Chiyu extract stimulates antioxidant defense ability in senescence-accelerated mice

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Abstract

The effect of chiyu extract on antioxidant defense alteration in senescence-accelerated mice (SAM) was examined. Comparison with AKR/N Slc mice, a strain consistent with SAM but exhibiting normal aging, showed a lower glutathione (GSH) level and glutathione/glutathione disulfide (GSH/GSSG) ratio in the kidney and liver of SAM, whereas malondialdehyde (MDA), a lipid peroxidation product, was increased significantly. Administration of chiyu extract increased the GSH level and GSH/GSSG ratio, and markedly suppressed MDA production. On the other hand, detection of renal enzymes related to the glutathione redox cycle showed that catalase and glutathione peroxidase activities were largely decreased in SAM, whereas chiyu extract reversed this tendency. The reduced activities of hepatic catalase and glutathione reductase were increased significantly by the extract. These findings suggest that a decline of the antioxidant defense system occurs in SAM, and that chiyu extract may have a beneficial effect in ameliorating oxidative stress or damage.

Key words Chiyu, *Sanguisorba officinalis* L., senescence-accelerated mice (SAM), glutathione redox cycle, malondialdehyde.

Abbreviations GSH, glutathione; GSH-Px, glutathione peroxidase; GSSG, glutathione disulfide; H_2O_2, hydrogen peroxide; LOOH, lipid hydroperoxides; MDA, malondialdehyde; O_2^-, superoxide; SAM, senescence-accelerated mice.

Introduction

Aging is associated with a progressive decline in the ability to respond to the stresses of a dynamic environment. Several theories have been proposed to explain the aging process, in which the free radical theory has received particular attention in recent years.[1,2] Physiologically, the formation and consumption of free radicals, specially oxygen radicals, in the body are balanced by antioxidant defense systems; when the antioxidant ability provided by this defense system is decreased due to various physiopathologic causes, reactive free radicals accumulate, and this induces lipid peroxidation, consequently leading to oxidative stress or damage. It has been widely accepted that oxidative stress plays an important role in degenerative senescence, and various studies have revealed increased oxidative modification of proteins and changes in plasma membrane lipids with aging.[3,4]

The free radical theory of aging suggests that intervention designed to retard the intrinsic aging process would be possible by administration of antioxidants or free-radical scavengers. A number of dietary antioxidants have been administered to different organisms in attempts to increase life expectancy, and very interesting results have been obtained.[5-8]

Tannins are a family of bioactive natural compounds, which have been intensively studied and reported to possess distinctive antioxidant and free-radical-scavenging properties.[9-15] These properties are associated with several of the bioactivities and

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pharmacological effects of tannins, but their possible anti-aging effects have received less attention. In view of these effects of free radicals and lipid peroxidation in accelerating senescence, we considered that tannins would be likely to have a beneficial effect in attenuating oxidative damage, thereby retarding the aging process.

Chiyu is a traditional hemostasis drug which contains a large quantity of both hydrolyzable and condensed tannins. We have shown that chiyu extract scavenges free radicals in *in vitro* assay systems,[16,17] and ameliorates renal injury in an ischemia-reperfusion model which is closely associated with excessive generation of reactive oxygen species.[18] This crude drug extract also attenuates the impairment of renal function caused by lipopolysaccharide.[19] In the present study, using senescence-accelerated mice, we examined whether or not chiyu extract has an effect against the oxidative stress related to the aging process.

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 chiyu 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 Shizuoka Agricultural Cooperative Association for Laboratory Animals (Hamamatsu, Japan), were used as a strain consistent with SAM, but exhibiting normal aging. After induction of anesthesia by intraperitoneal administration of sodium pentobarbital 50 mg/kg body weight, blood samples were obtained by cardiac puncture, and the serum was separated immediately by centrifugation. The kidney and liver 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.

Preparation of chiyu extract : Chiyu, the rhizome and root of *Sanguisorba officinalis* L., grown in China and supplied by Uchida Wakan-yaku Co., Ltd., Tokyo, Japan, were finely powdered and extracted with distilled water at 100°C for 1 h (Chiyu : water= 1 : 10, w/v). After removal of the insoluble portion by filtration, the filtrate was concentrated under reduced pressure and then lyophilized to yield a brown residue. The yield of the extract was 17.4 % by weight of the original material. It was composed mainly of tannin (about 46.3 %). The estimation was based on the method of Japanese Industrial Standard.

Glutathione (GSH) and glutathione disulfide (GSSG) assays : According to the method of Floreani *et al.*,[20] the tissue (about 250 mg) was homogenized in 1 ml 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 x *g* for 30 min at 4°C. Determination of GSH and GSSG in the supernatant was performed by the method of Hissin and Hilf,[21] 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 superoxide dismutase (SOD) was measured according to the nitrous acid method described by Elstner and Heupel [22] and Oyanagui,[23] 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 ($\triangle E_{240}$) per unit time was used as a measure of catalase activity.[24] Glutathione peroxidase (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).[25] 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 x *g* for 40 min at 4°C. Glutathione reductase activity was assayed in the

supernatant by the method of Tor-Agbidye *et al.*[26] Protein was determined by the method of Itzhaki and Gill,[27] with bovine serum albumin as a standard.

Determinations of malondialdehyde (MDA): MDA in serum was measured using the method of Naito and Yamanaka,[28] and that in kidney and liver tissue was assayed according to the method of Uchiyama and Mihara.[29] Protein content was determined by the method of Itzhaki and Gill[27] described above.

Statistics: Values are presented as 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 kidney

As shown in Table I, the content of reduced glutathione (GSH) was significantly lower in the kidney from SAM than in that from AKR/N Slc mice. The level of oxidized glutathione (GSSG) in SAM showed an elevation of about 24%. As a consequence, the GSH/GSSG ratio was largely lowered in SAM, from 6.44 to 4.50, indicating a peroxidative state. On the other hand, when SAM were treated with chiyu extract, significant reverse effects were observed. The reduced GSH level and GSH/GSSG ratio were markedly increased, and the GSSG level was decreased.

Enzyme activities participating in the glutathione redox cycle were examined, and the data are demonstrated in Table II. Significant differences in the activities of catalase and GSH-Px were observed. In AKR/N Slc mice, renal catalase activity was 190.3 U/mg protein, and this was lowered to 147.8 U/mg protein in SAM, and reversed to 157.8 U/mg protein in mice treated with chiyu extract. GSH-Px activity also showed similar variations, from 167.7 to 151.2 and to 163.0 U/mg protein in the three respective groups. In contrast to these enzymes, the alteration of SOD activity was less marked, and did not reach statistical significance.

Glutathione redox cycle in the liver

Consistent with the variations in the kidney, the GSH level and GSH/GSSG ratio were significantly lower in SAM, being about 90 % of the normal value for the former, and 78% for the latter; the GSSG level was increased from 0.97 to 1.09 μmol/g liver, as shown

Table I Effect of Sanguisorbae Radix extract on glutathione in kidney.

Group	GSH (μmol/g kidney)	GSSG (μmol/g kidney)	GSH/GSSG
AKR/N Slc mice	3.15 ± 0.04	0.50 ± 0.03	6.44 ± 0.77
SAM			
Control	2.66 ± 0.01[b]	0.62 ± 0.08[a]	4.50 ± 0.55[a]
Sanguisorbae Radix extract	2.99 ± 0.01[b,e]	0.48 ± 0.01[c]	6.39 ± 0.44[d]

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

Table II Effect of Sanguisorbae Radix 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	19.73 ± 2.65	190.3 ± 7.0	167.7 ± 3.4
SAM			
Control	16.20 ± 4.39	147.8 ± 4.3[a]	151.2 ± 2.8[a]
Sanguisorbae Radix extract	16.68 ± 2.01	157.8 ± 2.9[a,b]	163.0 ± 3.2[b]

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

in Table III. Administration of chiyu extract efficiently suppressed the oxidation of GSH in SAM. The content of the reduced form, GSH, was increased, and that of the oxidized form, GSSG, was lowered, the difference being significant for both parameters. The GSH/GSSG ratio in SAM given chiyu extract was elevated 37 % as compared with that in SAM given no extract.

Detection of the related enzyme activities showed significant decreases in both catalase and GSH-Px in the liver from SAM, while reductions in SOD and glutathione reductase were not significant. The administration of chiyu extract significantly increased the reduced catalase and glutathione reductase activities. Catalase activity rose from a control value of 264.5 U/mg protein to 274.7 U/mg protein, and glutathione reductase from 17.20 to 19.43 nmol/min/mg protein. There were no significant variations in SOD and GSH-Px activities after administration of chiyu extract (Table IV).

MDA in the serum, kidney and liver

In comparison with AKR/N Slc mice, the level of

Table III Effect of Sanguisorbae Radix extract on glutathione in the liver.

Group	GSH (μmol/g liver)	GSSG (μmol/g liver)	GSH/GSSG
AKR/N Slc mice	5.64 ± 0.01	0.97 ± 0.06	5.94 ± 0.40
SAM			
Control	5.05 ± 0.01[b]	1.09 ± 0.02[a]	4.63 ± 0.29[b]
Sanguisorbae Radix extract	6.25 ± 0.03[b,d]	0.99 ± 0.03[c]	6.33 ± 0.34[d]

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

Table IV Effect of Sanguisorbae Radix 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	27.38 ± 3.17	285.4 ± 3.5	170.6 ± 7.4	18.26 ± 0.36
SAM				
Control	26.45 ± 4.78	264.5 ± 4.3[a]	157.5 ± 3.9[a]	17.20 ± 0.65
Sanguisorbae Radix extract	29.38 ± 5.97	274.7 ± 2.3[a,b]	159.3 ± 6.0[a]	19.43 ± 0.76[a,c]

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

Table V Effect of Sanguisorbae Radix extract on malondialdehyde.

Group	Serum MDA (nmol/ml)	Kidney MDA (nmol/mg protein)	Liver MDA (nmol/mg protein)
AKR/N Slc mice	2.13 ± 0.13	0.16 ± 0.01	0.59 ± 0.04
SAM			
Control	2.90 ± 0.20[a]	0.18 ± 0.01[a]	0.92 ± 0.12[a]
Sanguisorbae Radix extract	2.34 ± 0.09[c]	0.16 ± 0.01[c]	0.70 ± 0.07[b]

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

MDA was significantly elevated in both serum and liver of SAM. As shown in Table V, the level in the serum of normal AKR/N Slc mice was 2.13 nmol/ml, compared with 2.90 nmol/ml in SAM, an increase of approximately 36 %. The rise was more marked in the liver, the MDA level in SAM being 56 % greater than that in normal AKR/N Slc mice. Although the increase in the kidney was not as large as that in serum and liver, the difference was significant. In contrast, oral administration of chiyu extract decreased the MDA level efficiently to near normality in serum and kidney.

Discussion

The antioxidant defense system, which includes antioxidant enzymes and non-enzymatic low-molecular-weight antioxidant molecules such as GSH, is present in the living body and plays a critical role in maintaining the balance between prooxidant and antioxidant, protecting cells and tissues against the potentially harmful effects of reactive free radicals and peroxidation. The system formed by SOD, catalase, GSH-Px, glutathione reductase and glutathione plays a major role in scavenging reactive oxygen radicals, including O_2^-, H_2O_2 and the hydroxyl radical, which have been extensively implicated in pathogenic mechanisms associated with aging.[30] Among these, it is generally considered that GSH is the most important antioxidant molecule in cells, possibly reacting directly with reactive oxygen species and also serving as a substrate in the enzymatic reduction of detoxified H_2O_2 and lipid hydroperoxides (LOOH), thus protecting cells against oxidative damage. The GSH/GSSG ratio is considered to be a sensitive measure of both tissue GSH status and oxidative stress.[31-33] As the kidney and liver are the most important organs involved in regulation of the glutathione redox cycle, we examined the alterations of antioxidant defense related to aging in these two tissues.

In the present study, SAM showed a lower GSH level and a higher GSSG level. Compared with normal AKR/N Slc mice, the GSH/GSSG ratio was a 30 % lower in kidney and 22 % lower in liver, suggesting that GSH is excessively consumed for direct and indirect scavenging of H_2O_2 or LOOH, and that the oxidative product accumulates in senescence-accelerated mice. In contrast, oral administration of chiyu extract significantly elevated GSH and decreased GSSG, and consequently the GSH/GSSG ratio was increased, in both the kidney and liver. These effects may be associated with increased glutathione reductase activity in the liver, although this activity was not detected in the kidney because of the smaller amount present.

We also examined the activities of several antioxidant enzymes. SOD is the most important enzyme providing defense against the deleterious effect of reactive oxygen species, and can rapidly scavenge O_2^- by converting it to H_2O_2. In addition, it has been demonstrated that SOD activity is associated with life span in various species and strains.[34,35] In the present study, SOD did not show significant alteration among the three test groups. However, the activities of catalase and GSH-Px, two enzymes responsible for scavenging H_2O_2, were shown to be significantly depressed in SAM, whereas chiyu extract reversed the decrease in enzyme activities. These results suggest that the antioxidant defense system in senescence-accelerated mice has been partly impaired, and that chiyu extract can partly stimulate antioxidant defense ability.

Lipid peroxidation has been proposed as a major mechanism by which free radicals induce tissue injury. The reactive free radicals attack polyunsaturated fatty acids, initiate lipid peroxidation in biological membranes, and consequently alter membrane structure and function. Furthermore, accumulated lipid peroxides leak from organs and tissues into the blood system, increasing their level in blood lipoproteins and promoting atherogenesis and cardiovascular disease, which are a major factor in morbidity and mortality among the elderly.[36] As a lipid peroxidation product, the level of MDA was measured in serum, kidney and liver. As predicted, MDA levels were markedly increased in SAM, being 36 % higher in serum, 13 % higher in kidney and 56 % higher in liver, providing direct evidence of peroxidative conditions in senescence-accelerated mice. Chiyu extract suppressed the lipid peroxidation mediated by these reactive free radicals, and lowered MDA formation markedly in the serum, kidney and liver.

It has been suggested that antioxidants prevent age-associated impairment of performance. Earlier reports have indicated that antioxidant supplementation increases the mean life span of flies to some extent.[37] Long-term dietary administration of the antioxidant vitamin E could ameliorate renal lipid peroxidation and accumulation of F2-isoprostanes in aging kidneys.[38] More recent studies have revealed the potential role of antioxidants in aging and age-associated degenerative diseases.[39] Previously, we found that chiyu extract possesses strong free radical-scavenging activity *in vitro*.[16,17] This crude drug protected renal function and inhibited apoptosis in an ischemia-reperfusion model, which is closely associated with excessive generation of reactive oxygen species.[18] We have also reported that chiyu extract contributed to the regulation of renal function under conditions where excessive generation of nitric oxide occurred.[19] In the present experiment, we showed that chiyu extract can suppress lipid peroxidation and stimulate antioxidant defense ability in senescence-accelerated mice, suggesting that this crude drug may be an effective agent for ameliorating the pathological conditions related to excessive generation of free radicals and oxidant damage, especially in the aging process.

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和文抄録

老化促進マウス (SAM) の抗酸化防御機構に対し，地楡エキスがいかなる影響を及ぼしているかについて，SAMと同じ系統ではあるが，通常の老化過程を辿るAKR/N Slcマウスと比較検討した。その結果，SAMの腎，肝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有意に上昇していたが，地楡エキスを投与したSAMでは，これらパラメーターがいずれも改善していた。一方，グルタチオン酸化還元サイクルに関係している酵素のうち，腎ではカタラーゼとグルタチオンペルオキシダーゼ活性がSAMで著しく低下し，地楡エキス投与によってこれら酵素の活性が回復していた。肝においても低下していたカタラーゼとグルタチオンレダクターゼ活性がエキス投与により著しく回復していた。これら所見は，SAMで認められる抗酸化防御系の低下が，地楡エキスによって是正されることを示唆するものである。

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