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Antioxidative and Hypolipidemic Effects of Barley Leaf Essence in a Rabbit Model of Atherosclerosis

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ABSTRACT—The antioxidative and hypolipidemic effects of barley leaf essence (BL) were investigated in a rabbit model of atherosclerosis. Twenty-four New Zealand White male rabbits were assigned randomly into four dietary groups. The normal group was fed regular rabbit chow and the control group was fed a chow containing 0.5% cholesterol and 10% corn oil. The BL group and the probucol group were fed the same diet as the control group plus 1% (w/w) BL or 1% (w/w) probucol, respectively. The plasma levels of total cholesterol, triacylglycerol, lucigenin-chemiluminescence (CL) and luminol-CL were increased in the control group compared to the normal group; and they were decreased in the BL group and the probucol group compared to the control group. The value of T_{50} of red blood cell hemolysis and the lag phase of low-density lipoprotein oxidation increased in the BL group and in the probucol group compared to the controls. Ninety percent of the intimal surface of the thoracic aorta was covered with atherosclerotic lesions in the control group, but only 60% of the surface was covered in the BL group. This 30% inhibition of hyperlipidemic atherosclerosis by BL was associated with a decrease in plasma lipids and an increase in antioxidative abilities (as measured by T_{50}, lag phase and CL). These results suggest that the antioxidant and hypolipidemic effects of BL could be useful in the prevention of cardiovascular disease in which atherosclerosis is important.

Keywords: Barley leaf essence, Plasma lipid, Red blood cell hemolysis, Chemiluminescence, Atherosclerotic lesion

Hypercholesterolemia is one of the major risk factors for coronary artery disease (1). Epidemiological and experimental data have shown that a high cholesterol-containing diet is highly related to the development of hypercholesterolemia. Rabbits develop hypercholesterolemia rapidly after excessive cholesterol feeding (0.5 – 1% cholesterol diet) (2, 3). Hypercholesterolemia increases the levels of the lipid peroxidation product malondialdehyde in blood (4) and the production of oxygen-free radicals (OFRs), which are induced by polymorphonuclear leukocytes (5). OFRs exert their cytotoxic effects by causing peroixdation of polyunsaturated fatty acids of membrane phospholipids, which can result in an elevation in membrane permeability and loss of cellular integrity (6, 7). Furthermore, free radical-induced lipid peroxidation has been implicated in the pathogenesis of atherosclerosis (8) and reactive oxygen species (ROS) are known to be the initiators of lipid peroxidation. Endothelial cells, smooth muscle cells, neutrophils, monocytes, and platelets may be the sources of ROS in atherosclerosis induced by hypercholesterolemia (5).

Recently, natural plants have received much attention as sources of biologically active substances including antioxidants, antimutagens, and anticarcinogens. Barley leaf essence (BL) is made from barley leaves harvested 2 weeks after seeding. The barley leaves were freeze-dried for 3 days and then ground. The ground leaves contain a variety of vitamins, minerals and polyphenolic compounds; and they have been shown to have antioxidant activity in the lipid peroxidation system (9). Probucol is an antioxidant which is used as a hypocholesterolemic agent and has an established inhibitory function in low-density lipoprotein (LDL) oxidation. It has been demonstrated to slow progression of atherosclerosis in Watanable heritable hyperlipidemia rabbits (10).

The present study compared the effects of BL to the effects of probucol on plasma lipids, free radical scavenging activities, susceptibility to red blood cell (RBC) hemolysis,

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lag phase of LDL oxidation and aortic atherosclerotic lesions in New Zealand White rabbits.

MATERIALS AND METHODS

Animal model and experimental design

A total of 24 male New Zealand White rabbits weighing 1 – 1.5 kg were obtained from the Laboratory Animals Service of the Department of Agriculture in Taiwan (Chiu-Nan, Taiwan). Rabbits were housed in individual cages in a room under temperature control at 23°C and kept on a 12-h light-dark cycle, with food and water supplied ad libitum. All experiments and procedures were performed in accordance with the procedures outlined in the Guidelines for the Care and Use of Laboratory Animal published by the Chinese Society for the Laboratory Animal Science, Taiwan. After a 2-week period of adaptation, the animals were randomly divided into four dietary groups of 6. The normal group was fed regular laboratory rabbit chow (100 g/day). The control group was fed the same amount of regular chow containing 0.5% cholesterol and 10% corn oil. The BL group and the probucol group were fed the same diet as the group control but containing 1% (w/w) BL (VIVA Products Co., Tustin, CA, USA) or probucol (Weida Chemicals, Taichung, Taiwan), respectively. BL contained antioxidants including β-carotene (2496.2 μg R.E./100 g), vitamin C (100 mg/100 g), vitamin E (10.8 mg α-tocopherol/100 g) and polyphenolic compound (191.6 mg gallic acid/100 g) (data not published). Blood samples were collected via ear venipuncture at the beginning, every 4 weeks, and at the end of the study. After 12 weeks of feeding, all rabbits were sufficiently anesthetized with an injection of 130 mg/kg pentobarbitol via the marginal ear vein. Blood samples were then collected by cardiac puncture and the aortas were removed for morphological studies.

Biochemical studies

The blood was centrifuged at 100 × *g* for 15 min at 4°C to obtain serum. Serum triacylglycerol (TG), total cholesterol (TC), LDL-C, high-density lipoprotein (HDL)-C, serum glutamate oxalacetate transaminase (GOT) and serum glutamate pyruvate transaminase (GPT) concentration were assayed by commercial kits (Merck Ltd., Darmstadt, FRG).

Measurement of free radicals in blood

Lucigenin and luminol amplified chemiluminescence (CL) were used to quantify superoxide radicals and OFRs in peripheral blood. The methods used for measuring luminol-CL or lucigenin-CL were similar to methods described previously (11, 12). 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.

Analysis of AAPH-induced hemolysis

Hemolysis was induced by the thermal decomposition of a water-soluble azo compound, 2,2'-azobis-(2-amidinopropane) dihydrochloride (AAPH). The method of Miki et al. (13) with minor modification was used to determine radical-mediated hemolysis. Briefly, a 10% suspension of RBC in phosphate-buffered saline (PBS) [125 mM NaCl and 10 mM sodium phosphate buffer (pH 7.4)] was mixed with equal volumes of sodium phosphate solution (10 mM) containing AAPH (125 mM). Suspensions were incubated at 37°C for 210 min under aerobic conditions and agitated gently throughout. The AAPH solution was incubated for 1 h at 37°C and the RBC suspensions were incubated for 5 min at 37°C before mixing. Aliquots were obtained at times ranging from 0 (corresponding to the time of mixing RBC suspensions with AAPH solution) to 210 min. Samples (0.2 mL) were diluted in 2.5 mL of PBS and centrifuged. The extent of hemolysis was measured spectrophotometrically at 540 nm, by comparing the extracellular hemoglobin content of aliquots with that of a fully hemolyzed reference sample, which was prepared in the same way except that the AAPH solution was replaced by distilled water. Percentage of hemolysis was measured using the equation: % hemolysis = A/B × 100, where A is absorbance of the sample aliquot at 540 nm and B is absorbance of the fully hemolyzed reference at 540 nm.

Lag phase of LDL oxidation

LDL (density = 1.1019-1.063) was isolated using a micro-ultracentrifuge in a NaBr-NaCl solution (14). LDL oxidation was performed after overnight dialysis against PBS, pH 7.4 at 4°C. Oxidation of LDL was determined as the production of conjugated dienes induced by Cu^{2+} by continuously monitoring the change in absorbance at 234 nm (15). The formation of conjugated dienes was measured by incubating 50 μmol/L copper sulfate in 1 mL PBS at 37°C. The length of the lag phase was defined as the time (min) to the intercept of the tangent of the absorbance curve in the propagation phase with baseline.

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 estimation of the percentage of Sudan IV stained regions (lipid infiltration) in photographs. The aortas were sliced open longitudinally, fixed in a formalin solution for 24 h, stained with Sudan IV lipophilic dye, and photographed. The intimal lipid lesions were determined quantitatively by estimation of the percentage of sudanophilic stained area in the total aortic intimal area

in photographs (5).

Statistical analyses

All data were expressed as mean ± S.D. All statistical analyses were performed using GB-STAT (Version 5.0; Dynamic Microsystems Inc., Silver Springs, MD, USA). Comparisons were done by one-way ANOVA among different groups. Turkey's post hoc test was used to analyze significant effects. A P value of 0.05 was taken as the threshold for statistical significance.

RESULTS

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.

Serum cholesterol and TG

As shown in Table 1, serum TG, TC and LDL-C levels increased in the control group, the BL group, and the probucol group compared with the normal group; and they decreased in the BL group and the probucol group compared with the control group (P<0.01). However, the post-experimental TG level was not different between the probucol group and the BL group. Serum HDL-C increased in the control group, the BL group, and the probucol group compared with the normal group, but remained unchanged after exposure to experimental diets. Serum GOT and GPT levels increased in the control group but remained unchanged in the BL group and the probucol group. As can be seen in Fig. 1, TC levels in the normal group remained unchanged during the study, but increased at week 4 in the control group, the BL group, and the probucol group compared with basal values. After consumption of the experimental diets for 12 weeks, the serum TC level in the BL group and the probucol group was significantly lower than in the control group (P<0.01, P<0.05 respectively). In the probucol group, post-experimental serum TC was lower than in the BL group.

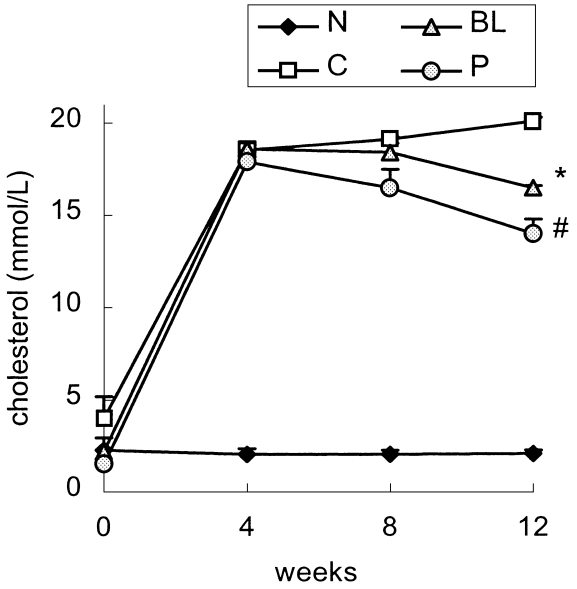

Fig. 1. Plasma cholesterol levels versus time in rabbits fed various diets. All values are the means ± S.D. In the normal group (N), rabbits (n = 6) were fed rabbit chow; in the control group (C), rabbits (n = 6) were fed rabbit chow plus 10% corn oil and 0.5% cholesterol; in the BL group (BL) and in the probucol group (P), rabbits (n = 6, respectively) were fed the same diet as control group plus 1% BL (barley leaf essence) or 1% probucol, respectively. *P<0.01, vs control, normal and probucol groups; #P<0.01, vs control, normal and BL groups.

Effect of experimental diet on free radical scavenging activities

After consumption of the experimental diets for 12 weeks,

Table 1. Serum GOT, GPT, TG and TC; LDL-C; and HDL-C levels from rabbits fed with experimental diet for 12 weeks

Groups	Normal	Control	BL	Probucol
GOT (U/mL)	41.0 ± 3.3[a]	121.2 ± 16.1[b]	62.5 ± 18.4[a]	66.9 ± 8.7[a]
GPT (U/mL)	49.5 ± 6.4[a]	133.1 ± 15.5[b]	54.9 ± 13.0[a]	68.7 ± 4.2[a]
TG (mmol/L)	0.5 ± 0.1[a]	3.1 ± 0.3[b]	1.4 ± 0.2[c]	1.9 ± 0.6[c]
TC (mmol/L)	2.1 ± 0.2[a]	20.1 ± 0.2[b]	16.5 ± 0.1[c]	14.0 ± 0.8[d]
LDL-C	1.1 ± 0.1[a]	17.1 ± 0.3[b]	14.1 ± 0.1[c]	11.8 ± 0.2[d]
HDL-C	0.6 ± 0.2[a]	2.2 ± 0.2[b]	2.0 ± 0.2[b]	2.0 ± 0.5[b]

All values are the means ± S.D. BL, barley leaf essence; GOT, glutamate oxaloacetate transaminase; GPT, glutamate pyruvate transaminase; TG, triacylglycerol; TC, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein. In the normal group, rabbits were fed rabbit chow; in the control group, rabbits were fed rabbit chow plus 10% corn oil and 0.5% cholesterol; in the BL and in the probucol group, rabbits were fed the same diet as control group plus 1% BL or 1% probucol, respectively. [a–d]: Data with different superscripts in the same row are significantly different at P<0.05.

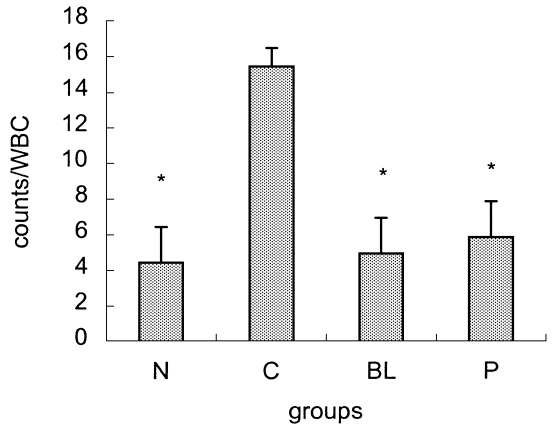

Fig. 2. Whole blood lucigenin-CL counts of rabbits fed various diets for 12 weeks. All values are the means ± S.D. In the normal group (N), rabbits (n = 6) were fed rabbit chow; in the control group (C), rabbits (n = 6) were fed rabbit chow plus 10% corn oil and 0.5% cholesterol; in the BL group (BL) and the probucol group (P), rabbits (n = 6, respectively) were fed the same diet as the control group plus 1% BL (barley leaf essence) or 1% probucol, respectively. **P*<0.05, vs control group.

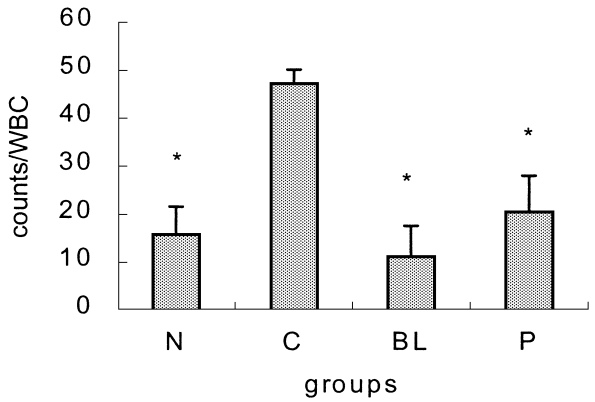

Fig. 3. Whole blood luminol-CL counts of rabbits fed various diets for 12 weeks. All values are the means ± S.D. In the normal group (N), rabbits (n = 6) were fed rabbit chow; in the control group (C), rabbits (n = 6) were fed rabbit chow plus 10% corn oil and 0.5% cholesterol; in the BL group (BL) and the probucol group (P), rabbits (n = 6, respectively) were fed the same diet as the control group plus 1% BL (barley leaf essence) or 1% probucol, respectively. **P*<0.05, vs control group.

the lucigenin-CL and luminol-CL levels in whole blood were higher in the control group than in the normal group and lower in the BL group and the probucol group than in the control group (Figs. 2 and 3). However, these differences were not significant.

Effect of experimental diet on AAPH-induced hemolysis and lag phase of LDL oxidation

Table 2 shows the time required to achieve 50% RBC hemolysis (T_{50}) and the lag phase of LDL oxidation in rabbits fed experimental diets. In the control group, post-experimental T_{50} and lag phase were significantly reduced compared to that of the normal group. In the BL group and probucol group, post-experimental T_{50} and lag phase were significantly higher than in the control group.

Table 2. The time required to achieve 50% red blood cell hemolysis (T_{50}) and lag phase of LDL oxidation from rabbits fed with experimental diet for 12 week

Groups	T_{50} (min)	Lag phase
Normal	145.5 ± 26.3^{a}	95.5 ± 8.0^{a}
Control	127.5 ± 15.0^{b}	60.0 ± 12.3^{b}
BL	151.7 ± 20.2^{a}	118.4 ± 23.1^{a}
Probucol	148.3 ± 15.3^{a}	130.4 ± 20.9^{a}

All values are the means ± S.D. In the normal group, rabbits were fed rabbit chow; in the control group, rabbits were fed rabbit chow plus 10% corn oil and 0.5% cholesterol; in the BL (barley leaf essence) and in the probucol group, rabbits were fed the same diet as control group plus 1% BL or 1% probucol, respectively. [a–b] Data with different superscripts in the same column are significantly different at $P<0.05$.

Morphological alterations of thoracic aorta

As shown in Fig. 4, A and B, the normal group did not develop any lipid lesions during the study. The average percentage of lipid lesions in the aorta of the control group, BL group and probucol group were 90.5 ± 2.5%, 61.7 ± 8.8% and 5.3 ± 1.0%, respectively. Rabbits in the BL group had fewer surface lipid lesions in the aorta than the control group, but had more lesions than the probucol group.

DISCUSSION

A high cholesterol diet has long been known to produce atherosclerosis in the aorta. In the present study, serum TG and TC increased significantly in rabbits receiving a high-fat and cholesterol diet (control group, BL group and probucol group). Moreover, the increases in blood TG and TC were higher in rabbits that received a high-fat and cholesterol diet (group control) than in those that received the same diet plus BL or probucol. These results suggest that BL and probucol reduced the extent of hyperlipidemia resulting from an atherogenic diet. In fact, the response was similar to that obtained in a previous study in rats (16), which indicated that hexacosyl alcohol and β-sitosterol, both isolated from BL, had hypocholesterolemic activities. It has been reported that the hypocholesterolemic action of β-sitosterol is due to the inhibition of intestinal absorption of cholesterol and the acceleration of catabolism of cholesterol to bile acid (16). Also, many polyphenolic compounds have been shown to possess hypolipidemic activity by increasing the fecal fat excretions and LDL receptor activity

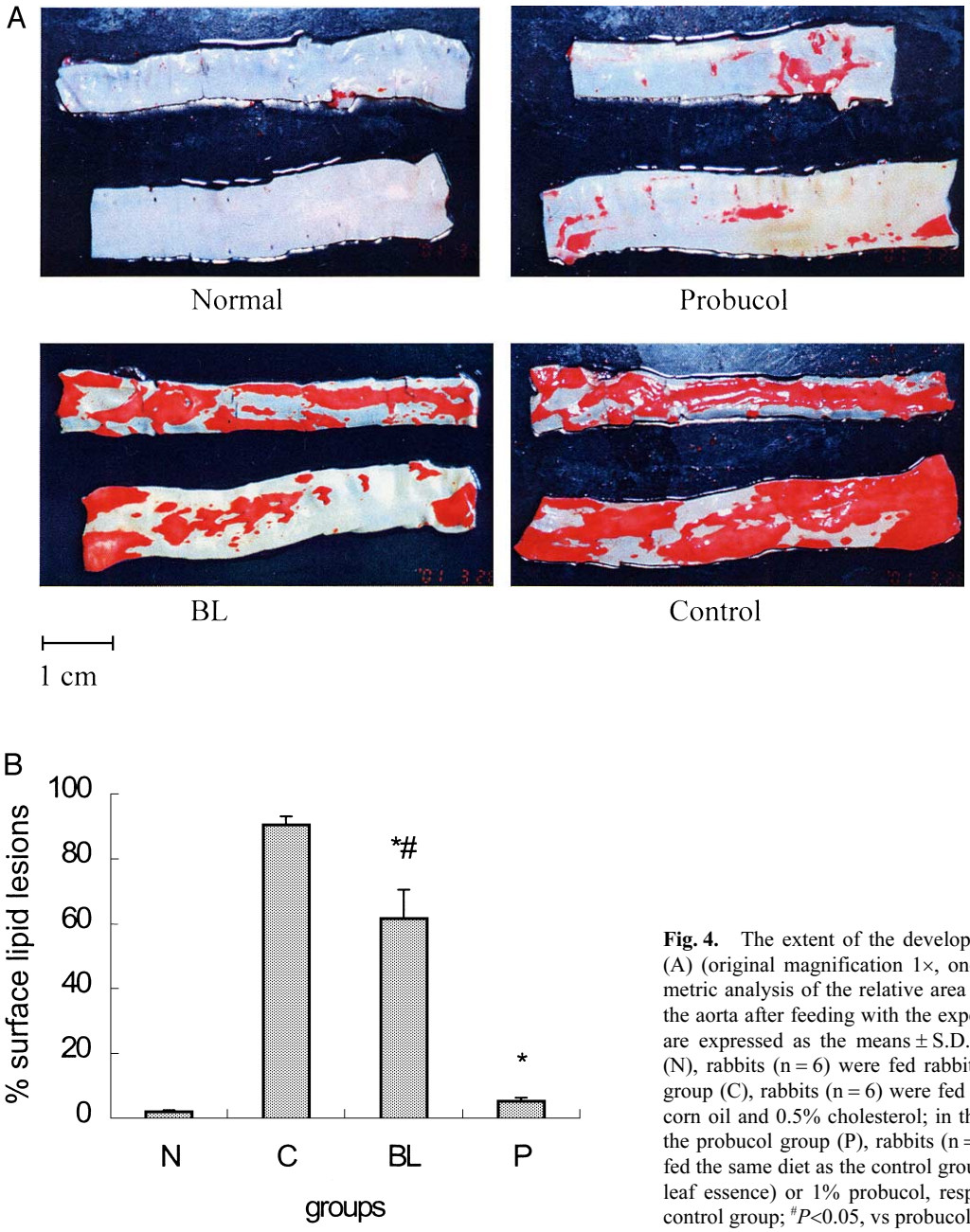

Fig. 4. The extent of the development of lipid lessions (A) (original magnification 1×, one of six) and densitometric analysis of the relative area of lipid lesions (B) in the aorta after feeding with the experimental diet. Results are expressed as the means ± S.D. In the normal group (N), rabbits (n = 6) were fed rabbit chow; in the control group (C), rabbits (n = 6) were fed rabbit chow plus 10% corn oil and 0.5% cholesterol; in the BL group (BL) and the probucol group (P), rabbits (n = 6, respectively) were fed the same diet as the control group plus 1% BL (barley leaf essence) or 1% probucol, respectively. *P<0.05, vs control group; $^{\#}P$<0.05, vs probucol group.

(17, 18). In our laboratory, we found that BL contained polyphenolic compounds suggesting that it may possess hypolipidemic action.

In biological systems, sources of oxygen free radicals may include superoxide radicals, hydrogen peroxide and hydroxyl radicals (19). The presence of luminol-dependent 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 (20). Thus, the level of luminol-amplified CL can be reduced by catalase pretreatment, and the level of lucigenin-amplified CL was found to be specifically inhibited by superoxide dismutase (SOD) but not catalase (21). In our study, post-experimental levels of luminol and lucigenin-CL in whole blood were higher in the control group than in the normal group and lower in the BL group and the probucol group than in the control group. This indicates that the production of oxygen free radicals by peripheral blood leukocytes was increased by the high-fat and cholesterol diet, and it was reduced after supplementation with BL. In this sense, BL seems to act like an oxygen radical scavenger. Its ability to scavenge

free radicals may be derived from the polyphenolic component of BL. This phenolic moiety of the structure can donate hydrogen atoms to deleterious oxy radicals and form the less-reactive phenoxyl radicals in the process (22, 23).

Results of this study also showed that the post-experimental T_{50} values were increased in the BL group compared with the control group, indicating that the antioxidant activity of BL can decrease the susceptibility of RBCs to AAPH-induced peroxidation. The purpose of using the method of AAPH-induced oxidation was primarily to assay the effect of free radicals on lipid peroxidation. Lipid peroxidation of RBC membrane was induced by peroxyl radicals, which were generated by thermal decomposition of the azo compound AAPH (22). The T_{50} of RBC hemolysis was prolonged in the BL group, suggesting that BL can scavenge hydroxyl radicals and inhibit AAPH-induced peroxidation. Previous studies indicated that free-radical-induced lipid peroxidation is involved in the pathogenesis of atherosclerosis (8). Therefore, the free radicals scavenging capacity of BL might be the mechanism responsible for slowing the progress of atherosclerosis. On the other hand, an increase in the blood level of free radicals might induce the oxidation of LDL particles, which increases their propensity to deposit in the vascular wall and facilitates the development of atherosclerosis. In our study, we found that the lag time of LDL oxidation increased in the BL group and the probucol group compared to the controls. This implies that LDL isolated from the blood of rabbits fed BL or probucol was more resistant to oxidation and that its uptake and deposition into the cells of the vascular wall might have been lower.

Some studies have shown that antioxidants such as tocopherol inhibit the development of atherosclerotic lesions in rabbits fed atherogenic diets (24). It has also been hypothesized that some antioxidants can prevent atherosclerosis by protecting LDL from oxidation (25) and an example is shown that their consumption is associated with a hypocholesterolemic effect (26). Yoshizumi et al. (2001) also demonstrated that natural polyphenolic compound quercetin inhibited vascular smooth muscle cell hypertrophy via the inhibition of mitogen-activated protein kinases (27). These previous findings led us to further investigate the influence of BL on the development of atherosclerotic lesions. Our histological study demonstrated that rabbits in the BL group had 30% fewer surface lipid lesions than controls. The anti-atherosclerotic effects of BL demonstrated in this study could be attributed to its cholesterol-lowing ability, free radicals scavenging capacity, as well as its inhibition of RBC hemolysis and LDL oxidation.

In clinical trials, probucol has been shown to have hypocholesterolemic, antioxidative and anti-atherosclerotic effects (10, 26, 28 – 30). Our results showed that 1% BL supplementation prolonged T_{50} values by 20 ± 4 min (Table 2), a result that was similar to that of the probucol group. This finding indicates that the antioxidative ability of BL was similar to that of probucol. We also found that the anti-atherosclerotic effect of probucol was more effective than that of BL, which may be due to other pharmacologic actions unrelated to its hypocholesterolemic and antioxidative effects. Previous studies also indicated that probucol might regulate some aortic gene expressions, such as vascular cell adhesion molecule-1 (31); preserve endothelium-derived relaxing factor action (32); inhibit HDL-mediated cholesterol efflux (33); increase the urinary excretion of oxidized cholesterol (34); inhibit the ox-LDL-induced adhesion of monocytes to aortic endothelial cells (35), to slow the progress of atherosclerosis.

In conclusion, the plasma levels of TG, TC and LDL-C decreased after 12 weeks supplementation of BL in rabbits fed an atherogenic diet. The decreased luminol-CL and lucigenin-CL levels in blood indicate that the production of oxygen free radicals was effectively inhibited by BL. The T_{50} of RBC hemolysis and lag phase of LDL oxidation were prolonged after supplementation with BL. However, the anti-atherosclerotic effect of BL was not as effective as probucol. Therefore, although BL may not be a suitable primary treatment for atherosclerosis, it may be useful to prevent or as an adjuvant to other treatments for cardiovascular diseases in which atherosclerosis plays a major role.

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