Abstract. – OBJECTIVE: To study the improving effect of atorvastatin on plaque stability in diabetes mellitus (DM) mice complicated with atherosclerosis.

MATERIALS AND METHODS: Apolipoprotein E (ApoE)-/- mice were used to establish the DM mouse model. Half of the mice received atorvastatin after successful modeling. ApoE-/- and C57BL/6J mice were used as controls. Oil red O staining and Masson staining were performed to detect the lipid and collagen components in mice. Immunohistochemical assay was used to observe the expressions of smooth muscle cell (SMC) and Ly-6c. The expressions of receptor for advanced glycation end products (RAGE), monocyte chemoattractant protein-1 (MCP-1) and nuclear factor-κB (NF-κB) in tissues were detected by Western blotting. Finally, the levels of serum soluble RAGE (sRAGE), advanced glycation end products (AGEs), malondialdehyde (MDA) and reduced glutathione (GSH) in mice were also detected.

RESULTS: Atorvastatin reduced the area of atherosclerotic plaque and improved the stability of arterial plaque through reducing lipid deposition, the number of macrophages and SMC, increasing collagen fibers. In mice in atorvastatin group, the levels of serum AGEs and sRAGE were decreased. Moreover, atorvastatin inhibited the downstream pathway of RAGE as well as DM, thus inducing the oxidative stress.

CONCLUSIONS: Atorvastatin improves plaque stability in diabetic atherosclerosis through the RAGE pathway.

Key Words: Atorvastatin, Atherosclerosis, RAGE.

Introduction

Diabetes mellitus (DM) is a kind of systemic disease that affects the quality of life and longevity of patients, and atherosclerosis is a major complication of type 1 and type 2 DM. Up to 75% patients diagnosed with DM eventually die of atherosclerosis-related cardiovascular diseases, such as myocardial infarction (MI), peripheral arterial disease and stroke. Both occurrence rate of cardiovascular events and death rate in DM patients complicated with coronary heart disease are significantly higher than those in non-DM patients. During the occurrence and development processes of atherosclerosis, the rupture of unstable atherosclerotic plaques can lead to acute coronary syndromes, such as MI. The pathological features of vulnerable plaques include the central lipid core area covered by thinner fibrous tissues and the infiltration of a large number of inflammatory cells.

During the course of DM, a variety of risk factors, including hyperglycemia, hyperlipidemia and disorders of angiotensin system, are associated with atherosclerosis. In recent years, studies have shown that oxidative stress and advanced glycation end products (AGEs) play important roles in the occurrence and development of vascular lesions of DM. AGEs interact with receptor for AGEs (RAGE), a receptor specific for vascular wall cells, to activate oxidative stress.

RAGE is a member of the cell surface molecule immunoglobulin superfamily and a multi-ligand receptor for vascular cells, which plays a key role in the inflammatory process. In addition to AGEs, other cytokines and inflammatory factors can also up-regulate the RAGE expression. The activation of AGEs-RAGE pathway will increase the level of oxidative stress in vivo, inducing the production of inflammatory factors, leading to vascular endothelial cell injury and apoptosis, and aggravating the occurrence and development of atherosclerosis.

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Atorvastatin, as a 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitor, is widely used in the treatment of patients with atherosclerosis. Statins can alleviate the coronary artery inflammatory changes and improve the plaque instability, thus reducing the mortality and morbidity rates of cardiovascular disease in DM patients. In addition, statins have many other effects, including anti-inflammation, anti-oxidation and improvement of vascular endothelial function. Non-antihyperlipidemic effects of these statins have also become research hotspots.

Materials and Methods

Animals and Reagents
A total of 60 clean-grade adult male apolipoprotein E (ApoE)−/− mice aged 8 weeks weighing (24.20 ± 0.50) g, and 20 C57BL/6J mice aged 8 weeks weighing (17.20 ± 0.48) g were provided by the Laboratory Animal Center of Nanjing University (Nanjing, China). Nutritional status and mental status of all animals were normal. This investigation was approved by the Animal Ethics Committee of Nanchang University Animal Center. Monocyte chemoattractant protein-1 (MCP-1) and nuclear factor-κB (NF-κB) antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). Oil red O, hematoxylin-eosin (HE) and Masson staining reagents and triglyceride, low-density lipoprotein, high-density lipoprotein, cholesterol, superoxide dismutase (SOD) and glutathione (GSH) enzyme-linked immunosorbent assay (ELISA) kits were purchased from Beyotime (Shanghai, China). Ly-6c and smooth muscle cell (SMC) antibodies were bought from CST (Danvers, MA, USA).

Modeling and Grouping
After 60 ApoE−/− mice were used for 2 d, 40 mice were randomly selected to receive intraperitoneal injections of sodium (120 mg/kg) and streptozotocin (STZ) (100 mg/kg) to induce the mild DM model. After 1 week, mice showed mild DM symptoms, and they were randomly divided into two groups with 20 mice in each group. Mice in DM + atorvastatin group received atorvastatin (10 mg/kg/d) intragastrically, while those in DM group received no treatment. In addition, 20 ApoE−/− mice were used as ApoE−/− control group, and 20 C57BL/6J mice as blank control group. All mice were fed with high-fat diet supplemented by 0.25% cholesterol and 20% lard oil with relative humidity of (50 ± 15) % at (21 ± 2°C) in 12-h light/dark cycle. Mice were executed after 20 weeks for further data analyses.

HE, Masson and Oil Red O Staining
Mice were fasted for 12 h and anesthetized, and the chest was opened to expose the heart, which, with the aortic root and aorta, were dissected, rinsed in phosphate buffered saline (PBS) at 4°C and fixed with 4% paraformaldehyde. Tissues were embedded into paraffin, and paraffin-embedded tissues were serially sliced into 4 cm-thick sections and placed at 60°C overnight, followed by dewaxing via xylene, dehydration via gradient alcohol, HE staining, Masson staining and oil red O staining. Three cross profiles of each section were detected using the medical image analysis software (Image-Pro Plus IPP, Mediaplayer, Silver Springs, MD, USA).

Immunohistochemical Staining
After paraffin-embedded arterial tissue sections were dewaxed via xylene, dehydrated via gradient alcohol and incubated with warm deionized water containing 0.3% H2O2 for 30 min, the endogenous peroxides were removed, and sections were sealed with serum and added with primary antibody at 4°C overnight. On the next day, immunoglobulin G (IgG) antibody-horseradish peroxidase (HRP) were added for incubation. Sections were incubated in the mixed solution prepared using the avidin-biotin complex (ABC) kit, followed by color development reaction via diaminobenzidine (DAB) for 10 min, hematoxylin re-staining, washing, dehydration, transparency and observation under an optical microscope (Olympus Optical Co., Ltd, Tokyo, Japan).

General Aorta Staining
Mice were fasted for 12 h and anesthetized, and the chest was opened to expose the heart, which, with the aortic root and aorta, were dissected, rinsed in phosphate buffered saline (PBS) at 4°C and fixed with 4% paraformaldehyde. After oil red O staining, the aorta was paved onto a wax plate under a microscope, and fixed with nails. After photography using a charge-coupled device (CCD) camera, images were integrated using Adobe Photoshop Version 7.0 (Adobe, San Jose, CA, USA), followed by calculation via ImageJ (Rawak Software, Inc., Hamburg, Germany).
**Western Blotting**

Tissues extracted were ground via liquid nitrogen, diluted with normal saline and placed on ice, the supernatant was taken using centrifugation at 4°C for 5 min. Then, the supernatant was discarded. The sediment was resuspended by radioimmunoprecipitation assay (RIPA) lysis solution containing phenylmethanesulfonyl fluoride (PMSF) and centrifuged at 16,000 x g and 4°C for 15 min. The supernatant was taken for protein quantification. The protein was added into loading buffer, heated and denatured, followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and membrane transfer. The membrane was sealed with 5% skim milk for 2 h, added with primary antibody for incubation at 4°C overnight and washed with Tris-buffered saline and Tween 20 (TBST) for 3 times (10 min/time). The corresponding secondary antibody was added for incubation at room temperature for 1 h; the membrane was washed again with TBST for 3 times (10 min/time), and the protein expressions in different samples were detected using the enhanced chemiluminescence (ECL) method.

**Statistical Analysis**

Data were presented as mean ± standard deviation and analyzed using paired or unpaired t-test. One-way analysis of variance was used for the comparisons among groups, and Student-Newman-Keuls (SNK) post-hoc test was used for pairwise comparisons. p < 0.05 suggested that the difference was statistically significant. Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) was used for data analysis, and GraphPad software (Version X; La Jolla, CA, USA) was used for drawing.

**Results**

**Atorvastatin Improved Atherosclerosis**

The aorta specimen in each group was treated with oil red O staining, and the areas of atherosclerotic plaque in the four groups were compared to observe the effect of statins on the atherosclerotic plaque size in DM mice. Figure 1A shows the oil red O staining of aorta specimens in the four groups of mice. Figure 1B shows the proportion of oil red O staining area in artery area. Almost no plaque was observed in the wild-type mice. In the aorta of untreated ApoE−/− mice, atheromatous plaques stained by oil red O were observed in the aortic arch, thorax and abdominal aorta, accounting for about 15%, while atheromatous plaques accounting for about 25% in STZ-treated ApoE−/− mice. In addition, the plaque area was reduced to 14% in atorvastatin group, which was similar to that in non-DM ApoE−/− mice. HE staining was performed for aorta sections (Figure 1C-D). There was almost no arterial plaque in mice in control group. In ApoE−/− mice, the area of atherosclerotic plaque accounted for about 25% of the aortic root, which was larger than that in DM group (30%). The fibrous cap of arterial plaque was thinner and the central necrosis area was larger in DM group, which, however, was improved in atorvastatin group, and the proportion of plaque area was also reduced to 21%. Statins reduced the size and composition of atherosclerotic plaques in DM mice. As shown in Table I, the body weight, triglyceride, cholesterol, high-density lipoprotein and low-density lipoprotein levels of mice in each group were also detected. Results showed that low-dose atorvastatin did not significantly reduce the lipid level in mice, and the effect of atorvastatin on atherosclerosis was independent of its lipid-lowering effect.

**Atorvastatin Improved the Stability of Arterial Plaque**

To further study the effect of statin on the stability of aortic plaque, the aortic root tissues of mice were further stained with oil red O (Figure 2). A large amount of lipid deposition could be seen in the artery of mice in DM group, while lipid deposition was improved in atorvastatin group. The production of inflammation is thought to be an important development process of atherosclerosis, and Ly-6c in macrophages is used as an index of detecting atherosclerotic inflammatory response. The number of macrophages in DM group was significantly increased compared with that in control group, while it was reduced by atorvastatin, suggesting that atorvastatin alleviates the degree of inflammation. In Masson staining, it was observed that the number of collagen fibers in arterial plaque was reduced in DM group, while it was significantly increased in atorvastatin group. The plaque stability mainly depends on the content of lipids, macrophages, SMCs and collagen. Therefore, STZ-induced hyperglycemia accelerates atherosclerosis, and atherosclerotic plaques are more vulnerable. The above phenomena of mice in atorvastatin group were improved, and the stability of atherosclerotic plaque was increased.
Atorvastatin improves plaque stability in diabetic atherosclerosis through the RAGE pathway

Atorvastatin Decreased AGEs-RAGE Expression

During the occurrence of DM, oxidative stress existed and AGEs were formed in addition to the increase in blood glucose. AGEs played roles through AGEs-RAGE pathway. The levels of AGEs and soluble RAGE (sRAGE) in mice were measured, and the level of RAGE in the aorta was also detected (Figure 3). The levels of serum AGEs and sRAGE in DM group were significantly increased, while they were decreased in atorvastatin group. The level of RAGE in the aorta in DM group was increased, but atorvastatin also decreased the expression of RAGE in the aorta, which was consistent with serological results.
Atorvastatin Inhibited the Downstream Pathway of RAGE as Well as DM Induced Oxidative Stress

As a downstream pathway of AGE-RAGE, MCP-1 also plays an important role in the recruitment and activation of monocytes. To further investigate the effect of statin on RAGE pathway, the MCP-1 expression was measured. As shown in Figure 4A-B, the expression of MCP-1 was significantly up-regulated in the aorta of DM mice, but it was decreased in atorvastatin group. NF-κB is a core factor in the activation process of oxidative stress pathway, and the total serum SOD and GSH levels can reflect the oxidative stress level in mice. The protein level of NF-κB in the aorta of mice was measured, and it was found that the protein level of NF-κB was increased in DM group, while atorvastatin could reduce NF-κB in the aorta of DM mice (Figure 4C-D). Meanwhile, in this experiment, the oxidative stress level in mice during the incidence of DM was increased significantly, and it was also observed that the serum malondialdehyde (MDA) level in mice was increased, but the GSH level was decreased. The serum MDA level in mice in atorvastatin group was decreased, but the GSH level was increased compared with those in DM group, indicating that atorvastatin reduces the oxidative stress level in DM mice (Figure 4E-F).
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**Discussion**

Atorvastatin, as a HMG-CoA reductase inhibitor, is widely used to lower the serum cholesterol level. Atorvastatin reduces total cholesterol and low-density lipoprotein cholesterol, thus reducing the risk and long-term morbidity and mortality rates of coronary heart disease. Besides, atorvastatin also has other functions than anti-hyperlipidemic effect, such as anti-inflammation and protection of endothelial cell function. We also found that low-dose atorvastatin did not significantly improve the blood lipid level in mice, and its effect of stabilizing atherosclerotic plaques was independent of its lipid-lowering effect.

We used immunohistochemical staining to observe the lesion area in atherosclerotic plaques and the expressions of macrophages, SMCs and collagen. It was found that atorvastatin treatment could increase the plaque collagen content. Previous research results have shown that the increase of collagen plays an important role in reducing the risk of plaque progression, thus improving the outcome of atherosclerotic diseases. Moreover, it was observed that the number of macrophages in plaques in atorvastatin group was significantly reduced compared with that in DM group, which is also consistent with the decreased MCP-1 content in the aorta. The decrease of macrophages can reduce the formation of foam cells as well as the deposition of cholesterol. Therefore, atorvastatin can improve the stability of atherosclerotic plaques.

AGEs play important roles in the occurrence and development of vascular complications of DM, which bind to the receptor on vascular wall, thereby depositing at the high-risk site of atherosclerosis. The interaction of AGES-RAGE can be blocked by sRAGE. These sRAGEs can bind to AGES and exert the endogenous protection effect to resist atherosclerosis. This work confirmed that in DM group, the levels of serum AGES, sRAGE and RAGE protein expression in...
vascular wall were increased. However, atorvastatin could reduce RAGE protein in the artery and serum sRAGE level.

During the occurrence of vascular inflammation, the recruitment and aggregation of blood mononuclear cells are important initiating links. RAGE can mediate the activation of MCP-1, recruit mononuclear macrophages, and initiate the inflammatory response in vivo, thereby increasing the cell apoptosis and accelerating the occurrence and development of atherosclerosis\(^1\). Through measurement of MCP-1, it was found that the anti-inflammatory effect of atorvastatin also reduced the monocyte aggregation via decreasing MCP-1 expression. The activation of RAGE is also proved to be associated with the activation of NF-κB pathway. The RAGE gene promoter contains three putative NF-κB-like binding sites, thereby stimulating the production of inflammation and increasing the oxidative stress level in vivo\(^1\). NF-κB activated by RAGE can further increase the expression of RAGE in inflammatory
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cells, forming a positive feedback. In this work, NF-kB in the artery and serum oxidative stress level in mice treated with atorvastatin were significantly decreased, suggesting that atorvastatin, in addition to the anti-oxidative stress effect, has the potential to break the positive feedback path produced by oxidative stress.

Conclusions

We suggest that atorvastatin improves oxidative stress level and reduces inflammatory response in DM mice through inhibiting AGEs-RAGE pathway and blocking downstream signals of RAGE, such as MCP-1 and NF-kB, thereby improving arterial plaque stability and reducing risks of cardiovascular diseases.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References