Prolongation of Life Span of Stroke-Prone Spontaneously Hypertensive Rats (SHRSP) Ingesting Persimmon Tannin

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The effects of persimmon tannin on pathophysiological changes in stroke-prone spontaneously hypertensive rats (SHRSP) were investigated. When the persimmon tannin was chronically ingested by SHRSP, the life span was significantly prolonged, yet the effect on blood pressure was slight. The incidences of brain hemorrhage and infarction were also significantly decreased by this treatment. To elucidate the mechanisms involved in these events, the effects of condensed tannins, including persimmon tannin, on free radicals and lipid peroxidation were examined *in vitro*. Using electron spin resonance analysis, we found that these tannins have a potent, concentration-dependent scavenging action toward active oxygen free radicals. These tannins strongly inhibited lipid peroxidation in rat brain homogenates, in a concentration-dependent manner. Persimmon tannin inhibited lipid peroxidation similarly to (−)-epigallocatechin. Persimmon tannin was 20 times more effective than α-tocopherol in terms of the 50%-inhibitory concentration. The radical scavenging action and inhibition of lipid peroxidation by persimmon tannin may explain, in part, the prolongation of the life span of the SHRSP ingesting persimmon tannin.

Keywords stroke-prone spontaneously hypertensive rat (SHRSP); persimmon; tannin; free radical scavenger; lipid peroxidation

Persimmon juice has been used in Japan as a traditional medicine for the treatment of hypertension and to prevent stroke. In 1955, Sasakawa[1] reported that persimmon juice was an effective antihypertensive. Funayama and Hikino[2] found that the active principles of persimmon leaves, as related to its hypotensive effect, were astragalin and isoquerucitrin. However, little is known of the active principles of persimmon juice.

Flavonoids, including condensed tannins, have various biological activities and these have been widely studied,[3-12] especially psychotropic activity,[3] inhibition of angiotensin converting enzyme (ACE),[4,12] improvement of nitrogen metabolism,[5] the improvement of renal disorders[6] and free radical scavenging action.[7] Inokuchi *et al.*[12] reported that the tannin fractions of Chinese crude drugs clinically prescribed to treat hypertensives markedly reduced the blood pressure of spontaneously hypertensive rats (SHR), inhibited the activity of ACE and also inhibited the pressor responses to both angiotensins I and II. Persimmon tannin is a mixture of various condensed tannins and one of the constituents of persimmon tannins is (−)-epicatechin.[13] We investigated whether persimmon tannin induce changes in blood pressure and in the pathophysiological functions of stroke-prone SHR (SHRSP).

We reported that condensed tannins isolated from persimmon, green tea leaves and Rhei Rhizoma had a potent scavenging action toward active oxygen free radicals.[7] Lipid peroxides formed by the reactions of free radicals are etiologic factors involved in the genesis of stroke.[14] To elucidate the mechanisms of improvement of pathophysiological changes in SHRSP, we examined the effects of condensed tannins isolated from persimmon, green tea leaves and Rhei Rhizoma on free radicals and lipid peroxidation *in vitro*.

Materials and Methods

Animals SHRSP inbred at the Laboratory Animal Center for Biomedical Research, Nagasaki University School of Medicine, were used. Groups of 3—5 rats were housed in plastic cages in an air-conditioned room (22±1 °C, 65±5%), with lights on 12 h per day (7:00—19:00).

Effect of Persimmon Tannin on Hypertension of SHRSP The experiment was started when the male SHRSP were 4 weeks of age. Persimmon tannin was dissolved in tap water at a concentration of 0.5% and animals were provided with this solution for drinking throughout the experimental period, up to age 41 weeks. The average intake of persimmon tannin calculated based on the daily consumption of water was 500 mg/kg/d. The blood pressure and heart rate were measured once a week up to age 35 weeks, by means of a plestimograph method (pre heat: 38—40 °C, 6—10 min). Values were statistically examined by the one-way analysis of variance (ANOVA) using the F-test, a p value of less then 0.05 was regarded as significant. Upon completion of the experiment, the rats were decapitated, and the brains, kidneys and mesenteries were fixed in a 10% formalin solution. The incidence of stroke (cerebral hemorrhage and/or infarction) was examined on the brain surface and sections of all of the rats, including those that had died during the experimental period. Histopathological changes of the kidney and mesenteric artery in SHRSP were also examined. The incidence of stroke was statistically examined by means of the Fisher exact probability test.[15] The survival rates in SHRSP, with and without treatment with persimmon tannin, were statistically examined by the Kaplan–Meier method. Persimmon tannin was isolated from persimmon by using Diaion HP-20 chromatography.[16] In brief, persimmon juice was applied to the Diaion HP-20 column, which was washed with water. The persimmon tannin fraction was eluted with 50% water–acetone and concentrated to dryness *in vacuo*, giving a brown amorphous material.

Effect of Condensed Tannins on Free Radicals We examined the effects of condensed tannins on free radicals, such as the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, the superoxide anion (O_2^-) generated by the hypoxanthine (HPX)–xanthine oxidase (XOD) system, and the hydroxy and hydroperoxy radicals (·OH and ·OOH) generated by Fenton's reaction,[17] by using the reported methods.[7]

Effect of Condensed Tannins on Lipid Peroxidation Estimation of the effect of condensed tannins on lipid peroxidation in rat brain homogenate was based on the method of Stocks *et al.*[18] Adult male Wistar rats weighing 200—250 g were decapitated, and the brains were immediately removed and homogenized in ice-cold phosphate buffer (50 mM, pH 7.4). The homogenate was centrifuged for 15 min at 1000×g and the supernatant was used. The assay mixture, containing 1 ml of the homogenate and 10 μl of the tannin solution, was incubated at 37 °C for 30 min. The reaction was halted by the addition of 200 μl of 35% $HClO_4$ and the mixture was centrifuged at 1300×g for 10 min. The malondialdehyde (MDA) in the supernatant was measured by the method of Suno and

Nagaoka.[19] One milliliter of the supernatant was heated with 0.5 ml of 0.5% thiobarbituric acid (50% acetic acid solution; Wako Chemical Co.) at 100 °C for 30 min. The absorbance of the mixture was read at 532 nm. As a control, lipid peroxidation in the absence of tannins was measured and the concentration required for 50% inhibition of lipid peroxidation was calculated. (−)-Epicatechin, (−)-epigallocatechin, (−)-epicatechin 3-*O*-gallate and (−)-epigallocatechin 3-*O*-gallate were isolated from green tea leaves. Procyanidins were isolated from Rhei Rhizoma, as described.[16]

Results

Effect of Persimmon Tannin on Blood Pressure, Heart Rate and Body Weight in SHRSP Chronic treatment with persimmon tannin did not inhibit the development of hypertension in SHRSP throughout the experimental period, though the blood pressure in this group was significantly lower than in the control at 13, 21, 25, 26 and 27 weeks of age (Fig. 1). There were no significant differences in heart rate and body weight between the control and the persimmon tannin-treated animals, throughout the experimental period.

Effect of Persimmon Tannin on the Histopathological Changes and Life Span of SHRSP The long-term administration of crude persimmon tannin significantly prolonged the life span of SHRSP (Fig. 2). In all the rats that died, cerebral hemorrhage or infarction at the brain surface or section was evident. The incidence of strokes was also significantly decreased by treatment with persimmon tannin (Table I). However, the treatment with persimmon tannin did not inhibit the histopathological changes of kidney and mesenteric artery in SHRSP.

Effect of Condensed Tannins on Free Radicals *in Vitro* All the condensed tannins we investigated scavenged DPPH, O_2^-, OH and OOH radicals, in a concentration-dependent manner. Table II shows the concentration required for 50% inhibition of these radicals. The monomers, (−)-epicatechin and (−)-epigallocatechin, were also scavengers of active oxygen free radicals. Among the various condensed tannins we tested, procyanidin C-1 3,3′,3″-tri-*O*-gallate was the most potent scavenger of the DPPH radical. This tannin was 50 times more effective than that of α-tocopherol. Persimmon tannin was 7 times more effective than α-tocopherol.

Effect of Condensed Tannins on Lipid Peroxidation in Rat

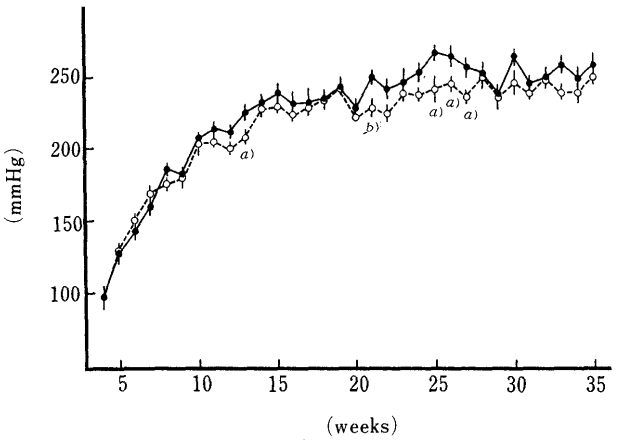

Fig. 1. Effect of Persimmon Tannin on Blood Pressure in SHRSP

A 0.5% persimmon tannin solution [persimmon tannin; $n=10$ (4—35 weeks of age)] or water [control; $n=10$ (4—31 weeks of age), $n=9$ (32 weeks of age), $n=8$ (33 weeks of age), $n=7$ (34—35 weeks of age)] was provided freely for drinking. Each point represents the mean ± S.E.M. *a*) $p<0.05$, *b*) $p<0.01$, significantly different from the control group. ●—●, control; ○---○, persimmon tannin.

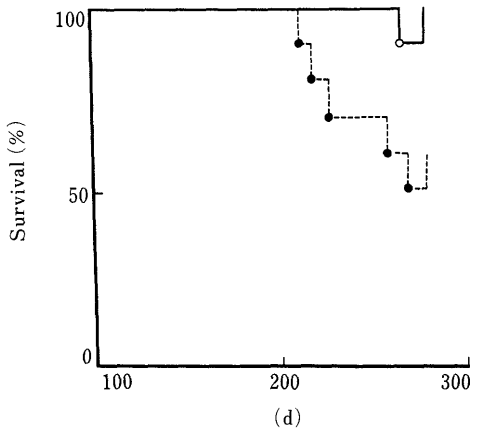

Fig. 2. Effect of Persimmon Tannin as to Prolongation of the Life Span of SHRSP

The survival rates were statistically examined by means of the Kaplan–Meier method. ●---●, control; ○—○, persimmon tannin.

Table I. Protective Effect of Persimmon Tannin against Stroke in SHRSP

	Survival (%)	Incidence of hemorrhage or infarction
SHRSP Control	5/10 (50%)	7/10 (70%)
SHRSP Persimmon tannin	9/10 (90%)	2/10 (20%)[a]

a) $p<0.05$, significantly different from the control group.

Table II. Effects of Condensed Tannins on Free Radicals

Tannin	Concentration for 50% inhibition: DPPH radical		Superoxide anion		OH radical		OOH radical	
	(μg/100 μl)	(μM)	(μg/100 μl)	(μM)	(μg/100 μl)	(μM)	(μg/100 μl)	(μM)
(−)-Epicatechin	0.58	20	6.6	227	1.2	41	2.2	76
(−)-Epigallocatechin	0.19	6.2	1.6	52	1.8	59	5.5	180
(−)-Epicatechin 3-*O*-gallate[a]	0.14	3.2	2.3	52	—	—	—	—
(−)-Epigallocatechin 3-*O*-gallate[a]	0.14	3.1	1.6	35	0.7	16	1.0	22
Procyanidin B-2 3,3′-di-*O*-gallate[a]	0.20	2.3	3.4	38	1.0	11	1.4	15
Procyanidin B-5 3,3′-di-*O*-gallate[a]	0.26	2.9	4.3	48	1.1	12	1.7	19
Procyanidin C-1 3,3′,3″-tri-*O*-gallate[a]	0.18	1.4	3.5	26	0.9	7	2.2	17
Persimmon tannin[a]	0.41	—	8.4	—	1.6	—	2.2	—
α-Tocopherol[a]	3.00	70	—	—	—	—	—	—

a) Documented.[7]

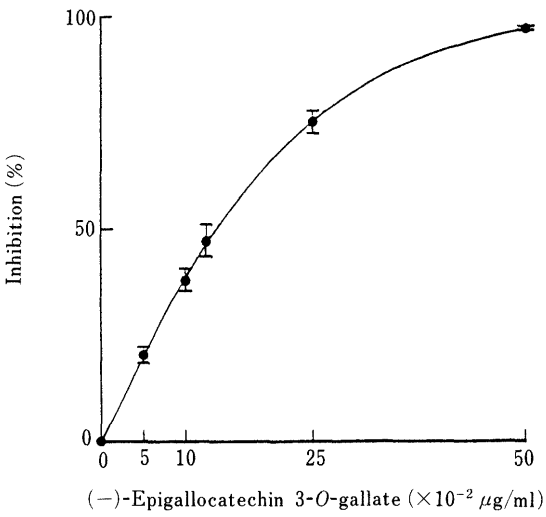

Fig. 3. Effect of (−)-Epigallocatechin on Lipid Peroxidation in Rat Brain Homogenates

Rat brain homogenates were incubated at 37 °C for 30 min in the presence of various concentrations of (−)-epigallocatechin 3-*O*-gallate. MDA, a secondary product of lipid peroxidation, was measured by the thiobarbituric acid method. Each point represents the mean ± S.E.M. ($n=5$).

TABLE III. Effects of Condensed Tannins on Lipid Peroxidation in Rat Brain Homogenates

Tannin	Concentration for 50% inhibition	
	(μg/ml)	(μM)
(−)-Epicatechin	6.3	22
(−)-Epigallocatechin	1.4	4.6
(−)-Epicatechin 3-*O*-gallate	0.16	0.36
(−)-Epigallocatechin 3-*O*-gallate	0.14	0.31
Procyanidin B-2 3,3′-di-*O*-gallate	0.44	0.48
Procyanidin B-5 3,3′-di-*O*-gallate	0.42	0.46
Procyanidin C-1 3,3′,3″-tri-*O*-gallate	0.62	0.45
Persimmon tannin	1.44	—
α-Tocopherol	30	70

Brain Homogenate The lipid peroxidation in rat brain homogenates was potently inhibited by all the condensed tannins, in a concentration-dependent manner. Figure 3 shows the effect of (−)-epigallocatechin 3-*O*-gallate on lipid peroxidation in rat brain homogenates. The concentrations required for 50% inhibition of lipid peroxidation are shown in Table III. Among the various tannins tested, (−)-epigallocatechin 3-*O*-gallate, isolated from green tea leaves, most potently inhibited lipid peroxidation in the rat brain homogenates. Persimmon tannin also inhibited lipid peroxidation similarly to (−)-epigallocatechin. All the condensed tannins tested were more effective in inhibiting lipid peroxidation than was α-tocopherol.

Discussion

The long term administration of persimmon tannin significantly prolonged the life span of SHRSP, but the effect on their blood pressure was only slight. The incidence of stroke was significantly decreased in the rats given persimmon tannin.

We have reported that Oren-gedoku-to, a Chinese herb that is clinically prescribed for hypertension, prevents strokes and prolongs the life span of SHRSP, without producing a reduction in blood pressure,[20] and that carteolol, a β-blocking agent, prevents secondary hypertensive lesions of SHRSP with slight depressor effects.[21] Maniwa *et al.*[22] reported that a small dose of arotinolol, an α,β-blocking agent, markedly prevented hypertension-related lesions, as well as strokes, and prolonged the life span of SHRSP, with no reduction in blood pressure. Although hypertension is an important etiologic factor in the genesis of stroke, the above-mentioned evidence indicates that other etiologic factors have to be considered.

Tomita *et al.*[14] reported that the level of MDA in the blood of SHRSP with cerebral lesions was high compared with that of SHRSP without stroke or Wistar Kyoto rats. Yoshikawa *et al.*[23] found that the levels of MDA in serum and cerebrospinal fluid of patients with cerebral apoplexy were significantly elevated compared with those in healthy persons. These findings suggest that lipid peroxides are closely linked to the etiology of stroke.

Lipid peroxides are formed by active oxygen free radicals generated in the living body.[14] Those radicals such as O_2^-, OH and OOH may cause peroxidation of the lipid membrane, and cell injury in neurons and blood vessels would ensue.[24] Making use of electron spin resonance (ESR) analysis, we found that condensed tannins exhibited a potent scavenging action toward the DPPH radical, O_2^-, and the OH and OOH radicals *in vitro*. Persimmon tannin also scavenged these active oxygen free radicals similarly to (−)-epicatechin, and it is more effective than α-tocopherol. A comparison of (−)-epicatechin and (−)-epigallocatechin with (−)-epicatechin 3-*O*-gallate and (−)-epigallocatechin 3-*O*-gallate suggested that the condensed tannins with the galloyl groups were more effective scavengers of free radicals.

The effect of condensed tannins on lipid peroxidation in rat brain homogenates was examined by measuring MDA, a secondary product of lipid peroxidation, *in vitro*. All the condensed tannins we studied potently inhibited lipid peroxidation in rat brain homogenates. Persimmon tannin also inhibited lipid peroxidation in rat brain homogenate similarly to that seen with (−)-epigallocatechin, and was 20 times more effective than α-tocopherol in terms of 50%-inhibitory concentration. Among the various condensed tannins tested, (−)-epigallocatechin 3-*O*-gallate was the most effective. The radical scavenging action and inhibition of lipid peroxidation by the condensed tannins may be involved in the prevention of strokes in SHRSP, without influencing the blood pressure.

The condensed tannins we used also specifically inhibited ACE *in vitro*.[4] The ACE in cerebral capillaries plays a pivotal role in regulation of blood-brain permeability to water and electrolytes; angiotensin II formed by ACE enhances blood-brain permeability.[25,26] Therefore, the action of condensed tannins as inhibitors of ACE may be associated with the prevention of brain tissue damage by hypertension in the SHRSP treated with persimmon tannins.

These biological activities of condensed tannins *in vitro* may improve the pathophysiological changes such as cell injury in blood vessels and neurons induced by severe hypertension in the SHRSP.

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