



Effects of naringin on hypercholesterolemia-induced atherosclerotic rabbits

Jiangyue Liu and Daijuan Zhang*

Department of Pathophysiology, Weifang Medical College, Weifang, Shandong Province, China

ABSTRACT

It's reported that Naringin might have the effect of protection in cardiovascular diseases. In this report, we further studied the mechanisms of naringin against atherosclerosis in hyperlipidemic rabbits. The rabbits were randomly divided into (n= 10): naringin group (50 mg/kg of naringin), simvastatin group (10 mg/kg of simvastatin), negative control group and positive control group. After 12 weeks, we found that there were not any differences in plasma total cholesterol (TC), triglyceride (TG), low density lipoprotein (LDL), oxidized LDL (ox-LDL), high density lipoprotein (HDL), and interleukin-1beta (IL-1 β) between naringin and positive control group. We did not find an obvious decrease in the expression of vascular cell adhesion molecule-1 (VCAM-1) protein in naringin group. In addition, aorta plaque instability was not significantly decreased by naringin, as demonstrated by a thinned elastic layer, less vascular smooth muscle cells (VSMCs) accumulation in the plaques, increased intima-media thickness and macrophage proliferation. In our study, these results suggest that naringin supplementation for 12 weeks cannot attenuate the aorta plaque vulnerability or the progression of atherosclerotic lesions in hyperlipidemic rabbits.

Key words: Naringin; Atherosclerosis

INTRODUCTION

Atherosclerosis (AS) is an important risk factor of cardiovascular diseases [1]. The rupture of vulnerable atherosclerotic plaques is currently regarded as a key cause of acute cardiovascular events and stabilization of vulnerable plaques has important clinical significance [2]. Vulnerable plaques are generally characterized as a large lipid core, a thin fibrous cap, and abundant macrophage infiltration [3, 4]. As we all know, inflammation plays a crucial role in the destabilization and development of atherosclerotic plaques. VCAM-1 is one kind of adhesion molecules; it can strengthen the adhesion and migration of monocytes and SMCs into subendothelial spaces [5, 6]. The process is facilitated by inflammation-associated proteins such as IL-1 β [7]. Statins, with potential anti-inflammatory and anti-oxidative properties, have been used to stabilize plaques [8,9]. However, liver dysfunction and myopathy as the side effects limit clinical application. Therefore, it is urgent to develop more safe and effective drugs to stabilize vulnerable plaques.

Naringin (4', 5, 7-trihydroxy flavanone 7-rhamnoglucoside, molecular formula: C₂₇H₃₂O₁₄, molecular weight: 580.5), is a flavanone glycoside of grapefruits, roots of *Cudrania cochinchinensis* and fruits of *Poncirus* [10]. It has been reported to have the properties of metal-chelating, free radical scavenging and antioxidant [11]. It has been reported that naringin has many pharmacological properties including anti-cancer, anti-inflammation and anti-atherogenesis [12-14]. However, the results of naringin on plasma lipids and atherosclerosis are conflicting. Here, we reported that naringin cannot decrease the plasma lipids, ox-LDL, IL-1 β , or stabilize plaques in hyperlipidemic rabbits.

EXPERIMENTAL SECTION

Materials

Naringin was obtained from Nanjing Debao biochemical equipment Co. Ltd. Commercial kits for TC, TG, LDL,

HDL were purchased from Sangon Biotech Co. Ltd. Elastic fiber staining solution was purchased from Beijing Yili Chemicals Co., Ltd.. HHF-35 and RAM-11 monoclonal antibody was bought from Guangzhou Qi Yun biotechnology Co. Ltd..

Animals and diets

New Zealand White rabbits were bought from experimental animal center of Weifang Medical College at the age of 8 weeks (weight 2.0- 2.3kg). All rabbits were kept in individual cages. After an adapted week, all the rabbits were randomly divided into four groups (n = 10 animals each) by randomized block design method. Negative control group was fed a standard diet. Positive control group received a high-cholesterol diet (containing 0.83% cholesterol, 7.5% yolk powder and 5% axungia porci). The simvastatin group received simvastatin (10mg/kg) with a high-cholesterol diet. The naringin group received naringin (50mg/kg) with a high-cholesterol diet. Each rabbit was fed with fresh rabbit food of 120g daily. Water was supplied ad libitum. All the animals were sacrificed with pentobarbital (120mg/kg) intravenously at the end of weeks 12. Plasma was obtained for future analysis. The aortas were rapidly excised for western blot and histological analysis of atherosclerotic lesions. The animal study was carried out according to the guide for the care and use of laboratory animals published by the Chinese National Institutes of Health. All surgeries were performed under pentobarbital anesthesia, and all efforts were made to minimize animal suffering. The study protocol was approved by the animal ethics committee of Weifang Medical College (Permitted number: 2012158).

Macroscopic analysis of aortic fatty streaks

The aorta was excised and fixed in PBS containing 10% neutral buffered formalin for 24h. After removal of outside connecting tissue and fat, the aorta was cut open longitudinally and stained using oil red O solution 15 min (Sigma Chemical Co.). The stained aorta was scanned and the total morphometric lesion area was evaluated by using IPP 6.0 (Media Cybernetics, USA). Data were expressed as percent lesion area.

HPLC conditions and Instrumentation

The HPLC apparatus included two pumps, a controller (Jasco, Groß-Umstadt, Germany), an UV spectrophotometric detector and a chromatopac (Merck, Darmstadt, Germany). The RP-18e column was equipped with a column (LiChrospher 100, 5 μ m). 1% acetic acid - acetonitrile (64:36) was used as mobile phase, the flow rate was set 1.0 ml/min, and the detection wavelength was set at 288 nm.

Lipids measurements

After blood coagulation, plasma was obtained from centrifugation at 8000rpm for 10 minutes. Determinations of plasma TC, TG, HDL and LDL were performed using an automatic blood chemical analyzer (Olympus, AU 600, Japan).

Enzyme-linked Immunosorbent Assay (ELISA) analysis

Plasma concentrations of ox-LDL and IL-1 β were measured by specific ELISA kit according to the instructions of the manufacturer (Wuhan Boster biotech co., Ltd., China). Optical density was obtained at 450nm using THERMO microplate reader. Standard curve was generated from standards of known concentration. IL-1 β and Ox-LDL concentrations were obtained according to the standard curve.

Western blot analysis

The aorta tissues were homogenized using lysis buffer (50mM Tris-Cl pH 7.4, 150 mM NaCl, 1% SDS, 150 mM NaCl, 1% Na-deoxycholate, 1% Triton, protease inhibitor cocktail, and 1mM EDTA.). Total protein contents were tested using a protein assay kit (BioRad, USA). After being separated on 10% SDS-PAGE, proteins (50 μ g) were transferred onto a nitrocellulose (NC) membrane (Millipore, MA, USA). The membranes were incubated with 5% skimmed milk in TBST buffer (TBS with 0.1% Tween-20) for 1 h at 25 $^{\circ}$ C. Protein samples were respectively probed with first antibody (VCAM-1, Sangon Biotech Co. Ltd., China) and glyceraldehyde-phosphate dehydrogenase (GAPDH, Sangon Biotech Co. Ltd., China) overnight at 4 $^{\circ}$ C, and then were washed with TBST. The membranes were incubated with secondary antibody (Sangon Biotech Co. Ltd., China). Finally, signal intensity was quantified by Sigma Scan Pro 5(USA).

Intima-media thickness

Intima-media thickness was obtained by hematoxylin and eosin staining. The aorta embedded segment was cut into 3 μ m sections. After routine deparaffinization and rehydration, the sections were stained with hematoxylin and eosin.

Elastic layer thickness

Elastic layer thickness was evaluated by elastic fibres staining kit. Cryosections (10 μ m thick) were mounted on slides and stained according to instructions for 10 minutes, washed with distilled water. Subsequently, with

differentiation medium they were differentiated for 10 seconds, then with counterstain redyed for 3 seconds and finally, with neutral gum mounted.

Immunohistochemical analysis

Immunohistochemical analysis was undertaken to evaluate the expression of macrophages and VSMCs. The thoracic aorta embedded segment was cut into 3 μ m sections. After routine deparaffinization and rehydration, the sections were incubated with 3% hydrogen peroxide for 10 minutes, and with 5% BSA serum for 20 minutes. Subsequently, they were immunostained with first antibody (RAM-11 or α -SM actin, Sangon Biotech Co. Ltd., China), respectively, for 12 h at 4 $^{\circ}$ C, and then with horseradish peroxidase complex system (R&D Laboratory Research, Santa Cruz, Calif, USA, 1: 200) at room temperature for 30 minutes. Then the sections were visualized under light microscopy using a DAB peroxidase substrate kit (Vector Laboratories). All the photographs were analyzed using IPP 6.0 (Media Cybernetics, USA) by an observer blinded to the study.

Statistical Analysis

One-way analysis of variance was used to analyze data. Data were expressed as Mean \pm SD. $P < 0.05$ was taken as statistically significant. All the analyses used SPSS (version 18.0)

RESULTS AND DISCUSSION

Results

Physiological and biochemical measurements

No rabbits died during the experimental process. All rabbits gained weight at weeks 0, 4, 8, 12, while there were no significant differences among any of the groups (Data not shown).

The percentage area of atherosclerotic lesions on the inner surface was not reduced in naringin group compared to control group ($P > 0.05$) (38.92 \pm 10.71% VS. 40.13 \pm 11.02%).

Plasma naringin levels were significantly elevated in naringin group for 12 weeks (0.38 \pm 0.11 μ M).

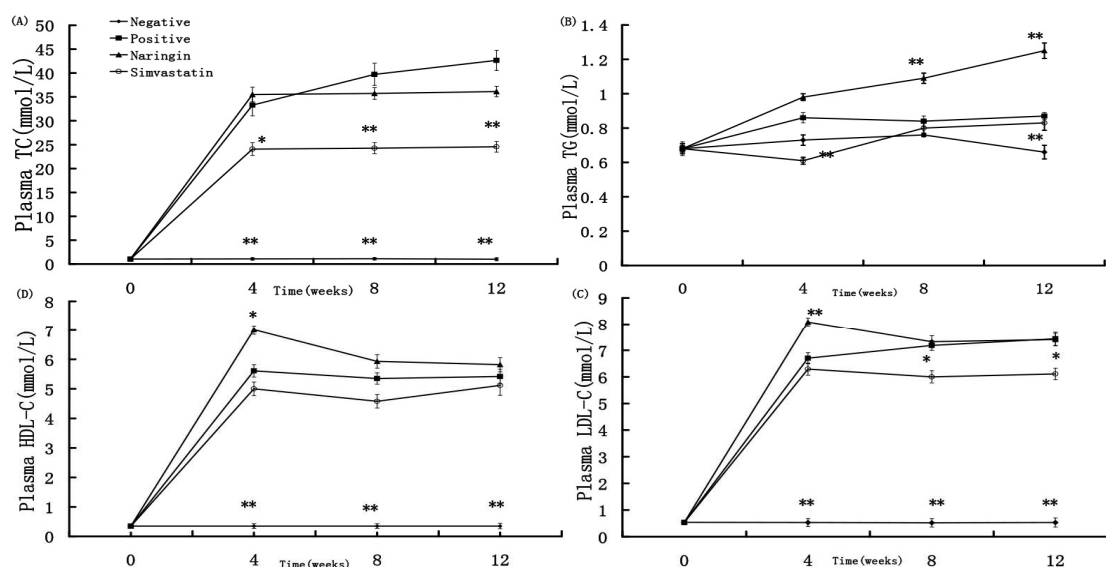


Figure 1.

Fig.1 Comparison of plasma lipids among each group (n=5, concentration expressed as mol/L) (*) Groups vs. Positive control group: $p < 0.05$. (**) Groups vs. Positive group: $p < 0.001$

Plasma lipid levels were shown in Fig. 1. TC, HDL-C and LDL-C concentrations in positive control group were increased significantly for weeks compared to normal group ($p < 0.001$), but TG concentration was increased significantly only at weeks 12 ($p < 0.001$). Simvastatin markedly decreased TC for weeks ($p < 0.05$), decreased TG ($p < 0.001$) at week 4, and reduced LDL-C ($p < 0.05$) at weeks 8, 12 compared to positive control group. The TC level in naringin group was not different from positive control group at weeks. At weeks 4, naringin significantly increased LDL-C and HDL-C ($p < 0.05$). At weeks 8, 12, naringin markedly increased TG ($p < 0.001$) compared to

positive control group. These findings suggest that naringin had no positive effects on plasma lipids in the hyperlipidemic rabbits.

Analysis of IL-1 β and ox-LDL level

The plasma IL-1 β and ox-LDL levels were detected using ELISA. At weeks 4, 8 and 12, in the positive control group, IL-1 β and ox-LDL levels were substantially increased ($p < 0.001$) compared to negative control group. At weeks 8 and 12, simvastatin group markedly decreased IL-1 β and ox-LDL levels ($p < 0.001$)

compared to positive control group. No differences were observed between naringin group and positive control group ($p > 0.05$) (Fig.2). These data suggest that naringin cannot decrease plasma IL-1 β and ox-LDL levels in the hyperlipidemic rabbits.

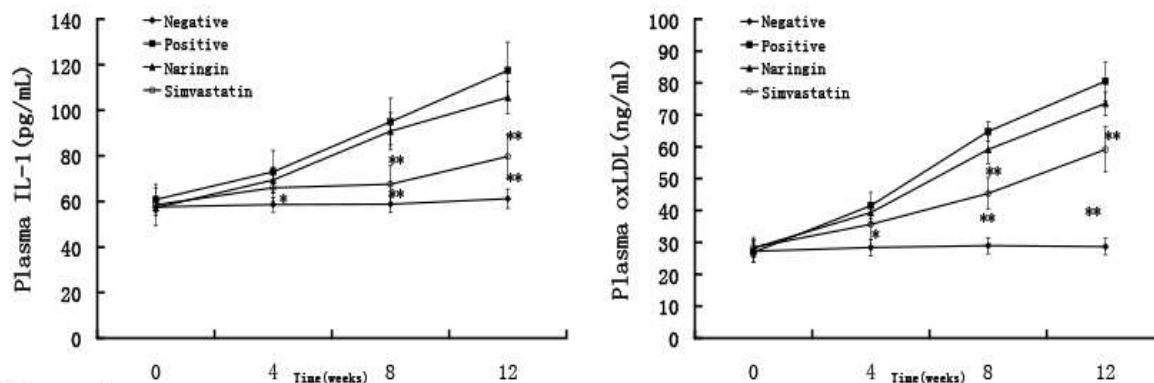


Figure 2.

Fig.2 The plasma IL-1 β and ox-LDL levels in four groups by ELISA.

(n=5, concentration expressed as pg/mL) (*)Groups vs. Positive control group: $p < 0.05$. (**) Groups vs. Positive control group: $p < 0.001$

Expression of VCAM-1 protein

The expression of VCAM-1 protein in the simvastatin group was markedly decreased ($p < 0.001$) compared to positive control and naringin group. But no differences were found between naringin and positive control group ($p > 0.05$) (Fig. 3). These data suggest that naringin cannot decrease VCAM-1 protein expression to attenuate thoracic aorta plaques.

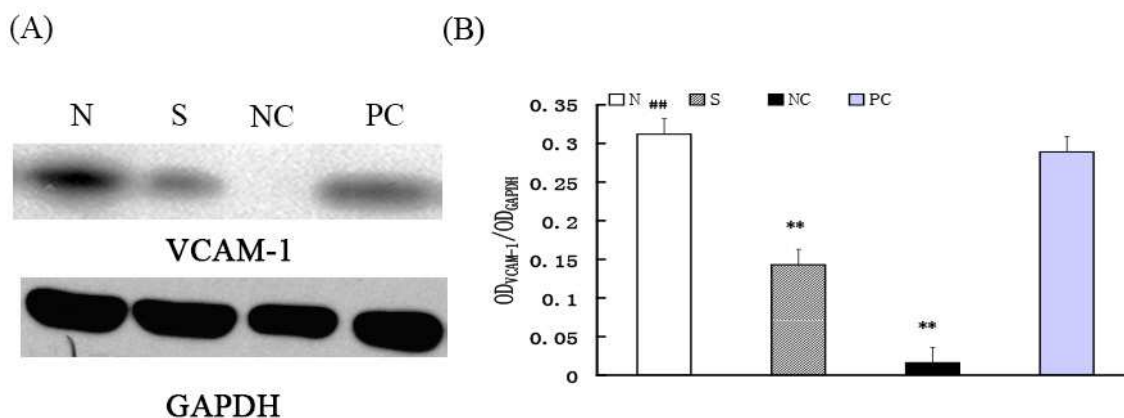


Figure 3.

Fig.3 Expression of VCAM-1 protein on thoracic aortae wall by western blot (**) Groups vs. Positive control group: $p < 0.001$. (##) Naringin group vs. Simvastatin group: $p < 0.001$

Histological and Immunohistochemical Discovery

Our findings showed that intima-media thickness was inhibited by simvastatin ($p < 0.001$) compared to positive control group, and positive control group was significantly increased ($p < 0.001$) compared to negative control group. But no marked difference was observed between naringin and positive control group ($p > 0.05$). Simvastatin

markedly increased the thickness of the elastic layer compared to positive control group ($p < 0.05$), and no sharply difference between naringin group and positive control group ($p > 0.05$). Content of VSMCs in plaques was remarkably increased in simvastatin group compared to positive control and naringin group ($p < 0.001$), but no marked differences were observed between naringin and positive control group ($p > 0.05$). Meanwhile, macrophage accumulation in naringin and positive control group sharply increased compared to negative control group, but no differences were observed between naringin and positive control group ($p > 0.05$). Simvastatin significantly decreased macrophage accumulation compared with positive control and naringin group ($p < 0.001$). (Fig. 4-1,2)

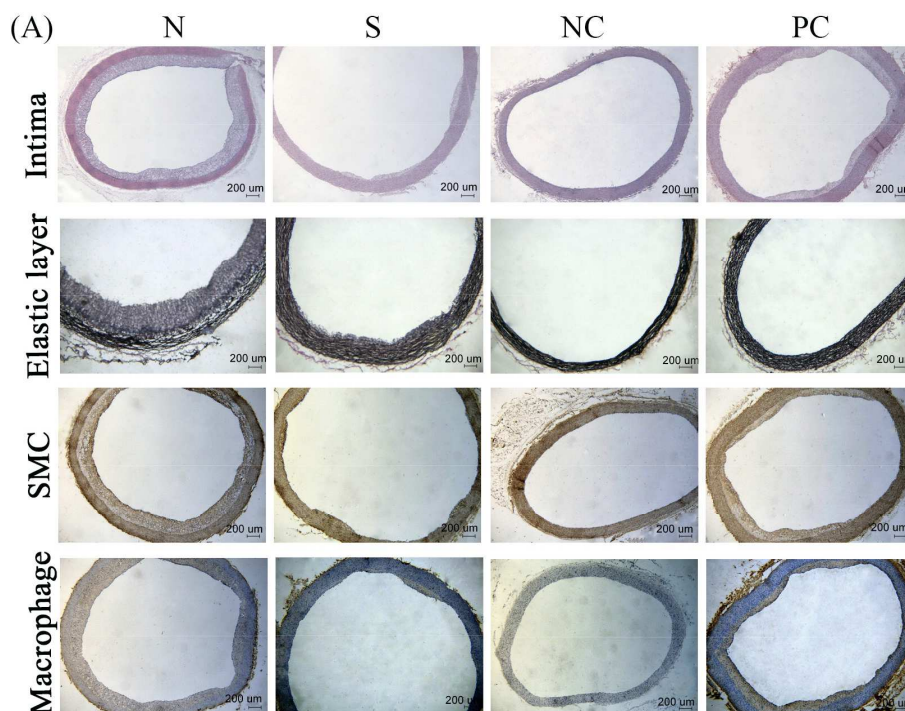


Figure 4-1.

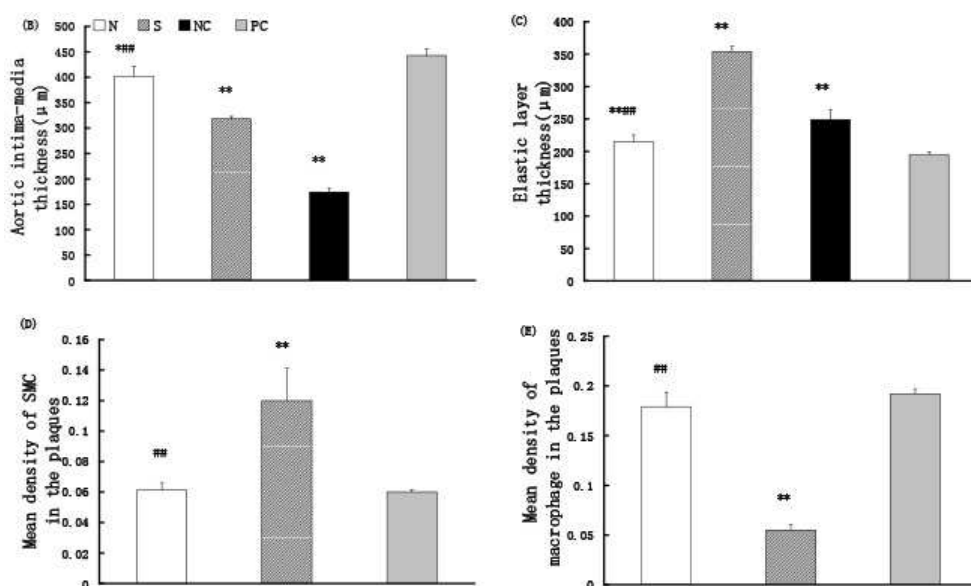


Figure 4-2.

Fig.4-1, 2 Histological and Immunohistochemical consequence. 4-1: (A) Intima of four groups by HE staining; Elastic layer of four groups; the expression of macrophages and VSMCs by immunohistochemical analysis.

4-2:(B)Comparison of Aortic intima-media thickness among four groups (C) Comparison of Elastic layer thickness among four groups (D) Comparison of mean density of SMC in the plaques among four groups (E) Comparison of mean density of macrophage in the plaques among four groups (*)Groups vs Positive control group: $p < 0.05$. (**)Groups vs. Positive control group: $p < 0.001$. (##) Naringin group vs. Simvastatin group: $p < 0.001$).

DISCUSSION

Naringin belongs to flavanone. In citrus fruits and citrus derived products, such as grapefruit, naringin makes it bitter [15]. Many studies of naringin focused on cardiovascular protection, insulin resistance, oxidative stress and inflammation, and so on. We are interested in the anti-atherogenic effect of naringin.

Atherosclerotic rabbit model and naringin dosage

In one study, the extent of atherosclerotic lesion was reduced by naringin compared with lovastatin [16]. However, we did not observe the anti-atherogenic effects of naringin. There are many differences in design between their studies and ours, which might explain why not in our study. Firstly, it could be discussed that naringin plasma levels were so low that could exert anti-atherogenic properties in our study. The dosage of 50mg/kg of naringin resulted in markedly increased naringin plasma levels ($0.38 \pm 0.11 \mu\text{M}$) in our study. Choe SC et al. used 500 mg/kg of naringin [16]. But in their references, Bok SH et al. used 100mg/kg body weight in rats and used 50mg/kg body weight in rabbits [17, 18]. They all didn't provide naringin plasma levels. Secondly, the difference of our study is the dose of cholesterol in diet. The hypercholesterolemia-induced atherosclerotic rabbit model has been widely used in studies of cardiovascular diseases. The hyper cholesterol diet consisted of 0.5-2% cholesterol [18-20]. They used 0.25% cholesterol diet, the plasma TC concentration increased to 1248mg/dL (32.32mmol/L) at weeks 8, whereas we used 0.83% cholesterol diet (1g/day each rabbit) in our study. At weeks 8, the plasma TC concentration increased to 40.72mmol/L in our study, as had been shown by Zhou G et al [21]. Thirdly, intervention time was different. Many studies finished at the end of 8 weeks, but we aimed at longer intervention times (12 weeks used in our study). Our major aim was to study the effects of naringin on cardiovascular parameters after the establishment of distinct atherosclerotic lesions in the vessels. But we did not observe decreased fatty streak in aorta after 12 weeks of high-cholesterol diet. The result suggested that longer intervention times may have no effects on atherosclerotic lesions.

To date, there are no human intervention studies of naringin on the progression of atherosclerosis. The anti-atherogenic effect of naringin was inconformity in various animal studies [16, 22].

Impact on plasma lipids

The studies of naringin on the blood lipids in human are not consistent. It's reported that healthy individuals received 400 mg/day of naringin for 2 months, but there were no changes in plasma lipids concentration [23]. In hypercholesterolemic subjects uptook the same dose of naringin, plasma LDL-C and TC concentrations were significantly lowered, and the HDL-C/TC ratio was increased. But several studies showed that naringin cannot decrease TC and LDL-C concentrations [24-27], namely, naringin had no effects on LDL-C and TC concentrations. A lipid-lowering effect of naringin has been reported in several animal models. Supplementation with naringin (0.05%) in rabbits or other rodents fed a cholesterol-rich diet reduced cholesterolemia [28, 29].

But in our study, we found that feeding the diet with 50mg/kg /d naringin did not affect diet-induced increases in plasma TC, LDL, HDL, TG levels. In addition, when prepared for plasma, we found that the plasma was ivory in naringin group, as same as the plasma of rabbits in positive control group. So we believed that naringin has no lowered-lipids effects in our study.

Impact on atherosclerosis

Previous studies in various animal models suggested that naringin might influence the formation of atherosclerosis. In rabbits fed with a high-cholesterol diet supplemented with naringin (0.5%), the atherosclerotic area was reduced [16]. In mice fed with a high-fat diet supplemented with naringin (0.02%), the atherosclerotic plaques were reduced, but the same dose naringin did not affect the area of the lesion in ApoE(-/-) mice [22].

Lipids deposited in the intima of arteries plays an important role at the beginning of atherosclerosis [30]. Triglycerides can elicit differential inflammatory responses in endothelium cell [31]. Kirii H et al reported that IL-1 β plays an important atherogenic role during atherosclerosis [32]. In our study, the plasma IL-1 β levels in naringin group and positive control group significantly increased compared with the negative control and simvastatin group. Iiyama K et al reported the expression patterns of the VCAM-1 in animal models of atherosclerosis [33]. The level of VCAM-1 in endothelial cells was induced by inflammatory cytokines [34]. Matsumoto et al reported in diet-induced rabbits the earliest morphological changes of atherosclerotic arteries included the activation of the

endothelium cells, the accumulation of foam cells in the intima, and focal adherence of monocytes to the endothelium [35], which was proven by us. In our study, the expression of VCAM-1 protein and macrophage accumulation in the thoracic aortic segment were found markedly increased in naringin and positive control group compared to the negative control and simvastatin group. The numbers of macrophages accumulation increased plaque vulnerability [36]. Macrophages, key component of atherosclerotic plaques, play a central role in the destabilization process; however, VSMCs facilitate plaque stabilization. Our study showed that the plaque content of VSMCs was higher in simvastatin group but lower in naringin group and positive control group.

In our study, there were no advanced anti-atherogenic changes: high level of proinflammatory cytokines IL-1 β and inflammatory cytokines VCAM-1, increase of intima-media thickness, thinness of the elastic layer, less VSMCs and more macrophage accumulation in the plaques led to unstable thoracic aorta plaque. In addition, when the thoracic arteries were excised, we observed them with the naked eye, and found that the vascular wall of rabbits in naringin group were thicker than the arteries of rabbits in positive control group.

CONCLUSION

All the findings in our study indicate that naringin has no anti-atherosclerotic effects. This represents an unexpected result.

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