Confirmation that Luobuma ameliorates the deterioration of antioxidant defense in senescence-accelerated mice

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Abstract

To determine whether Luobuma extract ameliorates the deterioration in antioxidant defense with aging, the effect of Luobuma extract was investigated in senescence-accelerated mice (SAM). In comparison with AKR/N Slc mice, a strain consistent with SAM but exhibiting normal aging, SAM treated with extract showed a lower glutathione (GSH) and glutathione/glutathione disulfide (GSH/GSSG) ratio in the liver and kidney, and increased levels of malondialdehyde (MDA), a lipid peroxidation product. Administration of Luobuma extract increased the GSH level and GSH/GSSG ratio, and suppressed MDA production. On the other hand, the reduced activities of hepatic superoxide dismutase (SOD), glutathione peroxidase and glutathione reductase participating in the glutathione redox cycle were increased significantly by administration of Luobuma extract. A significant increase in renal SOD activity was also observed. In addition, the increased level of MDA in hepatic tissue was reduced in SAM given Luobuma extract. These findings indicate that Luobuma extract helps to ameliorate oxidative stress in SAM.

Key words Luobuma, senescence-accelerated mice (SAM), glutathione redox cycle, malondialdehyde.

Introduction

In 1956, Harman [1] suggested that oxidation or peroxidation of the body is the cause of aging, and that free radicals are responsible for such oxidation and peroxidation. It was considered that as organisms get old, their defense mechanisms against free radical reactions deteriorate, leading to injuries to the tissues and thus manifestation of various aging phenomena. This theory became realistic after McCord and Fridovich [2] discovered superoxide dismutase (SOD) in 1969.

The living body, however, has defense mechanisms against aging-related peroxidation injury. It is believed that disorders are not manifested while such defense mechanisms are functioning efficiently, but become apparent when the mechanisms deteriorate. Factors contributing to the functioning of these defense mechanisms reportedly include antioxidant enzymes, such as SOD, catalase, and glutathione peroxidase (GSH-Px), and antioxidants including vitamins A, C, and E, carotenoid, and glutathione. Various studies have been carried out since the mid-1970s, on the assumption that administration of antioxidants may prevent various peroxidation injuries and thereby suppress aging if antioxidants prevent injuries from free radicals and if deterioration of their function initiates aging. Vitamin C, uric acid, and bilirubin have been cited as water-soluble radical scavengers. The known fat-soluble radical scavenger antioxidants include vitamin E, ubiquinol, β-carotene, and probucol.[3-5]

We have investigated the antioxidant activity of a number of medicinal and edible plants,[6-14] and found evidence of antioxidant activity in water extract of Luobuma leaves.[15-17] Luobuma is a wild plant growing gregariously in the central to northwestern part of

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China. In some areas of China, its leaves have been used as a tea for several hundred years. Although Luobuma leaves are rich in ash and minerals such as Ca, iron, and Na, they contain no caffeine unlike green tea leaves, arousing much attention as to their pharmacological actions.

The present study was aimed at investigating the effects of Luobuma, which is known to have antioxidant activity, on the defense mechanisms against free radicals in senescence-accelerated mice.

Materials and Methods

Animals : The senescence-accelerated SAM-P/1 substrain of mice (SAM) were originally obtained from Professor Masanori Hosokawa (Kyoto University). They were bred under conventional conditions, housed at 23±1°C with an alternating 12 h light/dark cycle, and supplied food and water *ad libitum*. At 7 or 8 weeks of age, male mice were used in each group. One group was given water, while the other was given Luobuma extract orally at a dose of 100 mg/kg body weight/day for 40 consecutive days. AKR/N Slc mice of corresponding age, obtained from Japan SLC, Inc., Hamamatsu, Japan, were used as a strain consistent with SAM, but exhibiting normal aging. After induction of anesthesia by intraperitoneal administration of sodium pentobarbital at 50 mg/kg body weight, the liver and kidney were subsequently extirpated from each mouse, and immediately frozen in liquid nitrogen. The tissues were kept at -80°C until analysis. Six mice were used for each experimental group. All experimental studies using animals were conducted in accordance with "Recommendations on the Establishment of Animal Experimental Guidelines" approved at the Toyama Medical and Pharmaceutical University.

Plant material : Luobuma, the leaves of *Apocynum venetum*, were collected in Shandong province, China, dried, and then roasted twice.

Preparation of Luobuma extract : Luobuma leaves were extracted with hot water at 70°C for 3 h. After filtration, the solution was evaporated under reduced pressure to give an extract at a yield of 17.8 %.

Glutathione (GSH) and glutathione disulfide (GSSG) assays : According to the method of Floreani *et al.*,[18] the tissue (about 250 mg) was homogenized in 1 ml of 25 % metaphosphoric acid plus 3.75 ml of 0.1 M sodium phosphate-5 mM EDTA buffer (pH 8.0), and then centrifuged at 105,000×g for 30 min at 4°C. Determination of GSH and GSSG in the supernatant was performed by the method of Hissin and Hilf,[19] using o-phthaldialdehyde as the fluorescent reagent.

Enzyme assays : The tissue was homogenized with a 9-fold volume of ice-cold physiological saline, and the activities of enzymes in the homogenate were determined. The activity of SOD was measured according to the nitrous acid method described by Elstner and Heupel[20] and Oyanagui,[21] based on inhibition of nitrite formation from hydroxylamine in the presence of superoxide (O_2^-) generators. Catalase activity was measured by following the decomposition of hydrogen peroxide (H_2O_2) directly by the decrease in extinction at 240 nm. The difference in extinction (ΔE_{240}) per unit time was used as a measure of catalase activity.[22] GSH-Px activity was obtained by colorimetry with 2-nitro-5-thiobenzoic acid, a compound produced through the reaction between glutathione and 5,5'-dithiobis(2-nitrobenzoic acid).[23] For glutathione reductase assay, tissue was homogenized with a 10-fold volume of ice-cold 1.5 % KCl and the homogenate centrifuged at 15,000×g for 40 min at 4°C. Glutathione reductase activity was assayed in the supernatant by the method of Tor-Agbidye *et al.*[24] Protein was determined by the method of Itzhaki and Gill,[25] with bovine serum albumin as a standard.

Determinations of malondialdehyde (MDA) : MDA in liver and kidney tissues was assayed according to the method of Uchiyama and Mihara.[26] Protein content was determined by the method of Itzhaki and Gill[25] described above.

Statistics : Values are presented as the mean±S.E. Differences among groups were analyzed by Dunnett's test. Significance was accepted at $p<0.05$.

Results

Glutathione redox cycle in the liver

As shown in Table I, the GSH level and GSH/GSSG ratio were significantly lower in SAM, being about 86 % of the normal value for the former, and 76 % for the latter; the GSSG level was increased

Table I Effect of Luobuma extract on glutathione in the liver.

Group	GSH (μmol/g liver)	GSSG (μmol/g liver)	GSH/GSSG
AKR/N Slc mice	6.24 ± 0.14	0.86 ± 0.06	6.80 ± 0.11
SAM			
Control	5.34 ± 0.42[a]	1.04 ± 0.06[a]	5.18 ± 0.40[a]
Luobuma extract	6.62 ± 0.17[c]	0.94 ± 0.02[b]	7.06 ± 0.05[c]

Statistical significance : [a]$p<0.001$ *vs.* AKR/N Slc mice values, [b]$p<0.05$, [c]$p<0.001$ *vs.* SAM control values.

Table II Effect of Luobuma extract on hepatic enzyme activities involved in the glutathione redox cycle.

Group	SOD (U/mg protein)	Catalase (U/mg protein)	GSH-Px (U/mg protein)	Glutathione reductase (nmol/min/mg protein)
AKR/N Slc mice	28.05 ± 2.00	290.4 ± 4.0	179.2 ± 5.9	18.97 ± 0.31
SAM				
Control	24.11 ± 1.20[a]	272.5 ± 24.6	147.8 ± 7.2[a]	16.64 ± 0.47[a]
Luobuma extract	27.26 ± 0.76[c]	263.2 ± 12.0	157.5 ± 2.3[a,b]	19.39 ± 0.46[c]

Statistical significance : [a]$p<0.001$ *vs.* AKR/N Slc mice values, [b]$p<0.05$, [c]$p<0.001$ *vs.* SAM control values.

from 0.86 to 1.04 μmol/g liver. On the other hand, when SAM were treated with Luobuma extract, the reverse was observed: the reduced GSH level and GSH/GSSG ratio were markedly increased, and the GSSG level was decreased.

Table II shows the effects of Luobuma extract on hepatic enzyme activities related to the glutathione redox cycle. In comparison with normal rats, enzyme activities were significantly decreased in SAM given no Luobuma extract, the values being 14 % lower for SOD activity, 18 % lower for GSH-Px and 12 % lower for glutathione reductase, while the reduction in catalase activity was not significant. In contrast, Luobuma extract significantly increased the SOD activity from 24.11 to 27.26 U/mg protein. A significant difference in the activities of GSH-Px and glutathione reductase were also observed. In SAM, GSH-Px activity was 147.8 U/mg protein, and this was increased to 157.5 U/mg protein in mice treated with Luobuma extract. Glutathione reductase showed similar variation, from 16.64 to 19.39 nmol/min/mg protein in both groups.

Glutathione redox cycle in the kidney

As shown in Table III, the GSH level was significantly lower in the kidney from SAM than AKR/N Slc mice. The level of GSSG in SAM was increased from 0.49 to 0.64 μmol/g kidney. As a consequence, the GSH/GSSG ratio was significantly lower in SAM, being about 67 % of the normal value. Administration of Luobuma extract efficiently suppressed the oxida-

Table III Effect of Luobuma extract on glutathione in kidney.

Group	GSH (μmol/g kidney)	GSSG (μmol/g kidney)	GSH/GSSG
AKR/N Slc mice	3.31 ± 0.06	0.49 ± 0.03	6.70 ± 0.45
SAM			
Control	2.88 ± 0.10[b]	0.64 ± 0.02[b]	4.52 ± 0.20[b]
Luobuma extract	3.51 ± 0.16[a,c]	0.62 ± 0.03[b]	5.66 ± 0.21[b,c]

Statistical significance : [a]$p<0.05$, [b]$p<0.001$ *vs.* AKR/N Slc mice values, [c]$p<0.001$ *vs.* SAM control values.

Table IV Effect of Luobuma extract on renal enzyme activities involved in the glutathione redox cycle.

Group	SOD (U/mg protein)	Catalase (U/mg protein)	GSH-Px (U/mg protein)
AKR/N Slc mice	20.51 ± 0.95	195.4 ± 6.0	170.1 ± 2.9
SAM			
Control	17.45 ± 0.53[b]	153.8 ± 7.1[b]	152.3 ± 4.5[b]
Luobuma extract	18.80 ± 0.44[a,c]	147.3 ± 4.7[b]	149.4 ± 4.1[b]

Statistical significance : [a]$p<0.01$, [b]$p<0.001$ *vs.* AKR/N Slc mice values, [c]$p<0.05$ *vs.* SAM control values.

Table V Effect of Luobuma extract on malondialdehyde

Group	Liver MDA (nmol/mg protein)	Kidney MDA (nmol/mg protein)
AKR/N Slc mice	0.512±0.033	0.155±0.009
SAM		
Control	0.858±0.070[b]	0.179±0.007[b]
Luobuma extract	0.701±0.022[b,c]	0.175±0.005[a]

Statistical significance : [a]$p<0.01$, [b]$p<0.001$ *vs.* AKR/N Slc mice values, [c]$p<0.001$ *vs.* SAM control values.

tion of GSH in SAM. The content of the reduced form, GSH, was increased, and the GSH/GSSG ratio in SAM given Luobuma extract was elevated 25 % as compared with that in SAM given no extract.

Enzyme activities participating in the glutathione redox cycle showed significant decreases in SAM, the values being 15 % lower for SOD activity, 21 % lower for catalase activity and 10 % lower for GSH-Px activity, as shown in Table IV. In contrast, the SOD activity, which was 17.45 U/mg protein, increased significantly to 18.80 U/mg protein in mice treated with Luobuma extract. There were no significant variations in catalase and GSH-Px activities after administration of Luobuma extract (Table IV).

MDA in hepatic and renal tissues

The level of MDA was significantly elevated in both hepatic and renal tissues of SAM as compared with AKR/N Slc mice, as shown in Table V. The administration of Luobuma extract resulted in a decrease of hepatic MDA from 0.858 to 0.701 nmol/mg protein. However, there was no particular variation in renal MDA after administration of Luobuma extract.

Discussion

The cause of aging can be intrinsic or extrinsic. Considering the genetically programmed mechanisms of aging to be intrinsic causes, the mechanisms of aging in which free radicals are involved in representative of extrinsic causes. To control aging has long been a dream of those searching for the secrets of perennial youth and long life. Harman himself, who proposed the free radical theory, examined the life-prolonging effects of various antioxidants in mice. His studies, however, were defective in reproducibility, and could not find antioxidants for controlling aging.[27] However, according to Epstein and Gershon[28] and Miquel *et al.*,[29] experiments using vitamin E showed a 31 % increase in 50 % survival and a 23 % increase in the maximum length of life in nematodes, and a 8-15 % increase in the average length of life and a 12 % increase in the maximum length of life in fruit-flies. On the other hand, Blackett and Hall[30] examined the effects of vitamin E in mice, and reported that the survival rate improved at 24 months, but there was no prolongation of the maximum length of life. Murano *et al.*[31] also examined the effects of vitamin E in rats with experimentally induced vitamin E deficiency, and found that the activity of the enzyme for hydrolyzing cholesterol was decreased in these rats, resulting in metabolic changes prone to atherosclerosis. Namely, free radicals seem to represent an important intrinsic factor which accelerates aging of the living body. However, the fact that there is no evidence of antioxidant-induced prolongation of the maximum length of life in animals of the higher orders suggests that some intrinsic genetic mechanisms determine the life span,

and that free radicals modify these mechanisms to produce aging-related pathological conditions, resulting in a shortening of the life span.

We have previously attempted to control aging by eliminating influences of free radicals, using SAM.[13,14] In this connection, the effects of Luobuma extract were investigated in the present study. Glutathione is a tripeptide composed of glutamic acid, cysteine, and glycine, and distributed extensively in the body. Its role in the oxidation-reduction system in the body has been known for some time, and the broad spectrum of its physiological actions has recently been attracting attention. Tateishi *et al.*[32] and Higashi[33] reported that the amount of cysteine is a rate-limiting factor for biosynthesis of glutathione, and that its concentration was highest in the liver, corresponding to 2-fold and 3-fold that in the kidney and lung, respectively, with the blood concentration being about 1/1000 the intracellular concentration. On the other hand, reduced glutathione GSH serves as a coenzyme of GSH-Px, and reduces the substrates H_2O_2 and LOOH to water and alcohol, respectively, while it is converted to oxidized glutathione (GSSG). GSSG is again converted to GSH by glutathione reductase, forming a glutathione redox cycle. GSH plays an important part in the metabolism of H_2O_2 and lipid peroxides catalyzed by GSH-Px, through an oxidation-reduction reaction and nucleophilic reaction, i.e., characteristic reactions of the SH group.[34] In the present study, in comparison with AKR/N Slc mice that follow the natural course of aging, the GSH level in liver tissue was decreased, the GSSG level was increased, and the activity of enzymes forming the glutathione redox cycle was decreased in SAM, showing that they were under oxidative stress. In contrast, in SAM given Luobuma extract, the antioxidant effect of the extract was apparent; as the level of reduced glutathione increased, the activity of GSH-Px and glutathione reductase increased. The activity of catalase, however, remained unchanged. Catalase is an enzyme which is also involved in the elimination of H_2O_2, like GSH-Px whose activity was increased. However, catalase is localized in peroxisome granules, whereas GSH-Px is present in the cytoplasm and mitochondrial matrix, serving as a scavenger for H_2O_2 and LOOH.[35] Since it is apparent that Luobuma extract exerted influences on TBARS, it is possible that the site of action of Luobuma extract is the cytoplasm and mitochondrial matrix. On the other hand, the glutathione redox ratio, {GSSG/(GSH+GSSG)×100}, an index for the degree of H_2O_2 generation,[36] was significantly decreased, and the glutathione reductase activity was significantly increased in SAM given Luobuma extract. These results suggest that Luobuma extract suppresses the generation of H_2O_2 and converts oxidized glutathione to reduced glutathione, steadily maintaining the antioxidative state of the body.

Lang *et al.*[37] have reported that the GSH concentration decreased in the brain, liver, and lung with aging, and that the blood concentration of GSH is decreased in approximately half of all people over 60 years of age as compared with healthy young people, showing a correlation with the progression of aging. Although there have been hardly any reports of antiaging treatment with GSH, an increase in the level of reduced glutathione, as observed in SAM given Luobuma extract, is believed to be helpful in preventing glutathione-related defense mechanisms from deteriorating with aging.

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和文抄録

羅布麻エキスが老化における酸化防御機構にいかなる影響を及ぼしているかについて，老化促進マウス (SAM) を用い検討した。SAM と同じ系統ではあるが，通常の老化過程を辿る AKR/N Slc マウスに比べ，SAM では肝，腎組織中のグルタチオンレベルとグルタチオン/グルタチオンジスルフィド比は低下し，脂質過酸化物のマロンジアルデヒドは逆に増加していた。一方，羅布麻エキスを投与した SAM では，これらパラメーターがいずれも改善し，グルタチオン酸化還元サイクルに関係している肝組織中のスーパーオキシドジスムターゼ，グルタチオンペルオキシダーゼ，グルタチオンレダクターゼ活性と腎組織中のスーパーオキシドジスムターゼ活性が有意に

上昇していた。また肝組織中のマロンジアルデヒドも低下し，SAMで認められた酸化的ストレス状態を羅布麻エキスが緩和していた。

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