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## Beneficial Effects of Natural Phenolics on Levodopa Methylation and Oxidative Neurodegeneration

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### Abstract

Levodopa (L-DOPA) is widely used for symptomatic management in Parkinson's disease. We recently showed that (–)-epigallocatechin-3-gallate, a tea polyphenol, not only inhibits L-DOPA methylation, but also protects against oxidative hippocampal neurodegeneration. In the present study, we sought to determine several other common dietary phenolics, namely, tea catechins [(+)-catechin and (–)-epicatechin] and a representative flavonoid (quercetin), for their ability to modulate L-DOPA methylation and to protect against oxidative hippocampal injury. A combination of *in vitro* biochemical assays, cell culture-based mechanistic analyses, and *in vivo* animal models was used. While both tea catechins and quercetin strongly inhibit human liver catechol-*O*-methyltransferase (COMT)-mediated *O*-methylation of L-DOPA *in vitro*, only (+)-catechin exerts a significant inhibition of L-DOPA methylation in both peripheral compartment and striatum in rats. The stronger *in vivo* effect of (+)-catechin on L-DOPA methylation compared to the other dietary compounds is due to its better bioavailability *in vivo*. In addition, (+)-catechin strongly reduces glutamate-induced oxidative cytotoxicity in HT22 mouse hippocampal neurons *in vitro* through inactivation of the nuclear factor- $\kappa$ B signaling pathway. Administration of (+)-catechin also exerts a strong neuroprotective effect in the kainic acid-induced oxidative hippocampal neurodegeneration model in rats. In conclusion, (+)-catechin is a dietary polyphenolic that may have beneficial effects in L-DOPA-based treatment of Parkinson patients by inhibiting L-DOPA methylation plus reducing oxidative neurodegeneration.

### Keywords

Levodopa; Catechol-*O*-methyltransferase (COMT); (+)-Catechin; Oxidative neuronal damage; COMT inhibition; Neuroprotection

## 1. INTRODUCTION

The clinical symptoms of Parkinson disease (PD) are largely due to the loss of nigrostriatal dopaminergic neurons and the decrease in striatal dopamine content (Riederer and

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Wuketich, 1976; Da Prada *et al.*, 1984; Huot *et al.*, 2007). Levodopa (L-DOPA), a natural precursor for dopamine biosynthesis, is commonly used for symptom management in many PD patients. This drug is always administered together with a peripheral dopa decarboxylase inhibitor (*e.g.*, carbidopa) to reduce its rapid conversion to dopamine in peripheral tissues (Bartholini and Pletscher, 1975; Fahn, 2006). When the peripheral dopa decarboxylase is inhibited, the hepatic catechol-*O*-methyltransferase (COMT)-mediated *O*-methylation of L-DOPA becomes a major metabolic pathway. Under such conditions, the use of a COMT inhibitor (*e.g.*, tolcapone or entacapone) has been recommended for some patients, which can further improve L-DOPA bioavailability by suppressing enzymatic conversion of L-DOPA to 3-*O*-methyldopa (3-OMD) (Mannisto and Kaakkola, 1999; Heikkinen *et al.*, 2002; Toulouse and Sullivan, 2008).

Clinical studies have shown that nearly 50% of patients using the L-DOPA + carbidopa treatment develop severe motor fluctuations (also called “wearing-off” phenomenon) and dyskinesia within the first 5 years of treatment (Koller *et al.*, 1999; Ahlskog and Muentzer, 2001; Toulouse and Sullivan, 2008). It has been suggested that some of the nervous system complications may result from a combination of the following two changes after chronic L-DOPA administration: One is the relatively large and rapid fluctuations in L-DOPA blood and CNS concentrations, and the other one is the adverse actions exerted by 3-OMD, which is a major L-DOPA metabolite formed in large quantities in both periphery and brain of patients treated with L-DOPA/carbidopa (Feuerstein *et al.*, 1977; Raches and Fahn, 1981; Lee *et al.*, 2008). In partial support of these suggestions, animal studies have shown that 3-OMD can interfere with L-DOPA utilization in the brain, and can also induce neuronal damage via oxidative stress (Raches and Fahn, 1981; Lee *et al.*, 2008). Moreover, 3-OMD was found at high levels in plasma as well as cerebral spinal fluid of PD patients treated with L-DOPA/carbidopa (Sharpless *et al.*, 1972; Tohogi *et al.*, 1991), and its plasma levels in patients with dyskinesia were significantly higher than patients without dyskinesia (Feuerstein *et al.*, 1977). Theoretically, addition of a COMT inhibitor to the L-DOPA/carbidopa therapy in PD patients will not only reduce L-DOPA concentration fluctuations, but will also greatly reduce 3-OMD levels, both of which would be beneficial to reducing complications. Indeed, many clinical studies have shown that such a three-drug therapy (*i.e.*, L-DOPA + carbidopa + a COMT inhibitor) improves L-DOPA bioavailability and also reduces the occurrence of adverse effects in PD patients (Müller, 2009).

We have previously shown that some of the catechol-containing bioflavonoids and tea catechins are exceptionally good substrates for human COMT (Zhu *et al.*, 2000). In addition, bioflavonoids and tea catechins are also strong inhibitors of human COMT (Lu *et al.*, 2003; Nagai *et al.*, 2004; van Duursen *et al.*, 2004; Chen *et al.*, 2005). In our recent study, (–)-epigallocatechin-3-gallate (EGCG) was found to have beneficial effect on the L-DOPA/carbidopa therapy by inhibiting COMT-mediated L-DOPA methylation (Kang *et al.*, 2010). In the first part of this study, therefore, we sought to further evaluate the effect of two other common tea catechins [(+)-catechin and (–)-epicatechin] and a common flavonoid (quercetin) on L-DOPA methylation to determine their relative effectiveness as naturally-occurring COMT inhibitors (Figure 1). We found that among these dietary compounds, (+)-catechin has the strongest *in vivo* effect in modulating L-DOPA methylation in a rat model, owing to its favorable pharmacokinetic properties.

It is known that PD patients usually also suffer from a variety of non-motor symptoms including depression and dementia (Yamamoto, 2001; Padovani *et al.*, 2006; Eskow *et al.*, 2010). Hippocampus is a brain region that often is associated with chronic damage in PD patients, and as a result, these patients have a six-fold higher risk than age-matched healthy control subjects for developing dementia (Padovani *et al.*, 2006). Deficits in hippocampal-dependent learning have also been observed in parkinsonian animals (Denenberg *et al.*,

2004; Costa *et al.*, 2012). It has been suggested that the use of L-DOPA may help partially restore hippocampal synaptic potentiation via activation of dopamine D1/D5 receptors and thereby ameliorate the cognitive deficit in parkinsonian animals (Costa *et al.*, 2012). Recently, we have reported that EGCG, which can beneficially inhibit L-DOPA methylation *in vivo*, also has a protective effect against oxidative hippocampal neurodegeneration (Kang *et al.*, 2010). This neuroprotective effect, which is not shared by the currently-approved COMT inhibitors (tolcapone and entacapone) for combination use with L-DOPA in PD patients, is highly desirable. Therefore, in the second part of this study, we also sought to determine whether (+)-catechin has protective effect against hippocampal oxidative neurodegeneration using both *in vitro* and *in vivo* models.

## 2. RESULTS

### 2.1. PART I – Effect of (+)-catechin, (–)-epicatechin, and quercetin on COMT-mediated L-DOPA methylation

**2.1.1. Inhibition of COMT-mediated L-DOPA methylation *in vitro***—The reaction conditions for the *in vitro* enzymatic *O*-methylation of L-DOPA were optimized by determining the effects of different incubation times and enzyme concentrations, and the data were described in our recent study (Kang *et al.*, 2010). Under optimized *in vitro* metabolism conditions, we then determined the modulating effect of two selected tea catechins and a flavonoid on L-DOPA methylation catalyzed by cytosolic COMT prepared from three representative human liver samples (HL4C, HL8C, and HL9C) (data shown in Figure 2). (+)-Catechin, (–)-epicatechin, and quercetin each inhibited COMT-mediated L-DOPA methylation in a concentration-dependent manner, with  $IC_{50}$  values of  $3.7 \pm 3.2 \mu\text{M}$  for (+)-catechin,  $10.7 \pm 6.7 \mu\text{M}$  for (–)-epicatechin, and  $1.9 \pm 0.4 \mu\text{M}$  for quercetin.

**2.1.2. Effect on L-DOPA methylation *in vivo***—To determine the *in vivo* modulating effect of these dietary compounds on L-DOPA methylation, rats were injected *i.p.* with 400 mg/kg (+)-catechin, (–)-epicatechin, or quercetin 2 h before oral administration of L-DOPA/carbidopa (20/5 mg/kg). The doses of L-DOPA and carbidopa were adopted from our recent study (Kang *et al.*, 2010). The plasma L-DOPA levels were not appreciably altered by co-administration of (+)-catechin, (–)-epicatechin, or quercetin (Figure 3A). Administration of (+)-catechin significantly reduced the plasma 3-OMD levels, but (–)-epicatechin or quercetin did not have a similar effect on plasma 3-OMD levels (Figure 3B). The striatal region of the brain was dissected at 6 h after L-DOPA/carbidopa administration and analyzed for dopamine and 3-OMD levels. In the striatum, 3-OMD levels were significantly reduced by (+)-catechin administration ( $P < 0.05$ ), although dopamine levels were only slightly changed (no statistical significance) (Figure 3C, 3D). In comparison, no significant change in striatal dopamine and 3-OMD levels was observed following administration of (–)-epicatechin or quercetin (Figure 3C, 3D). These data show that only (+)-catechin is an effective inhibitor of L-DOPA methylation *in vivo*. Notably, very similar observations were made with these dietary compounds in another experiment when slightly different time points were used in collecting blood samples and brain tissues (data not shown).

Earlier studies showed that the beneficial effect of COMT inhibition is more pronounced under conditions when a stronger inhibition of dopa decarboxylase is achieved (Kaakkola and Wurtman, 1993; Mannisto and Kaakkola, 1999). Therefore, we further evaluated the *in vivo* inhibitory efficacy of (+)-catechin on L-DOPA methylation when a 4-fold higher dose of carbidopa (at 20 mg/kg) was used. (+)-Catechin was orally administered 2 h before L-DOPA/carbidopa administration (20/20 mg/kg). Under this condition, a significant increase in L-DOPA  $AUC_{0-6}$  value was observed (from  $374.8 \pm 81.2$  to  $570.3 \pm 128.9 \mu\text{g}\cdot\text{min}/\text{mL}$ ) (Table 1). In addition, the time required to reach maximal L-DOPA plasma concentration

was modestly shifted to the right, indicating that L-DOPA stayed in circulation longer in rats treated with (+)-catechin than in control animals (Figure 4A). Also, 3-OMD AUC<sub>0-6</sub> was slightly increased by approximately 1.6-fold (Table 1). Under this experimental condition, a similar decrease in plasma 3-OMD level was observed in animals treated with (+)-catechin (Figure 4B, Table 1) compared with the experiment when 20 mg/kg L-DOPA and 5 mg/kg carbidopa were used (Figure 3B, Table 1). Although striatal dopamine levels were not significantly affected by (+)-catechin treatment (Figure 4C), striatal 3-OMD levels were markedly reduced by the treatment (Figure 4D).

To determine whether (+)-catechin would have a stronger effect in improving L-DOPA concentration in the striatum of dopamine-deficient rats, the animals were first treated with reserpine to induce CNS dopamine depletion (described in the Methods section). Following oral administration of L-DOPA/carbidopa in these reserpinized animals, plasma L-DOPA and 3-OMD levels were found to be increased to  $15.9 \pm 2.1 \mu\text{M}$  and  $16.4 \pm 1.9 \mu\text{M}$ , respectively, at 2 h (Figure 5A, 5B). In animals co-treated with (+)-catechin, the plasma levels of L-DOPA were slightly increased to  $21.6 \pm 3.7 \mu\text{M}$  (no statistical significance), but plasma 3-OMD levels were significantly reduced in comparison with animals not treated with (+)-catechin (Figure 5A, 5B). Striatal dopamine levels in reserpinized rats were decreased by over 80% compared to normal control animals, but the levels were restored to levels comparable to those in normal rats by co-treatment with (+)-catechin (compare Figure 5C with Figure 4C)). Similarly, striatal 3-OMD levels were also markedly reduced in rats co-treated with (+)-catechin (Figure 5D).

**2.1.3. Plasma levels of (+)-catechin, (–)-epicatechin and quercetin after *i.p.* administration**—The pharmacokinetics and blood-brain-barrier penetration of some of dietary polyphenolic compounds used in this study have been studied in recent years (Catterall *et al.*, 2003; Loke *et al.*, 2008; Moon *et al.*, 2008; Ferruzzi *et al.*, 2009). The observed stronger effect of (+)-catechin compared to that of (–)-epicatechin and quercetin appears to be consistent with the more favorable bioavailability of (+)-catechin *in vivo* (Manach *et al.*, 1999). To confirm that the differential bioavailability of these dietary compounds is the key determinant of their *in vivo* efficacy in modulating L-DOPA metabolism, we conducted an abbreviated experiment to compare the blood levels of these dietary compounds at three selected time points (0.5, 1 and 2 h) in rats injected *i.p.* with the same dose as used in L-DOPA metabolism experiments. We found that the plasma concentrations of unconjugated (+)-catechin at 30 min after *i.p.* injection were markedly higher than those of (–)-epicatechin and quercetin ( $190.6 \pm 118.5 \mu\text{M}$  vs.  $88.6 \pm 39.2 \mu\text{M}$  and  $12.6 \pm 0.1 \mu\text{M}$ ) (Figure 6).

## 2. 2. PART II – Neuroprotective effect of (+)-catechin

**2. 2.1. *in vitro* protection against glutamate-induced oxidative neuronal cell death**—To investigate the effect of (+)-catechin on oxidative stress-induced neuronal cell death, HT22 cells, an immortalized mouse hippocampal neuronal cell line sensitive to glutamate-induced oxidative cell death (Tan *et al.*, 1998; Fukui *et al.*, 2009; Choi *et al.*, 2011), was used as an *in vitro* model. After 24-h exposure of HT22 cells to 5 mM glutamate, cell viability was reduced by 80%, and co-treatment with (+)-catechin abrogated cell death in a concentration-dependent manner (Figure 7A). In addition, the glutamate-induced accumulation of intracellular ROS was reduced by (+)-catechin treatment (Figure 7B).

Our recent study showed that NF- $\kappa$ B activation is involved in glutamate-induced oxidative cytotoxicity in HT22 cells (Kang *et al.*, 2010). To test the mechanistic hypothesis that (+)-catechin protects against neuronal cell death through inactivation of the NF- $\kappa$ B signaling pathway, HT22 cells were selectively transfected with a NF- $\kappa$ B-Luc reporter gene. As

expected, NF- $\kappa$ B transcriptional activity was increased in cells treated with 5 mM glutamate (Figure 7C). The presence of (+)-catechin in the cell culture medium significantly attenuated the transcriptional activity of NF- $\kappa$ B in these neuronal cells (Figure 7C).

### 2.2.2. *in vivo* study using the kainic acid-induced hippocampal neurodegeneration model

—The observed *in vitro* neuroprotective effect of (+)-catechin was further assessed using the kainic acid-induced hippocampal injury model *in vivo*. (+)-Catechin was administered to rats *i.p.* 30 min before intracerebroventricular (*i.c.v.*) kainic acid injection. Kainic acid (1  $\mu$ L of 1 mg/mL solution) was injected into the right lateral ventricle of rats, and the animals were sacrificed 24 h later. All rats receiving the *i.c.v.* injection of kainic acid produced well-characterized seizures (*e.g.*, staring, immobility, and subsequent wet dog shake), and no appreciable behavioral differences were observed in animals that also received (+)-catechin administration. The shrinkage of neuronal cell bodies and formation of peri-neuronal vacuoles were detected in CA3 region at 24 h post kainic acid injection (Figure 8A). Different from the CA3 region, no similar changes were seen in the neighboring CA1 region (Figure 8A). These observations were in agreement with the known selective neurotoxicity of kainic acid in certain hippocampal regions (Nalder *et al.*, 1980). In animals co-treated with (+)-catechin, the extent of neuronal death was markedly reduced (Figure 8A, 8B). In addition, it was observed that there was a modest increase in the expression of GFAP in astrocytes in kainic acid-injected rat brain, and this increase was reduced by (+)-catechin administration (Figure 8A, 8C).

## 3. DISCUSSION

In recent years, a number of therapeutically-useful drugs have been rationally designed and synthesized according to the new knowledge gained from studying the actions of various biologically-active chemicals derived from plants and medicinal herbs (Matthews *et al.*, 1999; Schmidt *et al.*, 2008). One of the advantages of these naturally-occurring substances is their potential to interact with multiple targets in the body to elicit desirable beneficial outcomes (Schmidt *et al.*, 2008). With this paradigm in mind, we recently have reported the dual beneficial effects of EGCG on L-DOPA methylation and hippocampal neurodegeneration (Kang *et al.*, 2010). In the present study, we focused on evaluating whether these beneficial effects are associated with some of other well-known natural phenolics, which share certain degrees of structural similarity to EGCG. We found that although the three compounds tested in this study are highly effective *in vitro* for inhibiting COMT-mediated *O*-methylation of L-DOPA, only (+)-catechin was effective *in vivo*, due to its favorable bioavailability. In addition, (+)-catechin has a strong *in vivo* protective effect against oxidative hippocampal neurodegeneration, as it was also recently reported for EGCG (Kang *et al.*, 2010). Together, these results suggest that (+)-catechin may be another potential dietary compound for clinical use in PD patients. In addition, the results of this study may shed some light on the structural features in the design of novel COMT-inhibitors that also retain beneficial neuroprotective functions.

### 3.1. Effect of natural phenolics on L-DOPA methylation *in vitro* and *in vivo*

We found that (+)-catechin, (–)-epicatechin, and quercetin each can effectively inhibit, in a concentration-dependent manner, the *in vitro* methylation of L-DOPA catalyzed by human liver cytosolic COMT. Using a rat model, we also showed that the plasma and striatal 3-OMD levels (an *in vivo* marker of L-DOPA methylation) were effectively reduced by co-treatment with (+)-catechin, whereas (–)-epicatechin and quercetin did not show an appreciable effect. The stronger inhibitory effect of (+)-catechin than the other two dietary compounds on L-DOPA methylation *in vivo* agrees with the more favorable bioavailability of (+)-catechin.



Treatment of animals with reserpine sometimes has been used as a means to deplete catecholamine content in various brain regions, including a marked reduction in striatal dopamine content. This animal model has previously been used in evaluating some of the antiparkinsonian drugs (Dawson *et al.*, 2000; Segovia *et al.*, 2003). When the modulating effect of (+)-catechin on L-DOPA methylation and dopamine content was examined in reserpinized rats, we found that the increases in plasma and striatal 3-OMD levels following administration of L-DOPA/carbidopa were strongly blunted by (+)-catechin. Notably, the reduced striatal dopamine levels in reserpinized rats, which were approximately 20% of the levels found in non-reserpinized control rats, were markedly increased by co-treatment with (+)-catechin. This observation, along with earlier clinical studies in PD patients (Tedroff *et al.*, 1996), supports the notion that the ability of the administered L-DOPA to increase synaptic dopamine content depends on the degree of striatal dopamine deficiency. It is expected that in the striatum of PD patients where there is a severe dopamine deficiency, a greater increase in dopamine content likely will be seen when these patients are jointly treated with L-DOPA/carbidopa plus a dietary COMT inhibitor such as (+)-catechin or EGCG.

Compared to tolcapone and entacapone, the *in vivo* potency and efficacy of (+)-catechin in inhibiting L-DOPA methylation (based on inhibition of 3-OMD formation) are weaker, which likely is due to the following two factors: (i) (+)-catechin has a lower potency in directly inhibiting human liver COMT-mediated L-DOPA methylation, and (ii) (+)-catechin likely has a lower bioavailability *in vivo* (Chen *et al.*, 1997; Lu *et al.*, 2003) compared to tolcapone and entacapone.

Lastly, it is of note that while we observed a strong reduction in 3-OMD plasma level, there was no appreciable increase in L-DOPA plasma level in rats co-treated with (+)-catechin. Similar observations were also made in earlier clinical studies with tolcapone and entacapone in human subjects (Sêdek *et al.*, 1997; Cedarbaum *et al.*, 1991; Keränen *et al.*, 1993; Heikkinen *et al.*, 2001; Blandini *et al.*, 2003). Therefore, it is reasonable to suggest that while the use of (+)-catechin as an adjunct will help prolong the therapeutic effects of L-DOPA in PD patients, it will not increase the peak concentrations of L-DOPA nor aggravate its concentration fluctuations. These effects would be highly beneficial to reducing the occurrence of neural toxicity such as dyskinesia and hallucinations.

### 3.2. Neuroprotective effect of (+)-catechin *in vitro* and *in vivo*

To characterize the mechanism of the neuroprotective effect of (+)-catechin, HT22 cells, an immortalized mouse hippocampal neuronal cell line, was used. The mechanism of glutamate-induced oxidative stress and neurotoxicity in these neuronal cells has been well characterized in recent years (Tan *et al.*, 1998; Fukui *et al.*, 2009; Fukui *et al.*, 2010; Choi *et al.*, 2011). In addition, it is known that ROS-mediated cellular oxidative stress often results in NF- $\kappa$ B activation (Post *et al.*, 1998). NF- $\kappa$ B is usually sequestered in cytoplasmic compartment in complex with an inhibitory protein, I $\kappa$ B. Various extracellular signals can induce phosphorylation and rapid degradation of I $\kappa$ B to release NF- $\kappa$ B. After I $\kappa$ B is released, NF- $\kappa$ B then migrates into nucleus where it binds to specific DNA sequences and turns on gene transcription (Gloire *et al.*, 2006). Our results show that (+)-catechin can reduce glutamate-induced HT22 cell death by protecting these cells from ROS insult through reducing NF- $\kappa$ B activation, and its effect was slightly weaker than EGCG under the same experimental conditions (Kang *et al.*, 2010).

In addition, we have investigated the effect of (+)-catechin on neurodegeneration using the kainic acid-induced rat hippocampal injury model. Induction of oxidative stress has been identified as a major mechanism for hippocampal neurodegeneration (Nalder *et al.*, 1980; Floreani *et al.*, 1997; Shin *et al.*, 2009). Our results show that *i.c.v.* injection of kainic acid

induces neuronal loss as well as activation of glial cells in hippocampal CA3 region as described before (Nalder *et al.*, 1980; Matsuoka *et al.*, 1999), and treatment with (+)-catechin not only protects hippocampal neuronal cells, but also reduces GFAP-positive astrocytes. These data suggest that (+)-catechin has a strong neuroprotective effect against kainic acid-induced hippocampal neurodegeneration in rats. This protective effect is of considerable interest and clinical relevance because hippocampus is a brain region that often suffers chronic neurodegeneration in PD patients. It is estimated that PD patients have a six-fold higher risk than age-matched healthy control subjects for developing dementia (Padovani *et al.*, 2006). Hence, the observed neuroprotective effect of (+)-catechin may help alleviate hippocampal neurodegeneration in PD patients.

Here it is of note that in this study, we have also examined the effect of (+)-catechin in the 6-hydroxydopamine-induced neuronal damage model, and it did not show any appreciable protective effect (data not shown). Similarly, an earlier study by others also reported that EGCG, a major tea catechin, was ineffective in the 6-hydroxydopamine-induced neurotoxicity model (Leaver *et al.*, 2009). It is apparent that the neuroprotective effect of catechin is only seen under certain experimental conditions.

Lastly, it is noteworthy that recent epidemiological studies suggest that regular tea or coffee drinking is associated with a reduced risk of PD in humans (Hu *et al.*, 2007; Kandinov *et al.*, 2009). The potential beneficial effects of tea polyphenols (and possibly some of the coffee polyphenolic compounds) on brain catecholamine metabolism and neuronal survival as observed in this study may partially contribute to the epidemiological observations. Although the daily intake of tea and coffee polyphenols through regular tea and coffee drinking may not reach therapeutically-effective concentrations, it should be noted that tea and coffee also contain many other phenolic antioxidants (Scalbert and Williamson, 2000; Ferruzzi *et al.*, 2009). The collective neuroprotective effect exerted by these polyphenolic compounds could be significant. Moreover, many of these dietary phenolics (*e.g.*, catechins, quercetin, and caffeic acid) also contain the same catecholic structures as does L-DOPA, which make them good substrates and also effective inhibitors of COMT (Zhu *et al.*, 2000; Zhu, 2002 & 2004; Lu *et al.*, 2003; Nagai *et al.*, 2004; Bai *et al.*, 2007; Zhu *et al.*, 2008). Theoretically, a collective inhibition of COMT-mediated *O*-methylation of endogenous catecholamines (including dopamine and L-DOPA) by these dietary phenolics could be quite significant in the human body. In partial support of this suggestion, it is known that drinking tea and coffee is associated with a number of physiological effects in humans, such as increased heart rate and contractility, increased urination, and strong central nervous system stimulation. These effects are all known to be associated with elevated levels of endogenous catecholamines. While some of these effects associated with tea and coffee often are conveniently attributed to the small amounts of theophylline and caffeine contained in these beverages, the contribution of COMT inhibition has also been suggested in recent years (Zhu, 2002 & 2004).

**CONCLUSIONS**—In the present study, we showed that while both tea catechins and flavonoids can strongly inhibit L-DOPA methylation *in vitro*, only (+)-catechin was found to exert a significant inhibition of L-DOPA methylation *in vivo*. Notably, administration of (+)-catechin produces a greater increase in striatum dopamine content in dopamine-depleted rats than in normal rats, suggesting that this dietary polyphenol may have a greater beneficial effect in PD patients who have a reduced striatal dopamine content. The stronger *in vivo* effect of (+)-catechin compared to other dietary phenolics tested in this study is attributable to its more favorable bioavailability. In addition, we showed that (+)-catechin has a strong protective effect against oxidative hippocampal neurodegeneration *in vivo*. Based on these observations, it is suggested that (+)-catechin is a dietary compound that may

have beneficial effects in L-DOPA-based treatment of PD patients by jointly inhibiting L-DOPA methylation and reducing oxidative neuronal damage.

## 4. MATERIALS AND METHODS

### 4.1. Chemicals

(+)-Catechin, (-)-epicatechin, quercetin, L-DOPA, carbidopa, 3-OMD, dopamine, 3,4-dihydroxybenzylamine hydrobromide (DHBA), glutamate, kainic acid and 2',7'-dichlorofluorescein diacetate (H<sub>2</sub>-DCF-DA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). [<sup>3</sup>H-Methyl]AdoMet (specific activity = 11.2-13.5 Ci mmol<sup>-1</sup>, purity > 97%) was purchased from New England Nuclear Research Products (Boston, MA, USA). The plasmid pNF-κB-Luc carrying a firefly luciferase cDNA driven by 5× NF-κB-binding sites was purchased from Stratagene (La Jolla, CA, USA). All other reagents used in this study were obtained from standard suppliers and were of analytical grade or better.

### 4.2. *in vitro* modulation of L-DOPA methylation by tea catechins and flavonoids

The procedures for collecting human liver samples were approved by the Institutional Review Boards (IRBs). The methods for preparing cytosols from these collected tissue specimens were described in detail earlier (Nagai *et al.*, 2004). For assaying the *in vitro* O-methylation of L-DOPA catalyzed by human liver cytosolic COMT, the incubation mixture consisted of 10 μM L-DOPA as a substrate, 250 μM [<sup>3</sup>H-methyl]AdoMet (containing 0.2 μCi) as a methyl donor, 0.25 mg/mL of human cytosolic protein as the enzyme source, and a dietary inhibitor [(+)-catechin, (-)-epicatechin, or quercetin] in a final volume of 0.25 mL Tris-HCl buffer (10 mM, pH 7.4) supplemented with 1.2 mM MgCl<sub>2</sub> and 1 mM dithiothreitol. Incubations were carried out at 37°C for 10 min. Each measurement is the mean of duplicate determinations (with an average variation below 5%).

### 4.3. *in vitro* protective effect of (+)-catechin against oxidative stress-induced neuronal cell death

Glutamate-sensitive HT22 murine hippocampal neuronal cells (a gift from Dr. David Schubert at the Salk Institute, La Jolla, CA) were maintained in DMEM supplemented with 10% (v/v) FBS and antibiotics (penicillin-streptomycin), and incubated at 37°C under 5% CO<sub>2</sub>. Cells were subcultured once every 2 days. Cells were seeded in 96-well plates at a density of 5000 cells per well. Stock solutions of glutamate (1 M in DMEM without serum) and (+)-catechin (100 mM in 200 proof ethanol) were diluted in the culture medium immediately before addition into each culture well at desired final concentrations, and the treatment lasted up to 24 h.

The MTT assay was used for assessing cell viability as described in our recent study (Fukui *et al.*, 2009). The production of ROS in HT22 cells with or without glutamate treatment was detected using the H<sub>2</sub>-DCF-DA method. H<sub>2</sub>-DCF-DA (1 μM) was added to each well, and incubated for 20 min at 37°C. Then the supernatant was removed and PBS was added. Intracellular ROS accumulation was observed and photographed under a fluorescence microscope (AXIO, Carl Zeiss Corporation, Germany). For detection of transcriptional activity of NF-κB, HT22 cells in 24-well culture plates were transfected with 0.05 μg pNF-κB-Luc plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Twenty-four h after transfection, cells were treated with 5 mM glutamate with or without 40 μM (+)-catechin for 8 h. Then cells were harvested and the luciferase activity was determined by a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). Firefly luciferase activity was normalized to cellular protein concentrations.



#### 4.4. Animal experiments

**4.4.1. Animals**—All procedures involving the use of live animals as described in this study were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center, and strictly followed the guidelines for humane care of animals set forth by the U.S. National Institutes of Health. Male Sprague-Dawley rats, weighing 250–270 g, were purchased from Harlan (Indianapolis, IN) and kept in a plastic bottomed cage with a 12-h light/12-h dark cycle, with controlled room temperature (at 25°C) and humidity (60%). They were allowed free access to laboratory pellet chow and water. After arrival, the animals were allowed to acclimatize to the new environment for one week before they were used in the experimentation.

**4.4.2. Animal experiment I – Compare the effect of (+)-catechin, (–)-epicatechin, and quercetin on L-DOPA methylation**—Rats were randomly divided into various experimental groups with no significant difference in average body weights. Rats were injected *i.p.* 400 mg/kg (+)-catechin, (–)-epicatechin, or quercetin 2 h before oral administration of L-DOPA/carbidopa (20/5 mg/kg). The *in vivo* experimental conditions (dose, time and administration routes) were adopted from our recent study (Kang *et al.*, 2010). Control rats received the same *i.p.* and/or oral administrations of vehicle only. Blood samples were collected from the tail vein under light anesthesia (isoflurane inhalation) at 0.5, 1, 2, 3, 4 and 6 h after L-DOPA/carbidopa administration. About 100  $\mu$ L blood was drawn, collected in a heparin-coated tube, and plasma was obtained following rapid centrifugation and immediately frozen at –80°C until analysis. After the final blood collection, rats were euthanized with CO<sub>2</sub> followed by decapitation. The striatal region of the brain was dissected, weighed, and kept at –80°C until analysis.

Plasma L-DOPA and its metabolites were measured using the method of Karimi *et al.*, (2006) with minor modifications. Plasma samples (50  $\mu$ L) from each rat were spiked with 5  $\mu$ L internal standard (DHBA, 100  $\mu$ M). To precipitate proteins, 20  $\mu$ L of 1.2 M perchloric acid was added to each tube. The tubes were briefly vortexed, placed on ice for 10 min, and centrifuged for 4 min at 1250 g at 4°C. Thirty microliters of the supernatant were added to 60  $\mu$ L potassium citrate buffer (0.2 M, pH 3.8) to precipitate perchlorate. After that, each tube was again vortexed for 1 min, left on ice for 10 min, centrifuged for 4 min at 1250 g at 4°C, and an aliquot of the supernatant was injected into HPLC for analysis of composition. Similarly, to determine the striatal 3-OMD and dopamine levels, the thawed brain tissues were homogenized using a PowerGen-700 homogenizer (Fisher Scientific, Pittsburgh, PA, USA) in 0.4 M perchloric acid, and the homogenates were centrifuged and filtered for HPLC analysis.

The HPLC system consisted of a Shimadzu pump (LC-10AT model), an electrochemical detector (Coulchem III, ESA Bioscience, Chelmsford, MA, USA), and a HR-80 C-18 reverse-phase column (3  $\mu$ m, 80  $\times$  4.6 mm, ESA Bioscience). The mobile phase consisted of 50 mM sodium phosphate, 1 mM sodium dodecyl sulfate, 0.67  $\mu$ L triethylamine, 13.3  $\mu$ M EDTA, and 8% acetonitrile in water, adjusted to pH 3.0 with phosphoric acid. Before use, the mobile phase was filtered through the 0.45- $\mu$ m (pore size) filter (Millipore, Bedford, MA, USA), and degassed under vacuum. An isocratic elution at a flow rate of 1 mL/min was used. L-DOPA and its metabolites were identified by comparing their retention times with those of standard compounds. Concentrations were calculated with the aid of an internal standard (DHBA). Linearity of the detector responses were tested for all the compounds of interest, and the coefficients of correlation were >0.999. The relative standard deviation for the intra-day repeatability was well below 5%, representing good precision of the analytical method used.

For measuring the plasma levels of unconjugated (+)-catechin, (-)-epicatechin, and quercetin, a gas chromatography/mass spectrometry (GS/MS) method (Donovan *et al.*, 1999; Loke *et al.*, 2008) was adopted. Briefly, taxifolin (10 µg) was added to the collected plasma samples as an internal standard, and the plasma was extracted twice with ethyl acetate (1 mL). One microliter of transmethylated sample was injected into an Agilent gas chromatography (model-6890N) coupled with an Agilent mass spectrometer (model-5975B). A capillary column (HP5-MS, 30 m × 0.25 mm, 0.25 µm film thickness) was used, with helium as carrier gas. For elution, the column oven temperature was set at 180°C, ramped to 260°C at 4°C/min, then further ramped to 300°C at 5°C/min and held at 300°C for 5 min.

**4.4.3. Animal experiment II – Determine the influence of carbidopa dose on the inhibitory efficacy of (+)-catechin on L-DOPA methylation**—Rats were randomly divided into various experimental groups with no significant difference in average body weights. Rats were *i.p.* injected 400 mg/kg (+)-catechin 2 h before oral administration of L-DOPA/carbidopa (20/20 mg/kg). Control rats received the same *i.p.* and/or oral administrations of the corresponding vehicles only. Blood samples were collected from the tail vein with light anesthesia (isoflurane inhalation) at 0.5, 1, 2, 3, 4 and 6 h after L-DOPA + carbidopa administration. The plasma and striatal tissues were prepared and analyzed as described above in animal experiment I.

**4.4.4. Animal experiment III – Determine the effect of (+)-catechin on L-DOPA methylation in dopamine-depleted rats**—Rats were randomly divided into various experimental groups. To induce dopamine-depletion, reserpine (5 mg/kg) was solubilized in water containing lactic acid (1%, *v/v*) and given to rats (*i.p.*) 18-20 h before drug administration (Rivas *et al.*, 1999). The reserpinized rats were then injected *i.p.* 400 mg/kg (+)-catechin 2 h before oral administration of L-DOPA/carbidopa (20/20 mg/kg). Control rats received the same *i.p.* and/or oral administrations of vehicles only. Animals were euthanized at 2h after L-DOPA/carbidopa administration, and the plasma and striatal tissues were prepared and analyzed as described above in animal experiment I.

**4.4.5. Animal experiment IV – Determine the protective effect of (+)-catechin on kainic acid-induced rat hippocampal injury**—In these experiments, male Sprague-Dawley rats were randomly divided into multiple experimental groups, with very similar average body weight for each group. Animals were administered orally 100 or 400 mg/kg body weight of (+)-catechin 30 min before kainic acid injection. Kainic acid (1 µL of 1 mg/mL solution) was injected into the right lateral ventricle (anterior/posterior, -1.0; rostral, 1.6; dorsal/ventral, 4.5) using a microliter syringe under anesthesia with ketamine/xylazine (50/5 mg/kg, *s.c.*). The needle was withdrawn 5 min later and scalp was sutured. Control rats were injected with 1 µL of saline instead of kainic acid (He *et al.*, 2006). Neuronal loss in the hippocampus (based on morphological evaluations) was analyzed at 24 h after kainic acid treatment. At the end of the experiment right before the brain tissue was collected for analysis, the animals received ketamine and xylazine (50 and 5 mg/kg, *s.c.*) for anesthesia, and then perfused with 0.1 M neutral phosphate-buffered formalin (4% final concentration of formaldehyde) via the ascending aorta, with the descending aorta clamped off.

The collected brain tissues were post-fixed overnight in 0.1 M neutral phosphate-buffered formalin (containing 4% formaldehyde). After cryoprotection in 30% sucrose/phosphate buffer, tissues were frozen in liquid nitrogen and sectioned serially (30 µm) through the entire brain. The sections were collected in 0.1 M neutral phosphate buffer, mounted on slides, then air-dried on a slide warmer at 50°C for at least 30 min, and stained with hematoxylin and eosin (H/E) for histopathological analysis.

Fluoro-Jade B staining was performed by following the protocols described by Schmued and Hopkins (2000) with minor modifications. Briefly, the slides were transferred to a solution of 0.06% potassium permanganate for 10 min. The staining solution was prepared from a 0.01% stock solution of Fluoro-Jade B that was made by adding 10 mg of the dye powder to 100 mL distilled water. After 20 min in the staining solution, slides were rinsed and placed on a slide warmer until they were fully dry. The cell bodies of Fluoro-Jade B-positive neurons were clearly visible. The number of stained neurons was counted in the obtained images using the Axiovision image analysis software (Carl Zeiss, Inc., Thornwood, NY). For analysis of the hippocampus, stained neurons of three sections from each rat were counted in the dorsal hippocampal region (between  $-1.5$  and  $-2.5$  mm from bregma).

Since glial cell activation, as evidenced by anti-glial fibrillary acidic protein (GFAP) upregulation, often was present in neuronal damage, tissue sections were also incubated successively with a rabbit monoclonal anti-GFAP antibody (1:800, Sigma-aldrich), biotin-conjugated goat polyclonal anti-rabbit second antibody (1:250, Vector Laboratories, CA, USA), and horseradish peroxidase-conjugated avidin-biotin complex (Vector Laboratories) for detection. To perform quantitative analysis of GFAP immunostaining, 3-4 sections per animal were selected and images were captured and analyzed using the Axiovision image analysis software. One field ( $100\ \mu\text{m} \times 100\ \mu\text{m}$ ) in each slide within the midpoint of hippocampal CA3 regions were selected for quantification, and the intensity of GFAP immunoreactivity was evaluated by means of a relative optical density value (Georgievska *et al.*, 2006).

#### 4.5. Data analysis

Wherever possible, data were expressed as mean  $\pm$  S.D. (standard deviation). Statistical significance was determined through analysis of variance (ANOVA) followed by a multiple comparison test with a Bonferroni adjustment. *P* values of less than 0.05 were considered statistically significant.

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This study was supported, in part, by a grant from the National Institutes of Health (ES015242).

#### ABBREVIATIONS USED

<b>L-DOPA</b>	Levodopa
<b>PD</b>	Parkinson disease
<b>COMT</b>	catechol-O-methyltransferase
<b>3-OMD</b>	3-O-methyldopa
<b>CNS</b>	central nervous system
<b>H2-DCF-DA</b>	2',7'-dichlorofluorescein diacetate
<b>AdoMet</b>	S-adenosyl-L-methionine
<b>ROS</b>	reactive oxygen species
<b>MTT</b>	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
<b>EGCG</b>	epigallocatechin gallate

**GFAP** glial fibrillary acidic protein

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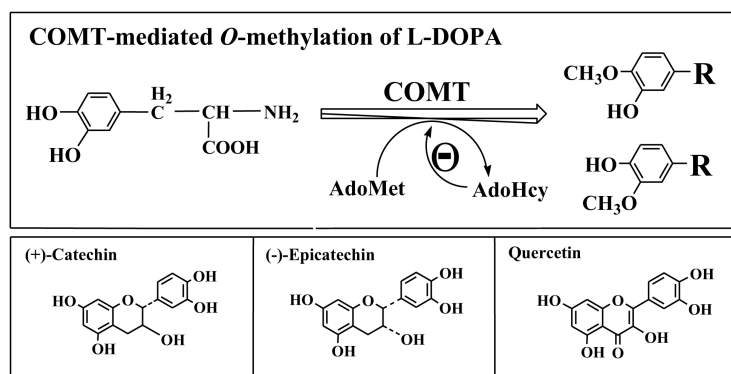


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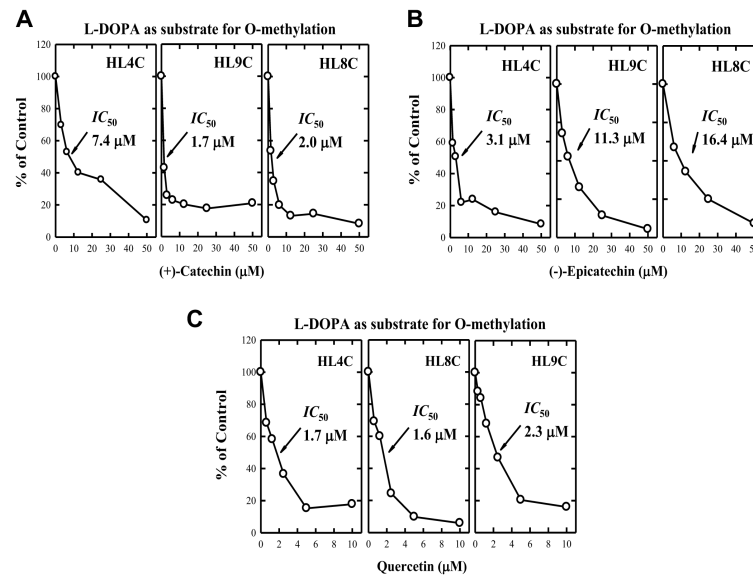
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**HIGHLIGHTS**

- (+)-Catechin strongly inhibits L-DOPA methylation metabolism in vitro and in vivo.
- (+)-Catechin also exerts a strong neuroprotective effect against oxidative damage in vitro and in vivo.
- (+)-Catechin may be used as an adjuvant in the treatment of human Parkinson disease.

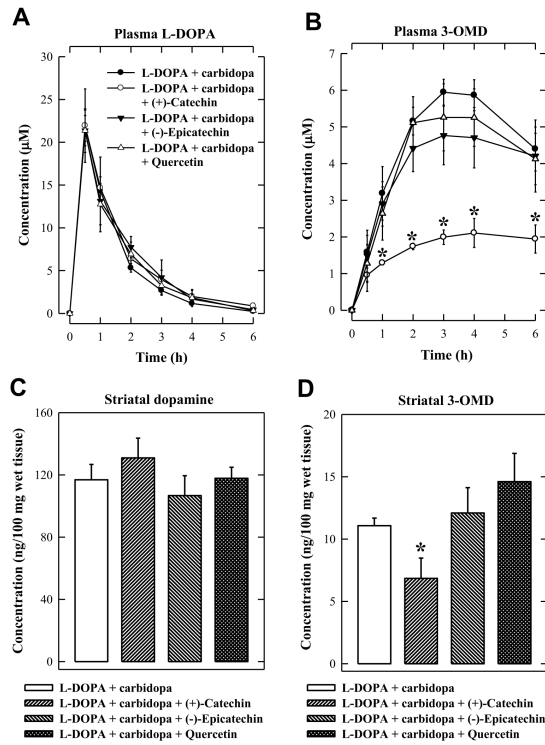
**Figure 1.**

Enzymatic methylation of L-DOPA and its potential modulation by certain dietary phenolics. The upper panel shows that the COMT-catalyzed *O*-methylation of L-DOPA, which results in the formation of two mono-methylated products. It is hypothesized that certain dietary phenolics such as tea catechins and flavonoids may serve as naturally-occurring inhibitors of human COMT-mediated *O*-methylation of L-DOPA *in vivo*. The structures of (+)-catechin, (-)-epicatechin, and quercetin are shown in the lower panel. Partly owing to their strong antioxidant activity, it is also suggested that some of these dietary phenolics may have additional neuroprotective effects. The potential dual beneficial effects of certain dietary phenolics, if found to be true, would be desirable in L-DOPA-based treatment of PD patients.

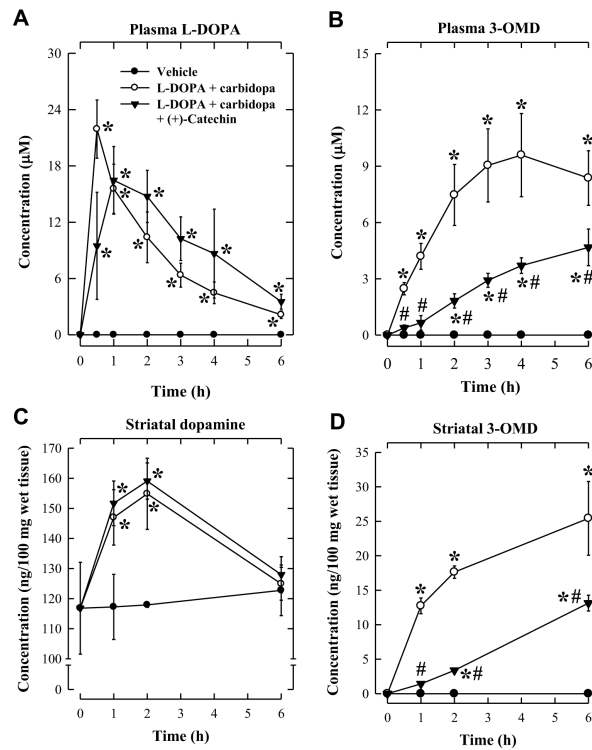


**Figure 2.** Inhibition of human liver COMT-mediated *O*-methylation of L-DOPA by two tea catechins, (+)-catechin and (-)-epicatechin (panels A and B), and a representative flavonoid quercetin (panel C). The incubation mixture consisted of 10 μM L-DOPA, 250 μM [<sup>3</sup>H-methyl]AdoMet (containing 0.2 μCi), 0.25 mg/mL of human cytosolic protein, 1 mM dithiothreitol, 1.2 mM MgCl<sub>2</sub>, and a dietary inhibitor (concentration as indicated) in a final volume of 0.25 mL Tris-HCl buffer (10 mM, pH 7.4). Incubations were carried out at 37°C for 10 min. Each point is the mean of duplicate determinations of the 3 human liver (HL) cytosolic samples named HL4C, HL8C and HL9C (with average variations below 5%).

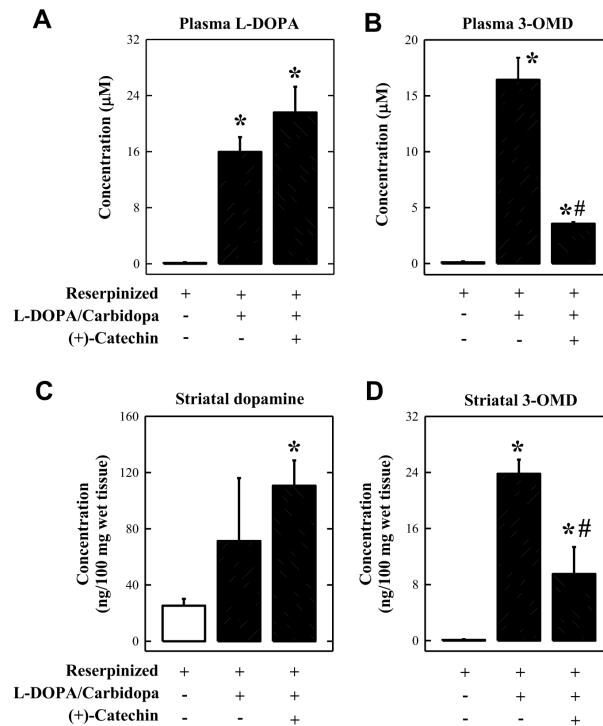




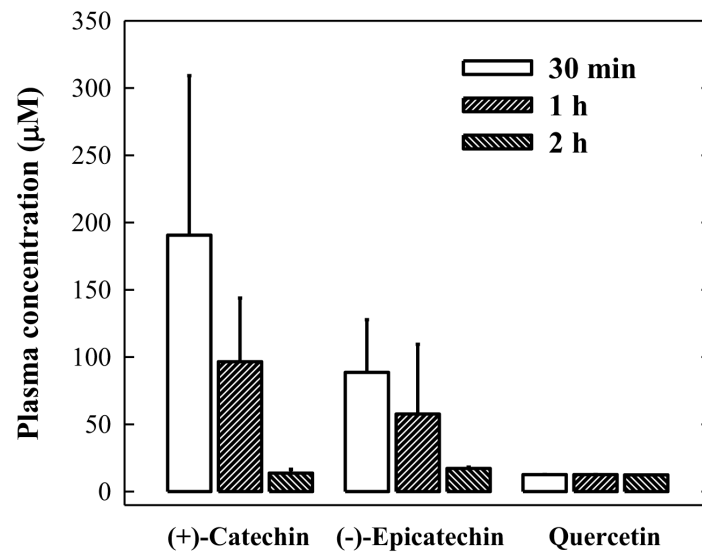
**Figure 3.** Comparison of the effect of (+)-catechin, (-)-epicatechin, and quercetin on the methylation of L-DOPA in rats. (A) Plasma L-DOPA concentrations. (B) Plasma 3-OMD concentrations. (C) Striatal dopamine concentrations. (D) Striatal 3-OMD concentrations. Rats were injected *i.p.* with 400 mg/kg (+)-catechin, (-)-epicatechin, or quercetin 2 h before oral administration of L-DOPA + carbidopa (20 + 5 mg/kg). Control rats received the vehicle administration only. Each value is the mean  $\pm$  S.D. \*  $P < 0.05$  compared to L-DOPA + carbidopa group.

**Figure 4.**

Effect of carbidopa on the degree of inhibition of L-DOPA methylation by (+)-catechin in rats. (A) Plasma L-DOPA concentrations. (B) Plasma 3-OMD concentrations. (C) Striatal dopamine levels. (D) Striatal 3-OMD levels. Rats were injected *i.p.* with 400 mg/kg (+)-catechin 2 h before oral administration of L-DOPA/carbidopa (20/20 mg/kg). The vehicle group received water administration only. Each value is the mean  $\pm$  S.D. \*  $P < 0.05$  compared to vehicle group. #  $P < 0.05$  compared to L-DOPA + carbidopa group.

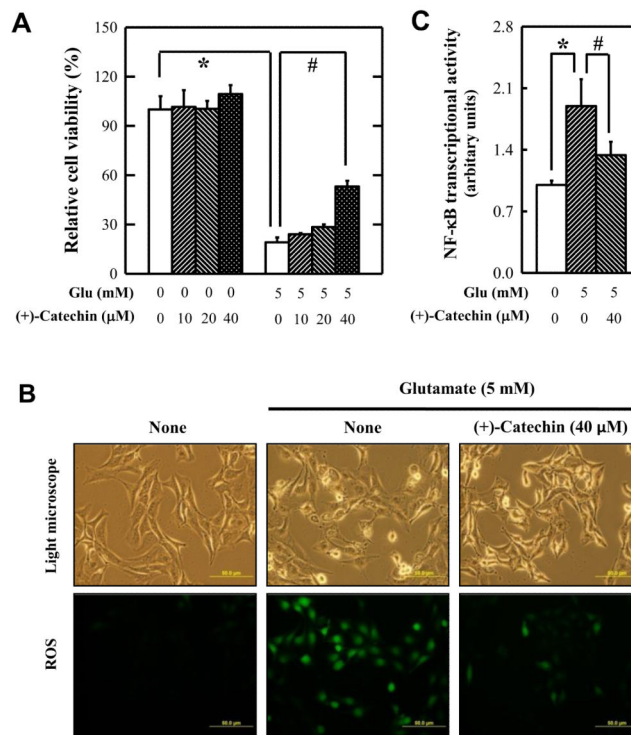
**Figure 5.**

Effect of (+)-catechin on the methylation of L-DOPA in dopamine-depleted rats. (A) Plasma L-DOPA concentrations. (B) Plasma 3-OMD concentrations. (C) Striatal dopamine levels. (D) Striatal 3-OMD levels. Reserpine (5 mg/kg) was solubilized in water containing lactic acid (1%, *v/v*) and given to rats 18-20 h before administration of L-DOPA and/or (+)-catechin. Rats were injected *i.p.* with 400 mg/kg (+)-catechin 2 h before oral administration of L-DOPA/carbidopa (20/20 mg/kg). Each value is the mean  $\pm$  S.D. \*  $P < 0.05$  compared to the reserpined group. #  $P < 0.05$  compared to L-DOPA + carbidopa group.



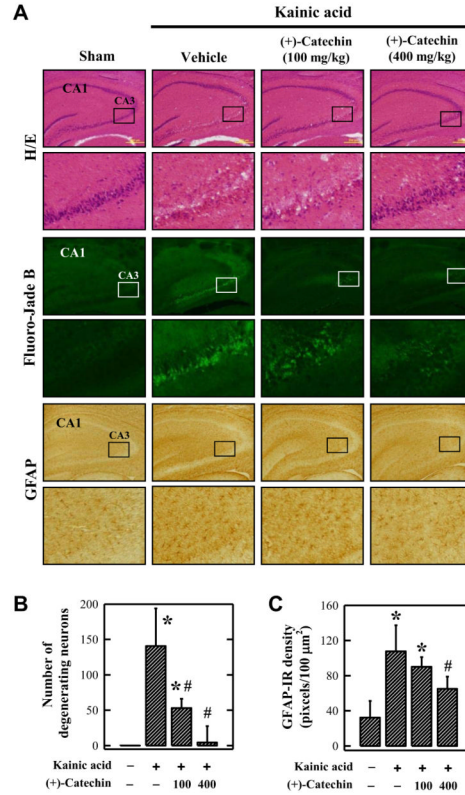
**Figure 6.**

Plasma concentrations of (+)-catechin, (-)-epicatechin, and quercetin in rats. Rats ( $N=4$  for each group) were injected *i.p.* with 400 mg/kg each dietary polyphenolic compound. Blood samples were collected from their tail veins at 0.5, 1, and 2 h after administration of the dietary compound. Each value is the mean  $\pm$  S.D.



**Figure 7.** Effect of (+)-catechin against glutamate-induced HT22 cell death. (A) Cell viability. (B) ROS accumulation. (C) Transcriptional activity of NF- $\kappa$ B. HT22 cells were treated with 5 mM glutamate in the presence or absence of (+)-catechin at indicated concentrations. Cell viability, transcriptional activity of NF- $\kappa$ B, and ROS accumulation were determined as described in the Materials and Methods. The experiment was repeated three times, and similar observations were made. DAPI staining was employed as a nuclear counter staining. Each value is the mean  $\pm$  S.D. \*  $P < 0.05$  compared to vehicle-treated group. #  $P < 0.05$  compared to glutamate-treated group.





**Figure 8.** Effect of orally-administered (+)-catechin on kainic acid-induced rat hippocampal injury. (A) Histopathological and histochemical analyses of brain tissue slides. (B) Number of degenerating neurons based on Fluoro-Jade B staining. (C) Relative density of GFAP-immunoreactive (IR) astrocytes in CA3 region. Rats were orally administered 400 mg/kg of (+)-catechin 30 min before kainic acid injection. Kainic acid (1 μL of 1 mg/mL solution) was injected into the right lateral ventricle (anterior/posterior, -1.0; rostral, 1.6; dorsal/ventral, 4.5) using a microliter syringe. Each value is the mean ± S.D. \*  $P < 0.05$  compared to vehicle-treated group. #  $P < 0.05$  compared to kainic acid-treated group.

**TABLE 1**

Comparison of the plasma AUC<sub>0-6</sub> values of L-DOPA and 3-OMD. Data were obtained from the animal experiments I and II.

Treatment groups	L-DOPA/carbidopa (at 20 mg/5 mg kg)		L-DOPA/carbidopa (at 20 mg/20 mg kg)	
	L-DOPA ( $\mu\text{g}\cdot\text{min}/\text{mL}$ ) ( $\mu\text{g}\cdot\text{min}/\text{mL}$ )	3-OMD ( $\mu\text{g}\cdot\text{min}/\text{mL}$ )	L-DOPA	3-OMD ( $\mu\text{g}\cdot\text{min}/\text{mL}$ )
<b>Control</b>	374.8 $\pm$ 81.2	377.9 $\pm$ 40.4	570.3 $\pm$ 128.9	600.4 $\pm$ 130.9
<b>(+)-Catechin</b>	427.8 $\pm$ 88.7	141.4 $\pm$ 21.8*	693.1 $\pm$ 227.4	215.1 $\pm$ 38.2*
<b>(-)-Epicatechin</b>	419.1 $\pm$ 79.6	321.3 $\pm$ 63.9	ND	ND
<b>Quercetin</b>	396.3 $\pm$ 104.2	343.8 $\pm$ 62.6	ND	ND

Data are expressed as the mean  $\pm$  S.D. ( $N=4$ ).

\*  $P < 0.05$  vs. the control group.

ND, not determined.