

Original Article

Rosuvastatin attenuates the progression of atherosclerotic plaque formation in ApoE^{-/-} mice

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Received August 18, 2016; Accepted May 12, 2017; Epub June 15, 2017; Published June 30, 2017

Abstract: The present study was conducted to investigate the effects of rosuvastatin on the atherosclerotic plaque formation and their underlying molecular mechanisms. Male Apolipoprotein E gene knockout (ApoE^{-/-}) mice fed high-fat diet were given saline or rosuvastatin for 8 weeks, respectively. Rosuvastatin significantly ameliorated the progression of atherosclerosis lesion formation without changes in body weight, blood pressure and heart rate, but with significant reductions in plasma total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C). Furthermore, rosuvastatin attenuated the MOMA2-labeled macrophage infiltration, and the secretion of vascular endothelial growth factor (VEGF) and placenta growth factor (PLGF) in atherosclerotic plaque. The results indicate that rosuvastatin impedes the progression of atherosclerosis plaque formation via reducing lipid accumulation and down-regulations of VEGF, VEGFR and PLGF.

Keywords: Atherosclerosis, apolipoprotein E, atherosclerotic plaque, VEGF, PLGF

Introduction

Atherosclerosis is characterized to be a polygenic pathology progressing of blood vessels, among which chronic inflammation plays a critical role in the formation of an atheromatous plaque in the aortic artery [1]. Atherosclerosis is the crucial contributor to cardiovascular diseases such as coronary heart disease and myocardial infarction, which is also considered as the leading cause of cardiovascular death [2]. Therefore, the prevention and treatment of atherosclerosis may be propitious to the prognosis of the subjects with cardiovascular diseases [3].

Lipometabolic disorder is one of the important events in the formation of atherosclerotic lesions [4]. The increased levels of total cholesterol (TC), triglyceride (TG), low-density lipoprotein-cholesterol (LDL-C) are present in the experimental atherosclerosis rats [5]. The dysregulated lipid metabolism is a hallmark of atherosclerosis, and the abnormal accumulation of TC, TG and LDL-C acts as a stress signal to accelerate the development and progression of atherosclerotic plaque [6]. Macrophages infil-

tration is pivotal to the development of atherosclerosis associated with the deterioration of chronic lipid-induced inflammation [7]. Activation of macrophages aggravates the uptake of LDL-C and induces the disruption in cellular cholesterol homeostasis, which stimulates the regression of foam cells in the artery [8]. High fat diet-fed ApoE^{-/-} mice exhibit the macrophage infiltration in the vessel wall during the progress of atherosclerosis [9]. Vascular endothelial growth factor (VEGF) family and placenta growth factor (PLGF) are two key factors for the physiological and pathological conditions of atherosclerosis [10]. VEGF-VEGFR system is involved in angiogenesis via modulation of non-inflammatory and inflammatory responses in atherosclerotic plaque, and it is an important target for suppressing vascular diseases including atherosclerosis [11]. PLGF is identified as a proinflammatory factor, and recognized as a potential therapeutic target in atherosclerosis [12].

Rosuvastatin is reported to retard the progression of atherosclerosis lesion formation via downregulation of pro-inflammatory cytokines and chemokines [13]. Long-term administration

Table 1. Body weight, blood pressure and heart rate in different groups after 8 weeks

	Body Weight (g)	SBP (mmHg)	DBP (mmHg)	HR (beat/min)
Saline	29±2.34	110±4.22	80.5±6.51	576.1±41.20
Rosuvastatin	30.37±1.56	109.2±5.63	78.8±3.34	557.2±9.30
t	0.071	0.135	0.274	0.092
P	0.721	0.336	0.112	0.683

Note: SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate. These parameters were analyzed by Student's-t test.

Table 2. TC, TG and LDL in different groups after 8 weeks

	TC (mmol/L)	TG (mmol/L)	LDL-C (mmol/L)
Saline	29.31±3.82	1.71±0.35	26.83±4.71
Rosuvastatin	17.94±4.07*	1.04±0.17*	13.62±4.39*
t	8.893	3.568	4.536
P	P < 0.001	0.017	0.011

Note: Values are mean ± SD. *P < 0.05 vs. Saline. n=20 for each group. TC, total cholesterol; TG, triglyceride; LDL-C, low density lipoprotein cholesterol. These parameters in different groups were analyzed by Student's-t test.

of rosuvastatin significantly improved the arterial stiffness and blood pressure in patients with inflammatory joint diseases [14]. In addition, rosuvastatin treatment depressed the macrophage infiltration, lipid deposition, proinflammatory cytokines levels in atherosclerotic plaques in rabbits [15]. Quantitative coronary angiography reveals that rosuvastatin therapy prevents coronary plaque progression in patients who undergo elective percutaneous coronary intervention [16]. In the present study, we investigated the effect of rosuvastatin on the progression of atherosclerosis lesion formation, and we also explored whether decreased lipid metabolism, macrophage infiltration and release of inflammatory mediators were responsible for the protective role of rosuvastatin during the progress of atherosclerosis.

Materials and methods

Animals

All of the procedures and protocols were conformed to Good Publishing Practice in Physiology (Persson & Henriksson 2011). The study was approved by the Animal Care Committee of the Second Affiliated Hospital of Shandong University of Traditional Chinese

Medicine and all the animal experiments were followed by the guidelines of Animal Management Rules of the Chinese Ministry of Health. Male Apolipoprotein E gene knockout mice (ApoE^{-/-}) aged 8-week-old were purchased from Peking University Animal Center (Beijing, China). The mice were housed in cages with a temperature-controlled and humidity-controlled room with free access to standard chow and tap water under a 12-h light/dark cycle.

Experimental design

Eighty male ApoE^{-/-} mice (age, 8 weeks) were fed a high cholesterol diet containing 10% fat, 1% cholesterol and 0.25% bile salt (Beijing science and Technology Co., Ltd. Beijing, China) for 12 weeks [17]. All the mice were randomly divided into two groups (n=20 for each group), which were subjected to intragastric administration of normal saline (0.9%, 0.1 ml/day) and rosuvastatin (AstraZeneca, UK, 1.5 mg/kg/day, 0.1 ml/day) treatment for 8 weeks. At the end of 8 weeks, the mice were killed with an overdose of pentobarbital sodium (50 mg/kg). Specimens for serum, aortic root, and aorta were collected.

Measurement of body weight and blood pressure

The body weight in different groups after 8 weeks was recorded. The systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate were measured with a computerized tail-cuff system (BP-2000, Visitech Systems, Apex, NC) in conscious animals as previous report [18].

Plasma lipids measurement

The plasma samples were collected from all ApoE^{-/-} mice and stored at -80°C as previously

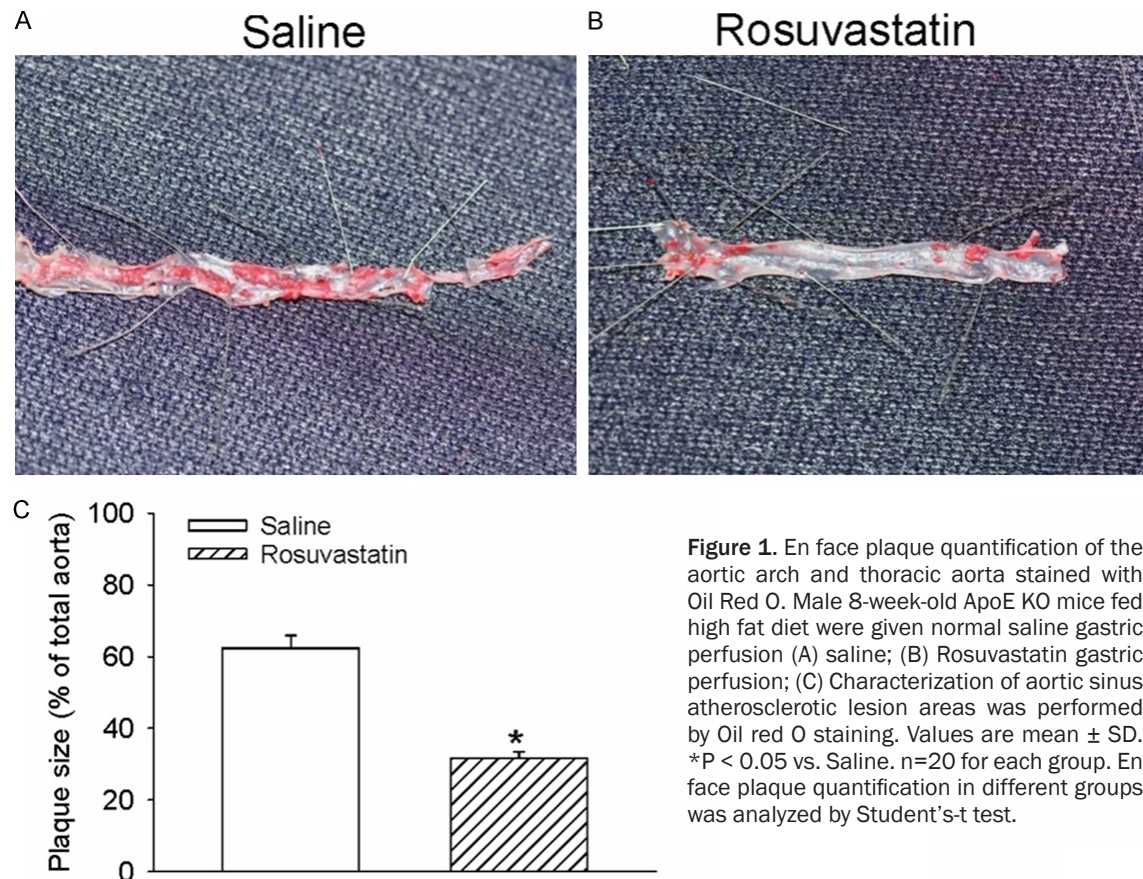


Figure 1. En face plaque quantification of the aortic arch and thoracic aorta stained with Oil Red O. Male 8-week-old ApoE KO mice fed high fat diet were given normal saline gastric perfusion (A) saline; (B) Rosuvastatin gastric perfusion; (C) Characterization of aortic sinus atherosclerotic lesion areas was performed by Oil red O staining. Values are mean \pm SD. *P < 0.05 vs. Saline. n=20 for each group. En face plaque quantification in different groups was analyzed by Student's-t test.

described [19]. The total cholesterol (TC), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-C) were determined by a colorimetric enzymatic assay system (Roche Modular P-800; Roche Diagnostics, Basel, Switzerland).

Enzyme-linked immunosorbent assay (ELISA) assay

The plasma levels of vascular endothelial growth factor (VEGF) and placenta growth factor (PLGF) were measured with the aid of commercial ELISA kits according to the manufacturer's protocols. The ELISA kit for VEGF was purchased from Cusabio Biotech Co., Ltd. (Wuhan, China). The ELISA kit for PLGF was obtained from R&D Co., Ltd. (Los Angeles, CA, USA).

Oil red O staining

Following 8 weeks of treatment, all mice were sacrificed with an overdose of pentobarbital sodium (50 mg/kg). The mice were perfused by

phosphate-buffered saline (PBS) and 4% (w/v) paraformaldehyde. The surrounding fat and connective tissue of aorta and heart were carefully removed under a stereomicroscope. The complete aorta was excised from the aortic arch to the common iliac artery, and then stained with Oil-red O (Sigma-Aldrich, St. Louis, MO, USA) as previously described. In addition, 5- μ m paraffin-embedded cross-sections of the aortic root were subjected to Oil Red O staining for subsequent histological examination. The photographs of Oil Red O staining were collected with a Leica DMI6000 microscope (Leica Microsystems, Wetzlar, Germany). Image Pro Plus analysis software was employed to assess the percentage of the plaque area indicated by percentage of the plaque area consisting of aortas [20].

Hematoxylin-eosin staining

5- μ m paraffin-embedded cross-sections of the aortic root were prepared for hematoxylin-eosin (HE, Beyotime Institute of Biotechnology, Shanghai, China) staining in order to determine

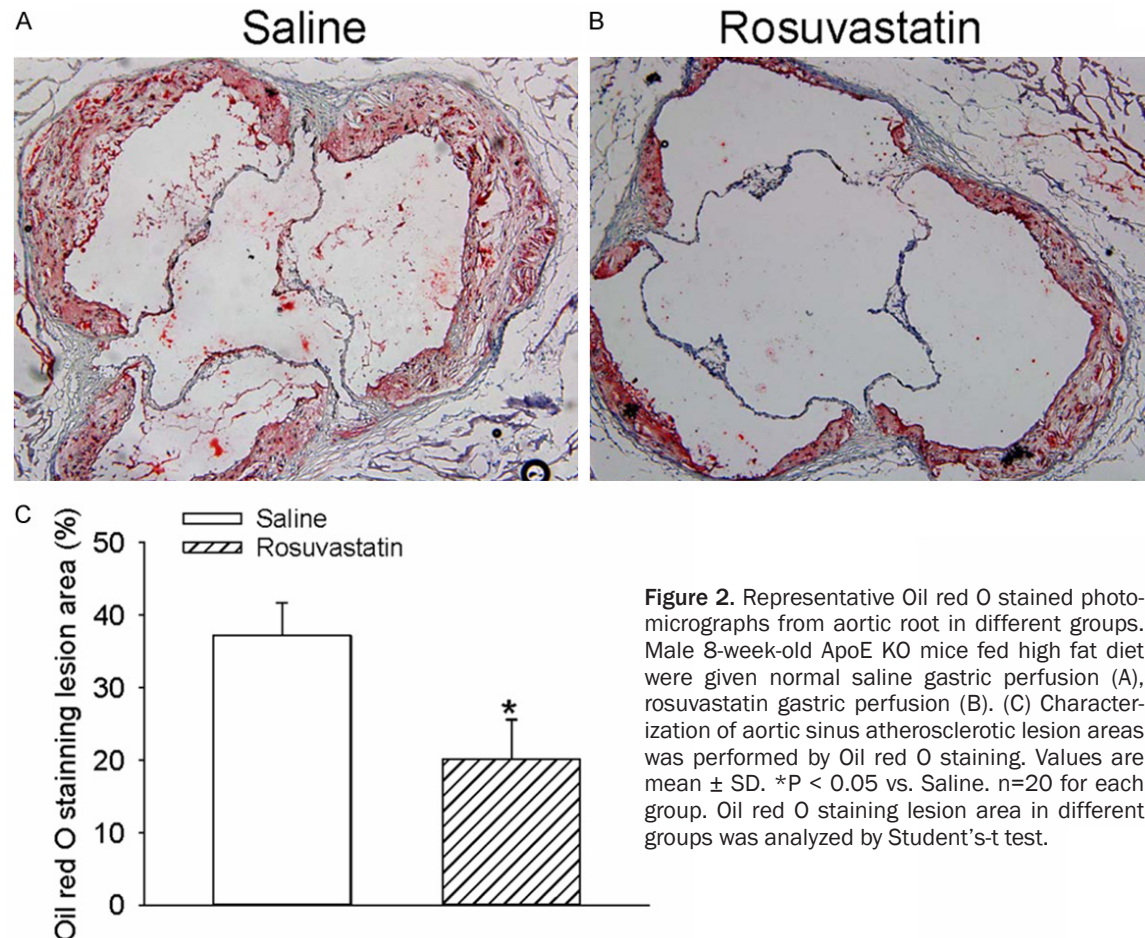


Figure 2. Representative Oil red O stained photomicrographs from aortic root in different groups. Male 8-week-old ApoE KO mice fed high fat diet were given normal saline gastric perfusion (A), rosuvastatin gastric perfusion (B). (C) Characterization of aortic sinus atherosclerotic lesion areas was performed by Oil red O staining. Values are mean \pm SD. * $P < 0.05$ vs. Saline. $n=20$ for each group. Oil red O staining lesion area in different groups was analyzed by Student's-t test.

the atherosclerotic lesion development. Images of the sections were captured with a Leica DMI6000 microscope. Lesion areas were detected by using Image Pro Plus software [21].

Immunostaining detection

The tissue cross-sections were dewaxed, rehydrated, and subjected to be heated for antigen retrieval with citric acid buffer (10 mmol/L, pH 6.0). The endogenous peroxidase activity was blocked by 3% hydrogen peroxide, and the 10% goat serum was employed to block nonspecific binding sites. The tissue cross-sections were then incubated with the antibodies against MOMA2 (1:500; BMA Biomedicals, Augst, Switzerland, specific marker for monocytes/macrophages), VEGF (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), VEGFR (1:200, Cell Signaling Technology Inc. Danvers, MA) and PLGF (1:100, Abcam, UK) overnight at 4°C. The sections were further incubated with a DAB

staining system kit (Santa Cruz, CA, USA). Specimens were counterstained with hematoxylin and the color reaction was visualized by using diaminobenzidine. The sections from each mouse were expressed as a percentage of total lesion area.

Statistical analysis

Statistical analyses were conducted using SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA). Continuous data were presented as mean \pm standard deviation. Student's-t test was employed to elevate the significance of differences between two groups. Values of $P < 0.05$ were regarded as statistically significant.

Results

General data and metabolic parameters

After treatment for 8 weeks, there was no significant difference in body weight, SBP, DBP or

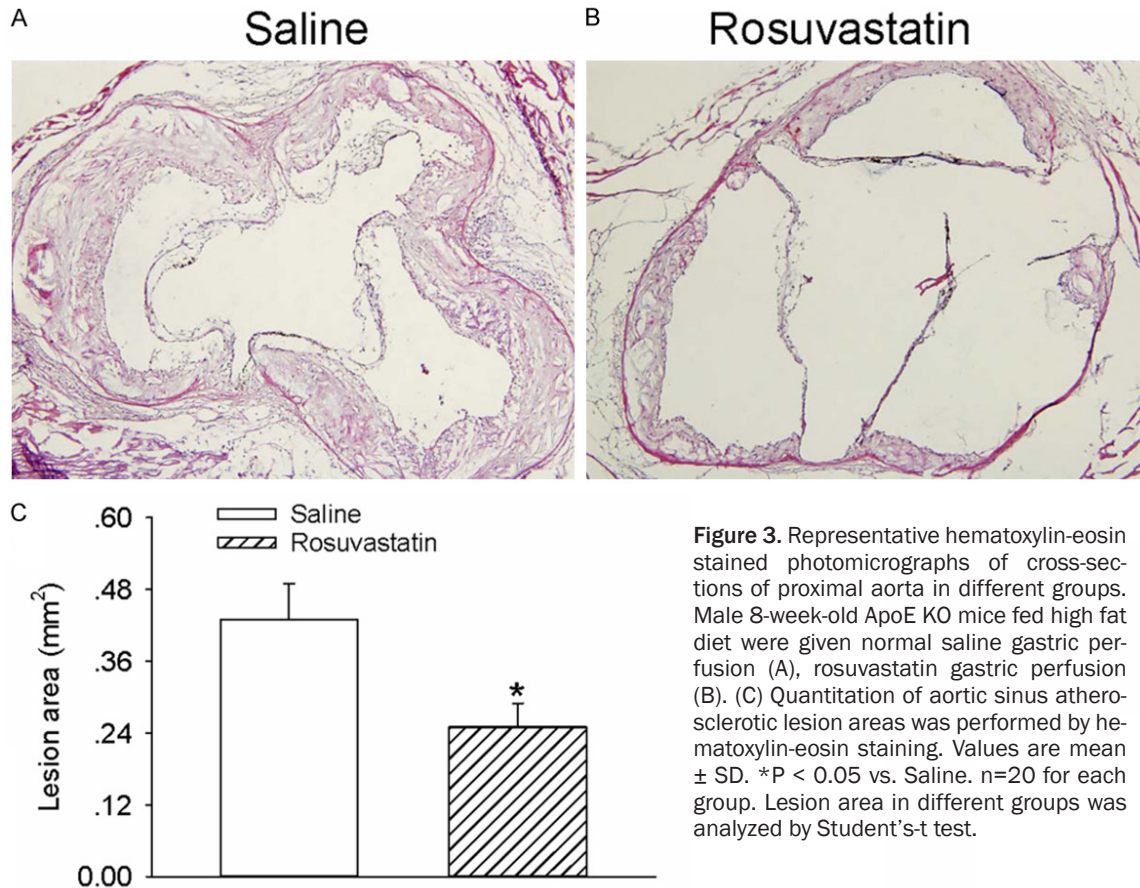


Figure 3. Representative hematoxylin-eosin stained photomicrographs of cross-sections of proximal aorta in different groups. Male 8-week-old ApoE KO mice fed high fat diet were given normal saline gastric perfusion (A), rosuvastatin gastric perfusion (B). (C) Quantitation of aortic sinus atherosclerotic lesion areas was performed by hematoxylin-eosin staining. Values are mean \pm SD. *P < 0.05 vs. Saline. n=20 for each group. Lesion area in different groups was analyzed by Student's-t test.

HR between two groups (**Table 1**). In comparison with mice in Saline group, the levels of TC and TG, as well as LDL in rosuvastatin group were obviously reduced (**Table 2**).

Progression of atherosclerosis in ApoE^{-/-} mice

To determine the effect of rosuvastatin on atherosclerotic plaque development, we quantified plaque area in the aortic root. The brachiocephalic artery and 5- μ m paraffin-embedded cross-sections of the aortic root were subjected to Oil Red O staining. In addition, the plaque morphology of atherosclerotic plaque was evaluated by hematoxylin- and eosin-staining. In the present study, en face plaque of the aortic arch and thoracic aorta stained with Oil Red O showed that male 20-week-old ApoE KO mice developed severe atherosclerotic plaque lesion ranging from the ascending aorta to the aortic arch after 8 weeks of fed high fat diet (**Figure 1A**). Rosuvastatin significantly retarded the atherosclerotic lesions of the whole aorta in en face. The lipid deposition in atherosclerotic lesions were evaluated with Oil red O (**Figure 1B**

and **1C**). The severity of lipid accumulation in cross-sections of the aortic root was remarkably alleviated in mice treated with rosuvastatin (**Figure 2**). Moreover, the plaque size in hematoxylin- and eosin-stained cross-sections of the aortic root was ameliorated in mice received with rosuvastatin treatment (**Figure 3**).

Expressions of MOMA2

Macrophages/monocytes infiltration into vascular wall critically contributes to the development of atherosclerosis and plaque instability, and MOMA2 was a specific marker for macrophages/monocytes [22]. Therefore, we examined whether rosuvastatin abated the infiltration of macrophages/monocytes into the vasculature, the MOMA2 expressions were detected by immunohistochemistry. We showed that rosuvastatin significantly inhibited the macrophage infiltration reflected by reduction in MOMA2 positive macrophages in plaques (**Figure 4**).

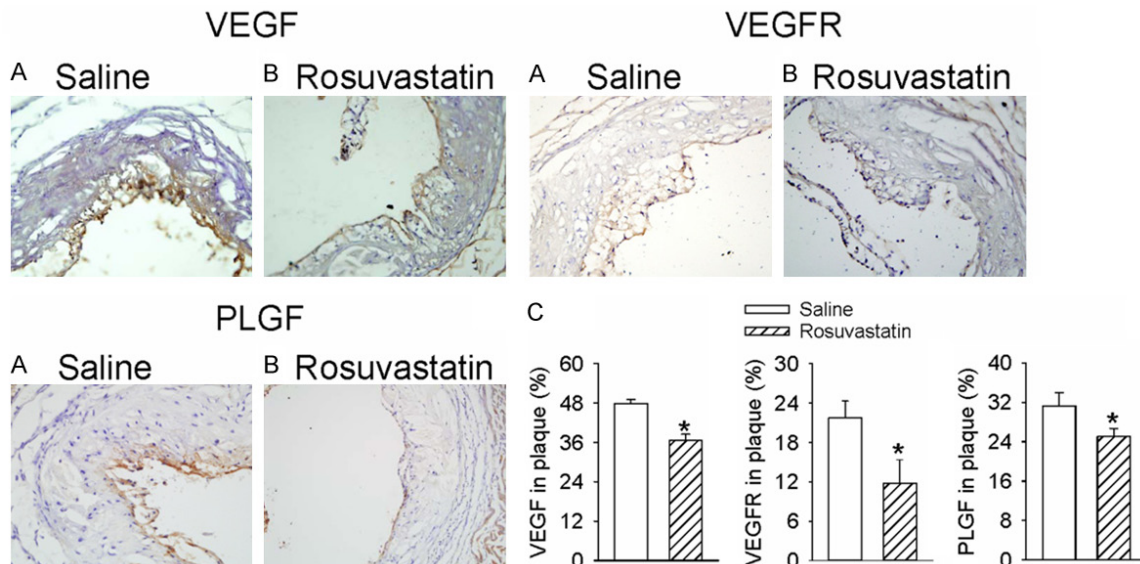
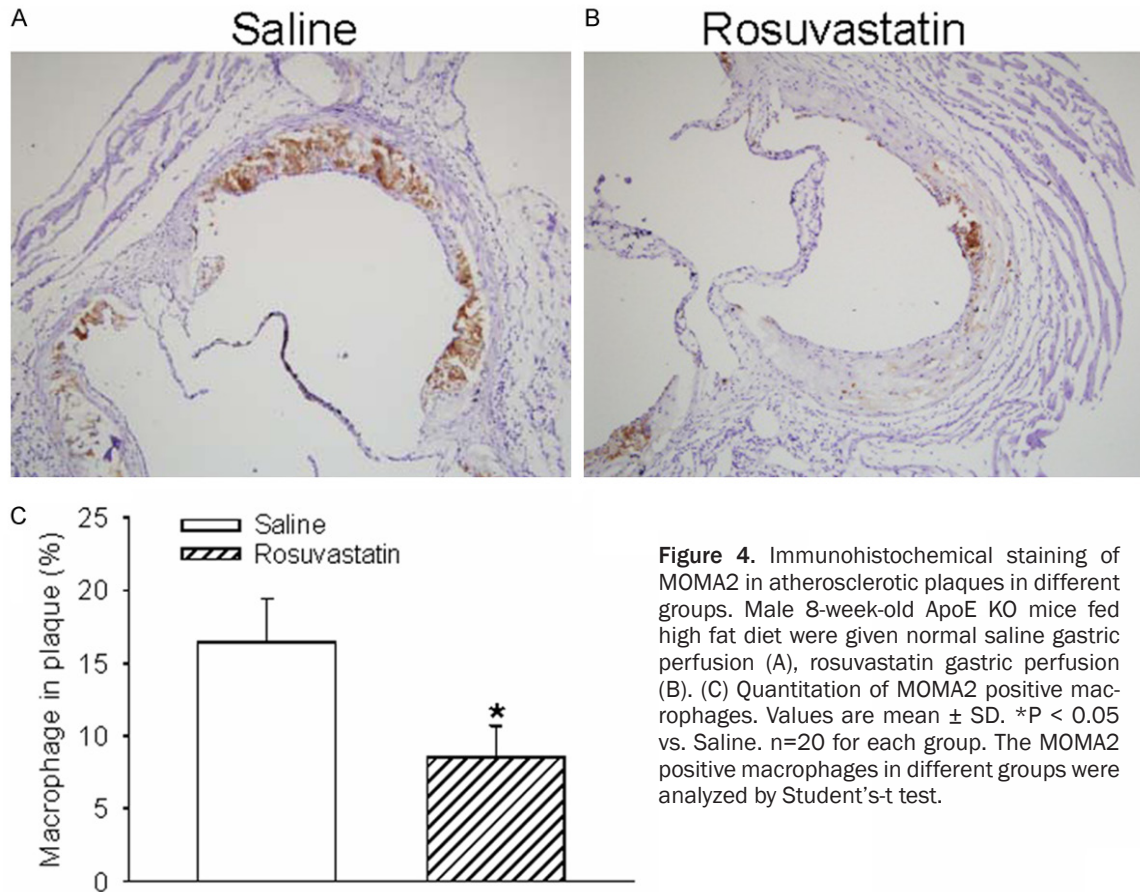


Table 3. VEGF and PLGF in different groups after 8 weeks

	VEGF (ng/L)	PLGF (ng/L)
Saline	160.34±22.96	5.12±0.76
Rosuvastatin	128.9±13.56*	3.71±0.73*
t	4.182	3.123
P	0.012	0.019

Note: Values are mean ± SD. *P < 0.05 vs. Saline. n=20 for each group. VEGF, vascular endothelial growth factor; PLGF, placenta growth factor. These parameters in different groups were analyzed by Student's-t test.

Expressions of VEGF, VEGFR and PLGF of atherosclerotic lesions

Immunohistochemical results displayed that the redundant expressions of VEGF, VEGFR and PLGF of atherosclerotic lesions in ApoE KO mice fed with high fed diet were obviously rescued by rosuvastatin gastric perfusion for 8 weeks (**Figure 5**). In addition, ELISA assay revealed that the VEGF and PLGF levels in ApoE KO mice treated with rosuvastatin were much lower than those in the ApoE KO mice with saline treatment (**Table 3**).

Discussion

Cardiovascular disease is ranked as the leading cause of death worldwide according to the report of the World Health Organization (WHO) in 2011 [23]. A host studies predicate that more and more people will suffer the worldwide deaths from cardiovascular disease. The report of Cardiovascular Disease in China reported that cardiovascular disease resulted in 41.1% deaths in the cities and 38.7% in the rural areas [1]. Atherosclerosis is the most common cause of cardiovascular disease, such as myocardial ischemia and hypoxia and coronary heart disease [24]. Therefore, the prevention and treatment of atherosclerosis are important topics of further research. The present study showed that rosuvastatin abated the progression of atherosclerosis plaque formation. The protective mechanism of rosuvastatin may be attributed to down-regulations of lipid accumulation and VEGF, VEGFR and PLGF expressions.

Rosuvastatin is clinically used to promote the reduction in coronary plaques in patients with coronary artery disease [25]. In this study, the plaque of the aortic arch and thoracic aorta stained with Oil Red O showed that rosuvastatin significantly retarded the atherosclerotic

lesions of the whole aorta in en face. The severity of lipid accumulation in cross-sections of the aortic root was remarkably alleviated in mice treated with rosuvastatin. Moreover, the plaque size in HE-stained cross-sections of the aortic root was ameliorated in mice received with rosuvastatin. These results indicated that rosuvastatin can dramatically ameliorate the atherosclerotic plaque in the ApoE^{-/-} mice fed with high-fat diet.

It is well established that subendothelial accumulation of macrophages is closely related with the development of atherosclerosis [3]. Vulnerable plaques are reflected by deposition of foam cells and macrophages [17]. The excessive lipids in macrophages are major contributor to the progression of atherosclerotic lesions [26]. In the present study, rosuvastatin significantly inhibited the macrophage infiltration reflected by reduction in MOMA2 positive macrophages in plaques. These results hinted that rosuvastatin alleviated the macrophage infiltration to obstruct the development of plaques in atherosclerosis.

The elevated serum TC, TG and LDL-C levels are hallmark feature of atherosclerosis [5]. The extracellular lipids were essential for the stability of atherosclerotic plaque [5]. The disorders of lipid metabolism are correlated with the prognosis of patients with atherosclerosis obliterans [27]. Our results showed that there was no significant difference in body weight, SBP, DBP or HR between two groups at the end of the eighth week. TC and TG, as well as LDL in rosuvastatin group were obviously reduced. These results suggested that rosuvastatin reduced atherosclerotic plaque areas partially through decreasing the blood lipid levels.

Atherosclerosis is recognized as a chronic inflammatory vascular disease, and inflammatory responses are verified to act on the vasculature to induce the atherogenic plaque [28, 29]. VEGF is confirmed to be an initial factor for early angiogenesis and vascular smooth muscle cell proliferation during the progress of atherosclerosis [30]. VEGF is an inflammatory cytokine and is a potential novel cardiovascular risk factor in atherosclerosis [31]. VEGF-VEGFR system is a main regulator of inflammatory monocytes in angiogenesis and atherosclerosis [32]. PLGF is an angiogenic cytokine, which belongs

to the VEGF family, and it is exerted a proinflammatory role in inflammatory response in vascular system in atherosclerosis [12]. PLGF is also a potential therapeutic target in atherosclerosis [33]. In this study, we showed that the redundant expressions of VEGF, VEGFR and PLGF of atherosclerotic lesions in ApoE KO mice fed with high fed diet were obviously rescued by rosuvastatin gastric perfusion for 8 weeks. In addition, ELISA assay revealed that the VEGF and PLGF levels in ApoE KO mice treated with rosuvastatin were much lower than those in the ApoE KO mice with saline treatment. These results proposed that the role of rosuvastatin may be related to inhibition of the inflammation inductions including VEGF, VEGFR and PLGF.

In conclusion, rosuvastatin ameliorated atherosclerosis plaque via inhibition of lipid accumulation and down-regulations of VEGF, VEGFR and PLGF. Rosuvastatin is an effective approach for the treatment of atherosclerosis.

Disclosure of conflict of interest

None.

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