

# Effects of withdrawing an atherogenic diet on the atherosclerotic plaque in rabbits

LIJUN ZHAO<sup>1</sup>, SHIFANG ZHANG<sup>2</sup>, QIAOLI SU<sup>1</sup> and SHUANGQING LI<sup>1</sup>

<sup>1</sup>Department of General Practice, West China Hospital of Sichuan University, Chengdu, Sichuan 610041; <sup>2</sup>Department of Pulmonary Disease, Institute of Respiratory Disease, Chengdu Second People's Hospital, Chengdu, Sichuan 610000, P.R. China

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**Abstract.** Lifestyle interventions and pharmacotherapy are the most common of non-invasive treatments for atherosclerosis, but the individual effect of diet on plaques remains unclear. The current study aimed to investigate the effect of withdrawing the atherogenic diet on plaque in the aortas of rabbits. Experimental atheroma was induced in 33 rabbits using a 1% high cholesterol diet for 30 days (H-30 d) or 90 days (H-90 d, baseline group). After 90 days of the atherogenic diet, the remaining animals were divided into four groups: A total of 10 rabbits continued to consume the atherogenic diet for 50 days (H-90 d & H-50 d; n=5) or 140 days (H-90 d & H-140 d; n=5). Another 13 rabbits were switched to a chow diet for 50 days (H-90 d & C-50 d; n=7) or 140 days (H-90 d & C-140 d; n=6). A total of 10 age-matched rabbits in the control groups were fed a chow diet for 90 and 230 days, respectively. The *en face* or cross-sectional plaque areas were determined using oil red O staining and elastic van Gieson staining. Immunohistochemistry analyses were used to assess the macrophages or smooth muscle cell contents. When fed an atherogenic diet for 90 days, the rabbits' abdominal aortas exhibited severe atherosclerotic lesions (the median *en face* plaque area was 63.6%). After withdrawing the atherogenic diet, the plaque area did not shrink with feeding the chow diet compared with the baseline, but increased to 71.8 or 80.5% after 50 or 140 days, respectively. After removing cholesterol from the diet, the lipids content in the plaques increased during the first 50 days, and then decreased compared with the baseline group. Furthermore, withdrawing the atherogenic diet increased the total collagen content and the percentage of the smooth muscle cells, alleviated macrophage infiltration, decreased the vulnerable index and promoted the

cross-linking of collagen. Feeding the rabbits an atherogenic diet followed by removal of cholesterol from the diet did not lead to the regression of established lesions but instead delayed the progression of the lesions and promoted the stabilization of the plaque.

## Introduction

Cardiovascular disease (CVD), which is the leading cause of death in China (1), accounted for ~40% of deaths in the Chinese population in 2017 (2). Atherosclerotic CVD (ASCVD), which includes ischemic heart disease and ischemic stroke, is the main form of CVD (2). In 2016, there were ~2.4 million deaths from ASCVD in China, which accounted for 61% of all deaths from CVD (2,3). A monitored atherosclerosis regression study revealed that quantitative coronary angiographic changes were associated with cardiovascular events, and optimization of therapies was required to promote the regression of atherosclerosis (4).

Atherosclerosis is characterized by lipoprotein retention, foam cell recruitment, vascular smooth muscle cell proliferation and matrix synthesis (5). The lumen may be occluded when the plaque is large or prone to rupture and cause thrombosis (5). As ASCVD is a huge health burden, therapies aiming to reduce the size of atherosclerotic plaque and open the luminal stenosis have become a popular research focus (6). Lipid-lowering diets and the administration of lipid-lowering agents such as statins, have been revealed to be effective in regressing atherosclerotic plaque (7). Of these, the lipid-lowering diet has been the most common non-interventional treatment for atherosclerosis (8). A number of trials assessing atherosclerosis do not evaluate diet as an independent intervention but rather combine it with other lifestyle changes, such as exercise or quitting smoking (9); this highlights the complex, multifactorial nature of the influence of lifestyle on atherosclerosis. Therefore, it is important to clarify the effects of dietary intervention on atherosclerosis.

A number of studies have demonstrated that switching from a high-fat or cholesterol-rich diet to a cholesterol-low diet reduced the plasma lipid levels and caused the regression of atherosclerotic plaque in rabbits (10-13), squirrel monkeys (14), rhesus monkeys (15) and swine (16). However, a previous study using the rabbit model reported that removing high cholesterol from the diet did not lead to regression but rather aggravated

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*Correspondence to:* Dr Shuangqing Li, Department of General Practice, West China Hospital of Sichuan University, 37 Guoxue Alley, Chengdu, Sichuan 610041, P.R. China  
E-mail: 1259594471@qq.com

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advanced atherosclerosis (17). This reason for this paradoxical response of rabbit arteries to the high cholesterol withdrawal treatment has not been fully determined, and is likely due to an inhibiting factor in the reversal of the atheromatous plaque. The effects of the lipid lowering diet on plaque requires further elucidation in animal studies.

The stability of plaque serves an essential role in acute coronary events and mortality (18). In nonhuman primates, the advanced arterial lesions in cholesterol-fed Rhesus monkeys underwent remodeling during a subsequent regression period of 40 months when the animals were switched to low-cholesterol diets (15,19). In atherosclerotic rabbits with aortic balloon injury, dietary lipid lowering promoted the stability of plaque via the maturation of smooth muscle cells (10), increased collagen content (20) and reduced endothelial activation (21). Collagen is the main structural protein of the fibrous cap, and its cross-linking provides mechanical strength to atherosclerotic lesions and determines the biomechanical stability of plaque (22). This supports the fact that collagen cross-linking serves an important role in determining the effect of a lipid-lowering diet on the stability of plaque. As the lipoprotein metabolism of rabbits is closer to that of humans than that of rodents, rabbits fed a simple high cholesterol diet have become the most popular animal model for the study of atherosclerosis (23,24). Therefore, the present study investigated the independent effect of diet intervention on atherosclerotic plaque and the cross-linking of collagen in cholesterol-fed rabbits.

## Materials and methods

**Animal protocol.** All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the West China Hospital of Sichuan University. Male New Zealand White rabbits ( $n=43$ ; age, 10 weeks) weighing  $\sim 2.0$  kg were obtained from Chengdu Dossy Experimental Animals Co., Ltd. The rabbits were acclimated to the laboratory condition for one week and housed individually in metal cages in rooms maintained with a 12 h light/dark cycle, a constant temperature of  $22\pm 2^\circ\text{C}$  and humidity at  $50\pm 5\%$ . All animals had access to water at all times and were fed *ad libitum* with a regular chow diet or a high-fat chew diet (100-120 g/day).

Atherosclerosis was induced using a high cholesterol diet (HCD) containing 1% (*w/w*) cholesterol (cat. no. 5915010104; Gen-View Scientific, Inc.). Cholesterol crystals were dissolved in peroxide-free diethylether, mixed with the chow and allowed to dry. The atherogenic, high cholesterol diet was given to rabbits for 30 (H-30 d) or 90 days (H-90 d) to determine whether plaque formed. A total of five rabbits were euthanized at 30 days and five rabbits were euthanized at 90 days, and these rabbits comprised the baseline group. A total of 13 animals continued to consume the atherogenic diet for 50 (H-90 d & H-50 d;  $n=7$ ) or 140 more days (H-90 d & H-140 d,  $n=6$ ). The remaining animals consumed a chow diet with no added cholesterol for 50 days (H-90 d & C-50 d;  $n=5$ ) or 140 days (H-90 d & C-140 d;  $n=5$ ). Age-matched rabbits in the control groups were fed a chow diet for 90, and 230 days, respectively. Each control group contained 5 rabbits. The experimental protocol is presented in Fig. 1.

**Blood sampling and cholesterol measurement.** Fasting blood was collected weekly via the left/right marginal ear vein and at the time of harvest after termination of the experiments in each group. The blood volume was  $\sim 1$  ml for each rabbit. Blood was centrifuged at  $1,123 \times g$  for 15 min at  $4^\circ\text{C}$ . Following centrifugation,  $\sim 500$  ml plasma could be obtained from each tube. Plasma samples were stored at  $-80^\circ\text{C}$  prior to analysis. The plasma lipid profiles were determined by the methods described as follows: Plasma total cholesterol (TC) by cholesterol oxidase peroxidase (CHOD-POD) method, high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) by the precipitation end point method, triglycerides (TG) by glycerol-phosphoric acid oxidase peroxidase (GPO-POD) method using a TC kit (lot no. 141615025), HDL-C kit (lot no. 142117009), LDL-C kit (lot no. 142016009), and TG kit (lot no. 141716001), respectively (Shenzhen Mindray Bio-Medical Electronics Co., Ltd.) (25). Each parameter for each sample was assessed in duplicate. All assays were followed according to the manufactures' protocol and were analyzed using an automatic chemistry analyzer (BS-120, Mindray).

**Tissue preparation.** Following termination of the experiments, the rabbits were anaesthetized using sodium pentobarbital (30 mg/kg intravenously; Rhone Merieux, Ltd.) and placed in the supine position (26,27). The perfusion process and collection of aortas was performed as previously described (6). An inguinal incision was made to access the aorta for the insertion of a cannula connected to a perfusion apparatus. The rabbits were then perfused with isotonic saline from the left ventricle using a 50 ml syringe needle (at a rate of 100 ml/minute/kg body weight). When the run-off liquid was clear, the aortas were isolated and cleaned of muscle, adherent fat and fascia. Finally, the abdominal aorta was cut into three segments for further analysis. The first segment, which was taken from the coeliac axis to the left renal artery, was longitudinally opened along the ventral midline for oil-red O staining to evaluate the gross atherosclerotic lesion area. The second segment, which was taken from the 3rd up to the 4th lumbar artery branch point, was embedded in optimal cutting temperature medium and serially sectioned at  $5\text{-}\mu\text{m}$  thickness for histopathologic examination or elastic van Gieson (EVG) staining. The remaining arterial tree was immediately frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  for later use.

**Quantification of the atherosclerotic lesions.** Oil-red O staining was used to quantify the *en face* atherosclerotic lesion area (6,28). The first segment of the aorta was fixed with 4% paraformaldehyde for 10 min at  $37^\circ\text{C}$ , stained with oil-red O (cat. no. SLBM4444V; Sigma-Aldrich; Merck KGaA) for 1 h at  $37^\circ\text{C}$ , and pinned out flat on a wet black cloth with 0.2-mm diameter stainless steel pins (Fine Science Tools, Inc.). The images of the inner surface of the aorta were captured using a Ziess digital camera (fiber-optic CL 1500 ECO cold light source; Carl Zeiss AG) mounted on a Ziess SteREO Discovery V8 stereo microscope (SteREO Discovery V8; Carl Zeiss AG). The total aortic surface and atherosclerotic lesion areas were analyzed *en face* using computerized quantitative morphometry by Image Pro Plus software (v6.0, Media Cybernetics, Inc.). The aortic lesion area was expressed as percentage of the total aortic area (29).

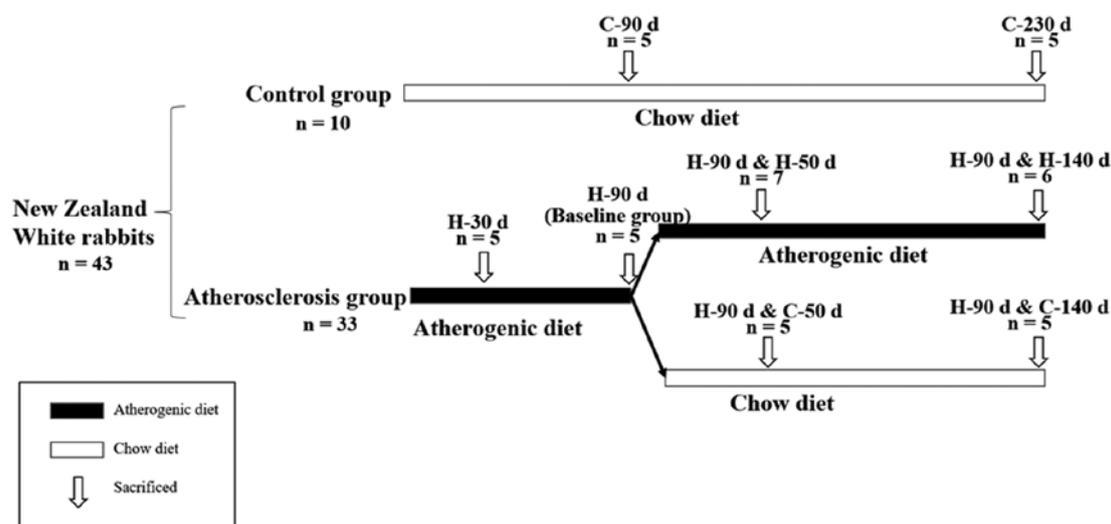


Figure 1. The experimental protocol of the current study. A total of 43 male rabbits were enrolled. Atherosclerosis was induced using a high cholesterol diet containing 1% (w/w) cholesterol.

To quantify the cross-sectional lesion area, the slices 5- $\mu$ m from the second segment were stained using EVG (28,30). Images were captured using a Nikon DXM 1200/NIS-Elements mounted on a Nikon Eclipse E600 light microscope (Nikon Corporation) and analyzed using Image Pro Plus software (v6.0, Media Cybernetics, Inc.).

**Quantification of the neutral lipids and total collagen in plaque.** To quantify the neutral lipid content of the plaque, frozen cross sections of aorta that taken from the 3rd up to the 4th lumbar artery branch point were stained with oil-red O for 30 min at 37°C, as described previously (28). To determine the total collagen fiber in the plaque, frozen sections were stained with Sirius red and fast green for 30 min at room temperature. At least 10 high-power fields (magnification, x200) were randomly used for each sample.

**Immunohistochemical staining.** Immunohistochemical studies were performed on the luminal aspect of the blood vessel through the plaque to the elastic lamina (to assess changes that also involved the media). The detailed procedure has been described previously (6). The 5- $\mu$ m-thick frozen sections were fixed in 10% paraformaldehyde solution for 15 min at room temperature, and then washed with phosphate-buffered saline (PBS) three times. Sections were blocked with 10% normal goat serum (Abcam) for 1 h at 37°C and incubated with primary antibody mouse anti-rabbit-macrophage monopoly antibody (1:400; CD68; cat. no. M0633; Dako; Agilent Technologies, Inc.); and primary antibody mouse anti-rabbit smooth muscle  $\alpha$ -actin monopoly antibody (1:50; HHHF-35; cat. no. ENZ-C34931; Enzo Life Sciences, Inc.) at 4°C overnight. After incubation, residual antibodies were removed using three washes with PBS. Subsequently, the tissue slides were incubated with a secondary antibody Horseradish Peroxidase (HRP) labeled goat anti-mouse antibody (ready-to-use; cat. no. 8125; Cell Signaling Technology, Inc.) at 37°C for 1 h. HRP binding sites were detected using the diaminobenzidine (DAB) substrate kit per protocol (cat. no. 8059S; Cell Signaling Technology,

Inc.). Tissues were subsequently counterstained with hematoxylin for 2 min at temperature for microscopic examination. Negative controls were realized by omission of the primary antibody. Images were captured with a digital camera (SPOT Flex Camera; Diagnostic Instruments, Inc.) mounted on a Nikon light microscope (Nikon Corporation) and analyzed using Image Pro Plus software (v6.0, Media Cybernetics, Inc.). At least 10 high-power fields (magnification, x200) were randomly used for each sample. The area of positive immunohistochemical staining was expressed as follows: Percentage of the stained area/total plaque area. The positive immunohistochemical staining of smooth muscle  $\alpha$ -actin area was used to determine the smooth muscle cell (SMC) content of the plaque.

**Evaluation of the stability of the plaque.** The stability of the plaque was evaluated using the vulnerability index, which was calculated as follows: [Macrophages staining (%) + lipids staining (%)]/[smooth muscle cells (%) + total collagen (%)] (31). At least 10 high-power fields (magnification, x200) were selected for each sample.

**Determination of collagen cross-linking.** The cross-linking of the collagen in the plaque was analyzed using Biocolor S1000 and Biocolor S2000 assay kit (Biocolor Ltd.). According to the kit, the tissues frozen with liquid nitrogen were put into a tube with a pepsin concentration of 0.2 mg/ml in 0.5 M acetic acid at 4°C overnight. After this process, the soluble collagen was released into the solution and the insoluble collagen was isolated and concentrated using reagents in the kit. The collagen was then stained by Sircol dye for 30 min at room temperature and eluted using Alaki Reagent according to the kit. The eluted solution was analyzed by a microplate reader under 555-nm wavelength. The insoluble collagen was analyzed according to the manufacturers protocol. The soluble collagen and the insoluble collagen were expressed as  $\mu$ g/mg wet tissue. The cross-linking of the collagen was described as the ratio of insoluble collagen to soluble collagen, according to the manufacturers protocol.

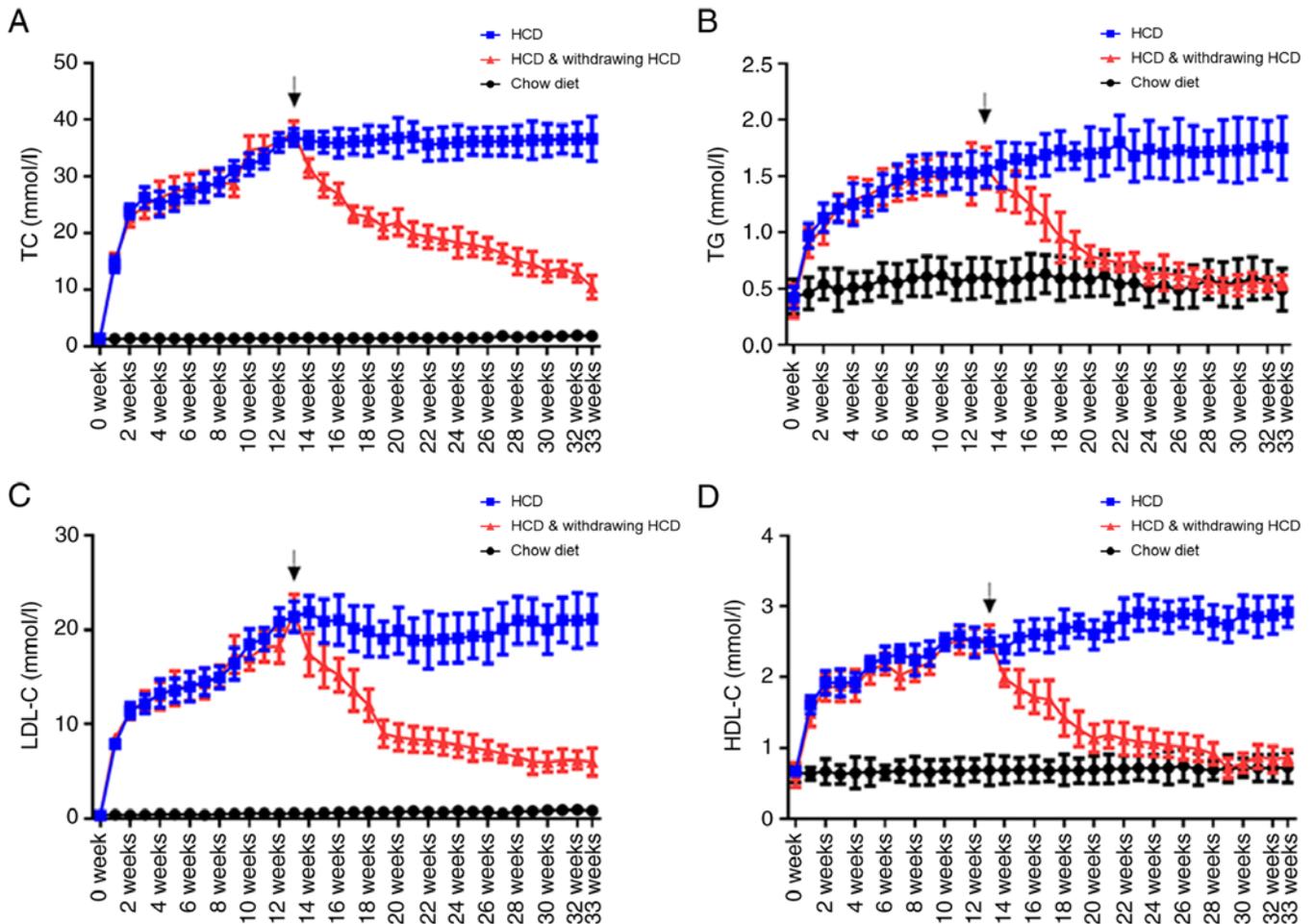


Figure 2. Plasma lipids level in rabbits during the atherogenic diet and after withdrawing the atherogenic diet. (A) Changes in concentration of TC. (B) Changes in plasma concentration of TG. (C) Changes in plasma concentration of LDL-C. (D) Changes in plasma concentration of HDL-C. The black arrow indicates the withdrawal of cholesterol from the diet. All experimental data were verified in two independent experiments. Mean  $\pm$  SD are presented. TC, Plasma total cholesterol; TG, triglycerides; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; HCD, high cholesterol diet.

**Statistical analysis.** Data are presented for continuous variables as mean  $\pm$  SD. Groups that had been fed the atherogenic diet were compared using one-way ANOVA, followed by Tukey test for multiple comparisons. All analyses were conducted using SPSS software (IBM Corp.; version 24.0).  $P < 0.05$  was considered to indicate a statistically significant difference. All figures were constructed using GraphPad Prism 8.0 Software (GraphPad Software, Inc.).

## Results

**Changes in the plasma TC, TG, LDL-C, and HDL-C concentrations after withdrawing the atherogenic diet.** Compared with the control groups, the plasma TC, TG, LDL-C and HDL-C concentrations markedly increased for 12 weeks and remained steady during the next 20 weeks of the atherogenic diet. After withdrawing the atherogenic diet, the plasma TG, LDL-C and HDL-C concentrations rapidly decreased while the plasma TC concentration decreased moderately. After 20 weeks of the chow diet, the plasma TG, and HDL-C concentrations returned to the baseline levels except for the plasma TC and LDL-C concentration (Fig. 2A-D).

**Changes to the atherosclerotic lesion areas after withdrawing the atherogenic diet.** After 30 days of the atherogenic diet (H-30 d), fatty streaks were induced in the rabbits' aortas. After 90 days of the atherogenic diet (H-90 d), widespread lesions were induced (Fig. 3A-D). Fig. 3A and B reveal the *en face* plaque areas of the aortas, which are determined by oil-red O staining. There was a significant difference between H-30 d group and all other groups (Fig. 3B). *En face* analysis of the aortas revealed that the plaque area was significantly increased from 18.4% in H-30 d group to 63.6% in H-90 d group (baseline group). When the atherogenic diet was continued, the *en face* plaque area increased to 72.6% in H-90 d & H-50 d group and 84.1% in H-90 d & H-140 d group. No significant differences were observed among the H-90 d group, H-90 d & H-50 d group and the H-90 d & H-140 d group. After the atherogenic diet was withdrawn for 50 days (H-90 d & C-50 d), the rabbits' *en face* lesion area was 71.8%, which was not significantly lower compared with the baseline group or the atherogenic diet group. Compared with the atherogenic diet group, the *en face* lesion area was reduced to 80.5% in the rabbits that had cholesterol withdrawn from their diet for 140 days (H-90 d & C-140 d).

Fig. 3C and D present the cross-sectional plaque areas of the aortic plaque sections, as determined by EVG staining. There

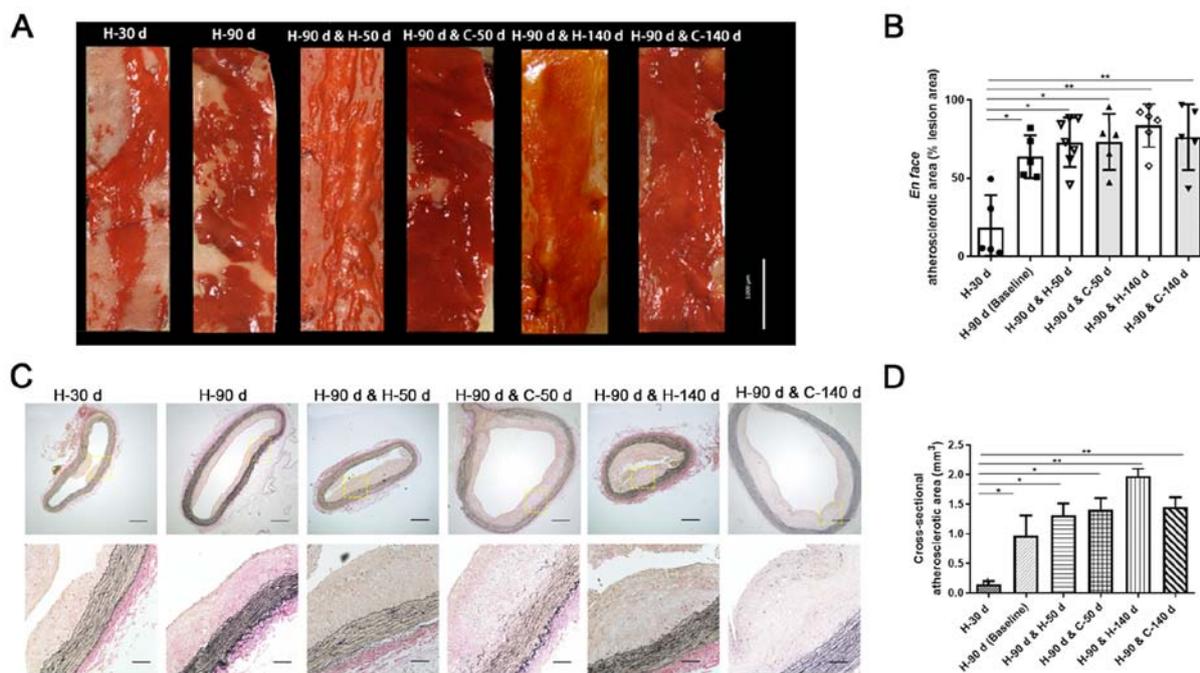


Figure 3. Atherosclerotic lesion areas did not shrink after withdrawing the atherogenic diet. (A) Oil-red O staining showed the *en face* atherosclerotic plaque on the inner surface of the abdominal aorta in rabbits.  $\times 8$ , bar=1000  $\mu\text{m}$ . (B) Quantification of the percentage of *en face* lesion areas. (C) Elastic van Gieson staining showed cross-sectional of plaque in the abdominal aorta in rabbits. Upper panel,  $\times 4$ , bar=500  $\mu\text{m}$ . Lower panel,  $\times 200$ , bar=100  $\mu\text{m}$ . (D) Quantification of the percentage of cross-sectional lesion area. All experimental data were verified in two independent experiments. Mean  $\pm$  SD are presented. \* $P < 0.05$ ; \*\* $P < 0.01$ .

was a significant difference between H-30 d group and all other groups (Fig. 3D). The median cross-sectional plaque area was 0.96 mm<sup>2</sup> in the H-90 d group (baseline group), 1.31 mm<sup>2</sup> in H-90 d & H-50 d group, and 1.97 mm<sup>2</sup> in H-90 d & H-140 d group, respectively. When the atherogenic diet was withdrawn, the cross-sectional plaque area increased slightly in the rabbits fed the chow diet for 50 or 140 days compared with the baseline group. However, compared with the atherogenic diet group (H-90 d & H-140 d), the cross-sectional plaque area was reduced in rabbits fed the chow diet for 140 days (H-90 d & C-140 d).

**Changes in the plaque components after withdrawing the atherogenic diet.** The changes in the plaque components are presented in Fig. 4. Fig 4A and B indicate the changes in the lipid content of the plaque. Oil-red O staining of the aortic plaque sections revealed that the lipids content increased during the atherosclerosis-inducing period. The lipids content was significant higher in H-90 d & H-50 d, H-90 d & C-50 d, H-90 d & H-140 d, H-90 d & C-140 d groups than H-30 d or H-90 d group (Fig. 4B). After withdrawing the atherogenic diet, the lipid deposition increased for the first 50 days of the chow diet (H-90 d & H-50 d vs. H-90 d & C-50 d). However, the lipid content of the plaque slightly decreased after 140 days of the chow diet (H-90 d & H-140 d vs. H-90 d & C-140 d; Fig. 4A and B).

Sirius red and fast green staining of the aortic plaques revealed that percentage of the total collagen content of the plaque did not change significantly in the rabbits fed the atherogenic diet from day 30 (H-30 d) to day 230 (H-90 d & H-140 d). However, after withdrawing the atherogenic diet, the total collagen content of the plaque gradually increased over time (Fig. 4C and D). The total collagen content of the plaque

was significantly higher in the H-90 d & C-50 d group and H-90 d & C-140 d group than the other groups (Fig. 4D).

Immunohistochemical analysis of the macrophage content in the aortic lesions revealed a significant increase in the macrophage-positive area in the H-90 d group compared with the H-30 d group. When the rabbits continued to consume the atherogenic diet, the macrophage content in the plaque remained steady from 90 days of atherogenic diet (H-90 d & H-140 d) of atherogenic diet (H-90 d & H-140 d). However, the macrophage-positive area in the lesions significantly decreased compared with the baseline when the diet was replaced with normal rabbit chow diet for 140 days (H-90 d & C-140 d; Fig. 4E and F).

Immunohistochemical analysis of the SMC content in atherosclerotic plaque demonstrated that the mean  $\alpha\text{SMA}$ -positive SMC area in the atherosclerotic lesions range from 5.48-6.80% during the atherogenic diet. When the atherogenic diet was shifted to the chow diet, the mean  $\alpha\text{SMA}$ -positive SMC area was 6.63% in H-90 d & C-50 d group and 9.26% in H-90 d & C-140 d group. The  $\alpha\text{SMA}$ -positive SMC area was significantly higher in the rabbits withdrawing from the atherogenic diet for 140 days (H-90 d & C-140 d) compared with those that continued with the atherogenic diet for another 140 days (H-90 d & H-140 d) (Fig. 4G and H).

**Plaque stability increased after withdrawing the atherogenic diet.** To evaluate the stability of the atherosclerotic plaque, the vulnerable index was calculated (Fig. 5A). When the rabbits were on the atherogenic diet, the vulnerable index of the plaque was 0.46 in the H-30 group, 0.59 in H-90 d group, 0.83 in H-90 d & H-50 d group, and 0.89 in H-90 d & H-140 d group, respectively. This result indicating that the formed plaque

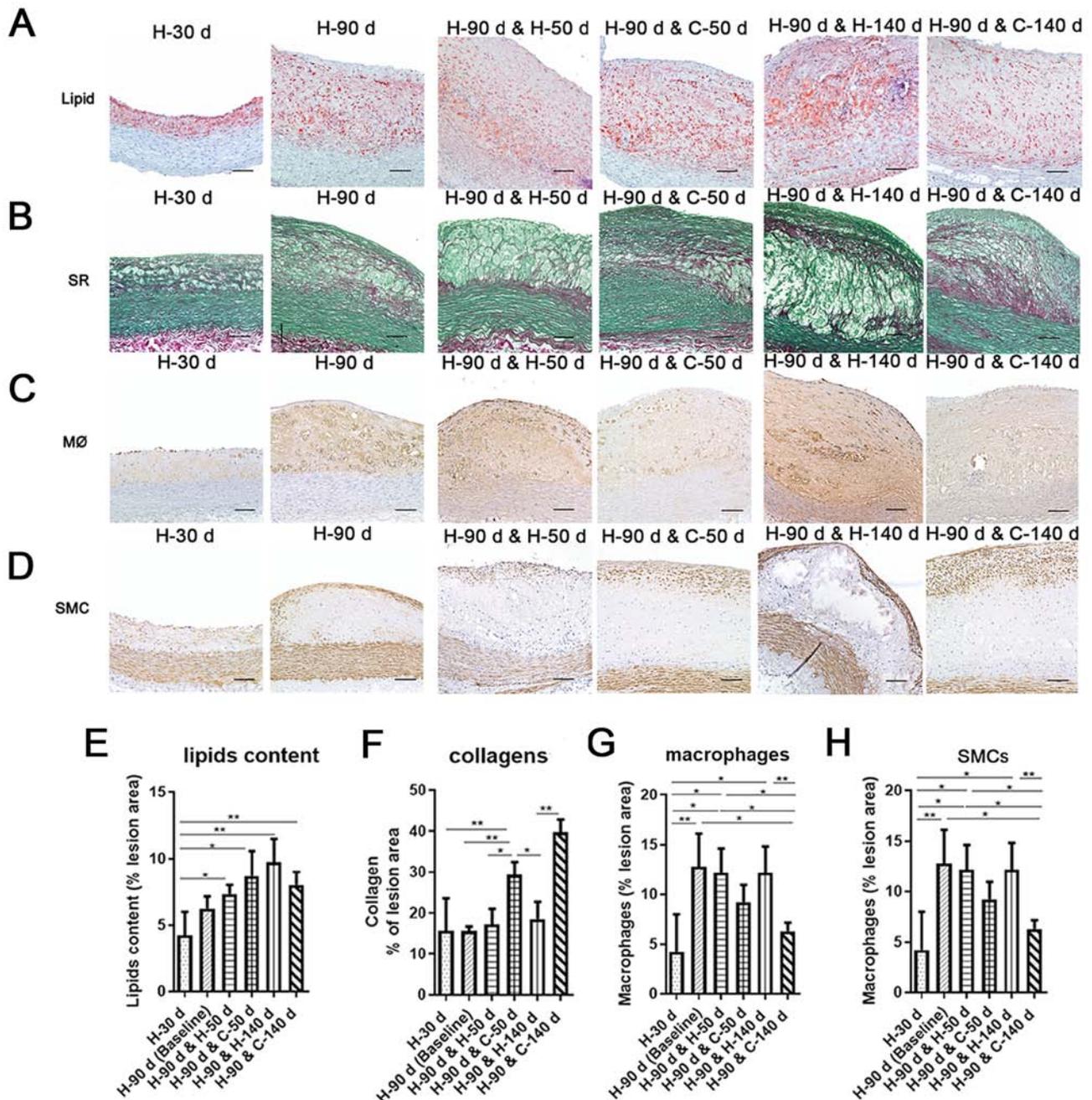


Figure 4. Changes in the plaque components during the atherogenic diet and after withdrawing the atherogenic diet. (A) Frozen cross sections were stained using ORO to quantify the neutral lipids in plaque.  $\times 200$ , bar= $100\ \mu\text{m}$ . (B) Frozen cross sections were stained with SR to quantify the total collagen in plaques.  $\times 200$ , bar= $100\ \mu\text{m}$ . (C) Immunohistochemical staining of macrophages in plaque.  $\times 200$ , bar= $100\ \mu\text{m}$ . (D) Immunohistochemical staining of SMCs in plaques.  $\times 200$ , bar= $100\ \mu\text{m}$ . (E-H) Quantitative data of the plaque components during the atherogenic diet and after withdrawing the atherogenic diet. Quantitative data for the percentage of lipid contents (E), total collagen (F), macrophage-positive areas (G), and SMC-positive areas (H). All experimental data were verified in two independent experiments. Mean  $\pm$  SD are presented. ORO, oil-red O; SR, Sirius red; SMCs, smooth muscle cells. \* $P<0.05$ ; \*\* $P<0.01$ .

became more vulnerable over time. However, the vulnerable index was 0.54 in the H-90 d & C-50 d group and 0.32 in H-90 d & C-140 d group after withdrawing the atherogenic diet. The vulnerable index decreased by 8% in H-90 d & C-50 d group compared with the rabbits in H-90 d group, and decreased by 46% in H-90 d & C-140 d group compared with the rabbits in the H-90 d & C-50 d group. Compared with the H-90 d group, the vulnerable index in the H-90 d & C-140 d group was significantly decreased. Moreover, the vulnerable index was the lowest in H-90 d & C-140 d group among all groups.

*Collagen cross-linking increased after withdrawing the atherogenic diet.* The cross-linking of collagen in the atherosclerotic plaque was further analyzed in the current study. Compared with the rabbits in H-30 d group, the cross-linking of collagen decreased significantly in the rabbits in the H-90 d & H-50 d group or H-90 d & H-140 d group. Conversely, when given the chow diet for 50 days or 140 days, the cross-linking of collagen in the lesions markedly increased compared with the atherosclerosis-inducing groups (Fig. 5B). The cross-linking of collagen was significantly higher in H-90 d &

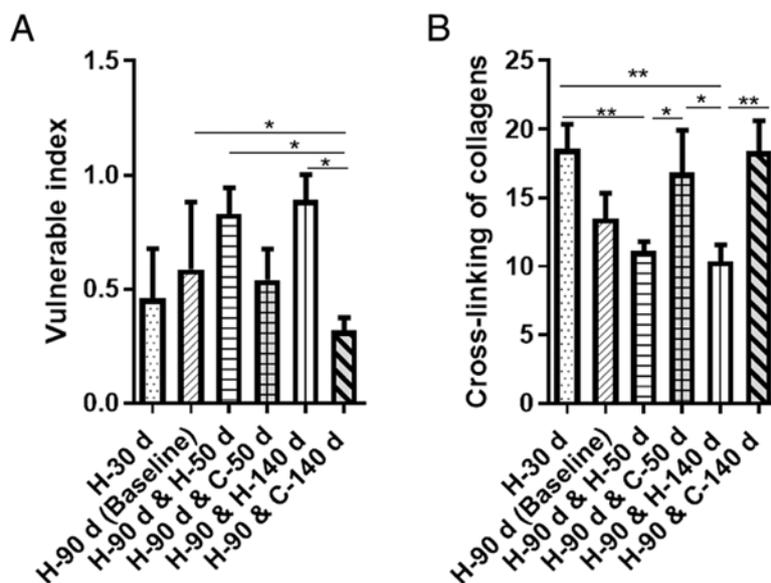


Figure 5. Changes in plaque stability and collagen cross-linking. The vulnerable index of the (A) plaque and (B) cross-linking of collagen in rabbits during atherogenic diet and after withdrawing the atherogenic diet. All experimental data were verified in two independent experiments. Mean  $\pm$  SD are presented. \* $P < 0.05$ ; \*\* $P < 0.01$ .

C-50 d group or H-90 d & C-140 d group than that in H-90 d & H-50 d group or H-90 d & H-140 d group.

## Discussion

The current study demonstrated that after 90 days of high cholesterol feeding, the rabbits' aortas exhibited severe atherosclerotic lesions accompanied by increased amount of lipids deposition, the accumulation of foam cells, overexpression of the extracellular matrix and decreased cross-linking of collagen. Withdrawing cholesterol from the diet did not lead to the regression of established atherosclerosis but delayed the progression of lesions compared with the baseline group. However, the plaque area was moderately reduced after withdrawing cholesterol from the diet for 140 days (vs. continuation of the atherogenic diet for 140 more days). Upon removal of the atherogenic diet for 50 days or 140 days, the lipid deposition, total collagen content, and SMC content increased in the plaque, and the macrophages content decreased in the lesions (vs. the baseline group). Further analysis demonstrated that the vulnerable index decreased and the cross-linking of collagens increased after withdrawing the atherogenic diet, which implied that the stability of the plaque increased.

Atherosclerosis is a lipoprotein-driven disease that leads to the formation of plaque at specific sites on the arterial tree with high stress (5). The plaque is characterized by lipoprotein retention, macrophage infiltration, smooth muscle cell proliferation and matrix synthesis (5). During the atherosclerosis-inducing period of the present study, lipid, collagen and macrophages were persistently deposited in the aortic plaque. The rates of macrophage infiltration and lipid deposition were higher than the total collagen expression rate, which was in line with the pathophysiology of xanthomas or fatty streaks (5). Xanthomas have been detected in fetal aortas and 6-month-old infants, which most likely reflects risk factors of the mother (32). However, xanthomas are

harmless and fully reversible if the stimuli that caused their formation dissipate (33). Previous clinical trials indicated that lipid-lowering drugs (for example, statins and proprotein convertase subtilisin kexin type 9 inhibitors) and HDL-raising therapy (for example, the infusion of reconstituted HDL) result in decreased atheroma volume (34-37). Experimental agents in preclinical studies, including intravenous injections of phospholipid liposomes or apoA-I, long-term administration of L-arginine or anti-inflammatory antibody combined with or without a normal diet, result in the attenuation of plaque progression compared with nontreated control animals (38-41). Nevertheless, lipid-lowering diets have always been the most common treatment for atherosclerosis.

The American Heart Association provides recommendations for diet modifications for cardiovascular disease risk reduction in the general population (42). The concept of regressing atherosclerosis in nonhuman primates has been reviewed by Malinow (43) and Feig (44). In the classic studies conducted in primates, the advanced arterial lesions in cholesterol-fed Rhesus monkeys underwent shrinkage and remodeling during the 40 months when their diet was switched to low-fat or linoleate-rich (15,19). More extensive work by Aikawa *et al* (10) indicated that cholesterol reduction by diet alone was able to shrink the plaque size and stabilize vulnerable plaque in rabbits subjected to balloon injury and cholesterol feeding (0.3%) for 4 months (10,12,20,21,45,46). Mice are relatively resistant to the development of atherosclerosis; Therefore, genetic manipulation of the lipid metabolism is routinely used in atherosclerosis studies involving these animals (47). In transgenic mice, studies on the effects of dietary intervention alone on atherosclerotic plaques are limited (48), whereas previous evidence has suggested that aortic transplant, the injection of recombinant apoA-I or a reconstituted statin-containing HDL particle combined with a chow diet resulted in a rapid lesion regression in apoE<sup>-/-</sup> or Ldlr<sup>-/-</sup> mice (49). Cessation of cholesterol feeding in animals demonstrates effectiveness in regressing

plaque progression (17,19). However, in the current study, the atherosclerotic plaque areas did not shrink but increased slightly in the rabbits with cessation of the atherogenic diet compared with the baseline group (H-90 d). The underlying reason for this result may be related to high cholesterol storage in the liver during the atherosclerosis-inducing phase. The diet-induced cholesterol storage supports the long-term persistence of hypercholesterolemia even after withdrawing cholesterol from the diet (Fig. 2A) (8). Compared with the rabbits that continued to consume an atherogenic diet, the plaque size was reduced in the animals from which cholesterol had been withdrawn for 140 days. Furthermore, the total collagen and  $\alpha$ SMC content increased and the macrophage content decreased, which resulted in an increased stability of the plaque after switching to a chow diet for 140 days. These results indicated that removal of cholesterol from the diet following a cholesterol-rich diet does not always lead to the regression of lesions but instead results in the slowing down of the lesion progression and stabilization of the plaque.

Plaque size and stability are other hallmarks of atherosclerotic vascular disease (50). Plaque rupture is the most common cause of coronary thrombosis and leads to ASCVD (51). The majority (~76%) of all fatal coronary artery thrombi are precipitated by plaque rupture (52). Ruptured and rupture-prone plaque is characterized by a large lipid-rich core, a thin and collagen-poor fibrous cap, the accumulation of macrophages, neovascularization and intraplaque hemorrhage (5). Collagen constitutes a major portion of the extracellular matrix in the plaque, where it contributes to the strength and integrity of the fibrous cap (53). Previous studies have indicated that lipid lowering reinforced the fibrous skeleton of the atheroma via the induction of collagen synthesis and the promotion of the transition of initial SMCs in atherosclerotic rabbits (10,12,20). The cross-linking of collagen is a pivotal process that ensures plaque stability and provides the tensile and elastic characteristics of connective tissues (22,54). This process is regulated by lysyl oxidase (LOX), which belongs to a family of copper-dependent enzymes (55). A previous study reported that LOX is strongly downregulated in different stages of the atherosclerotic process (56). In atherosclerotic plaque from human carotid endarterectomies, higher LOX levels, which suggest an enhanced cross-linking of collagen, were associated with a more stable phenotype of the plaque (22). The results from the current study revealed that the cross-linking of collagens was enhanced, and the vulnerable index was decreased after withdrawing the atherogenic diet, indicating that the plaque was becoming more stable. Therefore, it can be concluded that withdrawing the atherogenic diet changed the compositions of the plaque, resulting in increased plaque stability in atherosclerotic rabbits. The current preclinical study provides evidence for cholesterol restriction strategies in clinical practice.

Cholesterol-fed rabbits are the classical model for the study of atherosclerosis. New Zealand White rabbits are sensitive to dietary cholesterol and rapidly develop severe hypercholesterolaemia, leading to prominent plaque lesions when fed an atherogenic diet (1.0-1.5% cholesterol) for a short period time (about 8 weeks) (23,57). Under the atherogenic diet, plaque develops in the aortic arch, the thoracic aorta and the abdominal aorta in these rabbits (24,58). The data in the current study indicated that after 90 days of an atherosclerosis-inducing diet, the *en face* plaque areas of all aortic arches and thoracic aortas

were nearly 100%, whereas the *en face* plaque areas of the abdominal aortas fluctuated between 50 and 80% (Fig. 1B). The plaque changes in the abdominal aortas are able to be measured accurately using oil red O staining. In humans, plaque is commonly located in the abdominal aorta, so the rabbit abdominal aorta is a suitable atherosclerotic model that closely mimics the human lesions (59). Therefore, abdominal aorta tissue in rabbit was selected to investigate the effect of withdrawing atherogenic diet on atherosclerotic plaque in the present study.

The evaluation of the stability of plaques using the vulnerable index was one limitation of this study (31). This method was invasive. A number of invasive and noninvasive novel imaging modalities, such as MRI, optical coherence tomography, intravascular MRI and intravascular spectroscopy, have been investigated as being techniques that can be used to define the specific characteristics of vulnerable plaque (60). However, the majority of these techniques are undergoing constant refinement and have limited reliability in the identification of vulnerable plaque (60). The vulnerable index is a simple method and is still widely used for atherosclerosis in rabbits or transgenic mice (61-65).

In conclusion, the current study demonstrated that withdrawing the atherogenic diet did not regress the plaque but slowed the progression of atherosclerosis in the cholesterol-fed rabbits. Withdrawing the atherogenic diet increased the total collagen content, alleviated macrophage infiltration, enhanced the cross-linking of collagen and decreased the vulnerable index of plaque. These findings support the notion that a healthy diet should be the fundamental therapy for atherosclerotic cardiovascular disease in clinical practice (66).

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### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

LZ, SZ, QS and SL conceived and designed the study and reviewed the manuscript. LZ analyzed the data and interpreted the results. LZ and SZ drafted the manuscript. SL analyzed and interpreted data, edited/revised and approved the final version of the manuscript. LZ, SZ, QS and SL contributed to the discussion of this manuscript. SL and LZ were responsible for confirming the authenticity of

all the raw data. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the West China Hospital of Sichuan University.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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