Ruanmailing Oral Liquid Inhibit Atherosclerosis in ApoE-/− Mice by Regulation of TGF-β1/SMAD4 Signaling Pathway

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Research

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Abstract

Background

To investigate the effect of Ruanmailing oral liquid on atherosclerosis and TGF-\(\beta\)/SMAD4 signaling pathway in ApoE knockout mice induced by high-fat diet.

Methods

Forty ApoE\(^{-/-}\) mice were randomly divided into 5 groups, and mice fed with standard diets were the control group. ApoE\(^{-/-}\) mice high-fat diet induced atherosclerotic phenotype. After grouping and treatment, they were divided into high-fat feeding model group, low-dose and high-dose of Ruanmailing groups (1.75, 4.55 ml/kg/d), Lipitor Group (3.0 mg/kg/d). After 12 weeks of administration, blood was collected from the mice orbit to determine the levels of TC, TG, LDL-C, and HDL-C, and the pathological changes of thoracic aorta atherosclerosis were observed. Enzyme-linked immunosorbent assay (ELISA) was used to detect the concentration of serum TGF-\(\beta\), and RT-PCR and Western Blot were used to detect the expression of SMAD4 and GATA2 in the thoracic aorta of ApoE\(^{-/-}\) mice in each group.

Results

Compared with the high-fat model group, the serum lipids level of each administration group were reduced (\(P<0.01\) or \(P<0.05\)), and the ratio of plaque area to luminal area (W/L) was significantly reduced (\(P<0.05\)), and pathological examination indicated atherosclerotic lesions in thoracic aorta of ApoE\(^{-/-}\) mice were alleviated, and the high-dose Ruanmailing group had the most significant anti-atherosclerotic effect.

Conclusions

Ruanmailing oral liquid has an anti-atherosclerotic effect, and its mechanism may be related to the intervention of GATA2 in the TGF-\(\beta\)/SMAD4 signaling pathway to reduce the differentiation and proliferation of arterial smooth muscle cells.

Background

Atherosclerosis (AS) is an important pathological basis of cardiovascular and cerebrovascular diseases, and it is also an important cause of death in patients with cardiovascular and cerebrovascular diseases. The development of atherosclerotic plaque involves pathological mechanisms such as abnormal inflammatory cell recruitment, foam cell formation, smooth muscle cell proliferation, extracellular matrix synthesis, reactive oxygen species production, and arterial remodeling [1, 2]. Among these pathological changes, abnormal smooth muscle cell proliferation plays an important role in the occurrence and development of atherosclerosis. Numerous studies have shown that atherosclerotic lesions are caused by an excessive inflammatory-fibroproliferative response caused by vascular endothelial and smooth muscle cells damaged by various factors [3]. Therefore, inhibiting the excessive inflammatory-
fibroproliferative response is the key to reducing the development of atherosclerosis and delaying the stenosis of the arterial lumen.

Transforming growth factor β (TGF-β) is a cytokine with multiple biological functions, which can regulate cell growth, proliferation, differentiation and migration, stimulate the synthesis and secretion of active substances such as various cytokines and inflammatory mediators, and participate in the synthesis and degradation of extracellular matrix, and have the dual effects of inhibiting and promoting. Among them, TGF-β1 is related to the proliferation of vascular smooth muscle cells and vascular remodeling. It is the main regulator of the fibrogenesis process, and its role in anti-atherosclerosis has gradually attracted attention [4]. SMAD4, as a common pathway type SMADs protein, is the only signal molecule that mediates TGF-β1 signal from the cell membrane to the nucleus [5]. Studies have shown that the TGF-β1/SMAD4 signaling pathway plays an important role in cardiovascular and cerebrovascular diseases, especially in the development of atherosclerosis [6, 7].

The GATA transcription factor family is a type of transcription factor that functions by binding to the DNA sequence (G/A) GATA (G/A), and is an evolutionary conserved zinc finger protein transcription factor. It is widely expressed in different species and plays a very important role in proliferation, differentiation and gene regulation [8]. Among them, GATA2 is a transcription factor necessary for the expansion and maintenance of hematopoietic stem cells and pluripotent progenitor cells, and controls the proliferation and differentiation of stem cells. Studies have found that GATA2 negatively regulates the TGF-β signaling pathway in a SMAD4-dependent manner, and GATA2 specifically interacts with the N-terminal of SMAD4. With the overexpression of GATA2, the DNA binding activity of SMAD4 was significantly reduced, indicating that GATA2 is a new negative regulator of the TGFβ/SMAD4 signaling pathway [9].

Ruanmailing oral liquid is a pure Chinese medicine preparation, which has the effects of nourishing the liver and kidney, replenishing qi and activating blood. It is widely used in the clinical treatment of atherosclerosis and related cardiovascular and cerebrovascular diseases with remarkable curative effect. Numerous experimental studies have revealed the anti-atherosclerotic effects of Ruanmailing oral liquid, such as reducing PDGF-BB-induced vascular smooth muscle cell (VSMCs) migration and stress fibers formation [10]; enhancing the expression and redistribution of twinfilin-1 in VSMCs, and promoting the reorganization of the cytoskeleton [11]; reducing the expression of CD105 protein to inhibit angiogenesis and stabilize atherosclerotic plaque [12]. In this experiment, Ruanmailing oral liquid was used to interfere with the occurrence and development of atherosclerosis in the thoracic aorta of ApoE⁻/⁻ mice, to analyze the regulation of GATA2 on the TGF-β1/SMAD4 signaling pathway during atherosclerosis, and to explore the anti-atherosclerosis effect and mechanism of Ruanmailing oral liquid.

**Methods**

**Animals and Regents**
The experimental animals are from the Department of medicine, Peking University, license No. scxk (Beijing) 2014-0004. Forty 10-week-old ApoE\(^{-/-}\) mice, male, weighing 20-22g. Cholesterol 2.0%, bile salt 2.5% and lard 10.0% were added to the standard diet of mice. The feed was fully mixed and processed into granules by the experimental animal center of Fujian Medical University. Ruanmailing oral liquid was provided by Fujian xinwuyi Pharmaceutical Co., Ltd., and Lipitor (atorvastatin calcium, gyzz j20030047) was purchased from Pfizer Inc. (New York, NY, USA). All experiments were approved and conducted in accordance with the guidelines of the Animal Care Committee of Fujian Medical University.

**Main instruments**

Vortex shaker ql-902 haimenqi Linbei Instrument Manufacturing Co., Ltd.; biophotometer Eppendorf; fluorescence quantitative PCR, connect cfxtm bio rad; deionized water instrument, pall, Purelab plus USA; high speed centrifuge, Eppendorf company, Germany; transfer decolorization shaking table, ts-8 Haimen qilinbei instrument manufacturing company; electrophoresis instrument, Beijing 61 instrument factory; low temperature centrifuge, 5418r Eppendorf, Germany; vertical electrophoresis tank, dycz-20c Beijing 61 instrument factory; transfer tank, dycp-40c Beijing 61 instrument factory.

**Animal grouping, model preparation and administration method**

Forty ApoE\(^{-/-}\) mice were randomly divided into normal control group (control group), model group, low dose group, high dose group and Lipitor group, with 8 mice in each group. The mice were raised in a net cage with constant temperature \((22 \pm 2)^\circ C\) and humidity \((55\% \pm 5\%)\). The mice were illuminated by artificial light for 12 hours in light and dark, and were randomly fed with drinking water.

Control group: mice were fed with standard feed; model group: mice were fed with high-fat feed and fed with 0.2 ml/d distilled water; high-dose group: mice were fed with high-fat feed and fed with 4.55 ml/kg softmailing daily and 0.2 ml distilled water; low-dose group: mice were fed with high-fat feed and fed with 1.75 ml/kg softmailing daily and 0.2 ml distilled water; Lipitor group: mice were fed with high-fat feed, Lipitor was given 3.0 mg/kg per day and dissolved in 0.2 ml distilled water. Weigh every week and adjust the dosage according to the weight. Continuous feeding and medication for 12 weeks.

**Specimen collection and processing**

After 12 weeks of drug intervention, the mice fasted for 12 hours. The orbital venous blood of mice was taken, centrifuged at 2500 R/min for 10 mins, and the supernatant was stored in a refrigerator at \(-20^\circ C\). The cholesterol (TC), triglyceride (TG), low density lipoprotein cholesterol (LDL-C) and high density lipoprotein cholesterol (HDL-C) of ApoE\(^{-/-}\) mice were measured by Beckman automatic biochemical analyzer. After blood was collected from the orbital vein of mice, the thoracic cavity was opened and the aorta was removed from the root of the aorta. Some tissues were fixed with 10% paraformaldehyde, embedded in paraffin, sectioned and stained with he. The ratio of the area of vascular wall (middle membrane) to the area of vascular lumen (W/L) was measured with reference to the imaging quadrature method of image analyzer. Another part of the tissue is ground and the supernatant is taken. Trizol
reagent was used to extract RNA from the thoracic aorta of apoE−/− mice, and RT-PCR was used to detect the expression of GATA2 and SMAD4 genes. Their primer sequences are shown in Table 1. Western blot was used to detect the expression of GATA2 and SMAD4 proteins. Enzyme-linked immunosorbent assay (ELISA) was used to determine the concentration of TGF-β1 in serum of ApoE−/− mice, and strictly in accordance with the kit instructions.

**Statistical analysis**

SPSS software was used for analysis. The experimental data were expressed as means ± standard deviation, and the analysis of variance was used for the comparison between groups. $P < 0.05$, indicating significant difference, with statistical significance.

**Results**

**Ruanmailing oral liquid regulates the serum lipids**

The serum lipids were spontaneously elevated in ApoE−/− mice. Compared with control group, high-fat diet feed to ApoE−/− mice resulted in increase of serum lipids (Fig. 1). Compared with high-fat model group, total cholesterol (TC) (Fig. 1A), triglycerides (TG) (Fig. 1B) and low density lipoprotein cholesterol (LDL-C) (Fig. 1C) in RML groups decreased significantly ($P < 0.05$ or $P < 0.01$), and in a dose-effect manner. In terms of high density lipoprotein cholesterol (HDL-C), there was no difference between the low-dose RML group and the Lipitor group compared with the high-fat model group ($P > 0.05$), and only the high-dose RML group increased the HDL-C level (Fig. 1D) ($P < 0.05$).

**Ruanmailing oral liquid reduces atherosclerotic impairs**

Hematoxylin-Eosin staining was used to observe the pathological changes of the aorta taken out of the ApoE−/− mice aortic root (Fig. 2A). In the control group, the intima of the thoracic aorta was smooth and continuous, no lipid plaque bulge was found, and the cells of the middle membrane were arranged regularly with uniform thickness. In the high-fat model group, vascular intima was very irregular, there were large raised plaques in the lumen, 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 thickening of vascular wall was also seen at the bottom of the plaques. In the Lipitor group, only small lipid plaques were found in the intima, the outer layer of plaques 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 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 found. Moreover, the ratio of vascular wall (mesomembrane) area to vascular lumen area (W/L) is an important indicator to assess the severity of atherosclerosis. Compared with the high-fat model group, the high-dose RML group can significantly reduce the ratio of vascular wall (mesomembrane) area to vascular lumen area (W/L) in
ApoE⁻/⁻ mice, and there was no significant difference between the high-fat model group and Lipitor group (Fig. 2B).

**Effect of Ruanmailing oral liquid on the expression of TGF-β1**

The TGF-β1 signaling pathway has been demonstrated to play a central role in atherosclerosis. We next investigated whether the role of Ruanmailing oral liquid in the treatment of atherosclerotic impairs is modulated by the TGF-β1 pathway. As shown in Fig. 3, compared with the control group, the serum TGF-β1 of ApoE⁻/⁻ mice in the high-fat model group was significantly reduced ($P<0.01$), and Ruanmailing oral liquid can increase the serum TGF-β1 level in a dose-dependent manner ($P<0.01$), but 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 Ruanmailing oral liquid on mRNA and protein levels of GATA2 and SMAD4**

As shown in the Fig. 4, compared with the control group, the mRNA transcription and protein of GATA2 were significantly increased and the mRNA transcription and protein of SMAD4 were significantly decreased in the high-fat model group ($P<0.01$). Compared with the high-fat model group, both Ruanmailing oral liquid and Lipitor increased GATA2 mRNA transcription and decreased SMAD4 mRNA transcription ($P<0.01$) (Fig. 4A). As shown in Fig. 4B, the western blot experiment results showed 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 increased, and Ruanmailing oral liquid could dose-dependently reduce the expression of GATA2; The expression of SMAD4 was significantly reduced, and Ruanmailing oral liquid can increase 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**

Atherosclerosis is a chronic inflammatory disease related to many risk factors 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, leading to narrowing and hardening of lumen, which in turn affects the blood supply to the tissues. High-fat-fed ApoE⁻/⁻ mice have been widely used in the study of hyperlipidemia, atherosclerosis and its complications [13]. In this study, high-fat fed ApoE⁻/⁻ mice was used to induce the establishment of atherosclerosis model, and different drugs were continuously intervened for 12 weeks. The results showed that compared with the normal diet control group, the serum TG, TC and LDL-C levels of high-fat fed ApoE⁻/⁻ mice were significantly increased, HDL-C was decreased, and atherosclerotic plaque formation in the intima of the thoracic aorta and massive proliferation of the smooth muscle layer of the media. The occurrence of atherosclerosis in high-fat ApoE⁻/⁻ mice is not only related to dyslipidemia, but also the proliferation of smooth muscle layer cells in the vascular media plays an important role in its formation.
and development [14, 15]. Abnormally elevated blood lipids, vascular intimal lipid deposition and vascular media smooth muscle cell proliferation are the common pathological basis for the occurrence and development of atherogenesis [16]. In this study, after high-fat fed ApoE−/− mice were treated with Ruanmailing oral liquid, the results showed that Ruanmailing oral liquid can reduce serum lipids in a dose-dependent manner, inhibit 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 high-fat fed ApoE−/− mice. It shows that Ruanmailing oral liquid can inhibit the formation of atherosclerotic plaque and the proliferation of smooth muscle cells by regulating serum lipid levels, thereby delaying the process of atherosclerosis.

The pathogenesis of atherosclerosis is very complicated, in which the proliferation of arterial smooth muscle cells runs through the formation and development of atherosclerosis. Inhibiting cell proliferation and reducing migration and differentiation are important ideas and directions for preventing and treating atherosclerosis [13]. 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 [17]. As a multifunctional cytokine, TGF-β1 has a two-way regulatory effect. The change of TGF-β1 can promote the remodeling of blood vessel wall, the growth of damaged arteries and the transcriptional differentiation of vascular cells. On the other hand, TGF-β1 can also be used as an anti-inflammatory and anti-atherogenic factor to prevent the occurrence of atherosclerotic complications [18]. 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 atherosclerosis [9]. In this study, we observed that compared with the control group, the levels of TGF-β1 and SMAD4 protein in high-fat fed ApoE−/− mice were significantly decreased, and large raised plaques were also observed in the lumen of thoracic aorta, and 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 high-fat 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 group were significantly increased, and there was no significant difference from the Lipitor group. It indicates that the dose-dependent Ruanmailing oral liquid may increase the expression of TGF-β1 and downstream SMAD4 protein, enhance the transduction of the TGF-β1/SMAD4 signaling pathway, and inhibit the proliferation of arterial smooth muscle cells and the progression of atherosclerosis.

On the basis that TGF-β1 mediates the intracellular signal transduction of SMAD4 protein, whether it can regulate the proliferation of smooth muscle cells by intervening in the signal transduction process of the TGF-β1/SMAD4 signaling pathway in the cytoplasm or nucleus may be a new approach for anti-atherosclerosis 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 process of various organs such as early
neurodevelopment and bone metabolism [19, 20]. 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. This study shows that compared with the high-fat model group, Ruanmailing oral liquid can significantly decreased GATA2 levels and increased TGF-β1 and SMAD4 levels in high-fat ApoE⁻/⁻ mice, suggesting that GATA2 acts as a negative regulator of the TGF-β1/SMAD4 pathway may play an important role in reducing the differentiation and proliferation of smooth muscle cells and slowing down the process of atherosclerosis.

Compared with the use of statin drugs such as Lipitor to prevent and treat atherosclerosis, traditional Chinese medicine has the characteristics of multiple targets. It has synergistic effects such as lipid-lowering, inhibiting the proliferation and migration of vascular smooth muscle cells, and anti-oxidation, and has fewer side effects, providing a new coping strategy for the prevention and treatment of atherosclerosis. The main ingredients of Ruanmailing oral liquid are Chinese herbal medicine Shudi, Wuweizi, Gouqi, Fuling, Baiziren, Yuanzhi, Renshen, Danggui, Huangqi and so on. Taking a comprehensive view of the whole prescription, it has the effects of invigorating the kidney, promoting blood circulation and reducing phlegm. Elderly patients with atherosclerosis have pathological characteristics of deficiency of kidney essence, excessive phlegm and excessive blood stasis, and the pharmacological effects of Ruanmailing oral liquid are in line with this pathophysiological characteristic. This study confirmed from an experimental point of view that Ruanmailing oral liquid has significant lipid-lowering and anti-atherosclerotic effects.

**Conclusion**

In summary, Ruanmailing oral liquid has 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. It is worthy of further study and promotion.

**Abbreviations**

AS: Atherosclerosis; TGF-β: Transforming growth factor β; ELISA: Enzyme-linked immunosorbent assay; TC: Total cholesterol; TG: Triglycerides; LDL-C: Low density lipoprotein cholesterol; HDL-C: High density lipoprotein cholesterol; VSMCs: Vascular smooth muscle cell; W/L: The ratio of vascular wall (mesomembrane) area to vascular lumen area.

**Declarations**

**Acknowledgements**
Not applicable.

**Authors’ contributions**

JC and TW designed the study. WX, YH and LF contributed to literature review and data analyses. TW, XH and WX contributed to the project design and paper writing. All authors read and approved the final manuscript.

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**Availability of data and materials**

All the data used to support the findings of this study are available from the corresponding author upon reasonable request.

**Ethics approval and consent to participate**

All procedures were approved and conducted in accordance with the guidelines of the Animal Care Committee of Fujian Medical University.

**Consent for publication**

All the authors were concerned and agreed to publish before the submission.

**Competing interests**

The authors declare that they have no competing interests.

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Tables
Table 1 GATA2, SMAD4 and β-actin Primer sequence.

| Gene name | Sequence                                      | Product length |
|-----------|-----------------------------------------------|----------------|
| Gata2     | Upper reaches 5’-CACTCGGGCTCCCATCTCT-3’,       | 187            |
|           | downstream 5’-TGCCGCTTCCATCTTCAT-3’.           |                |
| Smad4     | Upper reaches 5’-CCTCCCATTTCCAATCATC-3’,       | 123            |
|           | downstream 5’-GCCATCCACAGTCAACAACA-3’.         |                |
| β-actin   | Upper reaches 5’-TGTGTCCGTCGTTGATCTGA-3’,      | 149            |
|           | downstream 5’-TTGCTGTTGAAGTGCAGGAG-3’.         |                |
Figure 1

Effects of Ruanmailing oral liquid on serum lipids in ApoE-/- mice. A. total cholesterol level, B. triglycerides level, C. HDL-C level, D. LDL-C level. Data are shown as mean ± SD. *P<0.05, **P<0.01, ***P<0.001, n.s. = non-significant.
Figure 2

Ruanmailing oral liquid treatment reduced atherosclerosis in the thoracic aorta of ApoE-/- mice. A. Pathological section of ApoE-/- mice thoracic aorta and H&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, n.s. = non-significant.

Figure 3

Effect of Ruanmailing oral liquid on the expression of TGF-β1. The serum TGF-β1 concentration of ApoE-/- mice in different treatment groups were measured by enzyme-linked immunosorbent assay (ELISA). Data are shown as means ± SD. ***P<0.001.
Effects of Ruanmailing oral liquid on mRNA and protein levels of GATA2 and SMAD4. A. The expression of GATA2 and SMAD4 were 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 were detected by western blot in different groups of thoracic aorta tissues of ApoE-/- mice.