Luobuma leaf inhibits oxidation of low-density lipoprotein in cholesterol-fed rats

Dong Wook KIM, Takako YOKOZAWA,* Masao HATTORI, Shigetoshi KADOTA and Tsuneo NAMBA

Research Institute for Wakan-Yaku, Toyama Medical and Pharmaceutical University

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Abstract

Peroxidation was found to be significantly reduced when low-density lipoprotein (LDL) isolated from the plasma of rats treated with Luobuma leaf extract orally was present in the incubation medium in comparison with plasma from untreated rats. Of the two Luobuma extracts tested, Luobuma B extract showed considerably strong suppression of the peroxidation induced by copper. It was also evident that Luobuma extract decreased the levels of free cholesterol and LDL-cholesterol in serum, and thiobarbituric acid-reactive substance in serum and liver. From these results, it seems likely that Luobuma extract has an antiatherosclerotic action.

Key words Luobuma, leaf, oxidized LDL, lipid peroxidation, atherosclerosis.

Introduction

Nutritional and clinical studies have revealed that high cholesterol levels in the blood may be one of the risk factors of coronary heart disease and atherosclerosis. It has been noted that there is a close relationship between atherosclerosis and increase and/or decrease of serum lipids. In particular, oxidative degeneration of low-density lipoprotein (LDL) may be a causative factor of atherosclerosis.[1,2]

On the other hand, the living body has various defense mechanisms against attack from free radicals and active oxygen species. Some of these mechanisms function in the plasma to inhibit the formation of radicals, capture existing radicals, and regulate repair and regeneration after injury caused by radicals.[3] According to Niki,[4] there is also an adaptive mechanism in the plasma by which antioxidants are produced and delivered to the required location as needed, although this mechanism is less effective than the corresponding one in the cell. In this regard, much attention has been focused on the development of antioxidants which inhibit the formation of radicals.

Currently available water-soluble radical scavengers include vitamin C, uric acid and bilirubin, and fat-soluble radical scavengers include vitamin E, ubiquinol, β-carotin and probucol.[4] We have studied the antioxidant activity of medicinal and edible plants, and provided data showing that Luobuma leaf extract possesses such activity.[5]

Luobuma is a widely distributed plant which grows gregariously in mid to northwestern China. Its leaves have been used for tea for several hundred years in certain parts of China, and the tea has been winning popularity among Chinese people as a readily available daily beverage. Luobuma leaves are rich in ash elements and minerals such as Ca, iron and Na, but differ from green tea leaves in that they contain no caffeine, arousing the interest of many researchers in their pharmacologic action. In our previous experiments, Luobuma extract efficiently inhibited the lipid peroxidation reaction generated either non-enzymatically or enzymatically *in vitro*. Spin-trapping also revealed the same ability. In the *in vivo* experiment, the activities of the antioxidation enzymes superoxide dismutase, catalase and glutathione peroxidase were higher in rats given Luobuma extract.[5]

*〒930-0194 富山市杉谷2630
富山医科薬科大学和漢薬研究所 横澤隆子
2630 Sugitani, Toyama 930-0194, Japan

Luobuma extract also decreased the serum total cholesterol, LDL-cholesterol and atherogenic index, as well as the hepatic total cholesterol levels in hypercholesterolemic rats,[6] suggesting an effect on the oxidation of LDL.

In order to investigate this issue, we isolated LDL from the plasma of rats after oral administration of Luobuma leaf extract, and examined its antioxidant effect.

Materials and Methods

Plant materials: Luobuma A, the leaves of *Apocynum venetum*, was collected in Shandong province, China, and dried. Luobuma B was the leaves of Luobuma A roasted twice.

Preparation of Luobuma extracts: Luobuma A and B were separately extracted with hot water at 70°C for 3 h. After filtration, each respective solution was evaporated under reduced pressure to give Luobuma A and B extracts at yields of 22.1 % and 17.8 %, respectively.

Animals and treatment: Male LWH : Wistar rats with a body weight of 150-160 g were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Hamamatsu, Japan). They were kept in a wire-bottomed cage under a conventional lighting regimen with a dark night. The room temperature (about 25°C) and humidity (about 60 %) were controlled automatically. The animals were pair-fed a laboratory chow diet (powdered CE-2 of CLEA Japan Inc., Tokyo, Japan) containing 1 % cholesterol and 0.5 % cholic acid. Luobuma A or B extract was dissolved in water, and given to the rats orally every day as drinking water. The dose was adjusted to 350 mg/kg body weight by regulating its concentration in response to water consumption, since this dose was shown in a previous study to produce satisfactory effects in rats fed a high-cholesterol diet.[6] Control rats were given access to water alone. Six rats were used for each experimental group. After 20 days, the rats were killed by decapitation between 1300 and 1400 to avoid any effect of circadian variation. The blood samples were allowed to clot at 4°C, and then centrifuged. The plasma obtained in this manner was used for determination of their chemical parameters. The liver was subsequently extirpated from each rat, and homogenized with a 9-fold volume of iced physiological saline to determine the content of thiobarbituric acid (TBA)-reactive substance in the homogenate.

Determination of plasma cholesterol: Total cholesterol and free cholesterol levels were determined using commercial assay kits (Cholesterol E-Test Wako and Free Cholesterol E-Test Wako obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan). The level of low-density lipoprotein (LDL)-cholesterol was determined by the method of Noma *et al.*[7]

Separation of LDL by density-gradient ultracentrifugation: LDL (density 1.019-1.063) was isolated from plasma by sequential ultracentrifugation as described previously.[8] The LDL preparation was dialyzed against two changes of at least 100 volumes of 0.15 M NaCl (pH 7.4) for 48 h at 4°C.

Determination of oxidized LDL: According to the method reported by Kuzuya *et al.*,[9] LDL was diluted with 0.15 M NaCl (pH 7.4) to a concentration of 150 μg protein/ml, and incubated with 10 μM $CuSO_4$ at 37°C for 4 h. The extent of lipid peroxidation was estimated fluorometrically as TBA-reactive substance.[10] For this purpose, oxidized LDL (20 μg protein) was mixed with 1.5 ml of 0.67 % TBA and 1.5 ml of 20 % TCA. After heating at 100°C for 45 min, the fluorescent reaction products were assayed on a spectrofluorometric detector (Shimadzu RF-550, Kyoto, Japan) with excitation at 515 nm and emission at 553 nm. TBA-reactive substance formed from 1,1,3,3-tetramethoxypropane was used as a standard. The values are expressed as nmol of malondialdehyde equivalents.

Determination of TBA-reactive substance in plasma and liver: TBA-reactive substance in plasma was measured using the method of Naito and Yamanaka,[11] and that in liver tissue was assayed according to the method of Uchiyama and Mihara.[12]

Determination of protein: Protein was determined by the method of Lowry *et al.*[13]

Statistics: Values are presented as mean±S.E. Statistical differences were considered significant at $p<0.05$, using Dunnett's test.

Results

Plasma cholesterol

Table I compares the levels of plasma cholesterol in the Luobuma-treated rats with those in the control rats. The total cholesterol, free cholesterol and LDL-cholesterol levels in control rats fed a high cholesterol diet were increased significantly compared with normal rats, reflecting hypercholesterolemia. Administration of Luobuma A extract tended to decrease the level of total cholesterol, although not significantly. A statistically significant difference in the level of free cholesterol between the Luobuma A extract-treated rats and non-treated rats was obtained. Similarly, administration of Luobuma A extract to rats resulted in a decrease of LDL-cholesterol. In addition, oral administration of Luobuma B extract reduced significantly the plasma levels of free cholesterol and LDL-cholesterol, although there was no significant difference in the level of total cholesterol between the control and Luobuma B extract-treated groups.

LDL peroxidation

LDL was oxidized by $CuSO_4$, leading to peroxidation. The peroxidation was suppressed when LDL isolated from the plasma of Luobuma extract-treated rats was present in the incubation mixture, as shown in Fig. 1. Although it was still higher than that under the corresponding copper-free condition, the peroxidation in the Luobuma A extract group was significantly reduced to 9.13±1.00 nmol/mg protein. A similar effect was also observed with the Luobuma B extract, being more potent than that of the Luobuma A extract. This inhibitory effect of Luobuma extract was also noted in the absence of Cu^{2+}, suggesting that Luobuma extract protects LDL against oxidative stress.

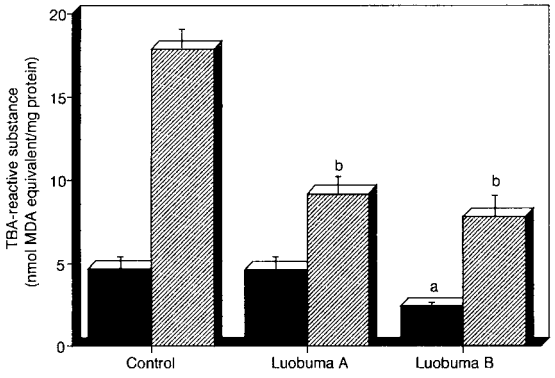

Fig. 1 Effect of Luobuma extract on LDL peroxidation. ■, Native LDL ; ▨, oxidized LDL. Statistical significance : $^{a}p<0.05$, $^{b}p<0.001$ *vs.* control rats.

TBA-reactive substance

The plasma TBA-reactive substance level was maintained at 0.02 nmol/mg protein in normal rats, whereas in cholesterol-fed rats, it was increased significantly to about 2.5 times the normal value (Table II). When the effect of oral administration of Luobuma extract A was examined, the plasma TBA-reactive substance level was decreased from 0.049 to 0.044 nmol/mg protein (a 10 % change, $p<0.05$). Luobuma extract B also significantly decreased the TBA-reactive substance level. In addition, the liver TBA-reactive substance level, which was 0.29 nmol/mg protein in normal rats, increased significantly to 0.61 nmol/mg protein in cholesterol-fed control rats. The TBA-reactive substance values in rats given

Table I Effect of Luobuma extract on plasma cholesterol levels.

Group	T. Chol. (mg/dl)	Free Chol. (mg/dl)	LDL-Chol. (mg/dl)
Normal rats	58.7±2.8	16.8±0.6	11.1±0.7
Cholesterol-fed rats			
Control	105.7±5.0[a]	29.0±1.7[a]	66.6±6.2[a]
Luobuma A	97.2±5.7[a]	24.7±1.6[a,c]	55.8±3.4[a,b]
Luobuma B	100.9±3.9[a]	25.9±1.2[a,b]	55.7±4.4[a,b]

Statistical significance : $^{a}p<0.001$ *vs.* normal rats, $^{b}p<0.05$, $^{c}p<0.01$ *vs.* cholesterol-fed rats.

Table II Effect of Luobuma extract on TBA-reactive substance levels.

Group	Serum TBA-reactive substance (nmol/mg protein)	Liver TBA-reactive substance (nmol/mg protein)
Normal rats	0.020±0.001	0.29±0.03
Cholesterol-fed rats		
Control	0.049±0.003[a]	0.61±0.08[a]
Luobuma A	0.044±0.004[a,b]	0.52±0.03[a,b]
Luobuma B	0.037±0.003[a,c]	0.46±0.05[a,c]

Statistical significance : $^{a}p<0.001$ *vs.* normal rats, $^{b}p<0.05$, $^{c}p<0.01$ *vs.* cholesterol-fed rats.

Luobuma extract A and B were significantly decreased to 0.52 and 0.46 nmol/mg protein, respectively, the latter value being 25 % lower than the control value.

Discussion

The initial phenomenon occurring in the development of atherosclerosis is the appearance of foam cells resulting from accumulation of lipids consisting mainly of cholesterol. However, much remains unclear about how foam cells are produced in LDL-hyperlipoproteinemia and how they induce atherosclerotic changes. However, since Goldstein *et al.*[14] discovered the scavenger receptor pathway to so-called degenerated LDL, monocyte-derived macrophages and degenerated LDL have drawn attention in relation to the formation of foam cells. Steinberg *et al.*[2] observed that probucol had an antioxidant activity and was more effective against atherosclerosis in WHHL rabbits than would be expected from the degree of the decrease in cholesterol. On the basis of these findings together with *in vitro* data on the antioxidant action of probucol on LDL oxidation, Steinberg *et al.*[2] proposed that oxidized LDL serves as the initiating factor for atherosclerosis.

The data supporting this hypothesis were obtained mainly from *in vitro* and *in vivo* animal experiments. However, this hypothesis is incomplete in that it is unclear where in the body LDL oxidation takes place and which cells and enzyme systems are involved in the oxidation.[15] In this connection, the peroxidation reaction of LDL after incubation with the oxidant $CuSO_4$ or with endothelial cells has been investigated in terms of the production of TBA-reactive substance,[16] the production of peroxides as determined by iodometry,[17] and the production of conjugated dienes (peroxides) as determined by the increase in absorbance at A_{234nm}.[18]

In the present study, the LDL fraction separated from rat plasma by ultracentrifugation was incubated at 37°C for 4 h in the presence of the oxidant $CuSO_4$. As a result, the production of TBA-reactive substance was about 2.3 times higher than that in the absence of $CuSO_4$, providing evidence of LDL oxidation by copper ions. However, when the LDL fraction separated from the plasma of rats orally administered Luobuma extract (unroasted or roasted) was used, the production of TBA-reactive substance was significantly lower, indicating the inhibition of LDL oxidation by Luobuma. The production of TBA-reactive substance with LDL incubated in the absence of copper ions and the plasma level of TBA-reactive substance were also significantly lower after Luobuma extract administration, suggesting that the extract prevented peroxidization in the body.

On the other hand, Morrow *et al.*[19] have demonstrated that the membrane phospholipid arachidonic acid is continuously peroxidized by free radicals *in vivo*, resulting in the formation of lipid peroxides, F_2-isoprostanes, *in situ*. They also observed that these lipid peroxides were increased markedly in the circulating blood of rats given CCl_4, and that the increase followed the same time course as that in the liver, with a lag of several hours. They therefore concluded that the F_2-isoprostanes in circulating blood were derived from the liver. Naito[20] also reported data suggesting that the liver was the source of lipid peroxides in plasma. We therefore investigated the involvement of the liver in the decrease of TBA-reactive substance in plasma, and found that the changes in TBA-reactive substance in the liver were similar to those in plasma, suggesting that the decrease in the plasma level of TBA-reactive substance was not associated with its uptake by the liver.

In the oxidative degeneration of LDL by copper ions, the copper ions initially bind to LDL molecules by coordinate bonding and decompose lipid hydroperoxides at the site.[4] The type of antioxidant most effective against LDL oxidation seems to depend on where the radicals are produced and where the reaction proceeds. Further investigation is necessary in order to clarify whether Luobuma extract, which was proved to have antioxidant activity in the present study, acts on radicals in the fluid system or plays a defensive role against radicals inside the lipid layer of LDL. In addition, the difference between the effects of roasted and unroasted leaves of Luobuma in the present study also suggests another area for future study, i.e., which component of Luobuma plays the major role in defense of LDL against oxidation.

In summary, the present study has revealed that

oxidation of LDL by Cu^{2+} is inhibited in the LDL fraction separated from the plasma of rats orally administered Luobuma leaf extract, thus demonstrating the antioxidant activity of Luobuma leaves.

和文抄録

ラット血漿から超遠心法で分離した低比重リポ蛋白(LDL)に，酸化剤としてCuイオンを加え，その酸化能をチオバルビツール酸反応物質を指標に検討した。その結果，あらかじめ羅布麻葉エキスを経口投与したLDLでは，酸化を著しく抑制する知見が得られた。また血中の遊離コレステロール，LDL-コレステロール，血漿，肝組織中のマロンジアルデヒドレベルの低下も認められ，羅布麻葉エキスに抗動脈硬化作用が示唆された。

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