Introduction

Oxidative stress has been implicated in the progression of neurodegenerative disorders, including Parkinson’s disease, Alzheimer’s disease and Huntington’s disease, as well as stroke and trauma (1–5). Oxidative stress is caused in situations where there is a marked disturbance in the balance between production of reactive oxygen species (ROS) and the level of antioxidants and the resulting excessive ROS causes cell damage. The brain is more vulnerable to oxidative stress than other tissues, due to high consumption of oxygen and the consequent generation of large amounts of ROS and limited antioxidant capacity (6, 7). Since many neurodegenerative disorders are closely related to free radical overloading and intracellular oxidative stress, anti-oxidants may have great relevance in the prevention and treatment of free radical–mediated neurodegeneration.

Flavonoids are naturally occurring polyphenolic compounds found in several kinds of fruits, vegetables, and medicinal plants (8–10). Flavonoids have multiple biological and pharmacological activities and consumption of flavonoid-rich foods, such as fruits and vegetables, is associated with a lower incidence of cancer, ischemic stroke, and neurodegenerative disorders (11–13). Luteolin is a flavonoid compound and usually found as glycosylated or aglycone forms in green pepper, celery, perilla leaf, and other plant species (Fig. 1) (14–16). It has been reported to possess antioxidant, anticancer, anti-inflammatory, and anti-allergic effects (17, 18). Recently several reports suggested that luteolin exhibits neuroprotective effects in vitro and in vivo (19–22). Moreover, luteolin has been reported to be effective against several neurological disorders such as anxiety, amnesia,
and depression (23 – 25). Furthermore, luteolin has been effective against myocardial infarction in a rat model of ischemia–reperfusion injury (26). However, there is no report to evaluate the effect of luteolin against oxidative stress induced by SNP in the brain.

We have previously established an in vivo brain oxidative stress model induced by intrastriatal microinjection of SNP (27), in order to evaluate the protective effects of antioxidant substances. In this study, using the oxidative stress model, we investigated the protective effect of luteolin against oxidative damage induced by SNP-toxicity. We found that striatal or oral administration of luteolin, exerted potent protective effects against oxidative stress induced by SNP in mouse brain.

Materials and Methods

Materials
ICR mice were obtained from (Nihon SLC, Shizuoka). Luteolin was obtained from LKT Laboratory (St. Paul, MN, USA). Sodium nitroprusside (SNP), 2,3,5-triphenyl-tetrazolium chloride (TTC), ferrous chloride tetrahydrate (FeCl2), ethylenediaminetetraacetic acid (EDTA), carboxymethyl cellulose sodium (CMC-Na), l-ascorbic acid, methanol, dimethyl sulfoxide (DMSO), acetonitrile of HPLC grade, 1-butanol, and trichloroacetic acid (TCA) were purchased from Nacalai Tesque (Kyoto). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (ferrozine), butylated hydroxytoluene (BHT), 2-thiobarbituric acid (TBA), and ferrous sulfate (FeSO4) were from Sigma (St. Louis, MO, USA). Reduced glutathione (GSH), β-glucuronidases, and trifluoroacetic acid (TFA) of high performance liquid chromatography (HPLC) grade were obtained from Wako Pure Chemicals (Osaka). Nembutal (sodium pentobarbital) was obtained from (Dainippon Sumitomo Pharmaceutical, Osaka). Malonaldehyde bis (dimethylacetal) was from Aldrich Chemical Company (St. Louis, MO, USA).

Animals and oral administration
Six-week-old male ICR mice (25 – 30 g) were used in the present study. The animals were housed in a controlled-temperature room; lighting was maintained on a 12-h light–dark cycle, with ambient temperatures maintained at 20°C – 22°C. Food was also freely available ad libitum. The experiments were conducted in accordance with the Ethical Guidance of the Kyoto University Animal Experimentation Committee and the Guidance of The Japanese Pharmacological Society. Mice were orally administered luteolin (dissolved in 0.5% CMC-Na aqueous solution) by gavage at the indicated doses. At 24-h after oral administration of luteolin, SNP (10 nmol/μl, dissolved in 0.9% saline) was injected into the right striatum, and one day after SNP injection, the behavior tests and TTC staining were performed.

Rotarod test
The rotarod test is used to assess motor coordination and balance in mice (28). In the present study, the rotational speed tested in the steady-state mode was 20 rpm for 180 s. The time the mice were able to walk on the rod before falling off was recorded.

Locomotor activity test
The locomotor activity test is used to assess spontaneous activity in mice (29). The locomotor activity was tested individually in an open field using boxes, equipped with infrared beams. The mice were placed into the box and their movements were measured for 5 min. The interruptions of photo beams for 5 min per mice were registered as the number of transitions (horizontal activity). In the same time, the number of rearings (vertical activity) was counted.

Surgery and intrastriatal microinjection
Mice were anesthetized with Nembutal (sodium pentobarbital, 60 mg/kg, i.p.) and placed in the stereotaxic frame. An incision was made along the midline of the skull to expose the skull. Coordinates for injections were: AP +0.2 mm, ML +2 mm, DV −3.5 mm. Then holes were drilled into the skull and a 30-gauge blunt-tip needle was inserted into the striatum. Luteolin (0.3 – 30 nmol) + SNP (10 nmol), in DMSO at a volume of 1 μl was injected into the right striatum, using a 10-μl Hamilton microsyringe mounted on a motorized pump at a rate of 0.2 μl/min over 5 min. After injection, the needle was held in place for an additional 5 min to prevent back flow and allow diffusion of the drugs.

Histopathological examination
Mice were decapitated one day after microinjection of drugs under deep anesthesia with sodium pentobarbital (60 mg/kg, i.p.). Brains were rapidly removed after intracardial infusion of phosphate-buffered saline (PBS). Then the brains were coronally cut into eight (1-mm
thick) slices, using a brain slicer. The brain slices were immediately incubated in TTC solution (2% solution in PBS) for 30 min at 37°C. Areas not stained red with TTC were considered to be damaged. Finally, the slices were photographed by scanning. The unstained areas in slices were quantified with the Image J 1.42 program, and the damage volume was calculated by summing up the damaged area in all slices.

High performance liquid chromatography
The determination of luteolin in plasma was performed with high-performance liquid chromatography using the LC-10AT system (Shimadzu, Kyoto) equipped with a C18 column (YMC-Pack Pro, 4.6 mm i.d. × 150 mm, particle size 5 μm; YMC, Kyoto). The separation was performed in a mobile phase of acetonitril/water (containing 0.3% TFA), under gradient condition (20% – 65% for 30 min) at flow rate of 0.8 ml/min. The detection wavelength was set at 350 nm and the column temperature was set at 50°C. Luteolin detection was found to be linear within the range of 0.5 – 15 μg/ml, with a detection limit of 0.5 μg/ml.

Sample collection and preparation
Mice were orally administered luteolin (dissolved in 0.5% CMC-Na aqueous solution) by gavage at the dosage of 600 mg/kg. Experimental mice were anesthetized by pentobarbital (60 mg/kg, i.p.) and 500 μl of blood sample was obtained by cardiac puncture with a heparinized syringe at 0 h (before administration) and at 1.0, 6.0, and 24.0 h after oral administration. The blood sample was centrifuged at 6000 rpm for 10 min at 4°C for plasma preparation, and the plasma sample was preserved at −20°C before analysis. For extraction of luteolin from plasma, a 50-μl sample of plasma was incubated with 50 μl of 0.1 M sodium acetate buffer (pH 5.0) containing 375 U β-glucoronidase for 30 min at 37°C and then the samples were extracted with 200 μl of pure acetonitril. After centrifuging at 12,000 rpm for 15 min, a 50-μl aliquot of supernatant was injected into the HPLC system for analysis.

Lipid peroxidation assay
Iron-induced lipid peroxidation assay was performed according to the method described previously (30). In brief, mice were killed by decapitation and brains were removed and homogenized (10% w/v) in 0.1 M PBS at pH 7.4. The homogenized brains were used freshly after decapitation. In 200 μl of brain homogenate, the indicated final concentrations of ascorbate (1 mM), EDTA (100 μM), H2O2 (200 μM), and FeSO4 (100 μM) were added. Then, luteolin was incubated with brain homogenate in the presence or absence of FeSO4. The homogenate samples were incubated at 37°C in a shaking water bath, and after 1-h incubation, 125 μl of BHT (0.5 mg/ml) and 250 μl of 25% TCA were added. Then the samples were heated in a boiling water bath for 10 min. After cooling, the samples were centrifuged at 2000 rpm for 20 min at 4°C. After centrifugation, 500 μl of the supernatant of each sample was transferred to another tube and 125 μl of 0.33% TBA was added to each sample and heated at 95°C for 1 h. After cooling, the samples were extracted with 500 μl of 1-butanol and the absorbance was read at 540 nm with a microplate reader (Bio-Rad, Hercules, CA, USA). MDA levels were determined from a standard curve of malonaldehyde bis (dimethylacetal) of different concentrations and results were measured as nmol/mg of tissue.

DPPH radical–scavenging activity
The free radical–scavenging activity of luteolin determined by the described previously method (30). In brief, a series concentration of drugs in methanol was prepared, and then 30 μl of each sample solution was mixed with 120 μl of 0.1 mM DPPH solution freshly prepared in methanol. The final concentrations of the compounds were 1 – 100 μg/ml. After incubation for 30 min at room temperature, the absorbance of reactant was read at 540 nm with a microplate reader (Bio-Rad). Lower absorbance of the reaction mixture indicates higher free radical–scavenging activity. The DPPH radical–scavenging activity was determined using the following equation: scavenging activity (%) = {(A0 – A1) / A0} × 100, where A0 is the absorbance of the control reactions (DPPH solution) and A1 is the absorbance in the presence of the test compound. For the positive control, we used antioxidants such as glutathione and ascorbic acid at the same final concentrations.

Iron-chelating activity
The chelating activity of the luteolin on Fe2+ ions was determined by the previously described procedures (30). In brief, a series of different concentrations of compounds was prepared and 100 μl of these solutions was added to 370 μl of nanopure water. Then, 10 μl of 2 mM FeCl2 was added and mixed by vortexing. Then, 20 μl of 5 mM ferrozine solution was added to this mixture. The final concentrations of the compounds were 1 – 100 μg/ml. The reaction mixture was allowed to stand for 30 min at room temperature. The absorbance was read at 570 nm with a microplate reader (Bio-Rad). Lower absorbance of the reaction mixture indicates higher iron-chelating activity. The percentage chelation activity on the Fe2+-ferrozine complex was calculated as follows: chelating activity (%) = {(A0 – A1) / A0} × 100, where A0 is the absorbance of the control reactions.

Effect of Luteolin on Oxidative Stress
(FeCl₂ + ferrozine) and A₁ is the absorbance in the presence of the test compounds. In this experiment EDTA was used as positive control.

Statistical analyses
The results were expressed as the mean ± S.E.M. One way analyses of variance (ANOVA) was used followed by the Tukey post test to determine statistically significant differences among three or more groups of mice. Between two groups of mice, Student’s t-test was performed to determine statistical significance. Results were considered statistically significant at \( P < 0.05 \). All statistical analyses were conducted using GraphPad InStat (GraphPad Software Inc., San Diego, CA, USA).

Results
Effect of intrastriatal microinjection of luteolin on SNP-induced motor dysfunction
To evaluate the effect of luteolin against SNP-toxicity, we first co-injected luteolin with SNP (10 nmol) into the mouse striatum, and after one day, the behavior and histological examinations were performed. In the rotarod test, co-injection of luteolin (30 nmol) into the striatum prevented SNP-induced impairment in mouse performance (Fig. 2A). In the locomotor test, co-injection of luteolin did not alter horizontal activities (transitions), but luteolin (3 – 30 nmol) dose-dependently prevented SNP-induced impairment in vertical activities (rearings) (Fig. 2: B and C, respectively). Quantitative analysis of TTC staining showed that co-injection of luteolin significantly protected against brain damage induced by SNP-toxicity (Fig. 3: B and C).

Determination of luteolin in mouse plasma
To evaluate whether luteolin is absorbed by the intestinal tract after oral administration, exerting its protective effects, HPLC analysis was performed. Figure 4 shows the plasma concentration–time curve of luteolin after a single oral administration of 600 mg/kg of luteolin to mice. The plasma concentration curve shows a rapid absorption following oral administration of luteolin and the maximum plasma concentration (11 \( \mu g/ml \)) was achieved 1 h after administration. However, after 24 h the plasma level of luteolin was below the limit of detection in our experiment. This result demonstrates that orally administered luteolin can be rapidly absorbed in the plasma.

Effect of oral administration of luteolin on striatal injection of SNP-induced motor dysfunction
Since luteolin is a common dietary constituent and the oral route is the preferred route of administration for food and drugs, we then investigated the effect of luteolin after oral administration. We first administered luteolin via oral gavage and after 24 h, SNP (10 nmol) was injected into the mouse striatum. One day after injection of SNP (10 nmol), the behavior examination and TTC staining were performed. In the rotarod test, luteolin (600 – 1200 mg/kg, p.o.) dose-dependently prevented SNP-induced impairment in mouse performance (Fig. 5A). In the locomotor test, luteolin did not alter horizontal or vertical activities (Fig. 5: B and C, respectively). However, luteolin tended to prevent the impairment of vertical activities. Quantitative analysis of TTC staining showed that oral administration of luteolin protected against brain damage induced by SNP-toxicity (Fig. 6: B and C).

![Fig. 2.](image-url)

Fig. 2. Effect of intrastriatal microinjection of luteolin on SNP-induced motor dysfunction. Behavioral changes were examined one day after microinjection of drugs. In the rotarod test, intrastriatal co-injection of luteolin (3 – 30 nmol) prevented SNP-induced impairment in mouse performance (A). In the locomotor test, luteolin did not alter horizontal activities (transitions) (B), but increased vertical activities (rearings) (C). ***(P < 0.001, compared with vehicle; *P < 0.05, compared with SNP treatment. Values represent the mean ± S.E.M. (n = 7).***
Effect of Luteolin on Oxidative Stress

Fig. 3. Representative photographs of mouse brain after microinjection of drugs. Mice were injected with 10 nmol SNP (A) or 30 nmol luteolin + SNP (B), at a volume of 1-μl, into the right striatum and TTC staining was performed one day after injection of drugs. The brains were sliced into coronal sections for TTC staining. C: Quantitative analysis showed a significant protective effect of luteolin against SNP. *P < 0.05, compared with SNP. Values represent the mean ± S.E.M. (n = 7). Scale bar = 1 cm.

Fig. 4. Representative chromatogram of the plasma concentration of luteolin (600 mg/kg) 1 h after oral administration (A). Mean plasma concentration–time curve of luteolin after oral administration of 600 mg/kg (B). Values represent the mean ± S.E.M. (n = 3).

Fig. 5. Effect of oral administration of luteolin on SNP-induced motor dysfunction. Behavioral changes were examined one day after microinjection of drugs. Mice were orally administered luteolin and after 24 h, SNP (10 nmol) was injected by intrastriatally. In the rotarod test, luteolin (600 – 1200 mg/kg, p.o.) prevented SNP-induced impairment in mouse performance (A). In the locomotor test, luteolin did not alter horizontal activity (B), but luteolin tended to prevent the impairment of vertical activities (C). ***P < 0.001, compared with the vehicle; *P < 0.05, **P < 0.01, compared with SNP treatment. Values represent the mean ± S.E.M. (n = 5 – 7).
To evaluate the effect of luteolin against oxidative stress in the brain tissue, we investigated the antioxidant activity of luteolin in mouse brain homogenate. As shown in Fig. 7, incubation of the brain homogenate with ferrous iron at 100 μM significantly induced lipid peroxidation. However, co-incubation of luteolin (30 – 100 μM) with ferrous iron significantly prevented Fe²⁺-induced lipid peroxidation in a concentration-dependent manner. This result indicates that luteolin potently protects against oxidative stress induced by iron-related radical reactions.

**DPPH radical–scavenging activity**

We previously reported that iron-related radical reactions had crucial roles in SNP-induced brain damage (27). Thus, in order to elucidate the protective mechanism of luteolin, we used the DPPH method to determine...
Effect of Luteolin on Oxidative Stress

The antioxidant effect of luteolin. The results shown in Fig. 8 revealed that all test compounds including luteolin potently scavenged the DPPH free radicals. However, in the lower concentrations (1 – 3 \( \mu \text{g/ml} \)), luteolin scavenged DPPH free radicals more potently than the well-known antioxidants ascorbic acid and glutathione. This result indicates that luteolin has a potent free radical–scavenging activity, showing that it is involved in the protective effects against SNP-induced toxicity.

Fe\(^{2+}\) chelating activity

The production of ROS such as hydroxyl radicals is catalyzed by free iron through the Fenton reaction (31). To evaluate the iron-chelating activity of luteolin, the ferrozine assay was performed. The results in Fig. 9 showed that formation of the ferrozine-Fe\(^{2+}\) complex was inhibited in the presence of luteolin and EDTA. EDTA potently chelated free iron ions (3 – 100 \( \mu \text{g/ml} \)) in a concentration dependent manner. However, luteolin chelated Fe\(^{2+}\) ions only at 30 – 100 \( \mu \text{g/ml} \) concentrations. This result indicates that luteolin has a chelating activity in the higher concentrations employed in our experimental conditions, showing that it is involved in the protective effects against SNP-induced oxidative damage.

Discussion

The present study revealed that not only intrastriatal but also oral administration of luteolin has protective effect against SNP-induced toxicity. Luteolin, a naturally occurring flavonoid found in vegetables, fruits, and medicinal plants has been reported to exhibit several biological and pharmacological activities including potent antioxidant, anticancer, and anti-inflammatory effects (17, 18). However, very few investigations have explored the effect of luteolin on the central nervous system associated with oxidative stress disorders. This is the first report demonstrating the protective effect of luteolin against oxidative stress induced by SNP-toxicity in mouse brain.

In this study, we first co-injected the luteolin with SNP into the mouse striatum and found that luteolin significantly protected against brain damage and motor dysfunction induced by SNP-toxicity. This result indicates that luteolin is quite effective in the prevention of the damage induced by SNP-toxicity. Next, since the results on striatal co-injection of luteolin indicate that luteolin protected against SNP-induced brain damage and motor dysfunction, we decided to determine the plasma level of luteolin after oral administration and then evaluated the protective effect of luteolin against SNP-induced toxicity. In our study, HPLC analysis showed that luteolin was absorbed by the gastrointestinal tract in sufficiently high concentration to exert its protective effect against oxidative stress induced by SNP-toxicity. As shown in Fig. 4, the maximum plasma concentration was achieved 1 h after oral administration of luteolin. It is generally believed that the absorption of flavonoids is very low following oral administration. Flavonoid-mediated neuroprotection in vivo will depend on its extent of absorption from the gastrointestinal tract and ability to cross the blood–brain barrier to reach the CNS. Furthermore, the absorption and excretion of flavonoids will play an important role on the effects of flavonoids in vivo. In a previous report, it was shown that luteolin in \textit{Chrysanthemum morifolium} extract was absorbed sufficiently in a rat model and the cumulative luteolin excreted in the urine and feces were 37.9% of the dose during 72 h after oral administration (32). It was explained that much of the luteolin might be metabolized to simple compounds or accumulated in the tissues. In another report, it was indicated that after a single oral administration of phenolic extract from olive cake in the rat model, luteolin was absorbed by the gastrointestinal tract and distributed across the blood–brain barrier (33). Taking together our results with these reports, we considered that luteolin can be absorbed and reach the CNS sufficiently to show protective effects against SNP-toxicity.

Considering that luteolin is absorbed by the gastrointestinal tract following oral administration to mice, we then investigated the effect of oral administration of luteolin against SNP-induced brain damage and motor dysfunction. Our result showed that oral administration of luteolin significantly protected against brain damage induced by SNP-toxicity. Furthermore, these results indicate that, oral administration of luteolin to the mice elicited an improvement of motor function, demonstrat-

![Fig. 9. Iron-chelating activity of luteolin and EDTA. Values represent the mean ± S.E.M. (n = 3).](image-url)
ing the protective effect of luteolin against the deficiency of behavioral performance induced by intrastriatal injection of SNP. This behavior-improving action of luteolin was confirmed by histological detections using TTC staining on brain slices (Fig. 6), indicating the potential protective effect of luteolin against oxidative stress. This means that an adequate level of luteolin may be reached in the brain, so that it can exert its protective effect, but determination of the level of luteolin in the brain has to be evaluated in future investigations.

Furthermore, we evaluated the effect of luteolin against iron-induced lipid peroxidation in mouse brain homogenate. Iron-induced ROS can cause lipid peroxidation, DNA damage, enzyme inactivation, and alteration in the structure and function of neuronal cells (7). Our result in Fig. 7 showed that luteolin can potently protect neuronal cells against Fe2+-induced lipid peroxidation in mouse brain homogenate, suggesting the antioxidant effect of luteolin in the brain.

In an attempt to explain the main mechanisms through which the luteolin protects against brain damage induced by SNP-toxicity, the iron chelating and radical scavenging abilities of luteolin were assessed. The radical scavenging activity based on the DPPH model system was thought to be due to the hydrogen-donating ability of antioxidants. The DPPH assay in Fig. 8 showed that luteolin reduced the DPPH free radical more potently than well-known antioxidants, glutathione and ascorbic acid, in lower concentrations (1 – 3 μg/ml). It has been reported that SNP could cause oxidative stress by inducing hydroxyl radical generation via the Fenton reaction (34). Furthermore, flavonoids are believed to be antioxidants or free radical scavengers in the biological system. The active polyphenolic hydroxyl groups in the flavonoids are the important functional groups responsible for the potent antioxidant and free radical-scavenging properties, capable of scavenging superoxide, hydroxyl, and peroxyl radicals in the cells (35). The results of the DPPH assay clearly showed that luteolin is a potent radical-scavenging agent that can prevent oxidative stress.

Moreover, we performed the ferrozine assay to evaluate the iron-chelating activity of luteolin. Our result showed that luteolin can bind iron, indicating that this compound may prevent the redox cycling of iron and Fenton reaction, consequently reducing oxidative damage. It has been proven that chelating complexes of flavonoids with metal ions can be formed between the dihydroxyl groups at the 3- and 4-positions on the B ring or the hydroxyl group at the 5-position and the neighboring ketone at the 4-position (35, 36). These results showed that part of the mechanisms through which the luteolin protected against brain damage may be due to its iron-chelating ability. However, under our experimental conditions, the chelating effect of luteolin is induced by higher concentrations.

In conclusion, these results suggest that luteolin can be absorbed sufficiently after oral administration and protects mouse brain from SNP-induced toxicity by radical-scavenging and iron-chelating effects. Moreover, the present results also suggest that luteolin has a potent antioxidant effect and could be a potent neuroprotective agent against oxidative damage.

Acknowledgments

This study was supported in part by JSPS KAKENHI grant number 24390139 and by a grant from the Smoking Research Foundation, Japan.

References

Effect of Luteolin on Oxidative Stress

117


33 Serra A, Rubió L, Borràs X, Macià A, Romero MP, Motilva MJ. Distribution of olive oil phenolic compounds in rat tissues after administration of a phenolic extract from olive cake. Mol Nutr Food Res. 2012;56:486–496.

