

Original Article

Pitavastatin attenuates atherosclerosis by suppressing NF- κ B signaling in a high-cholesterol diet plus balloon catheter injury rabbit model

Ming Long¹, Xiao Ke², Hongzhang Zhu³, Gan Liu¹, Jingguo Wu⁴, Xinxue Liao¹, Zhimin Du¹

¹Department of Cardiology, The First Affiliated Hospital, Sun Yat-sen University, Key Laboratory on Assisted Circulation, Ministry of Health, Guangzhou, Guangdong, China; ²Department of Cardiology, Shenzhen Sun Yat-sen Cardiovascular Hospital, Shenzhen 518112, Guangdong, China; ³Departments of ³Radiology, ⁴General Internal Medicine, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, Guangdong, China

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Abstract: Atherosclerosis (AS) induced by endothelial cell (EC) dysfunction significantly contributes to the onset and development of cardiovascular disease. Pitavastatin is a member of the lipid-lowering drugs, statins that are widely used in clinics. In the current study, we evaluated the effect of pitavastatin on AS and nuclear factor-kappa-light-chain-enhancer of activated B cells (NF- κ B) signaling in abdominal aortic ECs. We induced AS in rabbits by high-cholesterol diet plus balloon catheter injury. The anti-AS effect of pitavastatin was assessed by measuring the intima-media thickness of the abdominal aorta, minimal lumen area (MLA), minimal lumen diameter (MLD), and other hemodynamic parameters. In addition, we measured the production of total cholesterol (CHOL, high density lipoproteins (HDL), low-density lipoprotein cholesterol (LDL-c), and triglycerides (TG) in the rabbits. To explore the underlying mechanism of pitavastatin on atherosclerosis, we isolated abdominal aortic ECs and determined the activity of NF- κ B signaling. In our model, we found that the affected animals had structural impairments of the heart and arteries: reduced left atrium diameter, right ventricular internal diameter, MLA, and MLD and increased inter-ventricular septal thickness, left ventricular internal diameter, left ventricular posterior wall thickness, right atrium diameter, and intima-media thickness of abdominal aorta. Most of these changes were restored by administration of pitavastatin. Moreover, concentrations of plasma lipids were also attenuated by pitavastatin. At the molecular level, pitavastatin inhibited the expression of NF- κ B and Bax and induced the production of IL-1 β and Bcl-2. In addition, we demonstrated that the anti-AS effect of pitavastatin depends on restoring normal function of ECs and eliminating dysfunctional ECs by inducing apoptosis.

Keywords: Atherosclerosis, endothelia cells, NF- κ B, pitavastatin

Introduction

Cardiovascular disease (CVD) induced by atherosclerosis (AS) is one of the leading causes of mortality and morbidity worldwide. During the onset of AS, susceptible sites in arteries are impaired by endothelial cell (EC) dysfunction, which results in recruitment and accumulation of monocytes [1, 2]. Furthermore, the dysfunctional ECs themselves produce reactive oxygen species (ROS) and promote vascular inflammation and thrombosis [3]. Therefore, impaired flow-mediated vasodilatation (FMD), increased arterial stiffness, and carotid intima-media thickness associated with the progression of AS are important predictors of cardiovascular

events [4-6]. To better manage the symptoms and complications of AS, it is thus, crucial to elucidate the underlying mechanism driving the aberrant function of ECs in AS. From the perspective of hemostasis and thrombosis, the normal functioning endothelial lining is an ideal container for blood [7] and from the bioengineering perspective, individual ECs comprising the lining of various parts of the cardiovascular system [8]. Once challenged by pro-inflammatory cytokines or bacterial products, ECs activate gene expression that alters vital functional properties. This EC response is characterized by the activation of pleiotropic transcription factors, such as NF- κ B, subsequently, inducing the expression of various effector proteins with

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important pathophysiologic implications [9]. NF- κ B related signaling is critical in the initiation of inflammatory responses. It has been previously demonstrated that suppression of NF- κ B relieves ECs from impairments due to stimulation of cytokines and other pro-inflammatory factors [10, 11]. Therefore, NF- κ B is considered a central target in the development of anti-AS therapies in recent years.

Currently, statins are the first-line drugs used in the prevention and treatment of CVD based on clinical guidelines [12]. Statins are inhibitors of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) and primarily exert their anti-CVD effect by inhibiting the onset and progression of AS [13]. Statins are also implicated in restoring the function of ECs via multiple pathways. The third generation of statins, pitavastatin is known as "super statin" due to its pharmacokinetic characteristics [14]. As reported by Xu et al., pitavastatin reduces the progression of AS by inhibiting the expression of the vascular cell adhesion molecule-1 (VCAM-1) [14]. Jing et al. further showed that pitavastatin suppresses lipopolysaccharide-stimulated inflammatory response in human umbilical vein endothelial cells by up-regulating eNOS production [15]. Moreover, pitavastatin inhibits NF- κ B activation in hepatocellular carcinoma cells [16]. Based on these findings, we hypothesized that pitavastatin ameliorates AS by restoring EC function via NF- κ B suppression.

To test our hypothesis, we induced AS in rabbits, by the high-cholesterol diet plus balloon catheter injury method. We then assessed the effect of pitavastatin on AS symptoms and measured changes in animal body weight, hemodynamic parameters, structures of heart and abdominal aorta, and production of serum lipids. In addition, we isolated abdominal aortic ECs from rabbits in the different groups and determined the expression of NF- κ B and other related signaling molecules. Our data suggested that pitavastatin attenuated the pathological characteristics of atherosclerosis by suppressing NF- κ B signaling.

Materials and methods

Chemicals and agents

Antibodies against NF- κ B, IL-1 β , Bcl-2, Bax, and β -actin were purchased from Santa Cruz Biotechnology (CA, USA). Total Protein Extraction Kit was purchased from Thermo Fisher Sci-

entific. Kits for detection of serum levels of total cholesterol (CHOL), high density lipoprotein (HDL), low-density lipoprotein cholesterol (LDL-c), and triglyceride (TG) were purchased from Elabscience Biotechnology.

Animals

Healthy New Zealand rabbits were obtained from Laboratory Animal Center of Sun Yat-sen University and were housed individually in stainless steel cages at 20 \pm 3 $^{\circ}$ C with a 12-hour light/dark cycle and with free access to food and water. All assays conducted with the animals were in accordance with the Institutional Animal Ethics Committee and Animal Care Guidelines for the Care and Use of animals, at the First Affiliated Hospital of Sun Yat-sen University.

High-fatty diet plus balloon catheter injured rabbit model and administration of pitavastatin

Twenty animals were randomly divided into four groups (five animals per group) for assessing the effect of pitavastatin on AS: A. Control group, healthy rabbits. B. Blank group, rabbits subjected to high-fatty diet plus balloon catheter injury for induction of AS. C. Low dose group, AS rabbits administered with 30 mg/kg pitavastatin. D. High dose group, AS rabbits administered with 60 mg/kg pitavastatin. The high-fatty diet plus balloon catheter injury method was performed according to a previous study [17]. In brief, during the first four weeks of induction, animals in the control group were fed with standard rabbit chow, animals in the Blank group were fed with a high-fatty (HF) diet, and animals in the Low dose and High dose groups were fed with HF diet plus the corresponding concentration of pitavastatin. After four weeks of housing, rabbits in the Blank, Low dose, and High dose groups were injured by a balloon catheter with slight modification: a 3F Fogarty catheter was inserted into the right femoral artery and advanced to a position just below the diaphragm. The balloon was then inflated with 0.6 mL saline and the catheter was pulled 3 times until the bifurcation of the iliac arteries was reached. Finally, the balloon was deflated and the catheter withdrawn. All the animals were raised for another 12 weeks with the same diet, before the injuries were made. The changes in body weight of all the rabbits, before and after injuries were recorded.

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Table 1. Data of body weight, and hemodynamics parameters

	Weight	LA		RV	IVS	LV	LVPW	EF	RA	
		a	B						a	b
Control	1.96±0.05	8.4±0.54	10.4±0.54	5.22±0.30	1.92±0.10	13.8±1.30	1.92±0.10	64±6.04	9.2±0.44	6.8±0.83
Control (Four weeks later)	2.26±0.13	7.72±0.93	10.3±0.96	7.22±0.77	2.34±0.20	13.2±1.48	2.16±0.15	64.4±3.64	9.68±0.55	7.86±0.47
Blank	2±0	8±1.22	9.6±0.89	4.8±0.44	1.96±0.08	12.4±1.14	1.78±0.22	64.8±2.58	8.4±0.54	7.2±0.83
Blank (After injury)	2.36±0.11	8.04±0.55	9.6±0.51	6.82±0.39	2.52±0.10	13.5±0.47	2.42±0.14	63±2	9.24±0.55	7.98±0.37
Low dose	2.04±0.08	7.4±0.54	10±0	4.92±0.57	1.92±0.17	13.8±0.83	1.84±0.16	61.6±1.14	9.8±1.30	8±0.70
Low dose (After injury)	2.26±0.08	7.72±0.64	9.6±1.52	6.3±0.46	2.22±0.17	14.4±1.51	2.2±0.14	67±4.12	9±1.47	7.2±1.00
High dose	1.86±0.13	7.4±0.54	10±0.70	5.4±0.54	1.92±0.10	13.4±1.51	1.68±0.16	67.6±2.70	9±0.70	7.6±0.54
High dose (After injury)	2.44±0.15	8.82±0.72	9.62±0.91	5.8±0.81	2.18±0.20	14.6±1.81	2±0.14	63.6±5.27	9.28±0.71	7.04±1.19

LA, left atrium diameter. RV, right ventricular internal diameter. IVS, interventricular septal thickness. LV, left ventricular internal diameter. LVPW, left ventricular posterior wall thickness. EF, ejection fraction. RA, right atrium diameter.

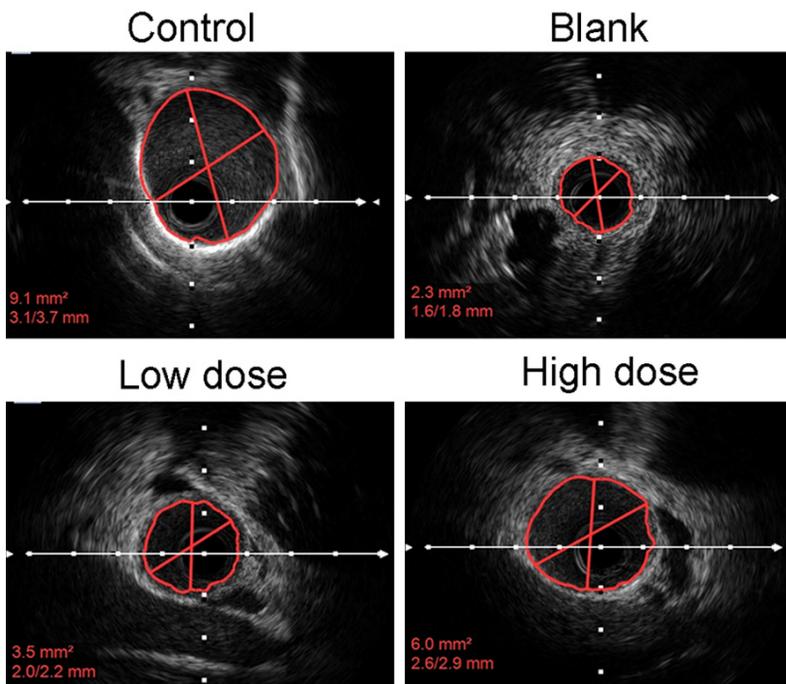


Figure 1. Administration of Pitavastatin improved stenosis caused by the induction of AS.

In vivo ultrasound imaging

The relative intima-media thickness of abdominal aorta, minimal lumen area (MLA), minimal lumen diameter (MLD), and hemodynamic parameters (including LA, left atrium diameter. RA, right atrium diameter. IVS, interventricular septal thickness. LV, left ventricular internal diameter. LVPW, left ventricular posterior wall thickness. EF, ejection fraction. RV, right ventricular internal diameter) of model rabbits before and after injuries were detected with an ultrasound system using Philips iE33 system (Philips Ultrasound, Bothell, WA).

Blood biochemistry

The concentrations of plasma lipids (including CHOL, HDL, LDL-c, and TG) of all the rabbits before and after injuries were measured using detection kits, according to the manufacturers' instructions.

Isolation of rabbit abdominal aortic endothelial cells

Rabbits were anesthetized with intraperitoneal injection of pentobarbital sodium (10 mg/mL) and the midline of the abdomen was incised and the thorax opened to expose hearts and lungs. The aortic endothelial cells were isolated from abdominal aorta following the standard procedures: abdominal aorta

was cut and washed with PBS. Afterwards, aorta was ligated at the proximal portion and infused with 0.1% collagenase and incubated at 37°C for 15 min before ECs were collected from the aorta by flushing with DMEM containing 20% fetal bovine serum. ECs were cultured in RPMI1640 medium before being subjected to any treatment.

Western blotting assay

Total cellular protein in ECs was extracted using the Total Protein Extraction Kit, according to the manufacturer's instructions. β-actin was

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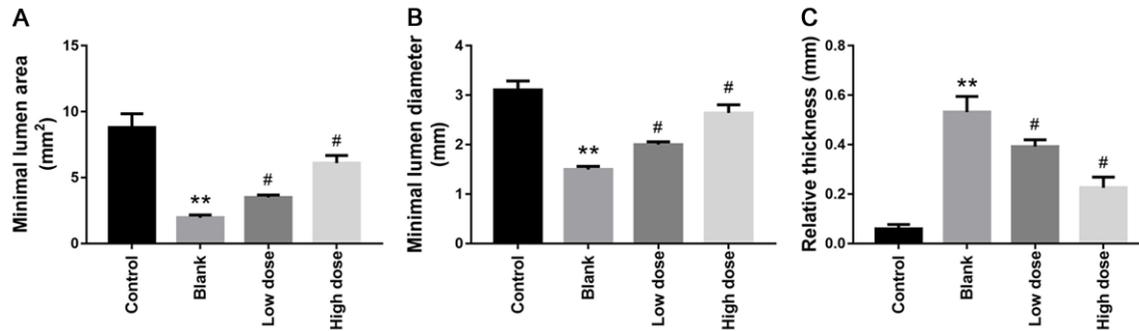


Figure 2. Administration of Pitavastatin increased minimal lumen area (A) and diameter (B) and decreased the relative intima-media thickness of the abdominal aorta (C) due to induction of AS. ** $P < 0.01$ vs. Control group. # $P < 0.05$ vs. Blank group.

used as the internal reference protein. Equal amounts of protein (40 μg) from different samples were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 80 V for 25 h and the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. After TTBS washes, the membranes were blocked with skimmed milk solution for 1 h. Thereafter, the membranes were incubated with the appropriate primary antibodies (against NF- κB , IL-1 β , Bcl-2, Bax, and β -actin) at 4°C overnight. After four washes with TTBS, the membranes were incubated with secondary HRP-conjugated IgG antibodies for 45 min at 37°C. After another six washes with TTBS, the blots were developed using the Beyo ECL Plus reagent and the images were recorded in the Gel Imaging System. The relative expression levels of proteins were calculated by the Gel-Pro-Analyzer (Media Cybernetics, USA).

Statistical analysis

All the data were expressed in the form of mean \pm SD. Student t-test, ANOVA, and post hoc tests with LSD method were performed with a significant level of 0.05 using GraphPad Prism 6 (GraphPad Software, San Diego, CA).

Results

Administration of pitavastatin improved heart and artery conditions impaired by HF diet plus balloon catheter injury

We induced AS using the HF diet plus balloon catheter injury method, in rabbits. We recorded the values of heart and artery structural parameters before and after AS was established, and

evaluated the changes to study the effect of pitavastatin on AS. As shown in **Table 1**; **Figures 1** and **2**, induction of AS caused structural changes in the hearts and arteries of model animals. We observed a reduction in LA and RV diameters, MLA and MLD and an increase in IVS thickness, LV internal diameter, LVPW thickness, RA diameter, and intima-media thickness of abdominal aorta. **Figure 1** and **Figure S1**, show the minimal lumen area, minimal lumen diameter, and the minimal lumen diameter, and the results suggest that pitavastatin alleviates the symptoms of AS. We further observed that induction of AS led to altered production of plasma lipids: CHOL, LDL-c, HDL, and TG (**Figure 3**). After pitavastatin treatment, we did not observe any difference in body weight of rabbits, LV diameter, and EF. However, we found that pitavastatin treatment decreased RA and RV diameters, IVS and LVPW thickness and increased LV diameter and HDL concentration (**Table 1**). Moreover, the changes in intima-media thickness of the abdominal aorta, MLA, MLD, CHOL, LDL-c, TG were reversed by pitavastatin (**Figures 1-3**). We did not observe any significant differences in effect between the two doses of pitavastatin. Although, we did not expect to observe some of the changes in hemodynamic parameters, we found that the overall effect of pitavastatin was to attenuate AS. We speculate that long-term administration of pitavastatin will achieve a better treatment outcome and additional studies to test this, need to be conducted in the future.

Administration of pitavastatin exerted its anti-AS effect by suppressing NF-κB signaling

To further explore the mechanism of how pitavastatin exerts its anti-AS effect, we isolat-

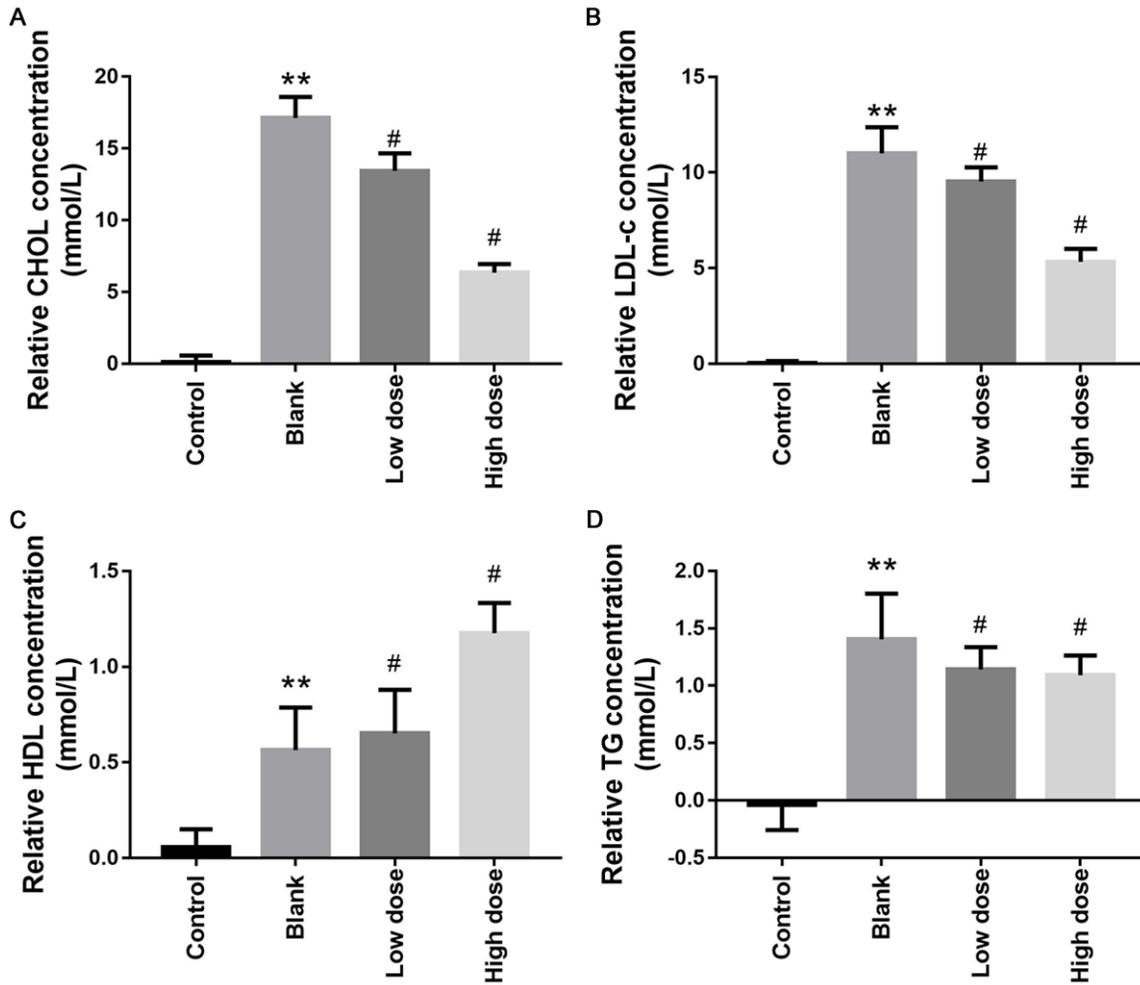


Figure 3. Administration of Pitavastatin attenuated the symptoms of AS by decreasing CHOL (A), LDL-c (B), TG (D) and increasing HDL (C) in model rabbits. ** $P < 0.01$ vs. Control group. # $P < 0.05$ vs. Blank group.

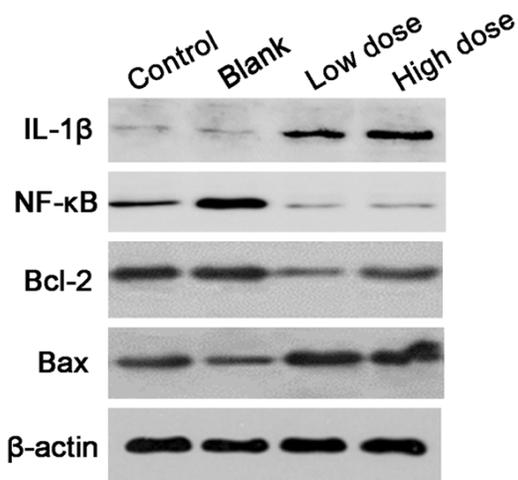


Figure 4. Pitavastatin exerted its effect on AS by inducing inflammation in dysfunctional ECs by inhibiting the NF-κB pathway.

ed abdominal aortic ECs from rabbits in the different groups. We detected the expression levels of NF-κB, IL-1β, the anti-apoptotic factor Bcl-2, and the pro-apoptotic factor Bax using Western blots. We found that pitavastatin administration inhibited the expression of NF-κB (Figure 4). However, consistent with a previous study, suppression of NF-κB was associated with the up-regulation of IL-1β [18], representing an augmented extension in inflammation, after treatment of pitavastatin. Moreover, administration of pitavastatin also increased the expression of the pro-apoptotic factor, Bax and decreased the expression of the anti-apoptotic factor, Bcl-2 (Figure 4). These results were surprising as they suggested that the positive effects of pitavastatin on AS were accompanied by negative effects on the pro-survival pathways of ECs.

Discussion

The major findings outlined in the current study are 1) administration of pitavastatin ameliorated stenosis associated with AS; 2) the anti-AS function of pitavastatin is due to the inhibition of NF- κ B signaling. These data are in accordance with earlier work that showed pitavastatin improved symptoms of AS [15, 19, 20] and provide an additional mechanism through which pitavastatin exerts its function.

AS is associated with both monocytes and the onset and progression of CVD. ECs are the central link in the development of AS [14], and represent the inflammatory entity of the disease [21-24]. In the current study, induction of AS in rabbits was accompanied by the activation of NF- κ B, partially supporting these previous studies. Therefore, suppressing NF- κ B signaling might be a promising therapeutic strategy for managing AS. We selected pitavastatin to treat rabbits with AS. Pitavastatin is a member of the cholesterol lowering class of drugs called statins and has a stronger CHOL eliminating effect than other statins [25-27]. Moreover, pitavastatin has been shown to restore EC function, and therefore alleviate AS. Jing et al., demonstrated that pitavastatin suppressed lipopolysaccharide-stimulated inflammation in human umbilical vein ECs by inhibiting the expression of the microRNA, miR-155 [15]. In another study conducted by Zhao et al., the authors concluded that pitavastatin improved EC function in patients with hypercholesterolemia [20]. In the current study, both doses of pitavastatin alleviated symptoms of stenosis associated with AS. However, we did not observe any effects of pitavastatin on body weight, LV diameter, EF and structural parameters of the heart and arteries. These could be because of the particular dose and time that we administered pitavastatin.

The inhibitory effect of pitavastatin on NF- κ B could represent the anti-inflammatory function of the drug. However, in the current study, we observed that the pro-inflammatory factor IL-1 β was also induced. In macrophages and neutrophils, enhanced pro-IL-1 β processing depends on caspase-1 and serine proteases respectively, and their activation is inhibited by NF- κ B-dependent gene products [18]. Therefore, effect of pitavastatin on inflammation needs to be

further assessed. Moreover, the expression levels of the pro-apoptotic factor Bax was also induced by pitavastatin, indicating an anti-survival function of pitavastatin, in ECs. We detected dysfunctional ECs in AS-induced rabbits and these were eliminated after treatment with pitavastatin, thus, contributing to the alleviation of AS.

In conclusion, the current study confirmed the anti-AS effect of pitavastatin in a high-fatty diet plus balloon catheter injury rabbit model. Moreover, our results showed that the anti-AS effect of pitavastatin depends on both restoring the normal function of ECs and eliminating dysfunctional ECs, by inducing inflammation and apoptosis. However, the effect of pitavastatin on inflammation in ECs is complicated and is not clearly demonstrated by our data. For a more comprehensive understanding of the mechanism associated with the anti-AS effect of pitavastatin, further work needs to be performed in the future.

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Disclosure of conflict of interest

None.

Address correspondence to: Xinxue Liao and Zhimin Du, Department of Cardiology, First Affiliated Hospital of Sun Yat-sen University, Key Laboratory on Assisted Circulation, Ministry of Health, No. 68 of Zhongshan Rd, Yuexiu District, Guangzhou 510080, Guangdong, China. E-mail: liaoxinx@mail.sysu.edu.cn (XXL); dujiaoshou7890@126.com (ZMD)

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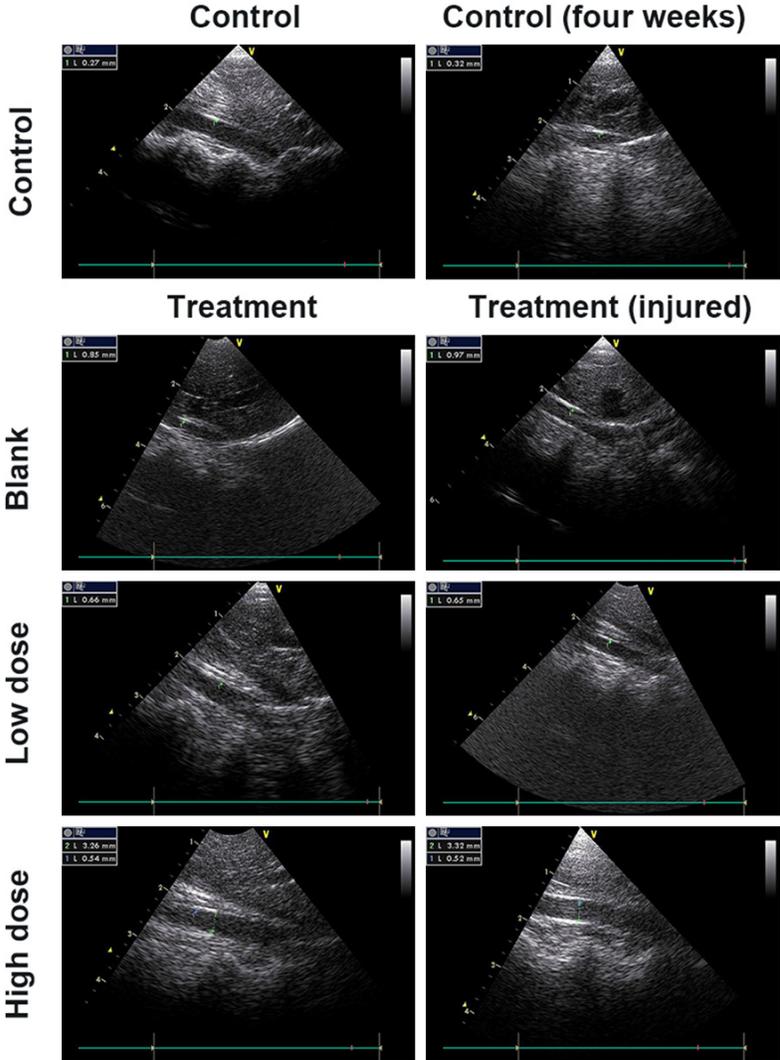


Figure S1. Pathological images of abdominal aorta using B-ultrasonography methods in rabbits before and after injuries.