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Original Article

Hawthorn Extract Alleviates Atherosclerosis through Regulating Inflammation and Apoptosis Related Factors: An Experimental Study*

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ABSTRACT **Objective:** To determine the effects of hawthorn extract on serum lipid levels, pathological changes in aortic atherosclerosis plaque, inflammatory factors, and apoptosis-related protein and mRNA expression in apolipoprotein E gene knockout (ApoE$^{-/-}$) mice. **Methods:** Thirty-six ApoE$^{-/-}$ mice were fed with a high-fat diet starting at the age of 8 weeks. Mice were randomly divided into 3 groups by a random number table including model group, hawthorn extract group, and simvastatin group, 12 mice in each group. Twelve 8-week-old C57BL/6 mice were fed a basic diet and served as control. The mice in the control and model groups were administered 0.2 mL saline daily, the mice in the hawthorn extract and simvastatin groups were administered with 50 mg/kg hawthorn extract or 5 mg/kg simvastatin daily for 16 weeks. After 16 weeks, plasma lipids including total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) were determined by an enzymatic assay. Aortic atherosclerotic lesions were observed by light microscopy, scanning and transmission electron microscopy, respectively. Plasma levels of monocyte chemoattractant protein-1 (MCP-1), interleukin-1 β (IL-1 β), adiponectin (APN), and hypersensitive C-reactive protein (hs-CRP) were measured by enzyme-linked immunosorbent assay (ELISA). Protein and mRNA expressions of Bax and Bcl-2 in the aorta were assessed by Western blotting and quantitative real-time polymerase chain reaction (qRT-PCR), respectively. **Results:** Compared to the control group, the plasma levels of TC, TG and LDL-C were significantly increased and HDL-C were significantly decreased in the model group (P<0.01). Compared to the model group, treatment with hawthorn extract significantly decreased the plasma levels of TC, TG, and LDL-C and increased the plasma level of HDL-C in ApoE$^{-/-}$ mice (P<0.01). The levels of MCP-1, IL-1 β, and hs-CRP in the model group were significantly increased and APN was significantly decreased compared with the control group (P<0.01). Compared to the model group, treatment with hawthorn extract decreased the levels of MCP-1, IL-1 β, and hs-CRP and increased the APN level (P<0.01). Compared to the control group, the protein and mRNA expression of Bax in the model group were significantly increased and the expression of Bcl-2 was significantly decreased (P<0.01). Hawthorn extract also reduced the protein and mRNA expression of Bax and increased the Bcl-2 expression in the aorta (P<0.01). **Conclusion:** Hawthorn extract has anti-atherosclerosis and stabilizing unstable plaque effects. The mechanism may be related to the inflammation and apoptosis signaling pathways.

KEYWORDS hawthorn extract, atherosclerosis, unstable plaque, inflammation factors, apoptosis-related protein

Cardiovascular and cerebrovascular diseases caused by atherosclerosis (AS) have become the leading cause of death in humans. Inflammation is among the main causes of AS. Inflammatory reactions accompany every stage of AS development. The pathological manifestations of AS also involve inflammation.[1] Apoptosis is a process in which cells automatically terminate their lives under physiological or pathological conditions regulated by an inherent genetic mechanism. Apoptosis was involved throughout the pathological process of AS, which can promote tissue resistance to endogenous or exogenous damage and

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influence the occurrence and development of AS.[2] Apoptotic macrophages that are not quickly eliminated induce the secretion of inflammatory factors in the intima and accelerate AS progression. The apoptosis of vascular smooth muscle cells (VSMCs) leads to the degradation of collagen components in the cells, thinning of the fibrous cap of the plaque, and formation of vulnerable plaques.[3] Therefore, regulating apoptosis and inhibiting inflammatory responses are key targets for treating AS. As a common treatment for AS, although simvastatin has shown some efficacy, statins may cause side effects, including myalgia, muscle weakness, elevated liver enzymes, hyperglycaemia, and diabetes risk.[4] Therefore, safe and effective alternative to statins are needed for treating AS. Alternative therapies, such as Chinese medicine, have attracted attention.

Hawthorn activates blood circulation to dissipate blood stasis, promote digestion to eliminate stagnation, and arrest diarrhea and treat dysentery. Recent studies showed that hawthorn flavonoids in hawthorn extract dilated the coronary artery, lowered the blood lipid and blood pressure, strengthened the heart, and excited the central nervous system.[5] A clinical study showed that hawthorn extract significantly reduced serum hypersensitive C-reactive protein (hs-CRP) and matrix metalloproteinase-9 (MMP-9) in patients with AS, suggesting that hawthorn extract can stabilize atherosclerotic plaques for treating AS. Its mechanism may be related to anti-inflammatory reactions and inhibiting extracellular matrix degradation.[6] Animal experiments also showed that hawthorn extract improved phagocytosis of the reticuloendothelial system and regulated the immune organ index as well as white blood cell count in mice.[7] Hawthorn extract inhibited the proliferation and promoted the apoptosis of HepG2 cells.[8]

The aim of this study was to investigate the effect of hawthorn extract on apoptosis and inflammation-related factors in an apolipoprotein E gene knockout (ApoE)$^{-/-}$ mice model of AS so as to explore the mechanism of hawthorn extract in anti-AS.

METHODS

Animals

Eight-week-old male ApoE$^{-/-}$ mice (n=36) and C57BL/6 mice (n=12), weighing 19–21 g, were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and bred by the Laboratory Animal Center of Beijing University. ApoE$^{-/-}$ mice were fed a high-fat diet containing 21% saturated fat and 0.15% cholesterol, while C57BL/6 mice were fed a basic diet. Mice were raised in a specific pathogen-free laboratory at a temperature of 18±1℃, relative humidity of 40%–50%, and free access to food and water. Experiments were conducted in accordance with the Guiding Opinions on Treating Experimental Animals and the Guidelines of the Animal Investigation Committee of Peking University.

Drug Preparation

A total of 5 g hawthorn extract (No. AKH12-2, Linyi Aikang Pharmaceutical Co., Ltd., Shandong, China) containing 30% total flavonoids of hawthorn was dissolved in 1 L distilled water, 0.5 g simvastatin tablet (No.100601-201003, Merck Pharmaceutical, Billerica, MA, USA) was smashed into powder and dissolved in 1 L distilled water. The above solution was ready for use.

Grouping and Administration

Thirty-six ApoE$^{-/-}$ mice were randomly divided into 3 groups by a random number table induding model group, hawthorn extract group and simvastatin group, 12 mice in each group. Twelve C57BL/6 mice were used as a control. The mice in the hawthorn extract group was administered 50 mg/kg hawthorn extract daily and the simvastatin group was administered 5 mg/kg simvastatin (equal to 20 times of the clinical dose), and the control and model groups were administered 0.2 mL saline per day. All mice were raised for 16 weeks.

Plasma Lipids Analysis

After 16 weeks, mice were fasted for 12 h before sacrifice. Intraperitoneal injection of pentobarbital was administered to the mice to minimize pain and 2 mL blood samples were collected from the retroorbital plexus. After allowing the blood samples to stand for 30 min, they were centrifuged for 10 min at 3,000 r/min and the serum was stored at –80 ℃. Total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) were tested using an enzymatic assay with an AD2700 automatic biochemical detector (Olympus, Tokyo, Japan).

Inflammatory Mediators in Plasma

The concentrations of monocyte chemoattractant protein-1 (MCP-1), interleukin-1β (IL-1β), adiponectin (APN) and hypersensitive C-reactive protein (hs-CRP) in plasma were measured by enzyme-linked

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immunosorbent assay (ELISA) using the mice serum samples. ELISA kits were purchased from Shanghai Senxiong Biological Co., Ltd. (China). A standard curve was drawn according to standard serum A_{492} value of different concentrations used to calculate inflammatory factor concentrations.

Hematoxylin and Eosin Staining

A piece of the tissue block prepared from the aorta was separated and placed in 10% formalin. The tissue was gradually dehydrated in different concentrations of ethanol and placed in xylene to make the sample transparent. The tissue was immersed in melted paraffin and fixed in a wax-embedded block. The paraffin block was cut into a 5-μm-thick sheet, placed on a glass slide, and incubated at 45 ℃. At last it was stained with Harris hematoxylin and eosin (HE) and the sample was sealed with gum.

Image Analysis of HE Slice

Lesion images were captured with a CK40-32ph microscope (Olympus, Tokyo, Japan) equipped with Image-Pro 6.0 software (MediaCybernetics, Rockville, MD, USA). HE slices were scanned by tissue slicing scanners (Pannoramic MIDI, 3D HISTECH Ltd., Budapest, Hungary). Slices were placed under the scanner lens and scanned into images. Then a file containing all the tissue information on the slice was formed and could be opened by Pannoramic viewer software. After the scopes of plaque and vascular lumen were selected by the researcher, the area of the plaque and vascular lumen and their ratio could be calculated by QuantCenter Software. All slices were scanned and analyzed.

Aorta Lesion Examination by Scanning Electron Microscopy

The glutaraldehyde settled vascular segments was stained with 1% osmium tetroxide and then was dehydrated using a gradient series of ethanol. After drying, the sample was fractured with liquid nitrogen, dehydrated and dried with a CO_2 critical point dryer. Samples were sprayed onto the sample holder with conductive adhesive for metal spray plating. The samples were observed and photographed under an acceleration voltage of 15 kV by scanning electron microscopy.

Aorta Lesion Examination by Transmission Electron Microscopy

After washing with physiological saline, the isolated aortic vascular tissue was immersed in 3% glutaraldehyde and dehydrated with gradient ethanol acetone. Ultrastructural changes in the aortic vascular were observed by transmission electron microscopy (TEM) after epoxy resin embedding, positioning, repairing, slicing by ultramicrocutting, and lead citrate staining.

Protein Expressions of Bax and Bcl-2 by Western Blotting

Five pieces of aortic vascular tissue of 2 cm in length preserved in liquid nitrogen were homogenized by adding an appropriate amount of cell lysates and the supernatant was collected. Using a tissue protein extraction kit (1:200; Abcam, Cambridge, UK), protein was extracted from the aortic vascular tissue. The protein concentration was measured by the bicinchoninic acid (BCA) method. Next, 20 μg denatured protein was separated by sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS-PAGE) gel and wet-transferred onto a polyvinylidene fluoride (PVDF) membrane. After blocking in skimmed milk powder for 1 h, anti-mouse Bax and Bcl-2 polyclonal antibodies were added to each membrane independently to detect the target protein and the corresponding reference overnight at 4 ℃. On the next day, the membranes were rinsed 3 times in Tris-buffered saline (TBS) containing Tween-20 for 5 min each time. A secondary anti-rabbit IgG antibody labelled with horseradish peroxidase was added and incubated for 1 h at room temperature. After rinsing the membrane 3 times in TBS containing Tween-20 for 5 min each time, protein was visualized by using the enhanced chemiluminescence system (ECL, Scientific Instrument Limited Company, Shanghai, China). β-actin (1:200, Abcam bioscience, Cambridge, USA) was used as an internal reference, and the grey level ratio of target protein to β-actin protein was calculated by semi-quantitative analysis.

mRNA Expression Levels of Bax and Bcl-2 by Quantitative Real-Time Fluorescence Polymerase Chain Reaction

Bax and Bcl-2 mRNA expressions in the aortic vascular tissue were detected by quantitative real-time fluorescence polymerase chain reaction (qRT-PCR). Aortic vascular tissue (20 mg) was weighed, total RNA was extracted with Trizol, and the concentration and purity of total RNA were determined. Reverse transcription cDNAs were prepared from 1 μg of total RNA using a two-step method (DNA removal and

reverse transcription). Using cDNA as a template, qRT-PCR reaction was conducted using SYBR Green. β-actin was used as an internal reference for analysis. The reaction conditions were as follows: predenaturation at 95 ℃ for 30 s, denaturation at 95 ℃ for 5 s, annealing at 60 ℃ for 20 s with a total of 40 cycles. The sequences of the mRNA primers were as follows: β-actin (266 bp): sense 5'-GTCCCTCACCCTCCCAAAAG-3', anti-sense 5'-GCTGCCTCAACACCTCAACCC-3'; Bcl-2 (230 bp): sense 5'-AGGAGCAGGTGCCTACAAGA-3', anti-sense 5'-GCATTTTCCCACCACTGTCT-3'; Bax (230 bp): sense 5'-AGGAGCAGGTGCCTACAAGA-3'; anti-sense 5'-GCATTTTCCCACCACTGTCT-3'. mRNA primers were entrusted to Beijing Huatai Boao Biotechnology Co., Ltd. to synthesize and purify. The PCR samples contained the following: 12.5 μL Maxima SYBR Green/ROX qRT-PCR Master Mix, 0.75 μL sense and antisense primers, 2 μL cDNA, and 9–25 μL nuclease-free water. An ABI7500 fluorescent qRT-PCR instrument (Applied Biosystems, Foster City, CA, USA) was used for the analysis. The Ct value was obtained from the amplification curve.

Statistical Analysis

All values were expressed as mean ± standard error of mean. Statistical analysis was performed by *t*-test for 2 groups or analysis of variance (ANOVA) for 3 or more subgroups using SPSS19.0 statistics software (SPSS Inc. Chicago, IL, USA). $P<0.05$ was considered significant in all analyses.

RESULTS

Modulations of Plasma Lipid Levels

Compared to the control group, the plasma levels of TC, TG and LDL-C were significantly increased and HDL-C was decreased in the model group ($P<0.01$). Compared to the model group, treatment with hawthorn extract or simvastatin significantly decreased the levels of TC, TG and LDL-C ($P<0.01$). In contrast, the HDL-C level was significantly increased in mice treated with hawthorn extract or simvastatin. There were no significant differences between the hawthorn extract and simvastatin groups ($P>0.05$, Figure 1).

Assessment of Aortic Atherosclerotic Lesions by Light Microscopy

No evidence of AS was detected in the aortas in the control group, endothelial cells were continuous and intact, the intima was not thickened, and the internal elastic membrane structure was clear and continuous

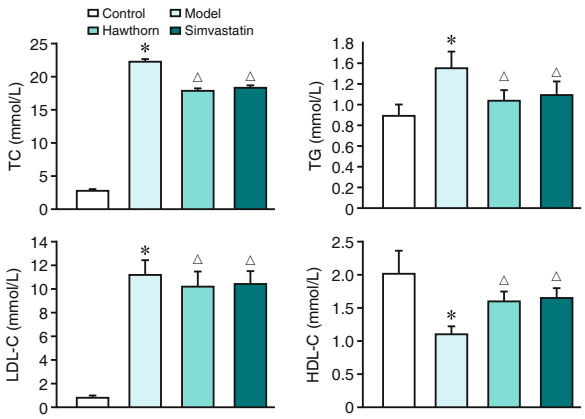

Figure 1. Modulations of Plasma Lipid Levels of Mice

Notes: *$P<0.01$, *vs.* control group; △$P<0.01$, *vs.* model group

with no stratification and rupture. The thickness of the vascular wall was uniform and the cells were arranged in order (Figures 2A and B). In the model group, endothelial cells were irregular and discontinuous. The internal elastic membrane was broken and unclear. Smooth muscle cells proliferated, and plaques protruded into the lumen of the vessel (Figures 2C and D). In the hawthorn extract and simvastatin groups, endothelial cells were morphologically intact, smooth muscle cells slightly proliferated, and the plaque protruded slightly into the vessel lumen (Figures 2 E–H). After calculating by QuantCenter software, the percentage of plaque area in the model group was 61.2%, compared to the model group, the percentage of plaque area decreased in the hawthorn extract group (20.3%) and the simvastatin group (24.8%, $P<0.05$).

Assessment of Aortic AS by Scanning Electron Microscopy

In the control group, the aortic intima was smooth and endothelial cells showed a regular shape and were closely connected to each other (Figure 3A). Atherosclerotic plaques appeared in the aortic arch intima in the model group. Some large plaques surfaces were not covered with endothelial cells, while other endothelial cells were withered, proliferative, or caducous. Endothelial cell lesions in the non-plaque area were similar to those area around the plaque (Figure 3B). The intima of the aorta was smooth in the hawthorn extract and simvastatin groups. Endothelial cells were tightly bound together in regular shapes. Small quantity of sediment was identified on them (Figures 3C and D).

Assessment of Aortic AS by Transmission Electron Microscopy

Endothelial cells and smooth muscle cells of the

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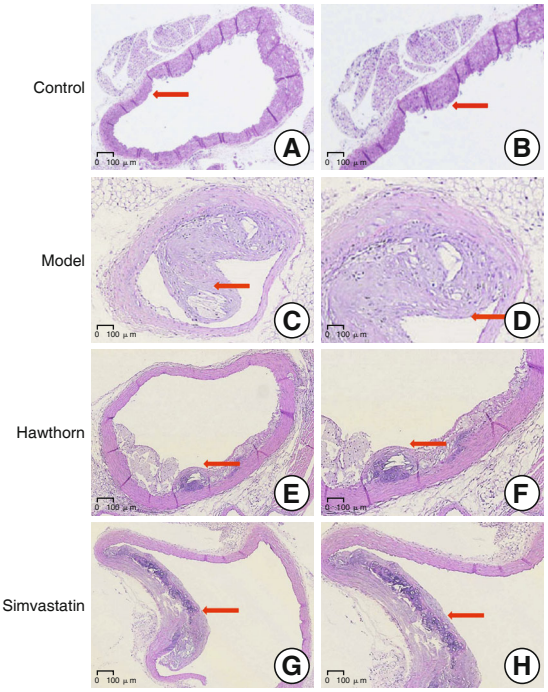

Figure 2. Assessment of Aortic Atherosclerotic Lesions in Mice by Light Microscope

Notes: A,B: smooth aortic lumen in the control group, the arrows indicate the vessel wall; C,D: arrows indicate the atherosclerotic plaque on the inner wall of the aortic in the model group; E, F: arrows indicate the plaque on the inner wall of the aortic in the hawthorn extract group; G,H: arrows indicate the plaque on the inner wall of the aorta in the simvastatin group; A, C, E, G, ×100 magnification; B, D, F, H, ×200 magnification.

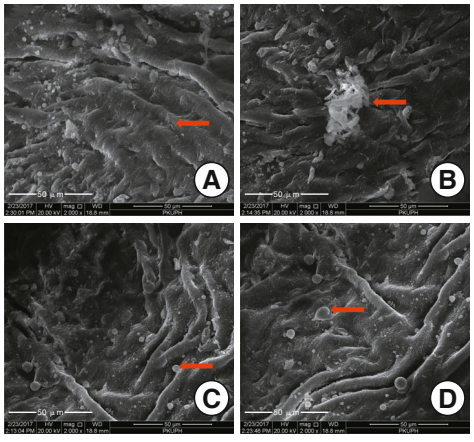

Figure 3. Assessment of Aortic AS in Mice with Scanning Electron Microscope (×1,000)

Notes: A: smooth inner wall of aorta in the control group, the arrow indicates the inner wall of aorta; B: plaque on the inner wall of the aorta in the model group, arrow indicates the plaque; C: adhesive substance in the hawthorn extract group, arrow indicates the adhesive substance on the inner wall of the aorta; D: adhesive substance in the simvastatin group, arrow indicates the adhesive substance on the inner wall of the aorta.

aorta in the control group exhibited a normal shape. The elastic plates were clear and arranged in rule with no lipid droplets and calcium deposits (Figure 4A). In the model group, most of the small plaques were covered with endothelial cells. These cells lost their normal morphology and showed a smooth surface and no villi. The cell body became thinner and the electron density of the nucleus decreased. Organelles in the cytoplasm were decreased and contained lipid droplets and lysosomes of different sizes. Smooth muscle cells and macrophages containing lipids were detected in the vascular wall. Calcium salt deposition was also observed in some areas, and most vascular smooth muscle cells showed a contractile phenotype (Figure 4B). In the hawthorn extract group and simvastatin group, lipid droplets and lysosomes in vascular cells of mice were decreased and minimal calcium salt was deposited (Figures 4C and D).

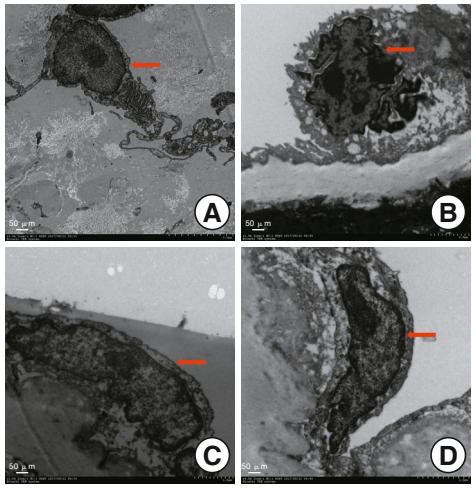

Figure 4. Assessment of Aortic AS in Mice with Transmission Electron Microscope (×4,500)

Notes: A: normal endothelial cell in the control group, the arrow indicates the endothelia cell; B: nuclear chromatin condensation and wrinkled nuclear membrane in the model group, arrow indicates the endothelia cell; C: endothelial cell with normal nuclear and cellular structure in the hawthorn extract group, arrow indicates nuclear chromatin condensation; D: endothelial cell with normal nuclear and cellular structure in the simvastatin group, arrow indicates nuclear chromatin condensation.

Levels of Inflammatory Factors in Plasma

Compared to the control group, the levels of MCP-1, hs-CRP and IL-1β were significantly increased and APN was decreased in the model group (all $P<0.01$). Compared to the model group, treatment with hawthorn extract and simvastatin decreased the levels of MCP-1, hs-CRP and IL-1β ($P<0.01$). In contrast, the APN level significantly increased in mice treated with hawthorn extract or simvastatin. However, there were no differences on effects between the hawthorn extract and simvastatin groups ($P>0.05$, Figure 5).

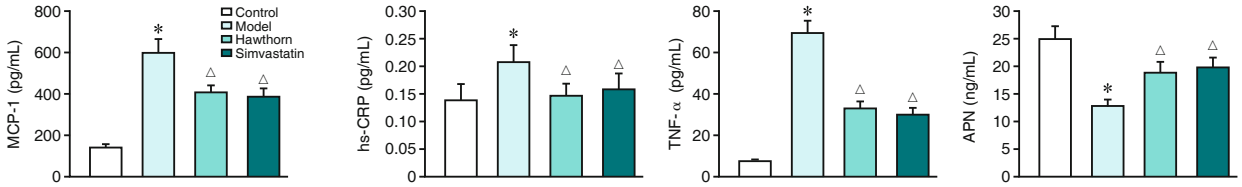

Figure 5. Levels of Inflammatory Factors in Mice Plasma among Different Groups

Notes: *P<0.01, *vs*. control group; △P<0.01, *vs*. model group

Bcl-2 and Bax Protein and mRNA Expression in Mice Aorta

Compared to the control group, the protein and mRNA expression of Bax increased, while Bcl-2 expression decreased (P<0.01). Comparison to the model group, the protein and mRNA expression of Bax decreased in the hawthorn extract and simvastatin groups (P<0.01). In contrast, Bcl-2 protein and mRNA expression levels were significantly increased in mice treated with hawthorn extract or simvastatin (P<0.01). However, there were no differences on the effects between the hawthorn extract and simvastatin groups (P>0.05, Figure 6).

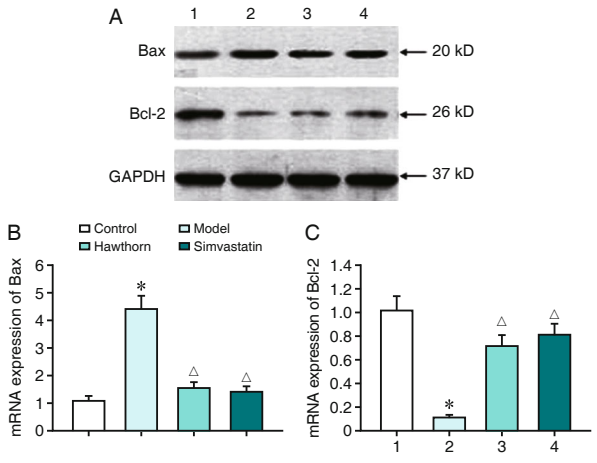

Figure 6. Bax and Bcl-2 Protein and mRNA Expression in Mice Aorta

Notes: A: Bax and Bcl-2 protein expression by Western-blot, 1: control group, 2: model group, 3: hawthorn extract group, 4: simvastatin group; B: Bax mRNA expression by qRT-PCR; C: Bcl-2 mRNA expression by qRT-PCR; *P<0.01, *vs*. control group; △P<0.01, *vs*. model group

DISCUSSION

The pathogenesis of AS is very complex and due to its aetiology varies, mainly including injury or apoptosis of the endothelium, abnormal lipid metabolism, hemodynamic damage, and physical and chemical damage, among other effects. Multiple complex factors acting on vascular wall lead to the occurrence of chronic inflammation of the vascular wall, resulting in AS.[9] Although a previous study showed that statins significantly reduce the size of lipid-rich plaque in patients with AS, statins are only effective in 15%–30% of AS patients[10] and some patients continue to have atherosclerotic plaques, plaque rupture, and stroke.[11] Additionally, statins had several severe side effects, such as myotoxity, hepatotoxicity and kidney damage.[12-15] Therefore, it is very important to find more safe and effective drugs for treating patients with AS.

Hawthorn is a common natural plant for both medicine and food which is safe with no side effects reported. At present, compositions found and extracted from hawthorn mainly include: flavonoids, flavanes and their polymers, organic acids, triterpenoids and steroids.[16] Hawthorn has strong pharmacological effects on cardiovascular immune and digestive systems.[17] Pharmacological studies have shown that flavonoids composition in hawthorn extract could lower blood lipids, protect liver and treat experimental atherosclerosis,[18] lower blood pressure, increase coronary flow, and improve arrhythmia.[19] Triterpenic acid in hawthorn extract could also improve circulation and increase coronary flow.[20,21] Ursolic acid could significantly enhance the phagocytic function of peritoneal macrophages in immunosuppressive mice, increase the number of peripheral blood leukocytes, promote the proliferation of splenic lymphocytes and increase the spleen index.[22] Organic acid in hawthorn extract could increase the secretion of digestive enzymes in the stomach, enhance the activity of lipase and protease, promote intestinal peristalsis and have obvious bidirectional regulation on intestinal dysfunction, which is helpful to mechanical and chemical digestion.[23,24] Other studies showed that Shanzha Xiaozhi Capsule (山楂消脂胶囊) significantly reduced the level of serum hs-CRP in patients with non-acute coronary heart disease with phlegm and stasis syndrome.[25] In our study, the levels of TC, TG, and LDL-C in the serum were increased and HDL-C decreased in the model group. Hawthorn extract and simvastatin reduced the levels of TC, TG, and LDL-C

and increased HDL-C level in the serum.

Meanwhile, we examined the effects of hawthorn extract by light microscopy and electron microscopy. The results showed that hawthorn extract protected the endothelial cells of the aorta, decreased the number of lesions of endothelial cells, and reduced lipid and calcium salt deposition in smooth muscle cells and macrophages. The size of the atherosclerotic plaque was also reduced.

MCP-1 is involved in the lipid stripe formation, plaque instability, and plaque rupture periods of AS. It combines with its receptor CC-chemokine receptor 2 to induce the expression of adhesion factors in monocytes and endothelial cells, thereby inflammatory cells migrate to the lesion and lead to inflammation during AS development.[26] Hs-CRP induces endothelial cells to secrete and express adhesion factors and chemokines.[27] The role of IL-1β in the AS inflammatory pathological mechanism are well-known. When an inflammatory reaction occurs, inflammatory corpuscles induce the secretion and maturation of downstream IL-1β, and IL-1β affects the development of AS.[28,29] APN protects the endothelium by exerting complex anti-inflammatory effects in AS. APN terminates the signal transduction pathway mediated by lipopolysaccharide, tumor necrosis factor-α, and IL-6 in macrophages and induces the expression of anti-inflammatory genes in macrophages.[30] We found that the levels of MCP-1, hs-CRP, and IL-1β in the serum were increased and APN in the serum was decreased in the model group. Hawthorn extract and simvastatin reduced the levels of MCP-1, hs-CRP, and IL-1β and increased the APN level in the serum.

An imbalance between the proliferation and apoptosis of VSMCs leads to the formation of AS plaques. In AS injury, VSMCs proliferation is dominant and apoptosis is reduced.[31,32] Apoptosis is strictly regulated by apoptosis genes. Bcl-2 is the most common antiapoptotic gene and is expressed in macrophages, VSMCs, and foam cells. Bcl-2 expression is increased in areas where inflammatory cells gather. Bax is also an essential signal for initiating apoptosis, which can resist the anti-apoptotic effect of Bcl-2 and promote apoptosis. In this study, we found that hawthorn extract and simvastatin reduced the protein and mRNA expression of Bax and increased Bcl-2 expressions.

In conclusion, our study suggests that hawthorn extract regulates lipids, inhibits proliferation, lipidosis, and calcium deposition of VSMCs, and resists inflammation and apoptosis and thus is useful for treating AS. Additionally, hawthorn extract decreased the levels of MCP-1, hs-CRP, and IL-1β, increased the level of APN in the serum, reduced the protein and mRNA expression of Bax, and increased the protein and mRNA expression of Bcl-2 in the aorta, suggesting that it functions by inhibiting inflammatory and apoptosis signalling pathways.

Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contributions

Wang SZ carried out the experiments, performed the data analysis, and drafted the manuscript. Sun J, Sun Z, and Ma H carried out the experiments and index detection. Chen KJ, Liu LT and Wu M were responsible for the conception, study design and data extraction. Liu Y participated in conception and study design and critically revised the manuscript.

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