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Reduction of oxidative stress and apoptosis in hyperlipidemic rabbits by ellagic acid

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Abstract

Oxidative stress is one of the major risk factors for coronary artery disease. Ellagic acid is a phenolic compound present in fruits and nuts, and has been found to have antioxidative property. Twenty-four New Zealand white (NZW) rabbits were assigned randomly into four dietary groups. The normal group was fed regular rabbit chow, and the cholesterol group was fed a high fat and cholesterol diet. The ellagic acid (E) group and probucol group were fed the same diet as the cholesterol group plus the addition of 1% (w/w diet) ellagic acid and probucol, respectively. Oxidative stress [as measured by plasma lipids, oxygen free radicals and thiobarbituric acid reactive substances (TBARS)] increased in the cholesterol group compared with the normal group; however, it decreased in the probucol and E groups compared with the cholesterol group. Forty-five percent of the intimal surface of the thoracic aorta was covered with atherosclerotic lesions in the cholesterol group, but only 2–3% was covered in the E and probucol groups. The aortic level of 8-(OH)dG and the expression of caspase-8, caspase-9 and Fas ligand were also suppressed after ellagic acid supplement. These results indicated that ellagic acid could prevent atherosclerosis via suppression of oxidative stress and apoptosis in hyperlipidemic rabbits.

Keywords: Antioxidant; Ellagic acid; Oxidative stress; 8-(OH)dG; Apoptosis; Atherosclerosis

1. Introduction

Atherosclerosis and its complications continue to be the major cause of premature death in the developed world. Oxidative stress is one of the major risk factors for atherosclerosis. Free radical-induced lipid peroxidation has been implicated in the pathogenesis of atherosclerosis, and reactive oxygen species (ROS) are known to be the initiators of lipid peroxidation [1]. Previous studies have shown that hyperlipidemia increases the plasma levels of oxygen free radicals [2], by reacting with lipids, proteins and DNA to produce oxidized compounds, such as malondialdehyde (MDA) and 8-hydroxy-2′-deoxyguanosin (8-OhdG). Therefore, the levels of oxidative DNA damage and activities of DNA repair enzymes were elevated in human atherosclerotic plaques [3,4]. The direct involvement of oxidative stress in apoptosis has also been demonstrated in a variety of cell types, such as aortic endothelial and smooth muscle cell [5]. Free radicals cause apoptosis [6,7] and can be inhibited by antioxidants such as vitamin E, Trolox, superoxide dismutase and catalase [6–9]. Furthermore, apoptosis has been seen in human atherosclerotic lesions [10,11]. It has been shown that the Fas ligand was expressed in the cells of the arterial wall of human atherosclerotic lesions [12,13]. The Fas/Fas ligand complex activates caspase-8, which causes nuclear apoptosis by caspase cascade pathway. Caspase-8 can also activate caspase-9 by the mitochondria-dependent pathway. In addition to the Fas/Fas ligand/caspase signal pathway, cytotoxic drugs and ionizing radiation can signal the activation of caspase-9, which leads to cell death [14–16].

Polyphenolic compounds are widely distributed in the vegetable kingdom and are often encountered in our daily

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lives, being contained in tea, red wine, fruits, etc. [17,18]. Ellagic acid is a phenolic compound present in fruits and nuts, including blueberries, blackberries, raspberries, strawberries and walnuts [19–21]. It has been found to have antimutagenic, antiviral and antioxidative properties [22–24]. Previous studies indicated that ellagic acid had a scavenging action against both oxygen and hydroxyl radicals, and inhibited lipid peroxidation and 8-OhdG formations in vitro and in vivo [25–28]. Probucol, 4,4-(isopropylidenedithio)bis [2,6-*tert*-butylphenol], is a lipid-soluble antioxidant drug with cholesterol-lowering properties and has been previously demonstrated to slow progression of atherosclerosis in most hyperlipidemic animals [29,30].

The present study was designed to examine the effect of ellagic acid on levels of plasma lipids, free-radical scavenging activities, thiobarbituric acid reactive substances (TBARS), aortic fatty streak, level of 8-(OH)dG and expression of apoptosis-related genes (caspase-8, caspase-9 and Fas ligand) in NZW rabbits fed a high fat and cholesterol diet.

2. Materials and methods

2.1. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging and inhibition of low-density lipoprotein (LDL) oxidation

The DPPH-radical scavenging activities of ellagic acid and probucol were determined. In brief, 1 vol of acetate buffer (100 mmol/L, pH 5.5), 1 vol of ethanol and 0.5 vol of freshly prepared DPPH ethanolic solution (500 nmol/L) were mixed. After adding the test compound, the mixture was incubated at 25°C for 90 min. The change in absorbance at 517 nm was then determined [31].

Low-density lipoprotein ($1.019<d<1.063$) was isolated using micro-ultracentrifugation in NaBr–NaCl solution as previously described [32]. Low-density lipoprotein was extensively dialyzed against phosphate-buffered saline (PBS) at 4°C under nitrogen for 24 h. In vitro LDL oxidation was carried out in a 96-well microtiter plate at 37°C [33]. A 50-μl aliquot of LDL in each well was preincubated with ellagic acid and probucol for 1 h. The final volume in each well was adjusted to 100 μl with PBS. Low-density lipoprotein oxidation was initiated by adding $CuSO_4$ to a final concentration of 10 μmol/L. After incubation, 150 μl of EDTA (2 mmol/L) was added. A 100-μl portion of the mixture was then transferred to a minivial containing 0.9 ml of 2-propanol. The precipitates were removed by centrifugation. The concentration of conjugated dienes in the supernatant was determined by absorption at 234 nm.

2.2. The trolox equivalent antioxidant capacity (TEAC) assay

The TEAC was determined according to the method of Miller et al. [34]. A value of 1 TEAC in a sample is defined as a concentration equivalent to 1 mmol/L Trolox, a water-soluble analog of α-tocopherol.

Table 1
Antioxidative capacity of ellagic acid and probucol

	Inhibition of LDL oxidation (IC_{50}, nmol/L)	DPPH radicals scavenging ability (IC_{50}, nmol/L)	TEAC
Probucol	12.57±2.16	1.45±0.19	2.38±0.54
E	18.21±0.49[a]	0.49±0.07[a]	2.54±0.15

All values are mean±S.D. IC_{50} values were obtained from the concentration response curves; $n=3$.
[a] $P<.05$ Group E vs. group probucol.

2.3. Animal models

Twenty-four 4–6-week-old New Zealand white rabbits with an average body weight of 1.5 kg were housed in individual cages with an environmentally controlled atmosphere (25°C) and a 12/12-h light/dark cycle. Food and water were provided ad libitum. All procedures were performed according to the *Guidelines of the Care and Use of laboratory Animals* published by the Chinese Society for Laboratory Animal Science (Taiwan). After a 2-week adaptation period, animals were randomly assigned to four experimental groups of six rabbits. The normal group was fed regular laboratory rabbit chow (100 g/day). The cholesterol group received an atherogenic diet consisting of chow enriched with 10% corn oil and 0.5% cholesterol. The ellagic acid and probucol groups were fed the atherogenic diet supplemented with 1% (w/w diet) ellagic acid (Sigma, MO, USA) and probucol (Weidar, Taichung, Taiwan). The selection of ellagic acid dose was based on its antioxidant activity comparable to that of probucol in TEAC and Cu^{2+}-induced LDL oxidation (Table 1). At the end of 8 weeks of feeding, all rabbits were sufficiently anesthetized with an injection of 130 mg/kg pentobarbital via the marginal ear vein after 12 h of fasting. Blood samples were then collected by cardiac puncture, and the aortas were removed for morphological studies.

2.4. Biochemical studies

The blood samples were centrifuged at 1000×*g* for 15 min at 4°C. The serum was then separated. Serum total cholesterol (TC), triacylglycerol (TG), LDL-cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) concentrations were assayed by commercial kits (Randox, San Diego, CA, USA).

2.5. Measurement of serum MDA level

Malondialdehyde in serum was determined according to the method devised by Ohkawa et al. [35] with minor modifications. The assay mixture consisted of 25 μl of serum, 2.5 μl of 60 mM $CuSO_4$ and 22.5 μl of H_2O, and was incubated for 4 h at 37°C. Thereafter, the mixture was added to 0.35 ml of 20% trichloroacetic acid and 0.35 ml

Table 2
Serum TG, TC, LDL-C, HDL-C and TBARS levels from rabbits fed an experimental diet for 8 weeks

	Normal	Cholesterol	Probucol	E
TG (mmol/L)	0.9±0.1[a]	4.2±0.6[b]	0.9±0.1[a]	1.7±0.2[a]
TC (mmol/L)	2.3±0.2[a]	23.9±2.3[b]	7.6±0.1[c]	10.3±2.1[c]
LDL-C (mmol/L)	1.3±0.1[a]	20.1±1.2[b]	6.3±2.1[c]	7.9±2.1[c]
HDL-C (mmol/L)	0.3±0.1[a]	3.7±0.1[b]	1.1±0.1[c]	2.4±0.1[c]
TBARS (MDA nmol/ml)	13.6±3.5[a]	57.1±4.9[b]	6.8±0.9[c]	12.7±5.6[a]

All values are mean±S.D. In the normal group, rabbits were fed rabbit chow; in the cholesterol group, rabbits were fed chow plus 10% corn oil and 0.5% cholesterol; in the probucol group and the E group, rabbits were fed the same diets as the cholesterol group plus the addition of 1% probucol or 1% ellagic acid, respectively.
[a–c]Data with different superscripts in the same row are significantly different at $P<.05$.

of 0.67% thiobarbituric acid, and was heated for 30 min at 70°C. After centrifugation at 10 000 rpm for 2.5 min, the supernatant was assayed spectrophotometrically at 540 nm. Serum MDA content was expressed as nanomoles per milliliter.

2.6. Measurement of serum free-radical level

Superoxide radicals and H_2O_2 in peripheral blood were quantified by lucigenin and luminal amplified chemiluminescence (CL). The methods for measuring luminal-CL or lucigenin-CL were similar to methods described previously [36,37]. The total CL counts were calculated by integrating the area under the curve and subtracting it from the background level. The production of CL per white blood cell (WBC) was calculated by dividing the blood CL levels by the WBC count and expressed as CL/WBC.

2.7. Morphological studies

The intimal lipid lesions in the thoracic region (from the distal end of the aortic arch to the mesenteric artery) were examined quantitatively by estimating the percentage of Sudan IV-stained regions (lipid infiltration) in photographs. The area of atherosclerosis was expressed as a percentage of the total aortic area.

2.8. Measurement of 8-hydroxy-deoxyguanosine (8-OhdG) level in aortic arches

The level of 8-OhdG was determined by the method devised by Imaeda et al. [38] with minor modifications. One gram of aortic arch was homogenized in a Polytron apparatus in 10 ml of PBS. The homogenate was centrifuged at 3200 rpm for 10 min at 4°C. The pellet containing nuclei was dispersed in 10 ml of lysis buffer (10 mM Tris–HCl, 25 mM EDTA, 75 mM NaCl, 1% SDS; pH 8.0), 250 μl of proteinase K and 50 μl of butylated hydroxytoluene, and incubated overnight at 37°C. The suspension was extracted twice with phenol/water/chloroform and twice with chloroform alone. DNA was precipitated from the aqueous phase with 2 vol of ethanol and 0.1 vol of sodium acetate trihydrate. Collected DNA was added to 3 ml of 10 mM Tris–HCl and digested with 6 μg RNase A and 150 U of RNase T1 at 37°C for 1 h. The suspension was extracted twice with phenol/water/chloroform and twice with chloroform alone. DNA was precipitated by ethanol as described above. The DNA pellet was resuspended in HPLC-grade water. Detection of 8-OHdG was performed with an HPLC/ECD (BAS, Indiana, USA) system. An aliquot was injected onto an Alltima C18 5U column (4.6 mm×15 cm; Alltech, Kentucky, USA). 8-OHdG and dG were separated by a mobile phase consisting of 50 mM sodium acetate. The levels of 8-OHdG were expressed as 8-OHdG/10^6 dG [38].

2.9. Measurement of Fas-ligand, caspase-9 and caspase-8 levels in aortic arches

After homogenization of the rabbit aortic arches by a Polytron PT3100 (POLYTRON, Luzernerstrasse, Switzerland) homogenizer and a sonicator (MISONIX, Farmingdale, Spain), protein content was determined by the Bradford method (Bio-Rad, Hercules, California, USA). A total of 80 μg of protein extract from aortic arches was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis using the Hoefer mini VE system (Amersham Biosciences, Piscataway, NJ, USA). Protein was transferred to a polyvinylidene difluoride membrane (Hybond-P Uppsala, Sweden). Following transfer, the membrane was washed with PBS and blocked for 1 h at 37°C with 5% fat-free milk in PBS with 0.1% Tween 20 (PBST). Western blot analysis was used to measure the binding of rabbit antibody to Fas ligand (1:1000; BD Pharmingen, California, USA), caspase-9 (1:1000; BD Pharmingen) and caspase-8 (1:1000; BD Pharmingen). Membranes were incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Amersham, Buckinghamshire, England). Following removal of the secondary antibody, blots were washed by PBST and developed by ECL-Western blotting system (Pierce, Rockford, IL, USA). Densities of the obtained immunoblots were quantified by Kodak digital science 1D (ver. 2.03; Kodak, Rochester, NY, USA).

2.10. Statistical analysis

All data were expressed as mean±S.D. Comparisons between the four groups were made by one-way ANOVA. Turkey's post hoc test was used to analyze significant

Table 3
Lucigenin-CL and luminol-CL of rabbits fed an experimental diet for 8 weeks

Groups	Lucigenin-CL (counts/WBC)	Luminol-CL (counts/WBC)
Normal	1.3±0.4[a]	0.2±0.1[a]
Cholesterol	7.0±0.1[b]	1.6±0.9[b]
Probucol	1.5±0.3[a]	0.2±0.1[a]
E	0.6±0.2[a]	0.3±0.2[a]

All values are mean±S.D.
[a–b]Data with different superscripts in the same column are significantly different at $P<.05$.

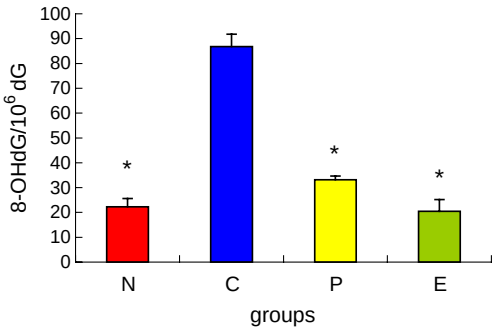

Fig. 1. The level of 8-(OH)dG in the aorta of rabbits fed an experimental diet for 8 weeks. In the normal group (N), rabbits were fed rabbit chow; in the cholesterol group (C), rabbits were fed rabbit chow plus 10% corn oil and 0.5% cholesterol; in the probucol group (P) and the ellagic acid (E) group, rabbits were fed the same diets as the cholesterol group plus the addition of 1% probucol or 1% ellagic acid, respectively. *P<.05 Groups N, P and E vs. Group C.

effects. A P value of .05 was taken as the threshold for statistical significance.

3. Results

3.1. Body weight and food intake

No differences were found among the four experimental groups with respect to weight gain at the end of the experimental period. All the animals ate a similar amount of food per day.

3.2. Antioxidative capacity of ellagic acid in vitro

The TEAC value of ellagic acid was similar to that of probucol. Both ellagic acid and probucol exhibited free-radical scavenging activity in DPPH assay and were effective in preventing Cu^{2+}-induced LDL oxidation (Table 1). On the basis of IC_{50} values, ellagic acid was three times more potent than probucol in DPPH assay. However, probucol was more effective than ellagic acid in the inhibition of LDL oxidation.

3.3. Plasma lipids and TBARS level

A high-fat atherogenic diet significantly increased serum TG, TC, LDL-C and HDL-C levels, as we expected (Table 2). Chow supplemented with ellagic acid and probucol suppressed the increase in TG, TC, LDL-C and HDL-C levels caused by an atherogenic diet. No difference in plasma lipids was found between the E and probucol groups. The plasma TBARS level had increased fourfold by the eighth week in the cholesterol group compared with that in normal group (Table 2). The increase in serum TBARS was significantly (P<.05) reduced by supplementation of an atherogenic diet with ellagic acid and probucol. Probucol showed the most effective reduction in TBARS levels by the eighth week.

3.4. Effect on free-radical scavenging activities

After consumption of the experimental diets for 8 weeks, the lucigenin-CL and luminol-CL levels in whole blood were higher in the cholesterol group than in the normal

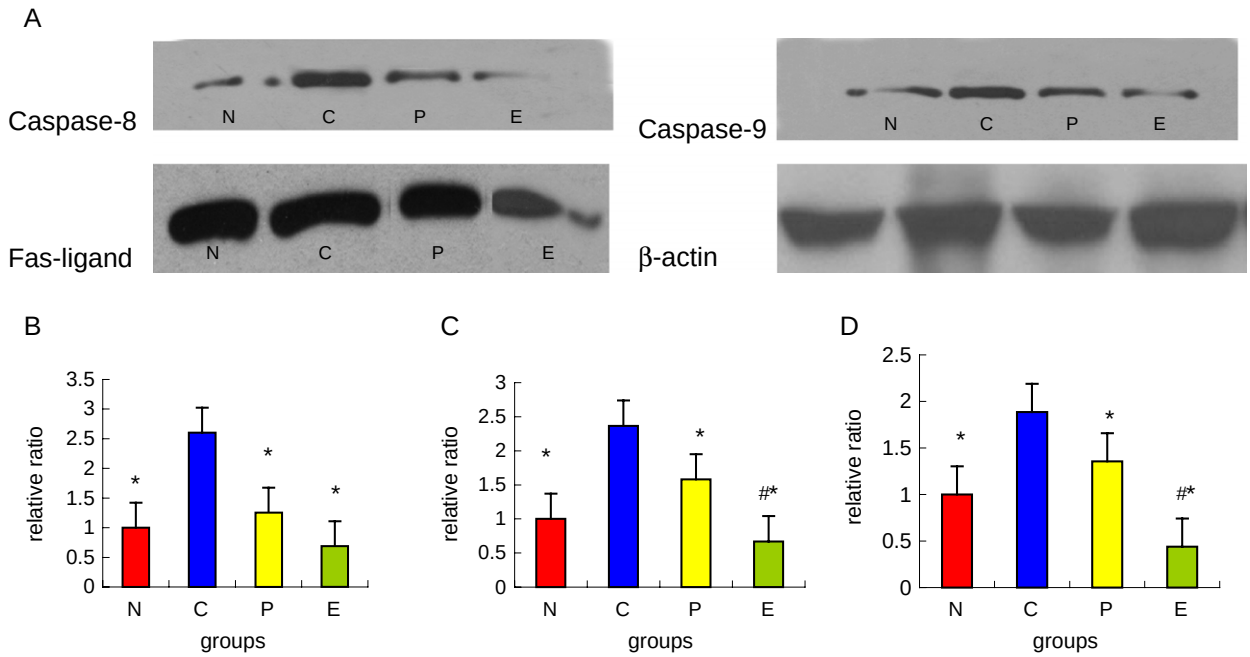

Fig. 2. Effect of ellagic acid on the expression of apoptosis-related protein levels of caspase-8, caspase-9 and Fas ligand in the aortic arches of rabbits. (A) Representative Western blot showing changes in the levels of caspase-8, caspase-9 and Fas ligand. The results of (B) caspase-8, (C) caspase-9 and (D) Fas ligand are expressed as x-fold increase over the normal group as measured by quantitative immunoblotting. *P<.05 Groups N, P and E vs. Group C. #P<.05 Group E vs. Group P.

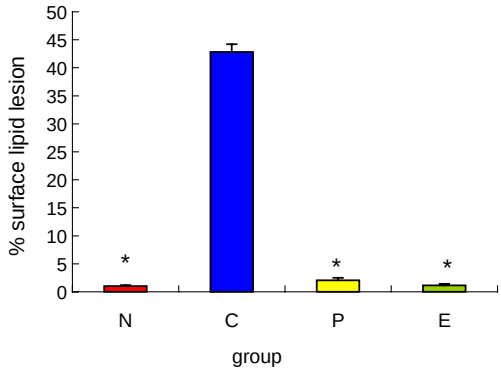

Fig. 3. The densitometric analysis of the relative area of lipid lesions in the aorta after the experimental diet. Results are expressed as mean±S.D. *P<.05 Groups N, P and E vs. Group C.

group and lower in the E group and the probucol group than in the cholesterol group (Table 3).

3.5. Effect on 8-OHdG levels

Aortic 8-OHdG levels were significantly lower in the group fed an atherogenic diet supplemented with ellagic acid and probucol compared with rabbits fed an atherogenic diet only (P<.05) (Fig 1).

3.6. Effect on the expressions of Fas ligand, caspase-8 and caspase-9

The caspase-8, caspase-9 and Fas-ligand protein levels in the aortic arches of animals were significantly higher in the cholesterol group than in the normal group, and lower in the E and probucol group than in the cholesterol group. The protein levels of caspase-9 and Fas ligand were lower in the E group than in the probucol group (Fig. 2).

3.7. Morphological change of the thoracic aorta

The lipid lesions on aortic areas were determined by Sudan IV staining (Fig 3). In the normal group, the rabbits, which were fed regular chow for 8 weeks, showed the fewest lipid lesions. In the cholesterol group, lipid lesions covered 42.8±1.4% of the aortic area, as predicted. However, in the ellagic acid and probucol groups, the extent of the lipid lesions had significantly decreased to 1.13±0.31% and 2.04±0.56%, respectively.

4. Discussion

In this study, we found that serum TG and TC increased significantly in rabbits receiving a high fat and cholesterol diet and decreased in rabbits receiving the same diet supplemented with ellagic acid. These results suggest that ellagic acid was effective at reducing serum TG and TC. The effectiveness of ellagic acid in decreasing plasma lipids was similar to that of probucol. Many polyphenolic compounds possess hypolipidemic activity and have been shown to increase the fecal fat excretion and LDL receptor activity [39,40]. Ellagic acid is a phenolic compound, which may also possess the same hypolipidemic action.

The critical features of atherosclerosis development are caused by the formation of free radicals (ROS), because ROS promote cell proliferation, hypertrophy, growth arrest, and/or apoptosis and oxidation of LDL [41,42]. In our study, we found that ellagic acid exhibits free-radical scavenging activities and inhibits LDL oxidation. The antioxidative ability is equal to two- to threefold of Trolox (Table 1). We also found that ellagic acid is more potent than probucol in scavenging DPPH radicals, and less potent in scavenging RO· or ROO· radicals. Therefore, both of them have similar TEAC values. Also as shown in Table 3, the increase in serum TBARS level was significantly reduced by supplementation of an atherogenic diet with ellagic acid. In addition to suppressing lipid peroxidation, ellagic acid also apparently scavenged free radicals in blood. The presence of luminol-CL reflects the production of hydrogen peroxide and singlet oxygen. Lucigenin was found to be insensitive to hypochlorite and hydroxyl radicals, and to specifically measure the level of superoxide radicals [43,44]. As shown in Table 3, lucigenin-CL and luminol-CL were lower in the E group than in the cholesterol group. These results indicate that ellagic acid functioned as a strong antioxidant and inhibited lipid peroxidation thereby improving atherosclerosis.

Oxidative DNA damage resulting from free-radical attack is another feature of atherosclerosis [41,42]. We therefore examined the amount of DNA damage marker 8-OHdG/dG in aortic arches. As seen in Fig 1, ellagic acid significantly decreased the amount of 8-OHdG produced. Hence, oxidative DNA damage was suppressed. In our study, we also found that ellagic acid has the ability to inhibit LDL oxidation (Table 1). It is well established that oxidative DNA damage and ox-LDL can induce apoptosis [41,42,45]. The activation of Fas/Fas ligand/caspase death-signaling pathway, Bcl-2 protein family/mitochondria, the tumor suppressive gene *p53* and the proto-oncogene c-*myc* in atherosclerotic lesions mediates vascular apoptosis during the formation of atherosclerosis [13]. It has been seen that the Fas ligand is expressed in the cells of the arterial wall of human atherosclerotic lesions [12,13]. The Fas/Fas ligand complex activates caspase-8 and then causes nucleus apoptosis [14,15]. In our study, ellagic acid was found to suppress the expression of the Fas ligand, the upstream signal of apoptosis and the expression of downstream caspase-8 and caspase-9 (Fig. 2). Therefore, ellagic acid seems to inhibit atherosclerosis by suppressing the activation of Fas/Fas ligand/caspase death-signaling pathway.

Our histological study demonstrated that 45% of the intimal surface of the thoracic aorta was covered with lipid lesions in the cholesterol group, but only 2% to 3% was covered in the ellagic group (Fig. 3). The anti-atherogenic effect is similar to that of probucol. In clinical trials, probucol has been shown to have hypocholesterolemic, antioxidative and anti-atherosclerotic effects [30]. Previous studies also indicated that probucol might regulate some aortic gene

expression, such as VCAM-1 [46], preserve endothelium-derived relaxing factor action [47], inhibit HDL-mediated cholesterol efflux [48], increase the urinary excretion of oxidized cholesterol [49] and inhibit the ox-LDL-induced adhesion of monocytes to aortic endothelial cells [50], to slow the progress of atherosclerosis. Therefore, further research is needed to investigate the anti-atherogenic mechanism of ellagic acid.

In conclusion, our studies found that ellagic acid significantly reduced aortic lipid lesions in rabbits fed an atherogenic diet. This anti-atherogenic effect seems to be closely involved with a decrease in oxidative stress and down-regulation of apoptosis genes. However, the exact mechanism by which ellagic acid induces an anti-atherogenic effect was not fully revealed by this study, and further investigation is underway.

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