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Therapeutic Effect and Mechanism of Action of Abnormal Savda Munziq in Development of Degenerative Atherosclerotic Aortic Valve Disease

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Data Interpretation D
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Background: The aim of this study was to investigate the therapeutic effect of abnormal Savda Munziq (ASMq) on the development of degenerative atherosclerotic aortic valve disease and its underlying mechanisms.


Material/Methods: We randomly divided 80 rabbits into 4 groups: a normal control group (group N, n=20); a high-fat diet group (group HC, n=20); a high-fat diet and Atorvastatin calcium intervention group (group AI, n=20); and a high-fat diet and ASMq intervention group (group MI, n=20). For evaluation of blood lipid profiles, blood samples were collected at week 0 and at the end of week 8. Aortic valve samples were taken at weeks 0, 2, 4, 6, and 8 for atomic force microscopy (AFM) examination of endothelial cell nanostructures, and at week 8 for pathological examinations.

Results: Triglyceride (TG), cholesterol (TC), low-density lipoprotein (LDL), and high-density lipoprotein HDL levels of rabbits in group HC were significantly different from those in group N (P<0.01). TG, TC, LDL, and HDL values of rabbits in group MI were significantly different from rabbits in group HC (P<0.05). Pathological examination revealed that the aortic valves from rabbits in group MI were visibly clear, with strong endothelial cell continuity. No infiltration of macrophages or other inflammatory cells nor subendothelial calcium deposition was found when compared with rabbits in group HC.

Conclusions: ASMq therapy can delay the onset of degenerative calcific aortic valve disease, and its effects are similar to those of Atorvastatin.

MeSH Keywords: **Aortic Valve • Hyperlipidemias • Microscopy, Atomic Force**

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Background

Degenerative valvular heart disease, also known as senile calcific valvular heart disease or senile cardiac calcification syndrome, is the most common valvular heart disease in the elderly. The incidence is rising with the worldwide increase in life expectancy. Gradual but progressive calcification of the aortic valves is characteristic of this disease. The initial stage resembles the pathogenesis of aortic atherosclerosis, which is characterized by damage of the basal layers, inflammation, cell infiltration, lipid deposition, and calcification. The presence of osteopontin (OPN) in the aortic valves indicates pathological calcification. Historically, research, genetic, and clinical evidence indicated that prevention of further calcification of the aortic valves, which leads to aortic stenosis and aortic regurgitation, can be achieved through understanding the pathogenesis and intervention of degenerative calcific aortic valve disease (DCAVD), as well as by understanding controllable risk factors that may lead to DCAVD [1]. Recent studies showed that the efficacy of drug treatment of middle- to late-stage valvular calcification is limited. Although surgery is the treatment of choice, it is often accompanied with high risk and complications. Therefore, early treatment of valvular calcification diseases is needed to improve outcome and rehabilitation rate. Animal studies, retrospective studies, and RAAVE studies have shown that statins can slow the progression of DCAVD [2]. However, 2 large prospective studies – SALTIRE and SEAS – have recently shown that statins do not prevent progression of DCAVD [3,4]. These 2 studies had different objectives from the RAAVE study: the objective of the RAAVE study was to review research usually associated with traditional statin indications, while the objective of the SALTIRE and SEAS studies was to assess possible ethics concerns regarding statin use. The RAAVE study and other retrospective studies included traditional statin indications. Therefore, clinical trials suggest that statin therapy delays the progression of DCAVD only in patients with hyperlipidemia. Pathological findings indicated that changes in DCAVD include activation of inflammatory cells, transformation of mature osteocytes, dysfunction of valvular interstitial cells, and alteration of the structure of the extracellular matrix, leading to calcified nodule formation. These findings led us to believe that lipid metabolism may play an essential role in the development and progression of DCAVD.

Histopathological studies on various stages of DCAVD have shown that disease development is accompanied by irreversible changes in the aortic valves. A number of risk factors for the development of atherosclerosis may also significantly affect the development and progression of DCAVD. Injured endothelial cells may result in lipoprotein deposition within the endothelium, activating the influx of inflammatory cells into the aortic valves. The development of valvular calcification involves multiple factors including lipid oxidation, cell migration

in the fibrosa and inflammatory cytokine release, all of which can significantly influence the calcification process. Therefore, lipid metabolism is an important factor in the pathogenesis and development of DCAVD [5].

In Uygur medicine, the human body is thought to have 4 kinds of body fluid – bile liquid substance, blood substance, mucus substance, and Savda Munziq [6] – which are present throughout human life, are naturally formed *in vivo*, and play key roles in health and disease. They are continuously consumed and produced in the body and are maintained in a delicate equilibrium. Disease is thought to occur when these 4 body fluids are not in proper balance (changes in quantity and quality). Similarly, the abnormal body fluids consist of 4 components – abnormal gallbladder hygroplasm, abnormal blood hygroplasm, abnormal lymphatic temperament, and abnormal Savda, which are present throughout human life, are naturally formed *in vivo*, and play key roles in health and disease. When the 4 abnormal body fluids are not in proper balance (changes in quantity and quality), they may become detrimental to health. ASMq is a mixture of 13 Uighur medicinal herbs: Kiku Ko, celery root, chicory root, moldavica dragonhead seed, fennel flower seed, fennel root bark, camomile, liquorice, citronella, basil fruit, seed of hollyhock, anise fruit, and *Peganum harmala* L [7]. According to the Uygur humoral medicine theory, abnormal Savda has a heavy mass and dense texture and easily attaches to the blood vessel wall, thereby resulting in stasis and obstruction and may eventually lead to refractory diseases [8]. Uygur medical practice has shown that abnormal Savda Munziq (ASMq) significantly affects anti-inflammation and immunomodulation, preventing calcium deposition, and improving blood circulation and anti-platelet aggregation [9]. Previous reports have demonstrated that ASMq plays a role in regulating blood viscosity, anti-calcium deposition, anti-inflammation, and anti-oxidation. Therefore, we established animal models of degenerative atherosclerotic aortic valve disease to determine whether ASMq delays the progression of DCAVD by affecting the development and underlying mechanisms of the disease.

Recent studies have provided some evidence that the pathomechanisms of atherosclerosis and DCAVD may be partly similar and include endothelial damage, accumulation of oxidized low-density lipoproteins, infiltration of monocytes, mast cells, and T lymphocytes associated with activation of local and systemic inflammation [10–12]. However, the final step in atherosclerosis is plaque formation in the intima of the blood vessels, while in DCAVD severe calcification of the aortic valve represents the end-stage of the disease [13,14]. Enhanced fibro-calcification of the valve leaflets limits their mobility and causes stenosis, which leads to high-pressure gradients through the aortic valve. C-reactive protein (CRP) and Interleukin-6 receptor (IL-6R) are biomarkers of inflammation

with predictive value for cardiac events. Wypasek et al. show that DCAVD patients carrying the CRP rs1205T allele and IL-6R Asp358Ala (A>C, rs2228145) are characterized by more severe aortic valve calcification. They also demonstrated that CRP rs1205 C>T polymorphism minor allele is associated with elevated CRP levels in DCAVD patients [15,16]. DCAVD is an active process involving lipid deposition, chronic inflammation, and progressive calcification. In contrast to the early pathological changes of atherosclerosis, the early pathological changes of DCAVD seldom involve smooth muscle cells.

Material and Methods

Drugs and reagents

Atorvastatin calcium, lot number L27840, was manufactured by Pfizer (New York, USA). ASMq comprised a combination of drugs, including *Cordia dichotoma* Forst fruit, *Anchusa italica*, *Adiantum capillus-veneris*, *Saccharum alhagi*, lemon balm, lavender, and humifusa. All herbs were inspected and approved by the Pharmacy Director of the Xinjiang Uygur Autonomous Region People's Hospital pharmacy. Preparation and quality control of ASMq was conducted in strict accordance with the requirements stated in Patent No. C082130082.8. High-fat diet was prepared by mixing 86.5% rabbit base food, 1% cholesterol, 7.5% egg yolk powder, and 5% lard. A Bruker Dimension Icon Atomic Force Microscope was purchased from ASTChina (Beijing, China).

Experimental animals and groups

All animals were purchased from the Xinjiang Medical Laboratory Animal Center. One hundred healthy male New Zealand white rabbits weighing 1.8~2.4 kg were included in the study. Twenty rabbits were pretreated before the start of the experiment and the remaining 80 rabbits were acclimated for 1 week then randomly divided into 4 groups: the normal control group (Group N, $n=20$) was fed regular rabbit pellet food; the high-fat diet group (group HC, $n=20$) was fed high-fat diet; the high-fat diet and Atorvastatin calcium

intervention group (group AI, $n=20$) was fed a high-fat diet and underwent daily gavage of Atorvastatin calcium; and the high-fat diet and ASMq intervention group N (group MI, $n=20$) was fed a high-fat diet and underwent daily gavage of 5.069 g/kg ASMq. Design and implementation of the experiments were audited and approved by the Animal Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University (approval number: IACUC-20130217777).

Experimental methods

- 1) Within-group comparison: N group of blood lipid index had no significant difference before and after the experiment. TC, TG, LDL, and HDL at 8 weeks were significantly higher than before the experiment in group HC, group AI, and group MI ($p<0.05$). The comparison between groups showed that compared with group N, blood lipid level at 8 weeks was not significantly different in group AI and group MI ($p>0.05$), and lipid levels in group HC were significantly higher than in group N ($p<0.05$) (Table 1).
- 2) Pathological examination of aortic valves was performed 8 weeks after the model was established, and the morphological characteristics of hematoxylin and eosin (HE)-stained aortas were observed under a light microscope. Calcium deposition of the aortic valves was evaluated by Alizarin Red staining and immunohistochemistry was conducted using the Envision two-step visualization system [17]. Aortic valves were stained with monoclonal antibodies directed against CD31, CD3 CD68, and OPN to detect expression of corresponding proteins. Aortic valves were collected from experimental animals at week 0, 2, 4, 6, and 8 and evaluated by AFM under $50\times 50\ \mu\text{m}$ and $500\times 500\ \text{nm}$ magnification.

Statistical analysis

SPSS19.0 software was used to analyze the data. The metering data are represented by means \pm standard deviation ($\bar{x}\pm s$). Comparisons between 4 groups were tested by one-way ANOVA and LSD test. $P<0.05$ indicates a statistically significant difference.

Table 1. Comparison of serum lipid levels at baseline and at 8 weeks.

Groups	TC	TG	LDL	HDL
N	3.01 \pm 1.84	1.68 \pm 1.01	1.74 \pm 1.17	0.64 \pm 0.15
HC	24.87 \pm 3.49**	2.86 \pm 1.26**	17.58 \pm 5.84**	3.58 \pm 0.67**
AI	14.38 \pm 3.63#	1.39 \pm 1.08#	7.41 \pm 3.33##	2.05 \pm 0.49##
MI	17.18 \pm 7.88#	0.84 \pm 0.50##	9.09 \pm 3.89##	1.80 \pm 0.52##

** $P<0.01$, highly significant compared with the normal group; # $P<0.05$, significant compared with group HC; ## $P<0.05$, highly significant compared with group HC.

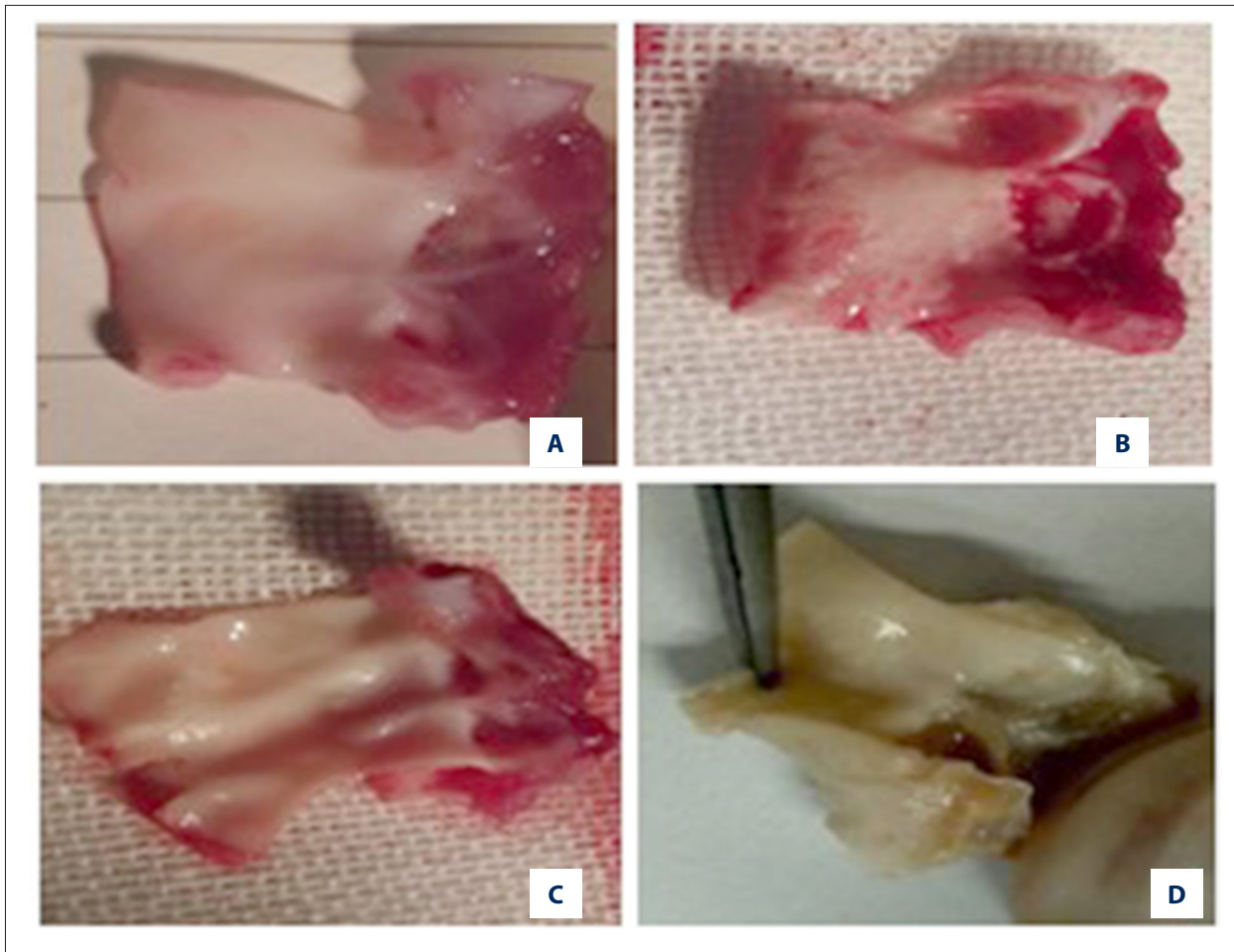


Figure 1. Visual observation of aortic valves from each group. (A) N group; (B) HC group; (C) AI group; (D) MI group.

Results

Changes in blood lipid profiles

No significant changes in blood lipids were observed in group N. However, blood lipid levels of TC, TG, LDL, and HDL were significantly increased at 8 weeks in groups HC, AI, and MI when compared to baseline levels and levels in group N. Blood lipid levels in group AI and group MI were significantly lower than those in group HC ($P < 0.05$), indicating that treatment with Atorvastatin (4 mg/kg/d) and ASMq (5.069 g/kg/d) had significant lipid-reducing effects (Table 1).

Gross examination of samples

The surface of the aortic valves from rabbits in group N appeared smooth and clear without patches or lumps (Figure 1A). Aortic valves of rabbits from group HC appeared thickened and translucent with large white plaques on the surface. Abnormal changes were primarily observed in the valvular base area (Figure 1B). Pathological changes were significantly reduced in

aortic valves from rabbits in group AI, in which only few white plaques on the valvular surface were observed (Figure 1C). Aortic valves from rabbits in group MI showed decreased pathology in the aortic membrane and a small number of white plaques (Figure 1D).

Histology and immunohistochemistry

HE staining

Aortic structures and the endothelial membrane appeared normal in group N (Figure 2A). In contrast, in group HC increased inter-endothelial cell gaps and loss of endothelial continuity were found, as well as widening of interstitial areas, loss of endothelial cells, deposition of foamy-like cells, and significant thickening of the fibrosa (Figure 2B). Although the aortic endothelial cells in group AI appeared intact and orderly, thickening of the fibrosa was observed when compared to group N. However, foam-like cells were significantly reduced compared with those in group HC (Figure 2C). Endothelial cells were absent in group MI; however, the cell count was higher

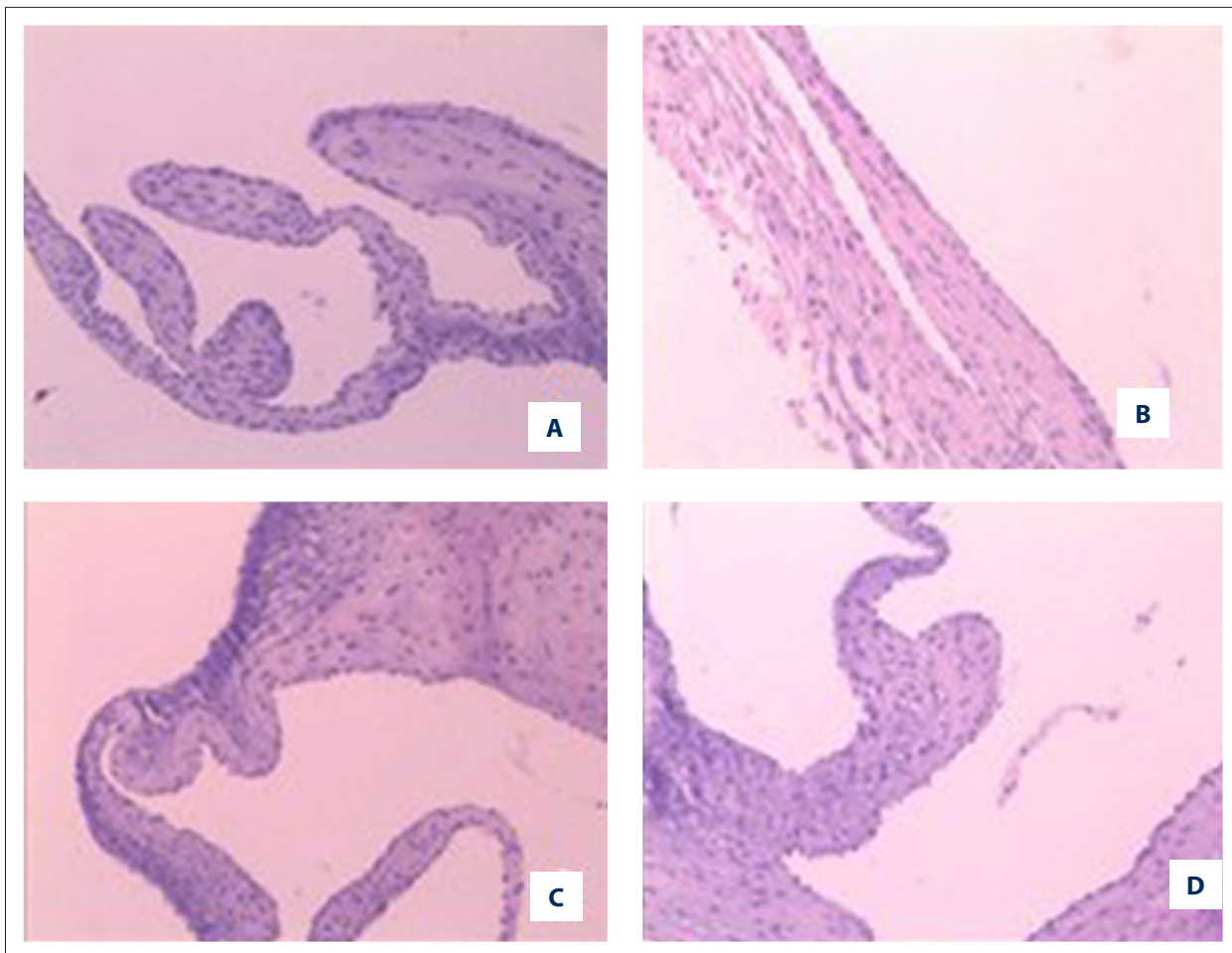


Figure 2. HE staining of the aortic valve in each group. (A) N group; (B) HC group; (C) AI group; (D) MI group.

than in group HC. The subendothelial fibrosa was also thickened, but the number of foam cells was decreased compared with group HC (Figure 2D).

Alizarin Red staining

No significant abnormalities were found in aortic valves from rabbits in group N (Figure 3A). In group HC, few orange-red calcium deposits were visible in the fibrosa of the aortic valves (Figure 3B). No significant calcium deposits were observed around the aortic valves in group AI (Figure 3C) and group MI (Figure 3D).

Immunohistochemistry

No positive staining of CD3, CD31, CD68, or OPN was observed in group N (Figure 4A). In group HC, a small number of CD3-positive T lymphocytes were visible in the interstitium, but most of these cells were located around foam cells. Foam cells found in the subendothelial region were CD68-positive, indicating their mononuclear phagocyte lineage. A small number

of OPN-expressing cells were found in the aortic valves and in the interstitial region. No CD31-positive cells were detected (Figure 4B). In contrast, no CD3, CD31, or CD68 positive staining was detected in group AI except for scattered OPN expression on cells and in the interstitial region of the aortic valves (Figure 4C). Comparably, no positive CD3, CD31, or CD68 staining was observed in group MI except for a small number of OPN-positive cells present in the fibrosa and interstitium of the aortic valves (Figure 4D).

AFM examination of aortic valve endothelial cell structures

Cell morphology examination by 50×50 μm AFM scanning

AFM 50×50 μm scanning showed significant changes in aortic valve endothelial cell morphology in rabbits in group HC after prolonged feeding with a high-fat diet. In addition to losing their normal fence-like arrangement, the disordered endothelial cells also lacked cellular structures and appeared as clusters and clumps. After Atorvastatin calcium intervention, aortic endothelial cells from rabbits in group AI demonstrated some

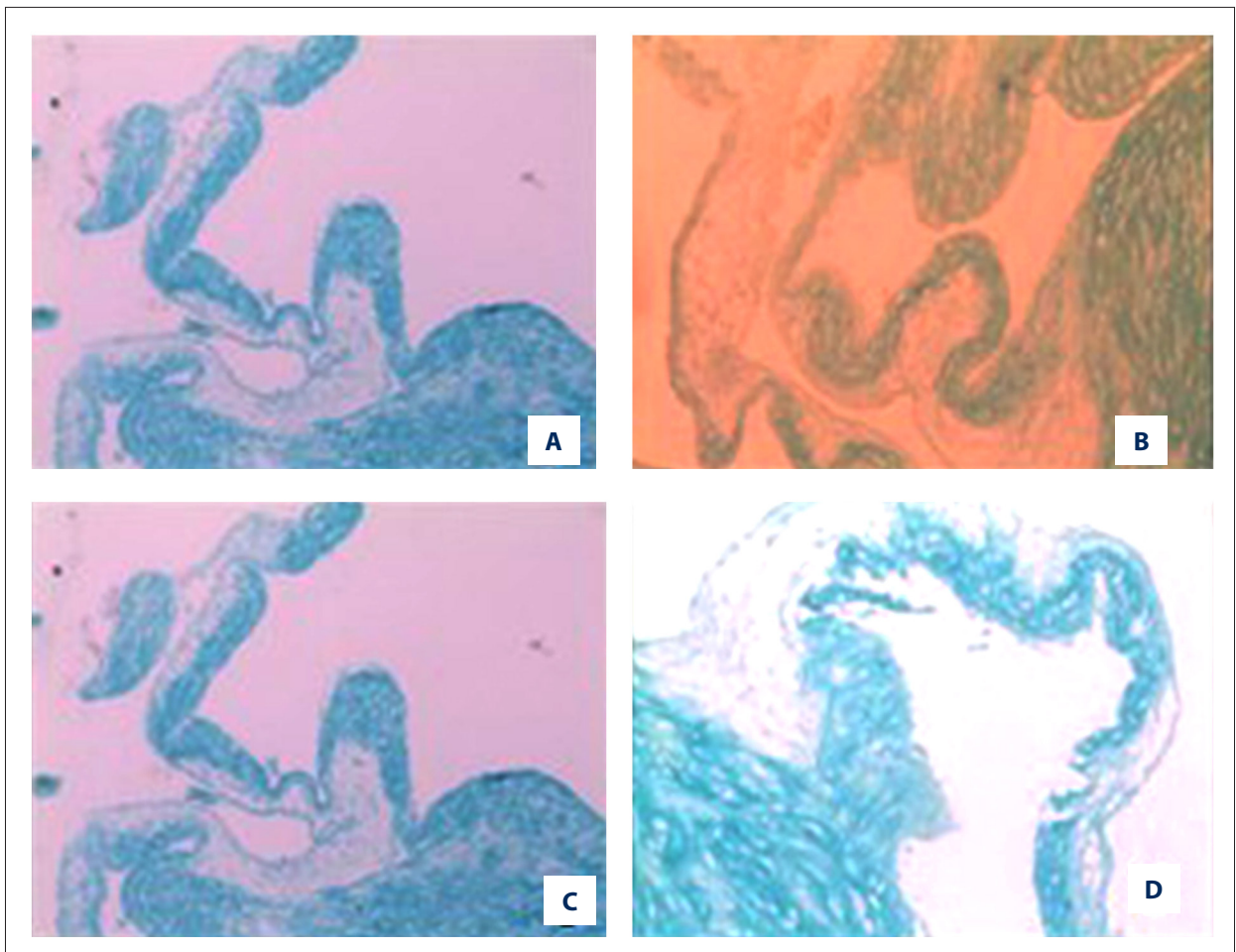


Figure 3. Alizarin Red staining of aortic valves. (A) N group; (B) HC group; (C) AI group; (D) MI group.

morphological abnormalities but maintained the basic fence-like arrangement between weeks 2–8. Similarly, various degrees of morphological abnormalities and reduced endothelial cell counts were observed in group MI at weeks 2–8, but the basic fence-like cell arrangement was maintained (Figure 5).

Morphological observation of cell-surface bulging by 500×500 nm AFM scanning

Examination using 500×500 nm AFM scanning indicated that prolonged high-fat diet resulted in significant structural changes of the surface of aortic valve endothelial cells in group HC, where disorderly scattered spherical bulges were seen. Over time, the inter-bulge gaps fused into sheets, resulting in fewer spherical bulges. Although there was less change in endothelial cell-surface bulges in group AI than in HC group during the same period, cell surface structures in group AI were different from those of group N due to the scattered structures and alignment of surface bulges with increased gaps. The changes in surface bulges in group MI were less prominent than those in group HC, but cellular fusions, loosened arrangement, and

widened gaps between the bulges were more obvious than in group N (Figure 6).

Discussion

In recent years, ASMq therapy of Uygur medicine has not only achieved major breakthroughs, but also established unique theoretical foundations in the fields of respiratory disease, digestive disease, and cancer. Through continued study, research, and establishment of various animal models of disease, we have created a new platform to investigate the efficacy of ASMq in treating cardiovascular diseases. Our work has set a solid foundation for the development of Uygur medicine and provided more treatment options for patients with degenerative atherosclerotic aortic valve disease.

In the current study, we demonstrated that a high-fat diet resulted in significant increase of blood lipid TG, TC, LDL, and HDL levels. Our study indicated that treatment with Atorvastatin (4 mg/kg/d) and ASMq (5.069 g/kg/d) had significant lipid-reducing

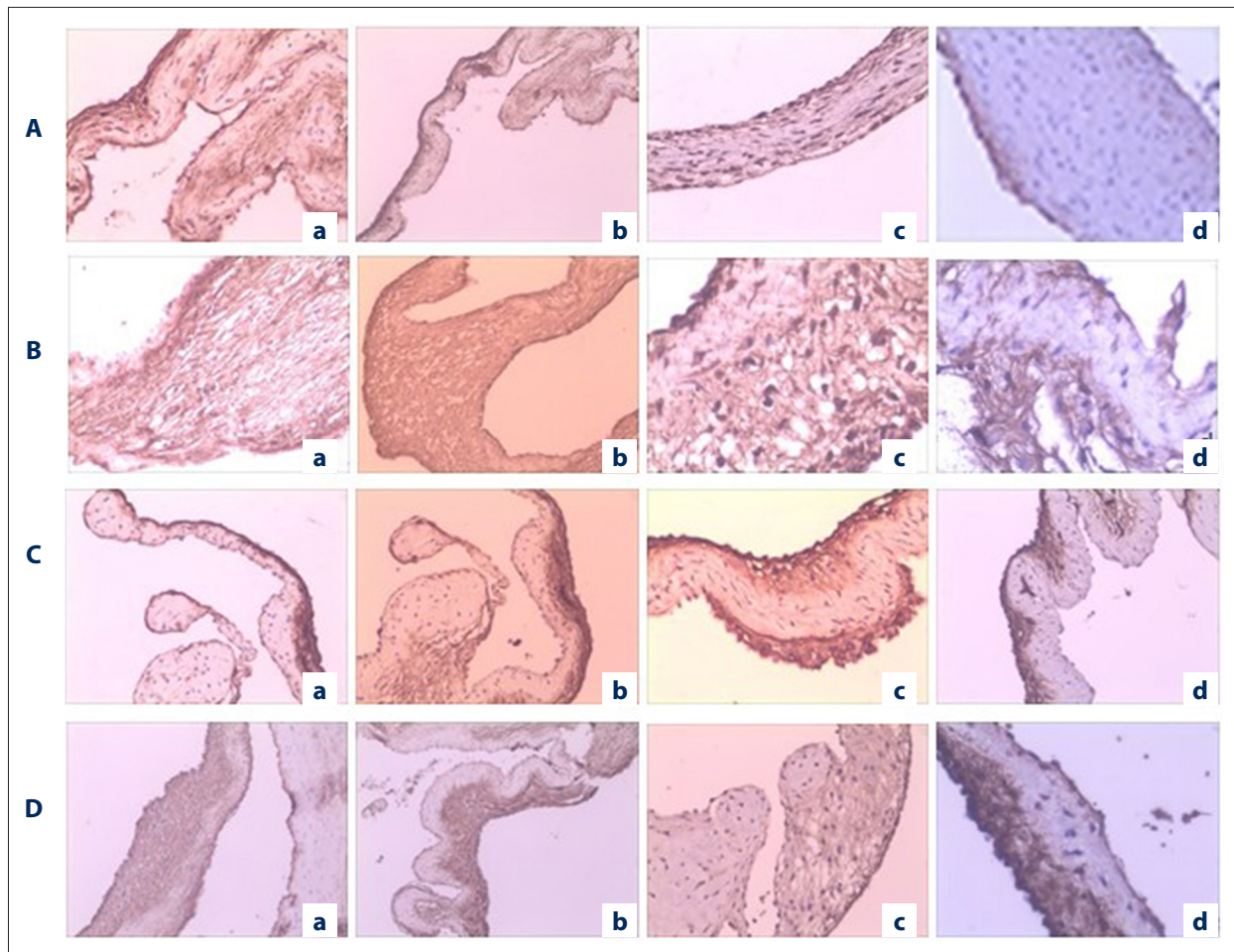


Figure 4. Expression of CD3, CD31, CD68, and OPN in each group. (A) N group; (B) HC group; (C) AI group; (D) MI group. (a) CD3; (b) CD31; (c) CD68; (d) OPN

effects. Pathological examination revealed the presence of aortic valve endothelial cell damage, loss of continuity, and total loss of endothelial cells in group HC. OPN-specific gene expression was demonstrated by the aortic valve interstitial cells. Positive expression of OPN by differentiated osteoblasts may be involved in bone mineralization of the cell [18]. The presence of subendothelial calcium deposition and infiltration of T lymphocytes, macrophages, and other inflammatory cells may be associated with the inflammatory responses within the aortic valves. Therefore, we suggest that there may be a significant correlation between hyperlipidemia and aortic valve calcification. Our study results are similar to those of other studies [19,20].

We also showed that aortic valve endothelial cell continuity was maintained in group AI and group MI, along with significantly reduced valvular interstitial OPN expression. Because we found no significant calcium deposition or presence of T lymphocytes, macrophages, and other inflammatory cells in group AI and group MI, we speculate that Atorvastatin and

ASMq can protect aortic valves by interfering with aortic inflammation induced by a high-fat diet.

Due to its high resolution, simple operation, and accurate observations of biological surface structures, AFM is widely used in biomedical fields [21,22]. AFM works by detecting changes of interaction between the probe and sample surface. In our study, we assessed the impact of high-fat diet on structural changes of aortic valve endothelial cells and examined the morphological changes in early stages of DCAVD through AFM analysis of endothelial cells and their surface nanostructures.

AFM scanning showed that endothelial cells in different parts of the aortic valve were significantly different in their surface structure and alignment. Hyperlipidemia may affect the base region of aortic valves and is therefore an important contributing factor for DCAVD. In this study, observations of the basal, middle and apical regions of the aortic valve were consistent with those reported in a previous study. Therefore, we

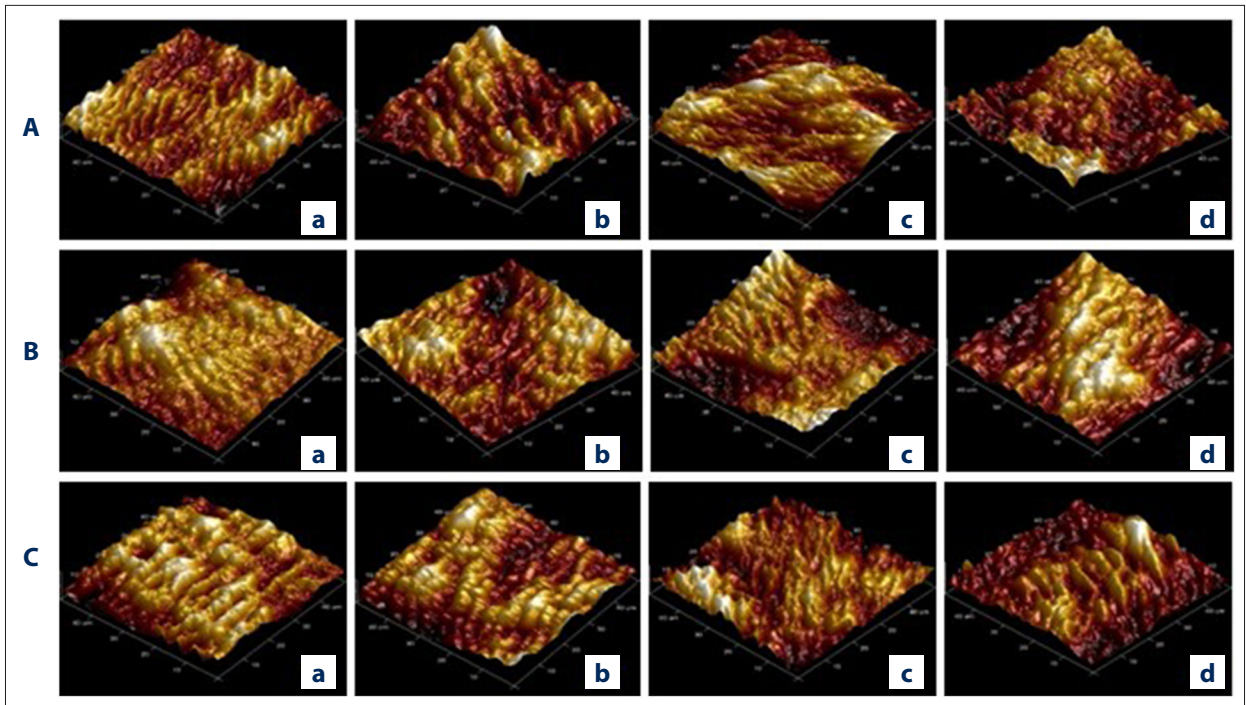


Figure 5. Changes of aortic endothelial cells over time in each group. (A) HC group; (B) AI group; (C) MI group. (a–d) represent weeks 2, 4, 6, and 8, respectively.

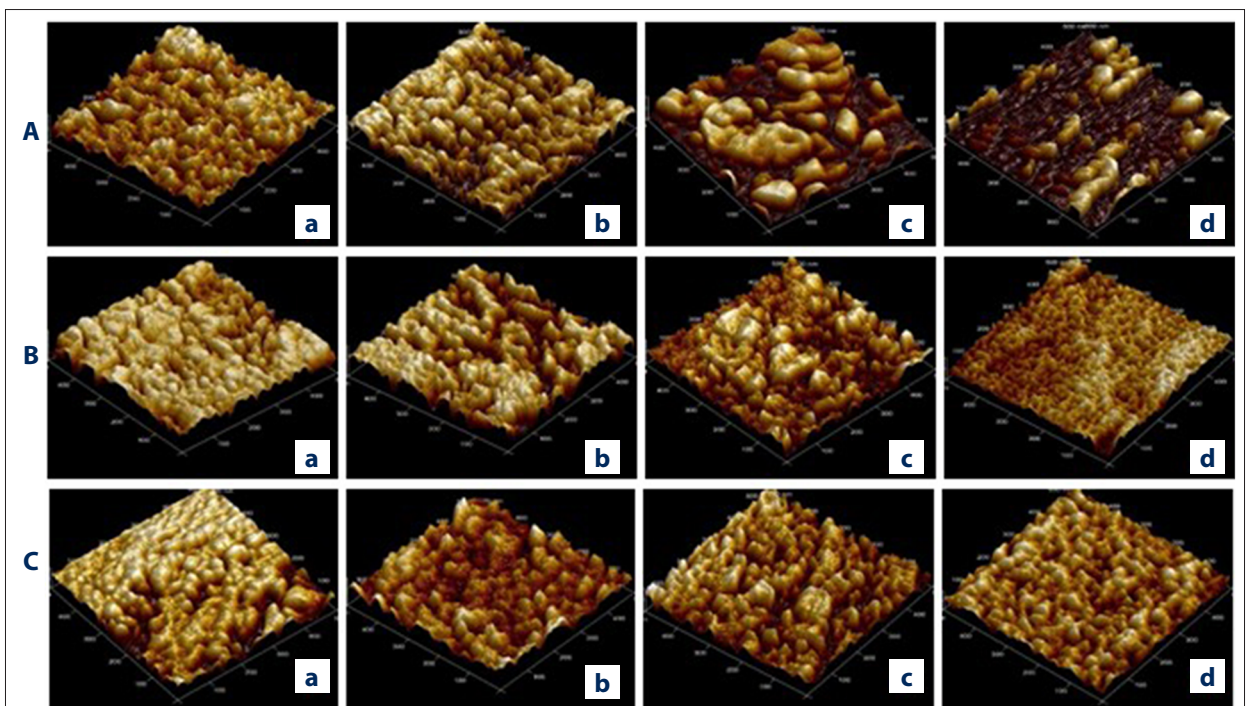


Figure 6. Changes in aortic valve endothelial cell surface over time in each group. (A) HC group; (B) AI group; (C) MI group. (a–d) represent weeks 2, 4, 6, and 8, respectively.

examined the changes on the surface structures of basal endothelial cells of aortic valves to test our hypothesis.

Results from 50×50 μm and 500×500 nm AFM scanning revealed that endothelial cells in different anatomical sites of the same aortic valve differed in their cellular alignment and surface nanostructures. Compared to middle and apical aortic valve endothelial cells, basal endothelial cells were more abundant and were arranged more tightly and orderly, with relatively uniform surface bulges. In addition, aortic valve endothelial cell length gradually reduced as the high-fat diet continued, resulting in merging and partial loss of endothelial cells. The number of endothelial cell surface bulges also gradually decreased, along with increased bulge diameter, reduced bulge height, and fusion of bulges into sheets. By comparing the changes in morphology observed via AFM, we conclude that Atorvastatin and ASMq can maintain the basic normal structures of aortic valve endothelial cells.

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Conclusions

ASMq can effectively protect aortic endothelial cells by decreasing blood lipid levels, reducing damage to aortic valve endothelial cells. ASMq therapy can delay the onset of “degenerative” atherosclerotic aortic valve disease and its treatment effects are similar to those of Atorvastatin.

Study limitation

This study has several limitations. First, our group of healthy subjects was rather small. Second, DCAVD and atherosclerosis have similar risk factors associated with age, hypertension, high cholesterol levels, and inflammation regulation, but we did not assess CRP or IL-6 levels in blood. Third, statins affect lipids and inflammation, but we did not measure CRP, IL-6 levels, or inflammatory markers in blood.

Disclosure of conflict of interest

None.