions, SYBR Green mix (TIANGEN Biotech Ltd.) was used for relative quantitative real-time PCR. The amount of RNA was calculated using the $2^{-\Delta\Delta Ct}$ method, and β-actin served as an internal control. Table 1 shows the primer sequences used.

Western Blot Analysis

The thoracic aorta tissues of ApoE$^{-/-}$ mice were lysed in Radio-immunoprecipitation assay (RIPA) buffer, and the protein concentration was measured by bicinchoninic acid (BCA) method. The quantity of total protein of each sample was kept consistent. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membrane. The bands were blocked with 5% skimmed milk for 1 h. Subsequently, the membrane was incubated with dilute primary antibodies (GATA2, SMAD4, and β-actin). The bound antibodies were then visualized using enhanced chemiluminescence (ECL) detection kit with an appropriate horse radish peroxidase (HRP)-conjugated secondary antibody. Protein bands were quantified with Gel Pro Analyzer software 4.0 (Media Cybernetics, Bethesda, MD, USA), and the intensity of the bands was normalized against β-actin.

Statistical Analysis

Statistical Package for the Social Sciences (SPSS) software was used to analyze the data, and the results are expressed as mean ± standard deviation. Analysis of variance was performed to compare the differences between the groups. $P < 0.05$ indicates a statistically significant difference.

RESULTS

RML Regulated the Serum Lipid Levels

The level of serum lipids was spontaneously elevated in ApoE$^{-/-}$ mice. Compared with the control group, HFD in ApoE$^{-/-}$ mice increased serum lipids [Figure 1]. Compared with the high-fat model group, the levels of TC [Figure 1a], TG [Figure 1b], and LDL-C [Figure 1c] in the RML groups decreased significantly in a dose-dependent manner ($P < 0.05$ or $P < 0.01$). In terms of HDL-C, there was no difference between the low-dose RML group and the Lipitor group compared with the high-fat model group ($P > 0.05$), but the level of HDL-C was elevated in high-dose RML group [Figure 1d] ($P < 0.05$).

Table 1: GATA2, SMAD4, and β-actin primer sequences

Gene name	Sequence	Product length (bp)
GATA2	Forward 5′-CACTCGGGCTCCCATCTCT-3′ Reverse 5′-TGCCGCCTTCCATCTTCAT-3′	187
SMAD4	Upper reaches 5′-CCTCCCATTTCCAATCATC-3′ Downstream 5′-GCCATCCACAGTCACAACA-3′	123
β-actin	Upper reaches 5′-TGTGTCCGTCGTGGATCTGA-3′ Downstream 5′-TTGCTGTTGAAGTCGCAGGAG-3′	149

RML Reduces Atherosclerotic Impairs

The tissue samples were stained using H and E to observe the pathological changes in the aorta of the ApoE$^{-/-}$ mice aortic root [Figure 2a]. In the control group, the intima of the thoracic aorta was smooth and continuous. There was no lipid plaque bulge, and the cells of the middle membrane were arranged regularly with uniform thickness. In the high-fat model group, vascular intima was highly irregular. There were large raised plaques in the lumen, and there were cavities in the center of the plaques. Proliferative cells and thick fibrous cap were seen in the outer layer of the lumen, and the vascular wall was also thickened at the bottom of the plaques. In the Lipitor group, only small lipid plaques were found in the intima. The outer layer of the plaque was thin and uniform, and only a few cells proliferated in the vascular layer of plaques. In the high-dose RML group, the intima was slightly uneven. Vacuoles and proliferative cells could be seen in the plaque. The smooth muscle of the middle membrane was arranged regularly, and the cells under the middle membrane proliferated. In the low-dose RML group, the intima was uneven. The plaques were prominent to the lumen, and some foam cells and proliferating cells were also found. Moreover, the ratio of the vascular wall (mesomembrane) area to vascular lumen area (W/L) is an important indicator to assess the severity of AS. Compared with the high-fat model group, the high-dose RML group can significantly reduce the W/L ratio in ApoE$^{-/-}$ mice, and there was no significant difference between the high-fat model group and the Lipitor group [Figure 2b].

Effect of RML on the Expression of TGF-β1

The TGF-β1 signaling pathway plays a central role in AS. Therefore, we next investigated whether the role of RML in the treatment of atherosclerotic impairs is modulated via TGF-β1 pathway. As shown in Figure 3, compared with the control group, the serum TGF-β1 level of ApoE$^{-/-}$ mice in the high-fat model group was significantly reduced ($P < 0.01$), and RML can increase the serum TGF-β1 level in a dose-dependent manner ($P < 0.01$). However, there was no significant difference in the TGF-β1 level between the Lipitor group and the high-dose RML group ($P > 0.05$).

Effects of RML on the mRNA and Protein Levels of GATA2 and SMAD4

As shown in Figure 4, compared with the control group, the mRNA transcription and protein synthesis of GATA2 and SMAD4 were significantly upregulated and downregulated, respectively, in the high-fat model group ($P < 0.01$). Compared with the high-fat model group, both RML and Lipitor upregulated the transcription of GATA2 mRNA and downregulated the transcription of SMAD4 mRNA ($P < 0.01$) [Figure 4a]. Western blot analysis revealed that compared with the control group, the expression of *GATA2* in the thoracic aorta of ApoE$^{-/-}$ mice in the high-fat model group was significantly upregulated, and RML downregulated the expression of *GATA2* in a dose-dependent manner; The expression of *SMAD4* was significantly downregulated, and RML upregulated the expression of *SMAD4* in the thoracic aorta of ApoE$^{-/-}$ mice in a dose-dependent manner. However, there were no significant differences in the expression of *GATA2* and *SMAD4* between the Lipitor group and the high-dose RML group.

DISCUSSION

AS is a chronic inflammatory disease. It is major risk factor for cardiovascular and cerebrovascular diseases, often involving multiple organs. Its pathological features are the formation of lipid plaques on the inner wall of blood vessels and the proliferation of smooth muscle cells,

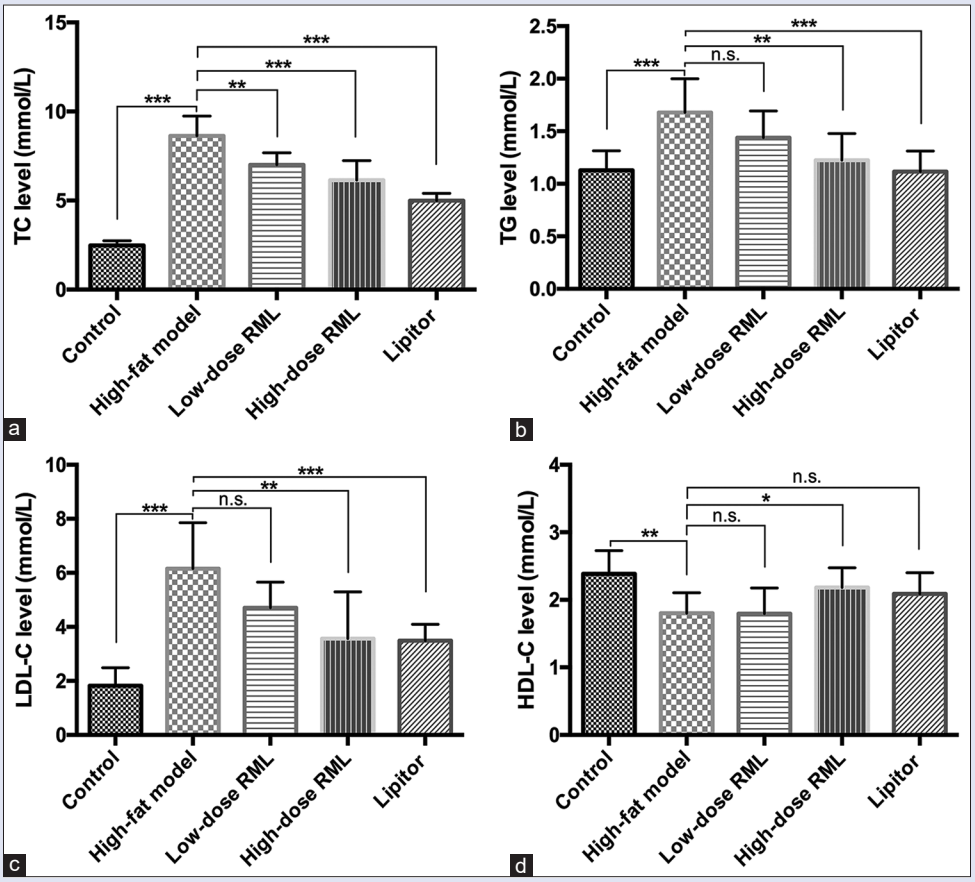

Figure 1: Effects of Ruanmailing oral liquid on the serum lipids in ApoE$^{-/-}$ mice. (a) TC level, (b) TG level, (c) HDL-C level, (d) LDL-C level. Data are shown as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001. HDL-C = high-density lipoprotein-cholesterol, LDL-C = low-density lipoprotein-cholesterol, n.s. = non-significant, RML = Ruanmailing oral liquid, SD = standard deviation, TC = total cholesterol, TG = triglyceride

leading to narrowing and hardening of the lumen, which in turn affects the blood supply to the tissues.[16] HFD-fed ApoE$^{-/-}$ mice have been widely used in the study of hyperlipidemia, AS, and its complications.[17] In this study, HFD-fed ApoE$^{-/-}$ mice were used to establish the model of AS, and RML or Lipitor was used for continuous intervention for 12 weeks. According to the results, compared with the normal diet control group, the serum levels of TG, TC, and LDL-C mice were significantly increased in HFD-fed ApoE$^{-/-}$ and the level of HDL-C was decreased. There was atherosclerotic plaque formation in the intima of the thoracic aorta, and there was massive proliferation of the smooth muscle layer of the media. The occurrence of AS in HFD-fed ApoE$^{-/-}$ mice is not only related to dyslipidemia, but also to the proliferation of cells in the smooth muscle layer in the vascular media.[18,19] Abnormally elevated blood lipids, vascular intimal lipid deposition, and vascular media smooth muscle cell proliferation are the common pathological bases for the occurrence and development of atherogenesis.[20] In this study, after the HFD-fed ApoE$^{-/-}$ mice were treated with RML, there was a reduction in the level of serum lipids in a dose-dependent manner. It inhibited the formation of lipid plaques in the intima of the thoracic aorta and the proliferation of smooth muscle cells in the media to reduce the area of atherosclerotic plaques in HFD-fed ApoE$^{-/-}$ mice. It shows that RML can inhibit the formation of atherosclerotic plaques and the proliferation of smooth muscle cells by regulating serum lipid levels, thereby delaying the process of AS.

The pathogenesis of AS is very complicated, in which the proliferation of arterial smooth muscle cells runs through the formation and development of AS. Inhibiting cell proliferation and reducing migration and differentiation are important ideas and directions for preventing and treating AS.[17] TGF-β1 has the highest proportion (>90%), the strongest activity, the most functions, and the widest distribution in somatic cell lines. It has become a hot spot in clinical and experimental research.[21] As a multifunctional cytokine, TGF-β1 has a two-way regulatory effect. The change in TGF-β1 can promote remodeling of the blood vessel wall, the growth of damaged arteries, and the transcriptional differentiation of vascular cells. However, TGF-β1 can also be used as an anti-inflammatory and anti-atherogenic factor to prevent the occurrence of atherosclerotic complications.[22,23] SMAD4 protein is an important signal molecule that mediates the transfer of TGF-β1 signal from the cell membrane to the nucleus. TGF-β1/SMAD4 is an important regulatory pathway for the differentiation and proliferation of arterial smooth muscle cells and the process of AS.[10] In this study, we observed that compared with the control group, the levels of TGF-β1 and SMAD4 protein in HFD-fed ApoE$^{-/-}$ mice were significantly decreased, and large raised plaques were also observed in the lumen of thoracic aorta. Furthermore, proliferative cells and thick fibrous cap were visible in the outer layer of the cavity. It shows that the arterial proliferation response is one of the important mechanisms of atherosclerotic plaque formation in HFD-fed ApoE$^{-/-}$ mice, and it is related to the inhibition of the TGF-β1/SMAD4 signaling pathway. Compared with the high-fat model group, the serum TGF-β1 levels and the SMAD4 mRNA and protein levels of the thoracic aorta in the low- and high-dose Ruanmailing groups were significantly increased, and there

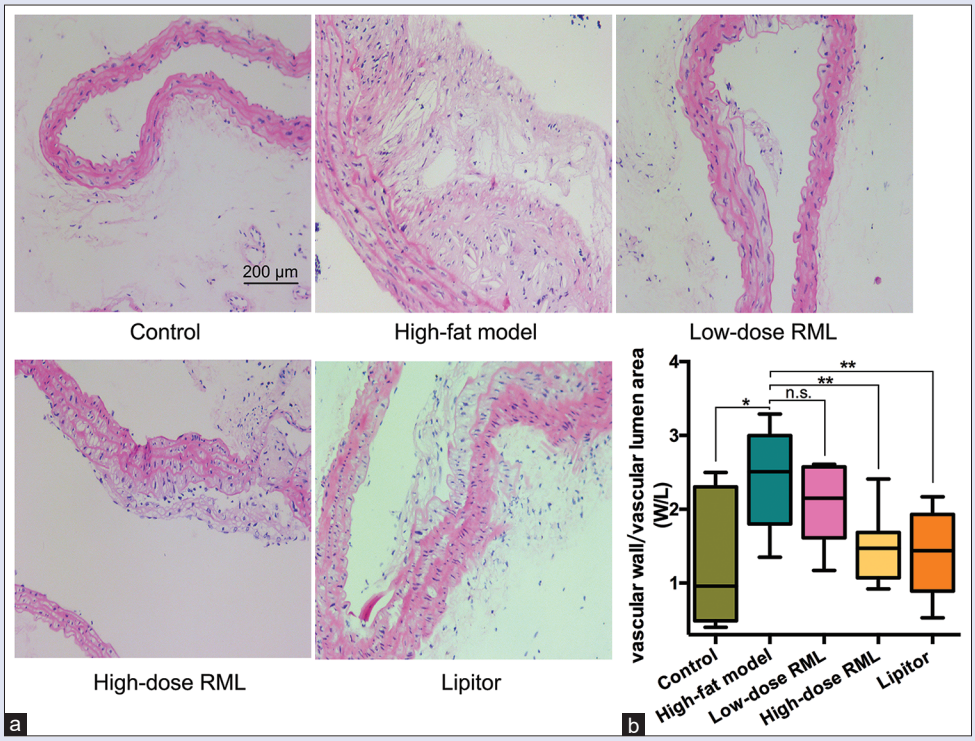

Figure 2: RML treatment reduced atherosclerosis in the thoracic aorta of ApoE$^{-/-}$ mice. (a) Pathological section of ApoE$^{-/-}$ mice thoracic aorta and H and E staining (200×). (b) Ratio of vascular wall (mesomembrane) area to vascular lumen area (W/L) in different groups of ApoE$^{-/-}$ mice. Data are shown as means ± SD. *P < 0.05, **P < 0.01. H and E, hematoxylin and eosin, n. s. = non-significant, RML = Ruanmailing oral liquid, SD = standard deviation

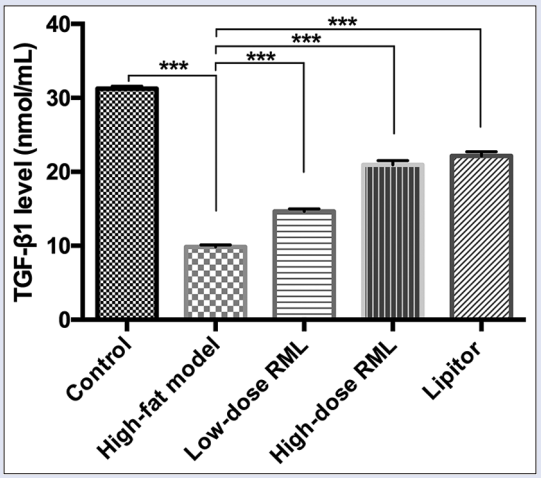

Figure 3: Effect of RML on the expression of TGF-β1. The serum TGF-β1 concentrations of ApoE$^{-/-}$ mice in different treatment groups were measured by ELISA. Data are shown as means ± SD. ***P < 0.001. ELISA = enzyme-linked immunosorbent assay, RML = Ruanmailing oral liquid, SD = standard deviation, TGF = transforming growth factor

was no significant difference from the Lipitor group. It indicates that RML upregulated the expression of TGF-β1 and downstream SMAD4 protein, enhanced the transduction of the TGF-β1/SMAD4 signaling pathway, and inhibited the proliferation of arterial smooth muscle cells and the progression of AS in a dose-dependent manner.

On the basis that TGF-β1 mediates the intracellular signal transduction of SMAD4 protein, we hypothesized that the regulation of proliferation of smooth muscle cells by intervening the process of signal transduction of the TGF-β1/SMAD4 signaling pathway in the cytoplasm or nucleus may be a new approach for anti-AS research. GATA2 is an important zinc-finger transcription factor. In addition to being an important regulatory factor in the differentiation of pluripotent hematopoietic stem cells into various mature lineages, it also participates in the regulation of various organs, such as early neurodevelopment and bone metabolism.[24,25] GATA2 can negatively regulate SMAD4, thereby promoting smooth muscle cell differentiation and proliferation in the process of atherogenesis. Studies have found that GATA2 can inhibit the transcriptional activity of endogenous SMAD4 and has a quantity-dependent effect. As the transfection dose of GATA2 increases, the inhibitory effect on SMAD4 activity increases.[10] This study shows that compared with the HFD-fed model group, RML can significantly decrease the levels of GATA2 and increase the levels of TGF-β1 and SMAD4 in HFD-fed ApoE$^{-/-}$ mice. This suggests that GATA2 acts as a negative regulator of the TGF-β1/SMAD4 pathway. Furthermore, it may play an important role in reducing the differentiation and proliferation of smooth muscle cells and slowing down the process of AS.

Compared with the use of statin drugs such as Lipitor to prevent and treat AS, traditional Chinese medicine has the characteristics of multiple targets. The medicines show synergistic effects of lipid-lowering activity, inhibiting the proliferation and migration of VSM Cs, and antioxidant activity, and have fewer side effects. These characteristics make traditional Chinese medicines a new coping strategy for the prevention and treatment of AS. The primary ingredients of RML are as follows: Shu Dihuang (*Radix Rehmanniae* Praeparata), Wuweizi (*Schisandra chinensis*), Gouqi (*Lycium barbarum*), Fuling (*Wolfiporia extensa*), Baiziren (Semen Platycladi), Yuanzhi (*Polygala tenuifolia*), Renshen (*Panax ginseng*), Danggui (*Angelica sinensis*), Huangqi (Astragalus), and so on. Taking a comprehensive view of

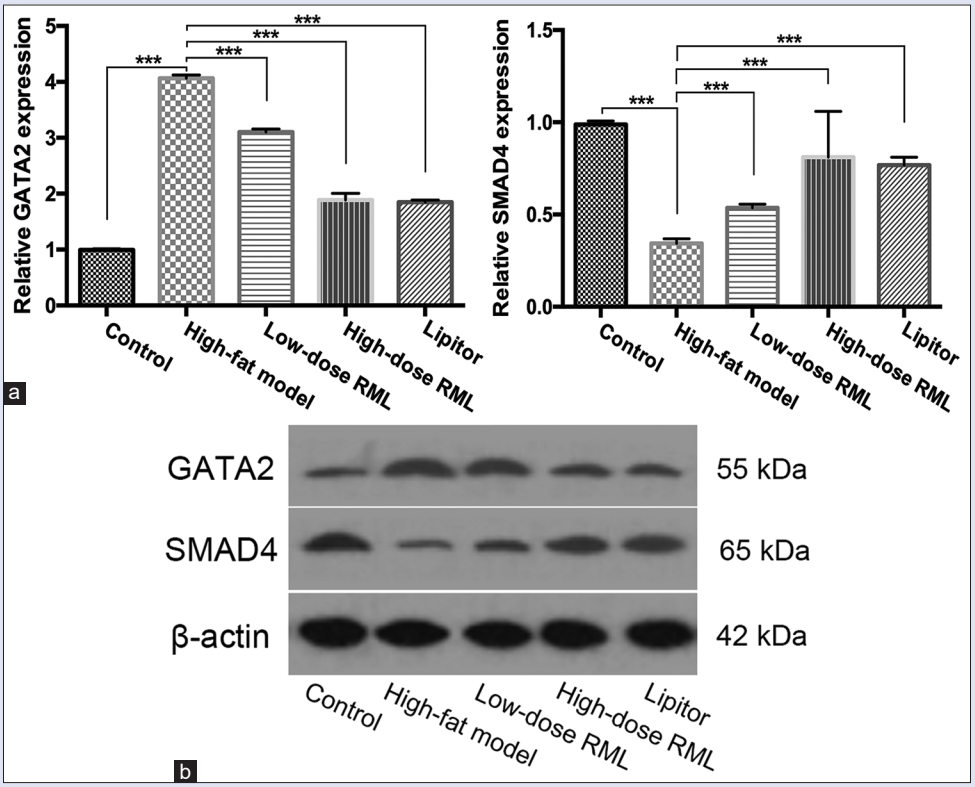

Figure 4: Effects of Ruanmailing oral liquid on mRNA and protein levels of GATA2 and SMAD4. (a) The expression of *CATA2* and *SMAD4* was detected by real-time PCR in different groups of thoracic aorta tissues of ApoE$^{-/-}$ mice. Data are shown as means ± SD. ***$P < 0.001$. (b) Protein expression of GATA2 and SMAD4 was detected by western blot in different groups of thoracic aorta tissues of ApoE$^{-/-}$ mice. PCR = polymerase chain reaction, RML = Ruanmailing oral liquid, SD = standard deviation

the whole prescription, it has the effects of invigorating the kidney, promoting blood circulation, and reducing phlegm. Elderly patients with AS have pathological characteristics of deficiency of kidney essence, excessive phlegm, and excessive blood stasis, and the pharmacological effects of RML are in line with these pathophysiological characteristics. This study confirmed from an experimental point of view that RML has significant lipid-lowering and anti-atherosclerotic effects.

CONCLUSION

In summary, RML exhibits a significant anti-atherosclerotic effect, and its mechanism may be related to inhibiting the expression of *GATA2*, promoting the TGF-β1/SMAD4 signaling pathway, and reducing the differentiation and proliferation of arterial smooth muscle cells, making it worthy of further research and promotion.

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Conflicts of interest

There are no conflicts of interest.

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