

Effects of Repeated Administration of Deer Antler Extract on Biochemical Changes Related to Aging in Senescence-Accelerated Mice¹⁾

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Several biochemical parameters related to aging were evaluated after a hot-water extract prepared from unossified pilose antler of *Cervus nippon* TEMMINCK var. *mantchuricus* Swinhoe (Rokujo) had been administered orally for 8 successive days to senescence-accelerated mice (SAM), a novel murine model of spontaneously promoted aging. It was found that the repeated oral administration of Rokujo had significant restoring effects on the physiological degenerations which were associated with the development of senile symptoms. These effects included (1) an increase in the plasma testosterone level in male mice; (2) a decrease in the contents of malondialdehyde in the liver and brain; (3) an increase in the liver protein contents; (4) an increase in the liver superoxide dismutase activity; and (5) a decrease in the activity of monoamine oxidase B in the liver and brain membranes. Most of these effects were selectively observed in the senile-prone strain of SAM (SAM-P) as compared to the normal, resistant strain (SAM-R). The present data suggest that Rokujo may exert an anti-aging action in male senile animals.

Keywords—Rokujo; *Cervus nippon* var. *mantchuricus* Swinhoe; senescence-accelerated mouse; testosterone; malondialdehyde; superoxide dismutase; monoamine oxidase B; anti-aging effect

Unossified horn or pilose antler cut from male deer which belong to the Cervidae is generally termed "Rokujo". Rokujo is one of the most famous Chinese traditional medicines and has been considered to possess sexual-reinforcing and anti-aging actions. In fact, Rokujo or its extracts have sometimes been compounded in recent Japanese commercial restoratives, although little is yet known about the pharmacological effects or active ingredients. To date, several modern studies have shown only that extract of Rokujo can improve the syndrome occurring after whiplash injury³⁾ and anemia⁴⁾ in rabbits, and that polysaccharides and lysophosphatidylcholines are responsible for anti-ulcer⁵⁾ and hypotensive⁶⁾ actions, respectively.

In early research to find unidentified pharmacological effects of Rokujo, we set out to investigate the specific effects of Rokujo on senile animals by using an established senile model, senescence-accelerated mice (SAM),⁷⁾ which rapidly acquire behavioral⁸⁾ and pathological⁹⁾ signs that are considered to develop in the normal aging process. Here, by comparing two series of SAM (SAM-P and SAM-R) which were prone to and resistant to self-promoted aging, the changes in several biochemical markers reflecting the senile grade of mammalian organs were evaluated after subchronic oral administration of a hot-water extract of Rokujo.

Materials and Methods

Animals—The substrations of SAM, SAM-P/8 and SAM-R/1 mice,⁷⁾ were originally obtained from Prof. T.

Takeda (Chest Disease Research Institute, Kyoto University) and bred under conventional conditions. In the present study, male SAM-P or SAM-R mice at 11 or 12 months of age were used. Groups of 5 mice per treatment were housed at $23 \pm 1^\circ\text{C}$ under an alternating 12 h light/dark cycle, and given food and water *ad libitum*.

Extract from *Cervus nippon* TEMMINCK var. *mantchuricus* Swinhoe (Rokujo)—Unossified pilose antler or *C. nippon* TEMMINCK var. *mantchuricus* Swinhoe (200 g) from the Jilin Province of China was finely pulverized and extracted with boiling water (1 l) for 2 h. The sludge mixture was filtered and the filtrate was evaporated to dryness under reduced pressure. The pale-yellow residue obtained (61.4 g) was stored at 4°C .

Administration of Extract—The water extract of Rokujo was freshly dissolved in physiological saline at a concentration of 10 or 20 mg/ml just prior to administration. Three groups of SAM-P and SAM-R, respectively, were given the solution or its vehicle orally at a volume of 10 ml/kg/d (representing 0, 100, or 200 mg/kg/d for each series) for 8 successive days. One hour after the last administration, the animals were decapitated and their blood was collected in heparinized tubes. The brain and liver were quickly dissected out and stored at -80°C until use.

Testosterone Assay—The concentration of immunoreactive testosterone (including a small amount of dihydrotestosterone) in the plasma was measured using a radioimmunoassay kit (Amersham, TRK 600).

Malondialdehyde Measurement—Portions (200 mg) of frozen brain and liver tissues were homogenized with 6% trichloroacetic acid by a Polytron homogenizer. The thiobarbiturate-reactive substance in the protein-free supernatant was extracted with butanol, and the optical density at 535 nm was determined.¹⁰⁾

Fractionation of Liver and Measurement of Protein Content—A mitochondrial pellet and its supernatant were prepared from liver sucrose homogenate by centrifugation.¹¹⁾ The protein content was determined by the method of Lowry *et al.*¹²⁾ using bovine serum albumin as the standard.

Superoxide Dismutase (SOD) Activity—The SOD activity was evaluated on the basis of the uneven subcellular distribution of distinct Cu/Zn-containing and Mn-containing enzymes in the liver.¹¹⁾ Using the mitochondrial pellet and its supernatant fractions separated as outlined above, selective inactivation of either the Cu/Zn-SOD or Mn-SOD activity was carried out as described previously.¹¹⁾ The total and separated SOD activities of the preparations were measured by a modified spectrophotometric method based on the xanthine oxidase/nitro blue tetrazolium reaction.¹³⁾ These assays were routinely standardized using purified SOD (3200 U/mg, Sigma).

Monoamine Oxidase (MAO) B Activity—Portions of frozen brain and liver tissues were homogenized with 10 volumes of ice-cold 0.32 M sucrose buffered with 10 mM sodium phosphate (pH 7.4). The homogenates were centrifuged at 1000 *g* for 10 min and the pellet was discarded. A 50 μl aliquot of the supernatant suspension was diluted with 50 mM phosphate buffer (pH 7.4) and incubated with the specific substrate for MAO type-B enzyme, [^{14}C]phenylethylamine (Amersham; final concentration = 0.55 mM), at 37°C for 20 min in a total volume of 0.3 ml. The reaction was terminated by adding 0.2 ml of 2 N HCl. Metabolites were extracted by vigorous shaking with 2 ml of toluene. The radioactivity in the upper toluene phase was measured with a liquid scintillation spectrometer.

Data Analysis—The values are given as the means \pm SEM obtained from 5 individual mice. Statistical significance of differences was determined by using Student's *t* test. Significant differences are indicated in the tables as $p < 0.05$ (#), $p < 0.01$ (##) or $p < 0.001$ (###) between the two control (at 0 dose) SAM-R and P groups, and as $p < 0.05$ a), $p < 0.01$ b) or $p < 0.001$ c) to indicate a significant effect of Rokujo treatment as compared to the control groups.

Results

General Behavior

Male SAM-P at 11–12 months of age presented a distinct appearance of senescence, particularly in terms of disorders of the skin and hair, loss of spontaneous activity and periophthalmic lesions, whereas SAM-R at the same age displayed fewer senile signs.¹⁴⁾ After treatment with Rokujo extract for 8 d, there was no marked improvement in appearance in the SAM-R and P strains in comparison with their control, saline-administered groups.

Plasma Testosterone

It is well known that most endocrine secretory functions tend to decline with aging process.¹⁵⁾ Table I shows that the plasma testosterone in SAM-P amounted to half the content in SAM-R. Repeated oral administration of Rokujo increased the testosterone concentration in both the R and P strains, but the effect was dose-dependent and significant only in SAM-P.

Malondialdehyde in the Liver and Brain

Free radical chain reactions which occur during lipid peroxidation¹⁶⁾ lead to the formation of malondialdehyde, the end product of the reactions. Table II shows that far more

TABLE I. Effects of Rokujo Treatment on the Plasma Concentration of Testosterone

Group (mg/kg/d)		Immunoreactive testosterone (pg/100 μ l plasma)	Group (mg/kg/d)		Immunoreactive testosterone (pg/100 μ l plasma)
SAM-R	0	58.4 \pm 9.0	SAM-P	0	30.2 \pm 4.6 [#]
	100	108.8 \pm 19.0		100	84.0 \pm 14.2 ^{a)}
	200	87.2 \pm 8.1		200	126.3 \pm 7.6 ^{c)}

#, a, c) See data analysis in Experimental.

TABLE II. Effects of Rokujo Treatment on the Contents of Malondialdehyde in the Brain and Liver

Group (mg/kg/d)		Malondialdehyde (OD ₅₃₅ /mg wet tissue)	
		Liver	Brain
SAM-R	0	15.1 \pm 1.0	111.5 \pm 6.9
	100	22.9 \pm 2.5 ^{a)}	85.5 \pm 9.3
	200	17.9 \pm 1.3	87.9 \pm 5.8 ^{a)}
SAM-P	0	29.0 \pm 2.5 ^{###}	126.9 \pm 5.7
	100	9.6 \pm 1.5 ^{c)}	90.0 \pm 8.1 ^{c)}
	200	12.2 \pm 5.4 ^{c)}	79.3 \pm 7.8 ^{c)}

OD₅₃₅: optical density at 535 nm. ###, a, c) See data analysis in Experimental.

TABLE III. Effects of Rokujo Treatment on the Liver Weight and Protein Contents

Group		Liver weight (g/10 g body weight)	Protein content (mg/100 mg wet tissue)	
			Pellet	Supernatant
SAM-R	0	0.44 \pm 0.03	1.22 \pm 0.23	2.14 \pm 0.05
	100	0.39 \pm 0.03	0.99 \pm 0.05	1.90 \pm 0.14
	200	0.42 \pm 0.02	1.13 \pm 0.05	1.99 \pm 0.30
SAM-P	0	0.48 \pm 0.12	0.92 \pm 0.09	1.84 \pm 0.06 ^{##}
	100	0.45 \pm 0.01	1.28 \pm 0.10 ^{a)}	2.12 \pm 0.17
	200	0.53 \pm 0.04	1.79 \pm 0.27 ^{a)}	2.72 \pm 0.04 ^{b)}

##, a, b) See data analysis in Experimental.

thiobarbiturate-reactive malondialdehyde-like substances were detected in the liver of saline-treated SAM-P than in that of SAM-R. Such a difference between SAM-R and P was not apparent in the brain. The Rokujo treatment did not affect the contents in SAM-R, however, they were markedly decreased in both the liver and brain of SAM-P.

Liver Weight and Protein Content

The data in Table III show that the wet weight of dissected whole liver was unaltered by the Rokujo treatment in both strains. After fractionation into membranes and cytosolic components, a decline in total protein content was noted in each fraction from SAM-P liver as compared to SAM-R liver. The Rokujo treatment produced a specific increase in the content of liver protein only in SAM-P.

SOD in the Liver

Table IV shows the Mn-SOD activity, which was predominant in the membrane fraction,

TABLE IV. Effects of Rokujo Treatment on the SOD Activities in the Liver

Group (mg/kg/d)		SOD activity (U/g wet tissue)			
		Mitochondria		Supernatant	
		Total-SOD	Mn-SOD	Total-SOD	Cu/Zn-SOD
SAM-R	0	21.9 ± 1.5	15.0 ± 2.1	22.6 ± 1.1	14.4 ± 0.7
	100	22.6 ± 0.5	16.4 ± 1.4	22.4 ± 1.2	13.2 ± 2.3
	200	21.7 ± 1.3	14.6 ± 1.6	23.2 ± 0.8	16.5 ± 1.4
SAM-P	0	9.8 ± 2.8 [#]	7.2 ± 2.0 [#]	15.0 ± 2.3 [#]	5.7 ± 1.8 ^{##}
	100	21.1 ± 1.4 ^{b)}	15.5 ± 0.9 ^{a)}	22.0 ± 0.8 ^{a)}	15.3 ± 1.0 ^{b)}
	200	22.4 ± 1.1 ^{c)}	12.1 ± 1.1 ^{a)}	25.9 ± 0.8 ^{a)}	20.2 ± 1.1 ^{c)}

[#], ii, a—c) See data analysis in Experimental.

TABLE V. Effects of Rokujo Treatment on the MAO B Activities in the Liver and Brain Membranes

Group (mg/kg/d)		Radioactivity of metabolite (× 10 ³ dpm/10 mg wet tissue)	
		Liver	Brain
SAM-R	0	5.29 ± 1.85	2.43 ± 0.41
	100	2.85 ± 0.31 ^{a)}	1.99 ± 0.14 ^{a)}
	200	2.47 ± 0.28 ^{a)}	1.21 ± 0.16 ^{a)}
SAM-P	0	13.45 ± 0.03 ^{##}	4.07 ± 0.49 [#]
	100	11.47 ± 0.52 ^{b)}	1.39 ± 0.18 ^{c)}
	200	10.22 ± 0.39 ^{c)}	0.81 ± 0.24 ^{c)}

[#], ^{##}, a—c) See data analysis in Experimental.

the Cu/Zn-SOD activity, which was predominant in the cytosol fraction, and the total SOD activities in both fractions from SAM liver. When expressed in terms of units per g wet tissue, all these radical scavenger activities were lower in the saline-treated group of SAM-P than in that of SAM-R, while the decreased SOD activities in SAM-P recovered significantly towards the normal level of SAM-R following Rokujo treatment.

MAO B in the Liver and Brain

MAO is one of the senescence-marker enzymes, since its activity increases with aging in various mammalian organs.¹⁷⁾ It has been shown¹⁸⁾ that MAO can be divided into two main subtypes, types A and B, in relation to a distinct selectivity for substrates. As shown in Table V, the MAO-B activities in the liver and brain membranes of saline-treated SAM were clearly increased in the P series, as compared to the R series. The Rokujo treatment inhibited the MAO-B activities significantly in both the SAM-R and P tissues, but the level of significance was higher in SAM-P than in SAM-R.

Discussion

The effects of repeated administration of Rokujo water-extract on several biochemical changes related to aging were evaluated comparatively using an animal model, SAM-R and P, at 11—12 months of age. Previous reports^{7-9,14,19)} have shown that the short-life-span strain SAM-P acquired severe forms of degeneration in its appearance accompanied with pathologi-

cal amyloidosis and memory dysfunctions even at one year after birth, while such senile signs did not emerge in the control SAM-R or other ordinary mice at this age. During the breeding of SAM, we noticed that, but could not explain why, such differences in aging syndromes between SAM-R and SAM-P were generally more prominent and promoted in males than females (unpublished observations). Accordingly, we chose male SAM first for the present study. The results obtained suggest that clear differences in several biochemical signs of aging also exist between the male R and P series at the same age, in agreement with the results on their behavior and appearance reported previously.

Eight-day oral Rokujo treatment had various significant effects on the male SAM. First, the plasma testosterone concentration was greatly increased. The effect was more obvious in SAM-P than in SAM-R. The results may reflect a potentiation of declined sexual function in aged male animals, as suggested through practical experience from ancient China. In this connection, a gonadotropic action of Rokujo extract leading to an increase in weight of the prostate and seminal capsule has been described.²⁰⁾ We have not yet evaluated the effect of Rokujo treatment in female animals, but it is quite possible that there may be some sexual differences in the effects of Rokujo.

The second category of effects of Rokujo is closely related to the oxygen radical reactions which are thought to underlie aging phenomena.²¹⁾ Interestingly, these effects, *i.e.* a decrease in malondialdehyde and an increase in SOD activity, were specific to the senile SAM-P series. We cannot fully explain our results at present; however, the effects of Rokujo treatment may not involve a direct interaction with these molecules. It seems likely that there may be induction or activation of the scavenger system, *e.g.*, the SOD enzyme itself, since the SOD activity (in units per g tissue) was potentiated by the Rokujo treatment in SAM-P. However, when the SOD activity was expressed in terms of units per mg protein, considering the results in Tables III and IV, there was no significant difference, indicating that the increase in protein content specific to the SAM-P liver may represent induction, or decreased decomposition, of various enzyme proteins by the Rokujo treatment.

In contrast to these effects, the inhibition of the MAO activity in the liver and brain membranes was also significant in the normal SAM-R. Advanced studies on the effects of Rokujo extract on MAO activity are currently in progress in our laboratories and several hydrophobic compounds, which have no promotional activity in protein synthesis, have been identified as MAO inhibitors *in vitro* (in preparation). The MAO-inhibiting action after repetitive Rokujo treatment therefore seems to be caused by such inhibitors that would remain in the tissues when the mice were decapitated one hour after the final oral administration.

Thus, subchronic administration of water extract of Rokujo reversed biochemical signs of aging selectively in the senile animals. Although no obvious improvement in the behavior or appearance of senescence was observed during the 8-d administration, some active components of the extract may be effective in counteracting physiological alterations of the long-term aging process. These heterogeneous effects observed in the present study suggest the existence of distinct active substances in the Rokujo extract. We must also examine the effects of Rokujo in normally aged animals, since we cannot assess whether or not the changes observed in the specific SAM-P might be identical with those of normally aged mice. Further studies are needed to clarify the detailed actions involved.

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References and Notes

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- 3) K. Takigawa, Y. Yabuuchi, M. Fujimoto and K. Harima, *Oyo Yakuri*, **5**, 747 (1971); K. Takigawa, N. Kokubu, M. Kajihara, M. Dohi and N. Tahara, *Nippon Yakurigaku Zasshi*, **68**, 489 (1972).
- 4) I. Y. Jae, *J. Korean Pharm. Sci.*, **6**, 20 (1976).
- 5) B. X. Wang, A. J. Liu, X. J. Cheng, Q. S. Wang, G. R. Wei and J. C. Cui, *Acta Pharm. Sinica*, **20**, 321 (1985).
- 6) H. Tsujibo, Y. Miyake, K. Maruyama and Y. Inamori, *Chem. Pharm. Bull.*, **35**, 654 (1987).
- 7) T. Takeda, M. Hosokawa, S. Takeshita, M. Irino, K. Higuchi, T. Matsushita, Y. Tomita, K. Yasuhira, H. Hamamoto, K. Shimizu, M. Ishii and T. Yamamuro, *Mech. Ageing Dev.*, **17**, 183 (1981).
- 8) M. Hosokawa, R. Kasai, K. Higuchi, S. Takeshita, K. Shimizu, H. Hamamoto, A. Honma, M. Irino, K. Toda, A. Matsumura, M. Matsushita and T. Takeda, *Mech. Ageing Dev.*, **26**, 91 (1982).
- 9) S. Takeshita, M. Hosokawa, M. Irino, K. Higuchi, K. Shimizu, K. Yasuhira and T. Takeda, *Mech. Ageing Dev.*, **20**, 13 (1982).
- 10) M. Uchiyama and M. Mihara, *Anal. Biochem.*, **86**, 271 (1978).
- 11) B. L. Geller and D. R. Winge, "Methods in Enzymology," Vol. 105, ed. by L. Packer, Academic Press, Orlando, 1984, pp. 105—114.
- 12) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
- 13) T. Imanari, M. Hirota, M. Miyazaki, K. Hayakawa and Z. Tamura, *Igaku No Ayumi*, **101**, 496 (1977).
- 14) M. Hosokawa, R. Kasai, K. Higuchi, S. Takeshita, K. Shimizu, H. Hamamoto, A. Honma, M. Irino, K. Toda, A. Matsumura, M. Matsushita and T. Takeda, *Mech. Ageing Dev.*, **26**, 91 (1984).
- 15) R. S. Swerdloff and D. Heber, "Endocrine Aspects of Aging," ed. by S. G. Korenmann, Elsevier, New York, 1982, pp. 119—135.
- 16) E. Sawaki, T. W. Stanley and H. Johnson, *Anal. Biochem.*, **35**, 199 (1963).
- 17) A. Nies, D. S. Robinson, J. M. Davis and C. L. Ravaris, *Adv. Behav. Biol.*, **6**, 41 (1973).
- 18) D. D. Schoepp and A. J. Azzaro, *J. Neurochem.*, **36**, 2025 (1981).
- 19) M. Miyamoto, Y. Kiyota, N. Yamazaki, A. Nagaoka, T. Matsuo, Y. Nagawa and T. Takeda, *Physiol. Behav.*, **38**, 399 (1986).
- 20) T. Iida, H. Ohno and T. Tamura, The 21st Annual Meeting of Pharmaceutical Society of Japan, Tokushima, Oct. 1965, p. 452.
- 21) K. Kator, *Jikken Igaku*, **4**, 1116 (1986).