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The Effects of Green Tea Supplementation on Behavioral Changes, Striatal Dopamine Level, and Hepatic Antioxidant Parameters of Parkinson's Disease Model Rats

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Green tea has attracted attention with respect to its potential for preventing and treating neurodegenerative disease. The neurotoxin, 6-hydroxydopamine (6-OHDA), was used to produce experimental Parkinson's disease (PD) model. The purpose of this study was to investigate the effects of green tea diet on behavioral changes, striatal dopamine content, and hepatic antioxidant parameters of PD model rats. In this study, we used male Sprague-Dawley rats weighing 200~220 g and injected 6-OHDA into the right substantia nigra and medial forebrain bundle of the brain. The supply of green tea diet was started at 2 weeks before 6-OHDA lesion and continually supplied during 0, 2, and 4 weeks after 6-OHDA lesion (GT-0, GT-2, GT-4). Behavioral disturbance was measured by the stepping and *d*-amphetamine drug-induced rotation tests. Then, we assayed the striatal dopamine content and the hepatic malondialdehyde (MDA), hydrogen peroxide (H_2O_2), and superoxide dismutase (SOD) activity. The percentage of lesioned forepaw to non-lesioned forepaw step scores was the highest in GT-4 group among all groups at both 3 and 4 weeks after 6-OHDA lesion. At 4 weeks after 6-OHDA lesion, the rotation score was the lowest in GT-2 group ($p<0.05$). However, increasing rate of the rotation score from 2 to 4 weeks after 6-OHDA lesion was the lowest in GT-4 group. The striatal dopamine content was not significantly different among four groups by green tea diet. The hepatic MDA level was the lowest in GT-4 group among four groups. The hepatic SOD activity was increased with the prolongation of green tea diet period. These results suggest that green tea diet affects behavioral changes in rats of PD model. It seems that continuous green tea supplementation has an influence on the reduction of behavioral disturbance and the hepatic MDA level. Accordingly, continuous green tea supplementation was recommended for the prevention and treatment of PD. However, further studies are needed to investigate the mechanisms and efficacy of green tea in PD.

Key words: Green tea, Parkinson's disease, Behavioral disturbance, Dopamine, Antioxidant

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INTRODUCTION

Green tea is a drink made from steamed and dried leaves of *Camellia sinensis* plant. It is a beverage that is widely consumed in Japan, China, and other Asian nations and becoming more popular in Western countries. Recently, green tea has attracted attention for its health benefits and pharmacological effects, particularly with respect to its potential for preventing and treating cancer, cardiovascular disease, inflammatory disease, and neurodegenerative disease.[1,2] These properties have been reported to be mediated by green tea polyphenols.[3] The biological properties of green tea polyphenols reported in the literature include antioxidant actions, free radical scavenging, and catechol-*O*-methyltransferase activity reduction.[3] Green tea polyphenols have been observed to be more efficient radical scavengers when compared to vitamin C and E.[1,4] The ability of these compounds to scavenge reactive oxygen species such as hydrogen peroxide (H_2O_2) and superoxide radicals ($\cdot O^{2-}$) depends on their phenolic chemical structures.[4]

A catecholaminergic neurotoxin, 6-hydroxydopamine (6-OHDA), has been used to produce experimental Parkinson's disease (PD) model both *in vitro* and *in vivo*.[2,5,6] Because it dose not cross the blood-brain barrier *in vivo*, it is administered directly in substantia nigra, medial forebrain bundle, or striatum of the brain tissue.[7] Intrastriatal injection with 6-OHDA injures selectively catecholaminergic neurons through the production of oxygen free radicals.[7]

Dopamine is synthesized by the dopaminergic neurons

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which make up the nigrostriatal system and it is a catechol neurotransmitter widely distributed throughout the brain.[8] However, most of dopamine in the brain is found in the striatum and severe dopamine depletion in the striatum causes motor symptoms.[8,9] Thus, the striatal dopamine content is involved in several diseases of the nervous system including PD.[10] It is one of the characteristic features of PD that dopamine neurons are lost because of the progressive degeneration of the nigrostriatal system.[11,12]

Since oxidative stress is the primary pathogenic mechanism involved in PD, naturally occurring polyphenolic catechins have attracted particular attention because of their abundance in tea extracts, their ability to pass through the blood-brain barrier, and their relatively high antioxidant capacity to offer neuroprotection.[13,14] Extract of green tea, which is well known antioxidant, has been shown to attenuate 6-OHDA-induced cell death *in vitro*.[14] However, the effects of green tea on PD *in vivo* are largely unknown despite numerous studies in recent years. Thus, in the present study, we investigated the effects of green tea supplementation before and after the brain injury by 6-OHDA on behavioral changes, striatal dopamine contents, and hepatic tissue in rats.

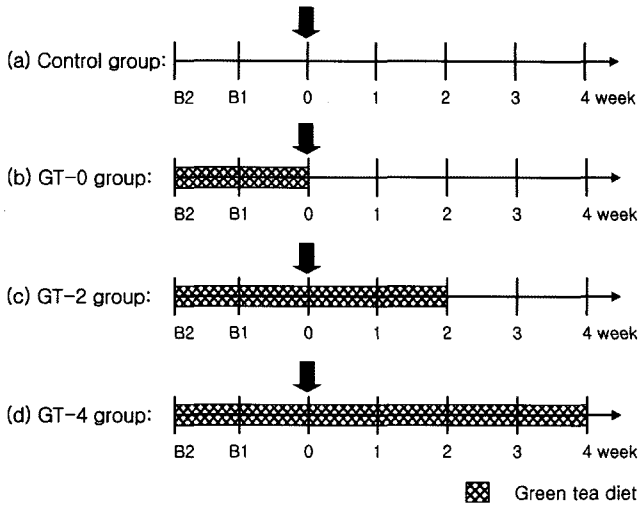

Fig. 1 Experimental design.
(a) Control group: Parkinson's disease (PD) model rats fed normal chow for 6 weeks. (b) GT-0 group: PD model rats fed the green tea diet (containing green tea powder 1 g/kg/day) for 2 weeks before 6-OHDA lesion. (c) GT-2 group: PD model rats fed the green tea diet for 2 weeks before and 2 weeks after 6-OHDA lesion. (d) GT-4 group: PD model rats fed the green tea diet for 2 weeks before and 4 weeks after 6-OHDA lesion. Before and after 6-OHDA lesion, behavioral tests were carried out. Stepping tests were carried out an onset of experiment and every week for 6 weeks. A period of the green tea diet supplementation was expressed as (▩) and an onset of 6-OHDA drug-induced lesion were expressed as thick arrow. Amphetamine-induced rotation tests were carried on 2 and 4 weeks after 6-OHDA lesion. Intact rats do not show turning behavior as well as clear preference in direction. After completion of the behavioral testing, the animals were killed and the striatum were processed for HPLC analysis as described in materials and methods.

MATERIALS AND METHODS

1. Chemicals and Reagents

All chemicals were purchased from Sigma Aldrich Korea Ltd. (Korea). unless specifically noted. HPLC grade methanol was obtained from Fisher Scientific Korea Ltd. (Korea).

2. Diets

Green tea diet was made with chow powder, green tea powder (Boseong, Korea), and water (10/0.2/3.5, w/w/v) freshly every morning. The rats were fed the green tea diet (containing green tea powder 1 g/kg body weight/day). After the rats finished eating green tea diet, they were fed chow and water *ad libitum*. The green tea diet was started at 2 weeks before the induction of the 6-hydroxydopamine (6-OHDA) lesion. Experimental design was schematized in Fig. 1.

3. Animals and Surgical Procedures

Animals were housed and treated according to the National Research Council's *Guide for the Care and Use of Laboratory Animals*. Male Sprague-Dawley rats weighing 200~220 g were anaesthetized intraperitoneally (i.p.) with pentobarbital sodium salt (50 mg/kg), and fixed on stereotaxic frame (51600 single manipulator model, Stoelting, Wood Dale, IL, USA). A volume of 5 μl 6-OHDA (8 μg/μl in normal saline with 0.2% ascorbic acid) was injected into the right substantia nigra [coordinates relative to bregma were antero-posterior, -4.4 mm; medio-lateral, 1.2 mm; dorso-ventral, 7.8 mm from dura] and the right media forebrain bundle [-4.8 mm; 1.8 mm; 8.2 mm], respectively.[15,16] Incisor bar was set at 2.5 mm. The injection was made via a 10-μl Hamilton syringe with a 28-gauge needle, at a flow rate of 1 μl/min.

4. Behavioral Tests

Stepping test

Forelimb akinesia was assessed by the 'stepping test'.[17] Animals were adapted to the test conditions during 5 days. One forelimb and two hindlimbs were fixed by the investigator, whereas the unrestrained forepaw was touching the table. The number of adjusting steps was counted while the rat was moved sideways along the table surface (90 cm in 10 s). Each stepping test consisted of three trials for each forepaw, alternating between forepaws. In all experiments, the average of the three trials for each forepaw was used for analysis.

Amphetamine-induced rotation test

The degree of amphetamine-induced rotation was assessed on day 0 before 6-OHDA lesion, to establish a baseline and assign surgery side. Typically, animals did not rotate in just one direction before a lesion, but there was a slight intrinsic bias.[1] Then, 2 and 4 weeks after the 6-OHDA lesion, animals were tested *d*-amphetamine (3 mg/kg, i.p.) drug-induced rotational behavior, which was monitored in automated rotometer bowls (Roto-Rat™, MED Associates, Inc., Albans, VT, USA) during 60 min.[18] Net rotational asymmetry score is expressed as 360° turns per minute.

5. Striatal Dopamine Determination by HPLC

The dopamine level in the striatum was determined by a modified method.[1] At 6 weeks after the experiment started, the rats were anaesthetized with pentobarbital sodium salt (50 mg/kg i.p.). Then, the striatal tissues were immediately collected as a fragment (2 mm×2 mm×2 mm size) in the same sites. The fragment was homogenized in 280 μl of 0.2 M perchloric acid containing 0.1 mM EDTA and 20 μl of 10^{-5} M L-3,4-dihydroxyphenylalanine as an internal standard. Homogenized samples were sonicated for 30 sec in an ice bath with a sonicator (GE 50; Sonics & Materials Inc., Danbury, CT, USA) and filtered through centrifugal filter devices (Microcon YM-10, Millipore Co., Bedford, MA, USA) by centrifuging at 13,000 rpm for 15 min at 4 ℃. After filtration, an aliquot of 100 μl of the sample was injected directly into an injector (7725(i), Rheodyne, Cotati, CA, USA) and analyzed by a high-performance liquid chromatograph HPLC) with electrochemical detection.[19] The HPLC system consisted of an electrochemical detector (ECD-300, EICOM, Kyoto, Japan), a solvent delivery system (515 HPLC-pump; Waters Co., Milford, MA, USA), a column oven (Waters Co.), and a data processor dsChrome software (Donam Int., Seoul, Korea). The separation column was a reverse- phase C18 column (3.0 mm i.d.×150 mm; EICOMPAK SC-50DS; EICOM) and guard column was a PREPAK (4.0 mm i.d.×5 mm, EICOM). The appendance potential of ECD-300 (carbon electrode vs. Ag/AgCl reference electrode) was set at +750 mV. The mobile phase consisted of 90 mM sodium acetate-100 mM citric acid buffer (pH 3.5)/methanol (83:17, v/v) containing 190 mg/L of 1-octanesulfonic acid (sodium salt) and 5 mg/L of disodium EDTA. The flow rate was set at 0.5 ml/min at 30 ℃. Dopamine levels were calculated using dopamine standard (3-hydroxytyramine; dopamine hydrochloride) and corrected by protein levels of the samples. Finally, dopamine levels were expressed as μg/mg protein. Chromatogram for mixture of standards (norepinephrine, epinephrine, dopamine, and L-3,4-dihydroxyphenylalanine) was obtained from the data processor of the HPLC system and shown in Fig. 2 (a). L-3,4-dihydroxyphenylalanine was used as internal standard for the quantification of dopamine concentrations. A typical chromatogram for dopamine in the striatum was shown in Fig. 2 (b).

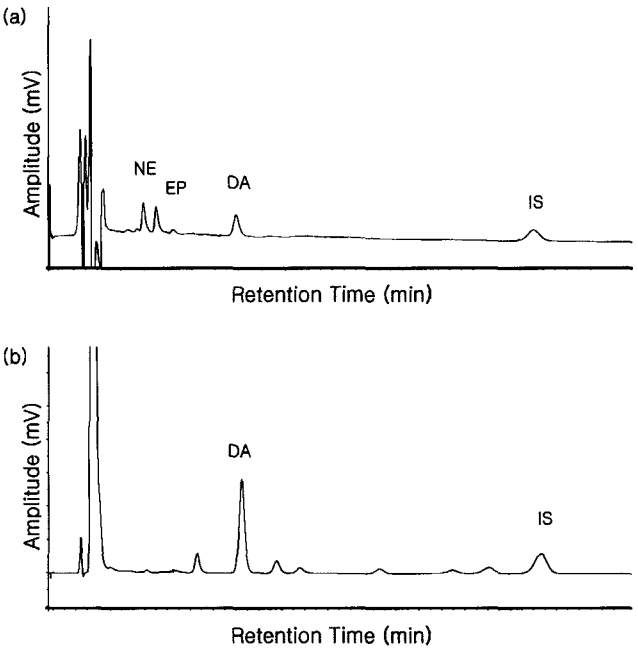

Fig. 2 (a) Chromatogram for mixture of standards (NE, norepinephrine; EP, epinephrine; DA, dopamine). IS represents the internal standard (L-3,4-dihydroxyphenylalanine) used for quantification of DA concentrations. (b) Typical chromatogram for dopamine in the striatum.

6. Hepatic Tissue Assays

Hepatic tissues were promptly excised and rinsed with cold isotonic saline. The tissues were frozen at -70 ℃ until analyzed. The 0.5 g of liver tissue was randomly collected and homogenized in phosphate buffer (10 mM, pH 7.4) (1:9, w/v) in an ice bath and sonicated for 20 sec with a sonicator (GE 50; Sonics & Materials Inc.) under an ice bath. This homogenate was centrifuged at 10,000×g and 4 ℃ for 20 min in an ultracentrifuge (Optima™ TL, Beckman Coulter, Inc., Fullerton, CA, USA). The supernatant was then recentrifuged at 100,000 ×g and 4 ℃ for 1 hr. Final supernatant was used as samples for the measurement of enzyme activity.[20]

The concentration of hepatic malondialdehyde (MDA) was measured by the method of Buege and Aust.[21] We added 20 μl of 0.2% butyl hydroxyl toluene to 500 μl sample homogenate. After addition of 500 μl of 17.5% trichloroacetic acid (TCA), 500 μl of 0.6% thiobarbituric acid was added. The mixture was heated at 100 ℃ for

15 min in boiling water bath. Then, solution was allowed to cool down at room temperature (RT). Sample mixture was reacted at RT for 20 min after addition of 500 μl of 70% TCA. The absorbance of the final supernatants after centrifugation at 3,000×g for 10 min was measured at 532 nm in a spectrophotometer (DU 600, Beckman Coulter, Inc., Fullerton, CA, USA). The thiobarbituric acid 1,1,3,3-tetramethoxypropane was used as a MDA standard.

Quantitative hydrogen peroxide of liver tissue was performed by H_2O_2-560^{TM} assay kit (OXIS International, Inc., Foster, CA, USA) according to the manufacturer's manual.

Superoxide dismutase (SOD) level in cytosolic fraction was measured by SOD-525^{TM} assay kit (OXIS International) according to the experimental procedures provided by the manufactures.

All hepatic parameters were corrected by protein levels of the samples.

7. Protein Assay

The protein concentrations of brain and hepatic tissues were determined by the method of Bradford.[22]

8. Statistical Analysis

For statistical analysis, SPSS/PC computer program (Statistical Package for Social Science 12.0) was used. Data were expressed as mean±standard error of mean (S.E.M.). The significance of differences among mean values was assessed by one-way analysis of variance (ANOVA) coupled with Duncan's multiple range test at p value < 0.05. Comparison between right and left striatum was analyzed by paired t-test at p value < 0.05.

RESULTS

1. Effects of Green Tea Diet on Behavioral Changes in Parkinson's Disease Model Rats

In the estimation of stepping and rotation scores before 6-OHDA lesion, no significant differences by green tea diet in the behavioral test were observed among four groups. As shown in Fig. 3 (a), at 1 and 2 weeks before 6-OHDA lesion, the percentages of lesioned (left) forepaw to non-lesioned (right) forepaw step scores (% step scores) were not significantly different among four groups. The average of intact paw performances was 20.13±0.11 steps (data not shown). Post-lesioning, all rats showed apparent motor disturbance. Thus, 1 week after 6-OHDA lesion, the % step scores were decreased dramatically at all groups. Also, data not shown, the average of impaired paw

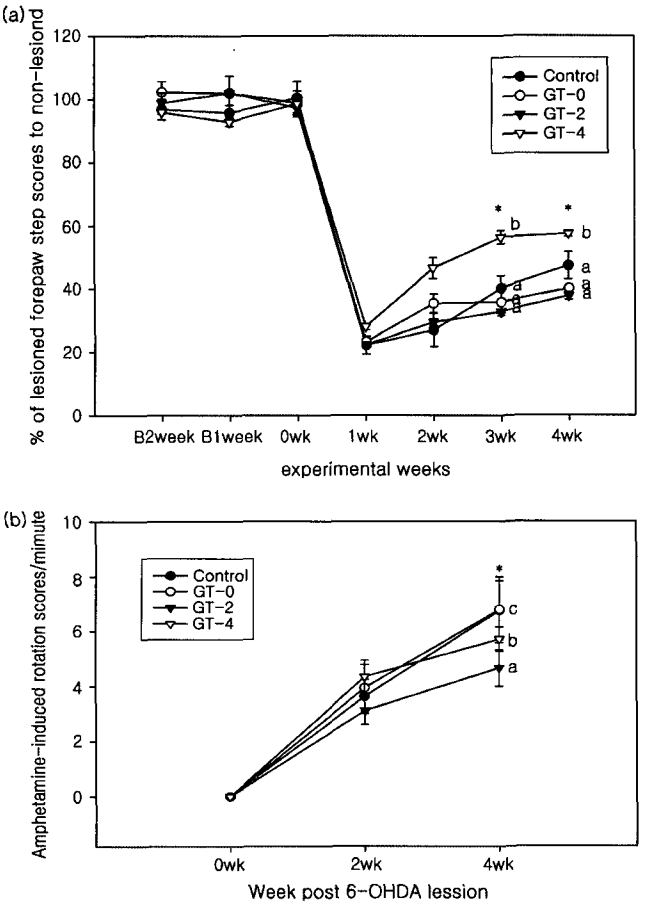

Fig. 3 Effect of green tea diet on behavioral changes in 6-OHDA lesioned rats.

(a) Stepping tests. The results are expressed as a percentage of the lesioned (left) paw step scores to the non-lesioned (right) paws step scores. (b) Amphetamine-induced rotation responses. The results are expressed as mean (plot)± S.E.M. of the number of rotation scores/minute.

* Significant difference among four groups at p<0.05 by one-way ANOVA. Small letters (a, b, and c) were significantly different by Duncan's multiple range tests. (●) Control group: Parkinson's disease (PD) model rats fed normal chow for 6 weeks; (○) GT-0 group: PD model rats fed the green tea diet (containing green tea powder 1g/kg/day) for 2 weeks before 6-OHDA lesion; (▼) GT-2 group: PD model rats fed the green tea diet for 2 weeks before and 2 weeks after 6-OHDA lesion; (▽) GT-4 group: PD model rats fed the green tea diet for 2 weeks before and 4 weeks after 6-OHDA lesion.

performances was 4.42±0.32 steps. The % step scores showed gradual improvement from 1 to 4 weeks after 6-OHDA lesion in control, GT-2, and GT-4 groups. The % step scores were the highest in GT-4 group among all groups at both 3 and 4 weeks after 6-OHDA lesion, 56.29±2.13% and 57.42±0.97% (p<0.05), respectively. From 2 to 4 weeks after 6-OHDA lesion, increasing rate of the % step scores in control group was higher than that in GT-2 group.

As shown in Fig.3 (b), immediately before 6-OHDA lesion (zero week), intact rats did not show turning behavior after each amphetamine administration. Sometimes the rats remained in the corner of the bowl with no turning behavior.

The number of rotation scores/minute of 2 weeks after 6-OHDA lesion was increased when compared to those of immediately before 6-OHDA treatment. At 4 weeks after 6-OHDA lesion, the rotation score was the lowest in GT-2 group ($p<0.05$). However, the increase of the rotation score from 2 to 4 weeks after 6-OHDA lesion tended to be the lowest in GT-4 group. The behavioral disturbance of GT-4 group was improved more than any other groups.

2. Effects of Green Tea Supplementation on Striatal Dopamine Levels in Parkinson's Disease Model Rats

Table 1 shows the effects of green tea diet on striatal dopamine level in 6-OHDA lesioned rats. As shown in Table 1, dopamine contents were significantly different between left (non-lesioned site) and right (lesiond site) striatum of all groups ($p<0.05$). However, there was no significant difference among four groups regardless of the green tea diet.

3. Effects of Green Tea Supplementation on Hepatic Tissues in Parkinson's Disease Model Rats

As shown in Table 2, the malondialdehyde (MDA) concentration was the lowest in GT-4 group among four groups ($p<0.05$). Hydrogen peroxide (H_2O_2) levels were not significantly different among four groups. Hepatic superoxide dismutase activities were increased with prolongation of green tea diet supply period ($p<0.05$).

DISCUSSION

This study focused on the effects of green tea diet on behavioral changes, striatal dopamine level, and hepatic antioxidant parameters in 6-OHDA lesioned rats. The testing of forepaw adjusting steps is a simple and quantitative measure, and can be used to evaluate the potential efficacy of treatments designed to restore dopaminergic function.[14] The deficits in forepaw adjusting steps were characterized as a function of the degree of dopamine depletion after medial forebrain bundle or striatal subregions lesions.[14] Drug-induced rotational behavior has conventionally been used to determine lesion-induced motor impairment.[11] It has known that rats with more than 50% surviving dopamine neurons showed little or no rotation in drug-induced rotation test. However, it has reported that rats with less than 50% surviving dopamine neurons showed precipitous rotation.[12] In this study, the percentage of lesioned (left) forepaw to non-lesioned (right) forepaw step scores (%) of 1 week after 6-OHDA lesion was decreased when compared to that of immediately before 6-OHDA lesion (zero week). Also, rotation score of 2 weeks after 6-OHDA lesion was predominately increased when compared to that of immediately before 6-OHDA lesion. Additionally, we found approximately 20-fold higher

Table 1. Effects of green tea diet on striatal dopamine level in 6-OHDA lesioned rats

	Control		GT-0		GT-2		GT-4	
	Left	Right	Left	Right	Left	Right	Left	Right
Dopamine (μg/mg protein)	17.55±0.24*	0.87±0.01	17.16±0.47*	0.89±0.05	17.92±0.34*	0.98±0.01	17.61±0.51*	0.89±0.04

Values are expressed as mean±S.E.M..
Data were analyzed by one-way ANOVA among four groups. But, those were not significantly different by green tea diet among four groups.
*: significantly different between left and right striatum of all rats at $p<0.05$ by paired t-test.
Control group: Parkinson's disease (PD) model rats fed normal chow for 6 weeks; GT-0 group: PD model rats fed the green tea diet (containing green tea powder 1 g/kg/day) for 2 weeks before 6-OHDA lesion; GT-2 group: PD model rats fed the green tea diet for 2 weeks before and 2 weeks after 6-OHDA lesion; GT-4 group: PD model rats fed the green tea diet for 2 weeks before and 4 weeks after 6-OHDA lesion.

Table 2. Effects of green tea diet on hepatic antioxidant parameters of Parkinson's disease model rats

	Control	GT-0	GT-2	GT-4
Malondialdehyde (μM/mg protein)	2.22±0.24[b]	2.41±0.15[b]	2.61±0.20[b]	1.89±0.08[a]
Hydrogen peroxide (H_2O_2) (nM/mg protein)	11.87±2.06	7.50±1.16	12.94±0.30	9.27±1.68
Superoxide dismutase (units/mg protein)	0.34±0.09[a]	0.37±0.06[a]	0.64±0.12[b]	0.99±0.07[c]

Values are expressed as mean±S.E.M..
Values with superscript letters (a, b, and c) in the same row were significantly different at $p<0.05$ by one-way ANOVA coupled with Duncan's multiple range tests.
Control group: Parkinson's disease (PD) model rats fed normal chow for 6 weeks; GT-0 group: PD model rats fed the green tea diet (containing green tea powder 1 g/kg/day) for 2 weeks before 6-OHDA lesion; GT-2 group: PD model rats fed the green tea diet for 2 weeks before and 2 weeks after 6-OHDA lesion; GT-4 group: PD model rats fed the green tea diet for 2 weeks before and 4 weeks after 6-OHDA lesion.

content of dopamine in left striatum (non-lesioned site) versus right striatum (lesioned site) as shown in Table 1. These results suggest that rats show apparent motor disturbance because of dopamine depletion after 6-OHDA lesion.

In the study of Nobre Júnior *et al.*[23] they found that catechin attenuated 6-OHDA-induced cell death. Also, Guo *et al.*[2] reported that green tea polyphenols had a protective effect on the SH-SY5Y neuroblastoma cells, cellular models of Parkinson's disease (PD), against 6-OHDA induced apoptosis through reactive oxygen species (ROS)-nitro oxide (NO) pathway. In the present study, from 2 to 4 weeks after 6-OHDA lesion, increasing rate of the % step scores in control group tended to be higher than that in GT-2 group. However, this result was not significantly different. The % step score was the highest in GT-4 group among all groups at 3 and 4 weeks after 6-OHDA lesion. At 4 weeks after 6-OHDA lesion, rotation score was the lowest in GT-2 group. However, increase of the rotation score from 2 to 4 weeks after 6-OHDA lesion tended to be the lowest in GT-4 group. Also, in the present study, the striatal dopamine contents were not significantly different according to green tea supplementation. It seems that green tea supplementation does not affect the behavioral disorder of rats immediately after 6-OHDA lesion because of toxic effect of 6-OHDA. However, at the final week of the experiment, the behavioral change tended to be the lowest in rats fed green tea diet for 4 weeks after 6-OHDA lesion. Accordingly, it seems that behavioral disturbances of the rats are occurred by the decrease of dopamine contents in the striatum after 6-OHDA injection. Also, it is thought that continuous green tea supplementation seems to reduce behavioral disturbances.

In the study of Komatsu and Hiramatsu[24], they reported that MDA levels in cerebellum and hippocampus were decreased because of catechin, one of the green tea polyphenol. In this study, the hepatic MDA level was the lowest in GT-4 group. In this PD model rats, we demonstrated that green tea had its ability to protect against hepatic lipid peroxidation. Since hydrogen peroxide (H_2O_2) is formed by spontaneous dismutation or by superoxide dismutase (SOD)[7,25], we expected the positive relationship between hepatic SOD and H_2O_2 in our results. But, in this study, the hepatic SOD activity was the highest in drug-induced PD model rats fed green tea diet for 6 weeks. However, H_2O_2 of the liver tissue was not affected by green tea diet. H_2O_2 is a reactive oxygen species (ROS) formed during normal metabolism. SOD converts $\cdot O^{2-}$ into H_2O_2, and then H_2O_2 is mostly degraded to H_2O by glutathione peroxidase (GSH) and catalase.[26]

Accordingly, other factors beyond green tea diet may have confounding influence on the relationship between H_2O_2 and SOD activity in liver tissue. It seems that more supplementary studies are required in this part. *In vivo* studies have shown that green tea catechins increase total plasma antioxidant activity.[27,28] Intake of green tea catechine also increases the activity of SOD in serum and the expression of catalase in the aorta, enzymes implicated in cellular protection against ROS. This action is combined with direct action on oxygen species by a decrease in the NO plasma concentration. Also, it was reported that green tea catechins supplementation have an increasing effect on GSH peroxide and GSH level of liver tissue.[28,29]

In conclusion, the present results indicate that green tea diet improves behavioral changes in rats lesioned by 6-OHDA. It seems that continuous green tea supplementation reduces the behavioral disturbance and the hepatic MDA level. Accordingly, continuous green tea supplementation is recommended for the prevention and treatment of PD. In the future, however, more studies are needed to investigate the mechanisms and efficacy of green tea in PD.

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