Bushen Jiangzhi formula reduces atherosclerosis in apoE−/− mice through autophagy

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Abstract

OBJECTIVE: To study the effect of Bushen Jiangzhi formula (BSJZF) on atherosclerosis (AS) in apolipoprotein E knockout (apoE−/−) mice and the underlying mechanism.

METHODS: We used a high fat diet to induce AS in apoE−/− mice. The mice were randomly divided into four groups: model, BSJZF, atorvastatin, and 3-methyladenine groups. Syngeneic C57BL/6 mice of the same age were used for the control group. Autophagosomes in the aorta were examined by transmission electron microscopy. Morphology, lipid accumulation, and collagen deposition in the aorta were examined by hematoxylin and eosin, Oil Red O, and Masson's staining, respectively. Serum levels of tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), and interleukin 10 (IL-10) were measured by enzyme-linked immunoassays. Protein expression of microtubule-associated protein light chain 3 (LC3), Beclin 1, and p62 in the aorta were examined by Western blot analyses.

RESULTS: ApoE−/− mice fed a high fat diet exhibited AS symptoms including less autophagosomes in the aorta, higher serum levels of TNF-α, IFN-γ, and p62, and lower serum levels of IL-10, LC3, and Beclin 1. Treatment with BSJZF significantly reduced the area of the aortic plaque, decreased expression of TNF-α, IFN-γ, and p62, and increased expression of IL-10, LC3, and Beclin 1.

CONCLUSION: Our findings suggest that BSJZF promotes autophagy and reduces inflammation by regulating the expression of autophagy-related proteins LC3, Beclin 1, and p62, thereby effectively treating AS.
rates lipid metabolism disorders and decreases the expression of inflammatory factors in patients with carotid atherosclerosis (CAS) and in apolipoprotein E knockout (apoE−/−) mice fed a high fat diet. In addition, treated CAS patients exhibit improved carotid artery elasticity.14 Based on the major role of autophagy in AS and preliminary results of effective treatment of AS by kidney-nourishing TCM, we investigated the effect of Bushen Jiangzhi formula (BSJZF) on regulation of AS in apoE−/− mice and the underlying mechanism.

METHODS

Experimental animals
Twelve-week-old specific pathogen-free (SPF) male apoE−/− mice [Animal license No. SCXK (Jing) 2016-0006] and wild type syngeneic male C57BL/6 mice [Animal license No. SCXK (Jing) 2016-0006] of the same age were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The mice were kept in an SPF grade animal facility at the animal center of the Shanghai University of Traditional Chinese Medicine (Shanghai, China). The room temperature was (24 ± 1 °C), relative humidity was 50%-70%, and the light-dark cycle was 12/12-h. All animal experiments strictly followed the rules and regulations of the Animal Experimental Ethics Committee of the Experimental Animal Center of Shanghai University of Traditional Chinese Medicine. This study was approved by the Experimental Animal Welfare Ethics Committee of Shanghai University of Traditional Chinese Medicine (Approval No. Pzhutcm 18113002).

Medicine and major reagents
Bushen Jiangzhi formula was purchased from Jiangyin Tianjiang Pharmaceutical Industry Co., Ltd. (Jiangsu, China). It consists of the following crude components: 10 g Tusizi (Semen Cuscutae), 10 g Shayuanzhi (Semen Astragali Complanati), 10 g Fupenzi (Fructus Rubi Chinigii), 10 g Nuizhenzi (Fructus Ligustri Lucidi), 10 g Gouqizi (Fructus Lycii), and 10 g Heidou (Semen Sajae Attircolor). The corresponding contents per granule were 0.5 g Tusizi (Semen Cuscutae), 1 g Shayuanzhi (Semen Astragali Complanati), 1 g Fupenzi (Fructus Rubi Chingii), 1 g Nuizhenzi (Fructus Ligustri Lucidi), 4 g Gouqizi (Fructus Lycii), and 0.5 g Heidou (Semen Sajae Attircolor). Atorvastatin tablets were produced by Pfizer Inc., (Pfizer Newyork, US), (20 mg/tablet, product batch number: H20051408). Anti-rabbit LC3 (Cell Signaling Technology, Boston, US), anti-rabbit Beclin 1 (Abcam, Cambridge, UK), and anti-mouse P62 (Abcam, Cambridge, UK) antibodies, a goat anti-rabbit IgG (H + L) secondary antibody (LI-COR, Nebraska, US), goat anti-mouse IgG (H + L) secondary antibody (LI-COR, Nebraska, US), BCA protein assay kit (Beyotime Biotechnology Co., Ltd., Shanghai, China), protein ladder (Thermo Fisher, Massachusettts, US), and SDS-PAGE Gel Preparation Kit (Beyotime Biotechnology Co., Ltd., Shanghai, China). Mouse Total Cholesterol (TC) ELISA Kit (Ek-Bioscience, Shanghai, China), Mouse Triglyceride (TG) ELISA Kit (Ek-Bioscience, Shanghai, China), Mouse High Density Lipoprotein (HDL-C) ELISA Kit (Ek-Bioscience, Shanghai, China), Mouse Low Density Lipoprotein (LDL-C) ELISA Kit (Ek-Bioscience, Shanghai, China), Mouse Tumor Necrosis Factor-alpha (TNF-α) enzyme-linked immunosorbent assay (ELISA) Kit (EK-Bioscience, Shanghai, China), Mouse Interferon-gamma (IFN-γ) ELISA Kit (Ek-Bioscience, Shanghai, China), and Mouse Interleukin-10 (IL-10) ELISA Kit (EK-Bioscience, Shanghai, China).

Modeling and grouping
A total of 96 apoE−/− mice at 12 weeks of age were fed a normal diet for 1 week before randomly divided into the following four groups using a random digital table: apoE−/− mice + high fat diet (model group), apoE−/− mice + high fat diet + Bushen Jiangzhi formula (BSJZF group), apoE−/− mice + high fat diet + atorvastatin (atorvastatin group), apoE−/− mice + high fat diet + 3-methyl-adenine group (3-MA group). Syngeneic C57BL/6 mice of the same age were used for the control group. The high fat diet consisted of a normal mouse diet with addition of 21% fat and 0.5% cholesterol. The 3-MA group was treated with 3-MA solution every other day at 15 mg per kg body weight via intraperitoneal injection.8 The BSJZF and atorvastatin groups were treated with BSJZF and atorvastatin solutions, respectively. The treatment was administered through daily oral gavage. The dosage for mice was calculated based on the corresponding dosage for a 60 kg adult human. The final BSJZF dosage was 12.33 g/kg body weight, and the final atorvastatin dosage was 2.05 mg active ingredient/kg body weight.9 Control and model groups were treated with equal volumes of distilled water via daily oral gavage. Treatments in all groups began at the same time and were continued for 12 weeks.

General morphological observation of mice
We observed the mouse body type, hair color, and behavioral response, recorded the mouse body weight fortnightly, and constructed a line graph of body weight changes.

Observation of pathological morphology, lipid accumulation, and collagen deposition in the mouse aorta
Mice were anesthetized with 2% pentobarbital sodium and infused with 4% paraformaldehyde. The heart and blood vessel between thoracic and abdominal aortas were removed and fixed in 10% formalin for embedding in paraffin or frozen sectioning. The paraffin-embedded tissue sections were stained with hemotoxylin and eosin or Masson’s trichrome. Frozen sections were
stained with Oil Red O. Morphology, lipid accumulation, and collagen deposition in the aorta were examined under an Olympus BH2 optical microscope. Images were acquired with a scanner (Pannoramic MID; 3D Histech, Hungary). Semi-quantitative analyses of the plaque area, lipid content, and collagen content were calculated as follows: plaque area (PA; mm²) = vascular area (VA; mm²) − lumen area (LA; mm²), corrected plaque area = PA/(VA-LA), lipid content = positively stained area/aorta luminal area, and collagen content = collagen area/aorta luminal area.

**Transmission electron microscopy to detect autophagosomes of the mouse aorta**

Mice were anesthetized with 2% pentobarbital sodium and the aortic arch was isolated immediately. Extravascular connective tissues were removed under an anatomical microscope (SZX7; Olympus, Tokyo, Japan). A small piece of the plaque was fixed in 2.5% osmium tetroxide for 2 h at room temperature, followed by ethanol dehydration, embedding in paraffin, sectioning, uranyl acetate staining, and lead citrate staining. Sections were examined under a transmission electron microscope (JEM1230; JEOL Ltd., Tokyo, Japan) to determine the autophagosomes in the aorta.

**Detection of the serum lipid levels in mice by ELISA**

Blood was collected from the orbital plexus after anesthesia with 2% pentobarbital sodium, which was set for 12 h, and then centrifugated at 13 523 × g for 30 min with the upper serum layer collected. Serum levels of TC, TG, HDL-C, and LDL-C were detected by the ELISA kits, according to the manufacturer’s instructions. OD values of samples were measured immediately at 450 nm. A standard curve was constructed according to the instructions with the corresponding concentration range obtained based on the standard curve.

**Detection of serum TNF-α, IFN-γ, and IL-10 levels in mice by ELISA**

Blood was collected from the orbital plexus after anesthesia with 2% pentobarbital sodium, which was set for 12 h, and then centrifugated at 13 523 × g for 30 min with the upper serum layer collected. Serum levels of TNF-α, IFN-γ, and IL-10 were detected by the ELISA kits, according to the manufacturer’s instructions. OD values of samples were measured immediately at 450 nm. A standard curve was constructed according to the instructions with the corresponding concentration range obtained based on the standard curve.

**Detection of LC3, Beclin 1, and p62 proteins expressions in the mouse aorta by Western blot**

Mice were anesthetized with 2% pentobarbital sodium and the blood vessel between thoracic and abdominal aortas was collected immediately. The tissues were rinsed in saline and snap-frozen in liquid nitrogen before storage at −80 °C. The mouse aorta was lysed in RIPA buffer containing phenylmethyl sulfonyl fluoride, and the protein concentration was measured by the BCA method. Then, 40 μg tissue protein was mixed with a 4-fold volume of loading buffer and boiled at 100 °C for 10 min to denature the proteins. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (80 V for 30 min followed by 120 V for 90 min) and then transferred to a PVDF membrane by the wet-transfer method (270 mA for 100 min). The membrane was blocked in 5% dry skim milk for 2 h at room temperature. Subsequently, the membrane was incubated with primary antibodies against the following proteins at 4 °C overnight: LC3 (1 : 1000), Beclin 1 (1 : 1000), and p62 (1 : 1000). Then, the membrane was washed six times (5 min for each wash) and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The membrane was scanned using the Odyssey infrared imaging system. ImageJ software was used to analyze the integrated absorbance (IA) of the protein band (IA = average light density value × area). The relative expression level of the target protein was measured by IA of the target protein/IA of glyceraldehyde-3-phosphate dehydrogenase.

**Statistical analysis**

Statistical analysis was performed using SPSS 23.0 software (SPSS Inc., Released 2014. SPSS Statistics for Windows, Version 23.0, Armonk, NY, USA). Figures were generated using GraphPad Prism 5 (GraphPad, CA, USA). Results are shown as the mean ± standard deviation ( ± s). One-way analysis of variance and the t-test were conducted to test differences between groups. P < 0.05 was the significance level in two-tailed tests.

**RESULTS**

**General conditions of the mice**

After 12 weeks of drug intervention, mice in the control group were well proportioned with hair color luster and agile movements. Mice in the model group were obese with withered fur and slow movements. Compared with the model group, mice in the BSJZF and atorvastatin groups showed well-proportioned limbs and even hair color with relatively agile movements, whereas mice in the 3-MA group were thin and slender with withered hair and slow movements. During the experimental intervention, mice in each group had good food intake.

The weight dynamic test of mice showed no significant difference in weights of each group at 12 weeks (P > 0.05), and the trend of weight growth in each group was the same at 12-20 weeks of age. At 14-16 weeks of age, there was a significant difference between control and model groups (P < 0.05). At 18-20 weeks of age,
there was a difference between the control group and model, 3-MA groups \((P < 0.05)\). At 20-24 weeks of age, the body weight of the 3-MA group showed a downward trend, and the weight of the other groups increased steadily. At 24 weeks of age, there was a significant difference between the model group and control, 3-MA groups \((P < 0.05)\), and there were no statistical differences between BSJZF and atorvastatin groups \((P > 0.05)\) (Figure 1).

**Figure 1 Dynamic body weights of mice**

Control: male C57BL/6 mice fed with normal diet; model: apoE-/- mice fed with high-fat diet; BSJZF: apoE-/- mice fed with high-fat diet and BSJZF granule; atorvastatin: apoE-/- mice fed with high-fat diet and atorvastatin; 3-MA: apoE-/- mice fed with high-fat diet and 3-MA. BSJZF: Bushen Jiangzhi formula; 3-MA: 3-methyladenine. Data are presented as mean ± standard deviation. \(^*P < 0.05\), model group compared to control group; \(^{ab}P < 0.05\), 3-MA group compared to control group; \(^{ac}P < 0.05\), 3-MA group compared to model group.

**Table 1 Comparison of serum lipid levels in each group (mmol/L, \(\bar{x} \pm s\))**

<table>
<thead>
<tr>
<th>Group</th>
<th>(n)</th>
<th>TC</th>
<th>TG</th>
<th>HDL-C</th>
<th>LDL-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>2.09±0.09</td>
<td>6.19±0.32</td>
<td>4.28±0.35</td>
<td>1.91±0.14</td>
</tr>
<tr>
<td>Model</td>
<td>6</td>
<td>6.81±0.11(^*)</td>
<td>6.46±0.09</td>
<td>3.18±0.09(^*)</td>
<td>4.68±0.05(^*)</td>
</tr>
<tr>
<td>BSJZF</td>
<td>6</td>
<td>4.15±0.18(^a)</td>
<td>6.19±0.05</td>
<td>3.59±0.35</td>
<td>3.26±0.09(^*)</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>6</td>
<td>4.11±0.25(^a)</td>
<td>6.20±0.08</td>
<td>3.59±0.21</td>
<td>3.23±0.05(^*)</td>
</tr>
<tr>
<td>3-MA</td>
<td>6</td>
<td>7.18±0.10(^*)</td>
<td>6.50±0.12</td>
<td>3.08±0.05</td>
<td>5.71±0.07(^*)</td>
</tr>
</tbody>
</table>

Notes: control: male C57BL/6 mice fed with normal diet; model: apoE-/- mice fed with high-fat diet; BSJZF: apoE-/- mice fed with high-fat diet and BSJZF granule; atorvastatin: apoE-/- mice fed with high-fat diet and atorvastatin; 3-MA: apoE-/- mice fed with high-fat diet and 3-MA. TC: total cholesterol; TG: triglyceride; HDL-C: high density lipoprotein-cholesterol; LDL-C: low density lipoprotein-cholesterol. The BSJZF dosage was 12.33 g/kg body weight, and the Atorvastatin dosage was 2.05 mg active ingredient/kg body weight. The 3-MA dosage was 15 mg/kg body weight. Treatments continue 12 weeks. Data are presented as mean ± standard deviation. \(^*P < 0.01\), compared to control; \(^{ab}P < 0.01\), compared to model, \(^{ac}P < 0.05\).

**Table 2 Comparison of serum inflammatory factor levels in each group (pp/mL, \(\bar{x} \pm s\))**

<table>
<thead>
<tr>
<th>Group</th>
<th>(n)</th>
<th>TNF-(\alpha)</th>
<th>IFN-(\gamma)</th>
<th>IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>0.341±0.012</td>
<td>0.355±0.007</td>
<td>0.283±0.005</td>
</tr>
<tr>
<td>Model</td>
<td>6</td>
<td>0.581±0.016(^*)</td>
<td>0.849±0.011(^*)</td>
<td>0.213±0.010(^*)</td>
</tr>
<tr>
<td>BSJZF</td>
<td>6</td>
<td>0.420±0.012(^a)</td>
<td>0.476±0.005(^*)</td>
<td>0.231±0.003(^a)</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>6</td>
<td>0.414±0.013(^a)</td>
<td>0.461±0.011(^*)</td>
<td>0.236±0.012(^a)</td>
</tr>
<tr>
<td>3-MA</td>
<td>6</td>
<td>0.605±0.014(^*)</td>
<td>0.866±0.010(^*)</td>
<td>0.200±0.004(^*)</td>
</tr>
</tbody>
</table>

Notes: control: male C57BL/6 mice fed with normal diet; model: apoE-/- mice fed with high-fat diet; BSJZF: apoE-/- mice fed with high-fat diet and BSJZF granule; atorvastatin: apoE-/- mice fed with high-fat diet and atorvastatin; 3-MA: apoE-/- mice fed with high-fat diet and 3-MA. TNF-\(\alpha\): tumor necrosis factor alpha; IFN-\(\gamma\): Interferon-gamma; IL-10: interleukin-10. The BSJZF dosage was 12.33 g/kg body weight, and the Atorvastatin dosage was 2.05 mg active ingredient/kg body weight. The 3-MA dosage was 15 mg/kg body weight. Treatments continue 12 weeks. Data are presented as mean ± standard deviation. \(^*P < 0.01\), compared to control; \(^{ab}P < 0.05\), \(^{ac}P < 0.01\), compared to model.
fbers in fibrous caps (P < 0.001), whereas the 3-MA group had thinner fibrous caps with fewer matrix fibers (P < 0.05). There was a significant difference in the phenotypes between BSJZF and atorvastatin groups (P > 0.05) (Figure 4).

Transmission electron microscopy: the control group had an intact aorta cell structure with no obvious lipid droplets and few autophagosomes. The model group exhibited visible accumulation of lipids and no autophagosomes. Compared with the model group, BSJZF and atorvastatin groups had significantly increased amounts of autophagosomes without accumulation of lipids, whereas the 3-MA group had no autophagosomes, but obvious lipid crystals (Figure 5).

**Expression of LC3, Beclin 1, and p62 proteins expressions in the mouse aorta**

The control group had high expression of LC3 and Beclin 1, but low expression of p62 in the aorta. Compared with the control group, the model group exhibit-

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**Figure 2 Effect of BSJZF granule on aortic plaque area in mouse**

A: aorta HE staining in control group (× 200); B: aorta HE staining in model group (× 200); C: aorta HE staining in BSJZF group (× 200); D: aorta HE staining in atorvastatin group (× 200); E: aorta HE staining in 3-MA group (× 200); F: statistical chart of aortic HE staining results. Control: male C57BL/6 mice fed with normal diet; model: apoE<sup>−/−</sup> mice fed with high-fat diet; BSJZF: apoE<sup>−/−</sup> mice fed with high-fat diet and BSJZF granule; atorvastatin: apoE<sup>−/−</sup> mice fed with high-fat diet and atorvastatin; 3-MA: apoE<sup>−/−</sup> mice fed with high-fat diet and 3-MA. The BSJZF dosage was 12.33 g/kg body weight, and the Atorvastatin dosage was 2.05 mg active ingredient/kg body weight. The 3-MA dosage was 15 mg/kg body weight. Treatments continue 12 weeks. Data are presented as means ± standard deviation. *P < 0.001, compared to control, BSJZF, atorvastatin and 3-MA groups.

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**Figure 3 Effect of BSJZF on the relative area of mouse aortic plaque lipids**

A: aorta Oil Red O staining in control group (× 40); B: aorta Oil Red O staining in model group (× 40); C: aorta Oil Red O staining in BSJZF group (× 40); D: aorta Oil Red O staining in atorvastatin group (× 40); E: aorta Oil Red O staining in 3-MA group (× 40); F: statistical chart of aortic Oil Red O staining results. control: Male C57BL/6 mice fed with normal diet; model: apoE<sup>−/−</sup> mice fed with high-fat diet; BSJZF: apoE<sup>−/−</sup> mice fed with high-fat diet and BSJZF granule; atorvastatin: apoE<sup>−/−</sup> mice fed with high-fat diet and atorvastatin; 3-MA: apoE<sup>−/−</sup> mice fed with high-fat diet and 3-MA. The BSJZF dosage was 12.33 g/kg body weight, and the Atorvastatin dosage was 2.05 mg active ingredient/kg body weight. The 3-MA dosage was 15 mg/kg body weight. Treatments continue 12 weeks. Data are presented as means ± standard deviation. *P < 0.01, compared to control, atorvastatin and 3-MA groups; *P < 0.05, compared to BSJZF group.
stress damage. Inhibiting autophagy causes apoptosis protecting VECs from inflammation and oxidative degrading degradation of damaged VEC structures and AS. Autophagy sustains the functions of VECs by indicating that autophagy is involved in the development (VSMCs), and macrophages undergo autophagy, indicating that autophagy plays a major role in the occurrence and development of AS. Autophagy reduces the secretion of inflammatory factors, promotes outflux of intracellular cholesterol, reduces accumulation of macrophages in the AS plaque, and consequently promotes stability of the plaque. These results demonstrate that autophagy inhibits the occurrence and development of AS effectively. TEM accurately identifies autophagic structures in cells and is the best method to detect autophagy. Autophagy involves a variety of autophagy-related proteins including LC3, Beclin 1, and p62. LC3 is the most commonly detected autophagy-associated protein, which is localized on the autophagosome and participates in extension of the membrane. Therefore, it is commonly used as a marker for autophagy. Beclin 1 activates the formation of autophagosomes. It forms a complex with Vps34 and induces formation of the two-membrane structure of autophagosomes. p62 is another marker that is widely used to measure the activ-

DISCUSSION

AS is a chronic inflammatory disease characterized by lipid accumulation in large and middle arteries. Autophagy plays a major role in the occurrence and development of AS. Upon AS induction, vascular endothelial cells (VECs), vascular smooth muscle cells (VSMCs), and macrophages undergo autophagy, indicating that autophagy is involved in the development of AS. Damage of VECs leads to the development of AS. Autophagy sustains the functions of VECs by inducing degradation of damaged VEC structures and protecting VECs from inflammation and oxidative stress damage. Inhibiting autophagy causes apoptosis of VECs, impairs the integrity of VECs, and promotes accumulation of lipids and the development of AS. VSMCs are the only cell type that produces collagen fibers in the AS plaque. Autophagy stabilizes vulnerable plaques by inhibiting senescence of VSMCs. In the early stage of AS, upregulation of VSMC autophagy facilitates induction of a static cell phenotype, which reduces cell proliferation and prevents the development of AS. Macrophages are the main source of foam cells. They secrete a variety of cytokines and play major roles in the occurrence and development of AS. Autophagy reduces the secretion of inflammatory factors, promotes outflux of intracellular cholesterol, reduces accumulation of macrophages in the AS plaque, and consequently promotes stability of the plaque. These results demonstrate that autophagy inhibits the occurrence and development of AS effectively.

Figure 4 Effect of BSJZF on the area of plaque collagen in apoE−/− mice
A: aorta Masson staining in control group (× 200); B: aorta Masson staining in model group (× 200); C: aorta Masson staining in BSJZF group (× 200); D: aorta Masson staining in atorvastatin group (× 200); E: aorta Masson staining in 3-MA group (× 200); F: statistical chart of aortic HE staining results. control: male C57BL/6 mice fed with normal diet; model: apoE−/− mice fed with high-fat diet; BSJZF: apoE−/− mice fed with high-fat diet and BSJZF granule; atorvastatin: apoE−/− mice fed with high-fat diet and atorvastatin; 3-MA: apoE−/− mice fed with high-fat diet and 3-MA. The BSJZF dosage was 12.33 g/kg body weight, and the Atorvastatin dosage was 2.05 mg active ingredient/kg body weight. The 3-MA dosage was 15 mg/kg body weight. Treatments continue 12 weeks.

Figure 5 Effect of BSJZF on autophagosomes of the mouse aorta
A: ultrastructure of cells in control group (× 2000); B: ultrastructure of cells in BSJZF group (× 2000); C: ultrastructure of cells in atorvastatin group (× 2000); D: ultrastructure of cells in 3-MA group (× 2000). BSJZF: Bushen Jiangzhi formula; 3-MA: 3-methyladenine. Autophagosomes are indicated by yellow arrows. id: lipid droplets and crystals are indicated by red arrows.

3-MA: 3-methyladenine; BSJZF: Bushen Jiangzhi formula; HE: hematoxylin-eosin. Data are presented as means ± standard deviation. P < 0.001, compared to control, BSJZF and atorvastatin groups; P < 0.001, compared to 3-MA group.

Figure 4 Effect of BSJZF on the area of plaque collagen in apoE−/− mice
A: aorta Masson staining in control group (× 200); B: aorta Masson staining in model group (× 200); C: aorta Masson staining in BSJZF group (× 200); D: aorta Masson staining in atorvastatin group (× 200); E: aorta Masson staining in 3-MA group (× 200); F: statistical chart of aortic HE staining results. control: male C57BL/6 mice fed with normal diet; model: apoE−/− mice fed with high-fat diet; BSJZF: apoE−/− mice fed with high-fat diet and BSJZF granule; atorvastatin: apoE−/− mice fed with high-fat diet and atorvastatin; 3-MA: apoE−/− mice fed with high-fat diet and 3-MA. The BSJZF dosage was 12.33 g/kg body weight, and the Atorvastatin dosage was 2.05 mg active ingredient/kg body weight. The 3-MA dosage was 15 mg/kg body weight. Treatments continue 12 weeks.
As an AS-inducing factor, IFN-γ derived from T cells and is highly expressed in the lesion tors of AS. This is a proinflammatory cytokine de- tory response, and is recognized as one of the risk fac-

tors of AS. IFN-γ promotes the accumulation of oxidized low-density lipoprotein and the formation of foam cells. IL-10 is a typical soluble anti-inflammatory factor produced by a variety of inflammatory cells. It inhibits local and systemic inflammation, and has been shown to inhibit the development of AS. In a clinical study, unstable plaque patients exhibited a significantly lower level of IL-10 in their serum compared with healthy controls. Autophagy affects key regulators of the immune response and plays a key role in regulating inflammatory responses. A study using mouse NG108-15 nerve cells showed that inhibiting autophagy significantly reduces the level of IL-10. Macrophages participate in the inflammatory response of AS. Autophagy regulates the polarization of macrophages, converts the proinflammatory M1 type to the anti-inflammatory M2 type, and reduces the levels of proinflammatory factors such as TNF-α and IFN-γ, thereby reducing inflammation. In this study, we used ELISA to analyze the serum levels of inflammatory cytokines TNF-α, IFN-γ, and IL-10 in mice. The model group exhibited significantly higher serum levels of TNF-α and IFN-γ, and significantly lower serum levels of IL-10 compared with the control group. The 3-MA group exhibited a further increase in the levels of TNF-α and IFN-γ as well as a further decrease in the level of IL-10 compared with the model group.

Pathological and mechanistic studies of AS in Western...
medicine have primarily focused on lipid metabolism disorder, inflammatory injury, and immune dysfunction. According to TCM theory, the pathogenesis of AS is frequently caused by deficiency in internal organs and effects of external factors. Thus, an increasing number of studies have focused on the role of kidney deficiency in AS. AS frequently occurs in elderly people and is closely related to aging. The kidney is the main reservoir of Jing (the essence of life) and the foundation of one’s body and growth. Theories of kidney deficiency leading to aging have been elucidated throughout the years. It is mentioned in “On art of health cultivation in ancient times” in Huang Di Nei Jing·Suwen that women at 7 years of age have full kidney energy, which is reflected by the fact that their permanent teeth come in and their hair grows long. However, at 49 years of age, the flow of menses ceases in women and they are not able to conceive anymore. Men at 8 years of age have full kidney energy, but at 64 years of age, their hair and teeth fall out. Moreover, it is mentioned in Yi Xue Zheng Zhanu·Mingmen Zhu Shoujian that full kidney energy prolongs lifespan and weak kidney energy shortens lifespan. Our previous study indicated that kidney-nourishing TCM improved AS symptoms in apoE	extsuperscript{−/−} mice fed a high fat diet by reducing expression of toll-like receptor 4 in the aorta and the levels of monocyte attractant protein 1 and intercellular adhesion molecule 1 in serum. The results of clinical trials have also revealed that kidney-nourishing TCM increases vascular elasticity of the carotid artery and reduces plasma interleukin 6 levels in CAS patients. These results suggest that kidney-nourishing TCM not only delays senescence, but also regulates the development of atherosclerosis. Therefore, in this study, we developed BSJZF by modifying and improving current TCM prescriptions with demonstrated anti-aging and AS-preventing effects. BSJZF consists of 10 g Tuisizi (Semen Cuscutae), 10 g Shayuanzi (Semen Atragali Complanati), 10 g Fupenzi (Fructus Rubi Chingsii), 10 g Nüzhenzi (Fructus Ligustri Lucidi), 10 g Gouqizi (Fructus Lycii), and 10 g Heidou (Semen Sojae Articulor). In BSJZF, Gouqizi (Fructus Lycii) replenishes the kidney essence and Tuisizi (Semen Cuscutae) invigorates the kidney. Their combination affects the “Jun” drug that treats the main cause of the disease. Shayuanzi (Semen Atragali Complanati) replenishes the kidney and nourishes Yang. Nüzhenzi (Fructus Ligustri Lucidi) replenishes the kidney and nourishes Yin. Fupenzi (Fructus Rubi Chingsii) invigorates the kidney and affects the “Chen” drug that enhances the actions of “Jun” or treats accompanying symptoms. Hei dadou (black beans) promote blood circulation, detoxify the inner toxin, and affect the “Zuo” drug that reduces or eliminates the possible toxic effects of Jun or Chen. Various herbs work together to replenish the kidney and lower blood lipids. Here, we showed that BSJZF significantly reduced AS symptoms in apoE	extsuperscript{−/−} mice fed a high fat diet by reducing lipid accumulation in the aorta root, enhancing collagen fiber content in the AS plaque, reducing serum levels of TC, TG, LDL-C, TNF-α, and IFN-γ, and elevating serum level of IL-10. The "congenital foundation" Jing in kidney turns to Qi. Therefore, the kidney is the source of Qi. The kidney is the reservoir of Jing and responsible for generating bones and bone marrow that is essential for immune functions. Therefore, the kidney and immunity are closely related. Kidney deficiency and aging lead to immune dysfunction. Kidney-nourishing formulas in TCM delay immune-related aging effectively. Autophagy is the microscopic manifestation of Qi transformation. In the process of autophagy, lysosomes degrade damaged structures and recycle cellular components and energy. Thus, autophagy not only maintains cellular homeostasis, but also eliminates the pathological factors in cells. This is consistent with the concept of Jing-Qi transformation in TCM, in which the Jing-to-Qi transformation promotes removal of endogenous pathological substances, maintains normal life activities, and sustains the balance of Yin and Yang. Moreover, autophagy activity may be reduced by aging, and the regulation of autophagy is tightly associated with aging. Therefore, autophagy can be considered as a feedback mechanism to vital Qi deficiency. Kidney nourishing enhances the activity of autophagy. Because AS is an age-associated autoimmune disease, it is very likely that BSJF treatment prevented AS effectively by enhancing autophagy activity and immune functions. Our study showed that BSJZF group exhibited more autophagosomes in the aorta compared with the model group. In addition, BSJZF treatment increased protein expression of LC3 and Beclin 1 significantly, and decreased protein expression of p62 significantly in the aorta. BSJZF and atorvastatin groups did not show significant differences in phenotypes including histological morphology, serum levels of lipids and inflammatory factors, the amount of autophagosomes, and expression of autophagy-related proteins LC3, Beclin 1, and p62. These results suggest that BSJZF functions similarly to atorvastatin in promoting autophagy and preventing inflammation and AS. Collectively, our results showed that BSJZF effectively inhibited AS possibly by enhancing autophagy. Our results provide solid experimental evidence to develop kidney-nourishing therapy against AS. In conclusion, BSJZF improves the pathological morphology, lipid accumulation, and inflammatory response of apoE	extsuperscript{−/−} mice induced by a high fat diet and enhances the level of autophagy. These data suggest that BSJZF effectively treats AS in apoE	extsuperscript{−/−} mice and enhancement of autophagy may be involved in the mechanism of its action.

REFERENCES

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