Protective Effects of Green Tea Polyphenols in the 6-OHDA Rat Model of Parkinson’s Disease Through Inhibition of ROS-NO Pathway

Shuhong Guo, Jingqi Yan, Tangbin Yang, Xianqiang Yang, Erwan Bezard, and Baolu Zhao

Background: Nitric oxide (NO) and related pathways are thought to play an important role in the pathogenesis of Parkinson’s disease (PD). Our in vitro experiments suggested that green tea polyphenols (GTP) might protect dopamine neurons through inhibition of NO and reactive oxygen species (ROS).

Methods: Immunohistochemistry, terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling assay, electron spin resonance spin trapping, enzyme linked immunosorbent assay, and molecular biological methods were used to investigate the effects of GTP in an unilateral 6-hydroxydopamine (6-OHDA)-treated rat model of PD.

Results: GTP treatment dose-dependently protected dopaminergic neurons by preventing from midbrain and striatal 6-OHDA-induced increase in 1) both ROS and NO levels, 2) lipid peroxidation, 3) nitrite/nitrate content, 4) inducible nitric oxide synthase, and 5) protein-bound 3-nitro-tyrosine. Moreover, GTP treatment dose-dependently preserved the free radical scavenging capability of both the midbrain and the striatum.

Conclusions: These results support the in vivo protection of GTP against 6-OHDA and suggest that GTP treatment might represent a neuroprotective treatment of PD.

Key Words: Electron spin resonance, natural antioxidants, neurodegenerative disease, nitric oxide, reactive oxygen species, stereology

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Parkinson’s disease (PD) results from an unrelenting process of cell death affecting the pigmented dopaminergic (DA)-containing neurons of the substantia nigra pars compacta (SNc) (1,2). A major commitment of preclinical research is to identify drugs or strategies that might slow or stop the neurodegenerative process. To date, however, no drug has been clinically validated for such neuroprotective indication (3). The etiology of PD is still not fully understood, but animal models, human postmortem material, and genetic analyses have provided important clues (4). For instance, data from human postmortem tissue indicate that reactive oxygen species (ROS), nitric oxide (NO), and decrements in mitochondrial complex 1 activity are important in the pathogenesis of sporadic PD (5), suggesting that compounds interfering both with ROS and NO production and with impaired mitochondrial complex 1 activity might be protective.

Green tea is a beverage that is popular worldwide and possess many pharmacological effects, such as antimutagenic, antiproliferative, anticarcinogenic properties, and, more importantly for our purposes, neuroprotective indications in models of degenerative disorders (6–10). These properties are thought to be mediated by the green tea polyphenols (GTP), the four main components of which are (-)-epigallocatechin gallate (EGCG), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC), and (-)-epicatechin (EC). The classified protective effects of the four catechins on PC12 cell against 6-hydroxydopamine (6-OHDA)-induced apoptosis are in the following order: ECG > EGCG > EC > EGC (9). In searching for a largely available neuroprotective compound, we recently demonstrated that GTP in combination and EGCG alone protected SH-SY5Y cells against apoptosis induced by the pro-parkinsonian neurotoxin 6-hydroxydopamine 6-OHDA (8). When we investigated further the mechanisms underlying this phenomenon, we showed that the protective action was achieved through inhibition of the ROS-NO pathway known to be involved in both 6-OHDA-induced cell death (8,9) and idiopathic PD neuronal loss (5).

In light of these findings, this study was designed to 1) assess the putative neuroprotective properties of GTP in vivo in the 6-OHDA rat model of PD and 2) determine whether the protective mechanisms involve inhibition of the ROS-NO pathway suggested by in vitro data.

Methods and Materials

Reagents

New-born calf serum, 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES), 5,5-dimethyl-pyrroline-1-oxide (DMPO), N-tetra-butyl-a-phenylnitrone (PBN), and 3,3-diaminobenzidine (DAB) were purchased from Gibco BRL (Grand Island, New York). 6-OHDA, 2',7'-dichlorofluorescin diacetate (DCF-DA), 4,5-diaminofluorescein diacetate (DAF-2DA), DNase I, ethylendiaminetetraacetic acid (EDTA), glutathione reduced (GSH), N0-methyl-L-arginine acetate salt (L-NMMA), trypsin, penicillin, and streptomycin were purchased from Sigma Chemical (St. Louis, Missouri); Fluo-3 acetoxymethyl ester (Fluo-3 AM) was purchased from Calbiochem (San Diego, California). Rabbit polyclonal antibody for neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS) were purchased from Santa Cruz Biotechnology (Santa Cruz, California). Green tea...
polyphenols (98% purity) containing 50% EGCG, 22% ECG, 18% EG, and 10% EC (analyzed by high-pressure liquid chromatography), were a gift from Sichuan Full-green Biology Technology (Mianyang, China).

**Animals and Treatment Protocol**

Experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were accepted by the local animal care committee. Male Sprague–Dawley rats (220–230 g, Beijing Experimental Animal Center, China) were maintained under standard housing conditions with constant temperature. Food and water were available ad libitum. Unilateral mesencephalic lesions were performed as previously described (11,12). Rats were pretreated with desipramine (25 mg/kg, administered intraperitoneally [IP]) and pargyline (5 mg/kg, IP), anesthetized with chloral hydrate (400 mg/kg, IP), and then placed in a stereotaxic frame. Four microliters of 6-OHDA (2 mg/mL, Sigma) or vehicle (sterile water with .1% ascorbic acid) was injected into the right medial forebrain bundle (anterior: −4.0 mm, lateral: 1.1 mm, ventral: −7.6 mm) (13) at a rate of .5 μL/min. The injection cannula was left in place for 5 min before removal.

Seven days before surgery, animals were randomly divided into seven groups, depending on the preoperative gavage regimen: vehicle-fed and sham-lesioned (A); vehicle-fed, 6-OHDA-lesioned (B); GTP 150 mg/kg/day-fed, 6-OHDA-lesioned (C); GTP 450 mg/kg/day-fed, 6-OHDA-lesioned (D); vehicle-fed unlesioned (E); GTP 150 mg/kg/day-fed, unlesioned (F); and GTP 450 mg/kg/day-fed, unlesioned (G). Groups E, F, and G were set as unlesioned control groups; GTP was given throughout the experimental period.

Animals for biochemistry were decapitated, and the brain was removed and rinsed with ice-cold physiologic saline and then homogenized in 5:1 (v:w) ice-cold .1 mol/L phosphate buffered saline (PBS) pH 7.4. For immunohistochemistry, animals were anesthetized with urethane (1300 mg/kg IP) and sacrificed by perfusion transcardially with .1 mol/L phosphate buffer (pH 7.4) and .8% thiobarbituric acid then boiled for 1 hour at 95°C and centrifuged at 4000 g for 10 min. The absorbance of the supernatant was measured by spectrophotometry (Hitachi, Tokyo, Japan) at 650 nm.

**Rotational Behavior**

Three weeks after surgery, rats were placed in circular bowls and allowed 15 min for habituation. The total number of contralateral turns was then counted over a 30-min period following IP apomorphine (Sigma) administration (.25 mg/kg, IP).

**Tyrosine Hydroxylase Immunohistochemistry**

Coronal mesencephalic sections (20 μm) were processed for tyrosine hydroxylase (TH) immunohistochemistry and then counterstained with cresyl violet (Nissl staining) as previously described (14–16). Briefly, unbiased stereologic techniques (17,18) were used to estimate mesencephalic DA-containing neurons by an investigator blind to experimental protocols. The boundaries of the SNC were determined by examining the size and shape of the different TH-immunoreactive neuronal groups (13,19,20). The volume was calculated by using the formula: \( V = \sum S \, t \), where \( S \) is the sum of surface areas, \( t \) is the average section thickness, and \( d \) is the number of slices between two consecutives analyzed sections measured (21). Seven sections were used; optical dissectors were distributed using a systematic sampling scheme. Dissectors (80 μm long, 60 μm wide) were separated from each other by 50 μm (x) and 40 μm (y). The following formula was used to estimate the number of TH-IR neurons: \( N = V_{SNc} \left( \sum Q^+ \, \sum V_{dis} \right) \), where \( N \) is the estimation of cell number, \( V \) is the volume of the SNC, \( \sum Q^+ \) is the number of cells counted in the dissectors, and \( \sum V_{dis} \) is the total volume of all the dissectors (21). Mean estimated number of neurons and SEM were then calculated.

**TUNEL assay**

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was applied to adjacent cryostat sections. Apoptotic cells were identified using a modified end-labeling technique (22,23). Briefly, sections were rinsed twice in Tris-buffer solution and .5% peroxide of methanol to quench endogenous peroxidase and rinsed three times in distilled water. The sections were then permeabilized with proteinase K (2 mg/mL) labeled with terminal deoxynucleotidyl transferase containing digoxigenin-dUTP (.3 EU/mL) and incubated with blocking buffer containing bovine serum albumin. The sections were incubated with anti-digoxin-biotin for 30 min and then incubated with avidin peroxidase for 30 min. The staining was developed with DAB as chromogen. The incubating sections with DNase I served as a positive control, and omission of the terminal transferase from the reaction mixture served as a negative control.

**Lipid Peroxidation**

Lipid peroxidation was assessed by measuring the TBARS content in the brain homogenate (24). An aliquot of the homogenate was added to a reaction mixture containing 20% acetic acid (pH 3.5) and .8% thiobarbituric acid then boiled for 1 hour at 95°C and centrifuged at 4000 g for 10 min. The absorbance of the supernatant was measured by spectrophotometry (Hitachi, Tokyo, Japan) at 543 nm.

**Nitrate/Nitrite Content**

The nitrate/nitrite level of brain homogenate was measured as described elsewhere (25). Briefly, an aliquot of the brain homogenate was first incubated with nitrate reductase (.2 U/mL), flavin adenine dinucleotide (FAD; .005 mmol/mL), and nicotinamide adenine dinucleotide phosphate hydrate (NADPH; .1 mmol/mL) in a total volume of 500 μL to reduce nitrate to nitrite. Then lactic dehydrogenase (1500 U/mL) and 100 mmol/mL pyruvic acid were added and incubated to oxidize any unreacted NADPH. The nitrite concentration was measured using the Griess reaction, by adding .1% naphthylethylene diamide and 2% sulphanilamide in 5% concentrated phosphate buffer. After 10-min incubation, the absorbance was determined by spectrophotometry at 543 nm.

**Associated Antioxidant Levels**

The scavenging ability of brain homogenates on superoxide anion and hydroxyl radical were assessed with electron spin resonance (ESR) spectrometer and a spin trapping method using DMPO (26). Superoxide anion scavenging ability was determined in a riboflavin system containing .3 mmol/mL riboflavin, 5 mmol/mL EDTA, .2 mol/mL DMPO, and brain homogenate for 30 μL of total volume at pH 7.4. Thirty seconds after light exposure, DMPO-OH was measured by ESR spectrometer. The hydroxyl radical generation system contained .1 mol/mL DMPO, .025 mmol/mL ferrous, .01% H2O2, brain homogenate for 50 μL of total volume in PBS solution at pH 7.4. Seventy seconds after the solution was mixed, DMPO-OH was measured by electron paramagnetic resonance (EPR) spectroscopy (26).
Simultaneous Detection of ROS and NO

The midbrain and striatum were used to detect ROS and NO according to a previously method with minor modifications (27). Briefly, the hemisphere was homogenized in a spin trap solution 100 mmol/mL of PBN and 2 mmol/mL diethyldithiocarbamate, 10 mmol/mL HEPES in ice-cold PBS; .5 mol/mL NaS2O4, 10 mmol/mL arginine, .5 mol/mL FeSO4, and 10 mmol/mL CaCl2 was then added to the system and vortexed. This was then isolated by ethyl acetate for ESR study. The height of the ESR spectra of diethyldithiocarbamate-Fe and PBN spin adducts were measured for the NO and ROS signal intensity (28). The Bruker ER-200 X-band ESR spectrometer settings for oxygen radicals were as follows: scan range, 200 G; microwave frequency, 9.47 GHz; modulation frequency, 100 kHz; microwave power, 20 mW; modulation amplitude, 1 G. The ESR conditions for detecting ROS and NO were the same except for the following: scan width was 400 G; modulation amplitude was 3.2 G.

Measurement of Neuronal and Inducible NO Synthase (nNOS and iNOS) Levels

The midbrain and striatum were homogenized on ice in buffer (50 mmol/mL Tris-Cl, 150 mmol/mL NaCl, .02% NaN2, 100 g/mL phenylmethanesulfonyl fluoride, 1 g/mL aprotitin, and 1% Triton X-100) at 0°C for 30 min. The lysates were then centrifuged at 12,000 g for 25 min at 4°C. The supernatant was used for sodium nitroprusside-polyacrylamide gel electrophoresis, and protein content was estimated using Bradford’s (29) method. Approximately 50 g total proteins were loaded. Proteins were separated on 8% polyacrylamide gels and transferred to a nitrocellulose membrane. Blots were blocked in a blocking buffer (containing 5% bovine serum and 1% Tween 20 in .1 mmol/mL PBS, pH 7.4) and incubated with primary antibody (rabbit anti-nNOS and anti-iNOS polyclonal antibodies; Santa Cruz Biotechnology) for 1 hour. The blots were then washed three times with .1% Tween-20 in .1 mmol/mL PBS buffer, incubated for 1 hour with horseradish peroxidase–conjugated goat antirabbit antibody and stained with DAB(3,3’-dia minobenzidine tetrahydrochloride). The level of nNOS and iNOS was estimated by analysis of the density of each band using a computerized densitometer and National Institutes of Health imaging software.

Measurement of Protein-Bound 3-NT

Protein-bound 3-NT was detected with a competitive enzyme linked immunosorbent assay as described by Rawlingson et al. (30) with some modifications. Each well of a 96-well plate was precoated with bovine thyroglobulin-nitrotyrosine. The cell lysates were incubated with mouse anti-3-NT antibody (1:8000) for 15 min, and then a 100 mL mixture was added in the plate to incubate for 1 hr. Following incubation, the plate was thoroughly washed with PBS (.1% v/v Tween 20) to remove unbound primary antibody. The secondary antibody (Santa Cruz Biotechnology) was then added and incubated for 1 hour. The plate was washed again, and orthophenylenediamine was added and incubated in darkness. After 30 min, the reaction was stopped with .2 mmol/mL sulfuric acid and the absorbance was read at 492 nm. The protein-bound 3-NT was calculated by comparing their absorbance with that of a standard calibration curve obtained from 3-NT. All samples and standards were assayed in triplicate.

Statistical Analysis

All experiments were done in triplicate. One-way analysis of variance was used to estimate overall significance followed by post hoc Tukey’s tests, corrected for multiple comparisons (31). Data are presented as mean ± SEM. A probability level of 5% (p < .05) was considered significant.

Results

GTP Treatment Partially Protects Dopaminergic Neurons

Three weeks after 6-OHDA injection, apomorphine-induced contralateral rotations were tested and pharmacologic treatment affected the number of rotations \[ F(3,31) = 865.8, p < .0001; \text{Figure 1, left}. \] GTP 450 mg/kg/day-fed 6-OHDA-lesioned rats had significantly fewer rotations than the vehicle-fed 6-OHDA-lesioned animals (p < .05; \text{Figure 1, left}), suggesting a relative sparing of the nigrostriatal neurons.

Indeed, stereologic counting of TH-immunopositive neurons in the SNc revealed a significant effect of treatment \[ F(3,31) = 75.1, p < .0001 \] of the side of the brain \[ F(1,31) = 546.9, p < .0001 \] and of their interaction \[ F(3,31) = 70.6, p < .0001; \text{Figure 1, right} \] (94.9% loss caused by 6-OHDA). Post hoc analysis showed that although the unlesioned left sides were identical among the four groups, the GTP 450 mg/kg/day-fed 6-OHDA-lesioned rats still had a significantly higher number of TH-immunopositive neurons than the vehicle-fed 6-OHDA-lesioned animals (p < .05; \text{Figure 1, right}; 88.7% and 80.8% loss caused by 6-OHDA for the GTP 150 and GTP 450 treatment, respectively).

Qualitative analysis of TUNEL staining in the same animals (\text{Figure 2}) further suggested that GTP 450 mg/kg/day-fed 6-OHDA-lesioned rats (\text{Figure 2D}) presented fewer TUNEL-positive cells than the vehicle-fed 6-OHDA-lesioned animals (p < .05; \text{Figure 1}, right; 88.7% and 80.8% loss caused by 6-OHDA for the GTP 150 and GTP 450 treatment, respectively).

GTP Treatment Prevents 6-OHDA-Induced Generation of ROS and NO

Using the ESR spin trapping technique, generation of ROS and NO can be simultaneously detected in brain tissues (26). Examples of ESR spectra for both ROS and NO free radicals trapped by

![Figure 1](https://www.sobp.org/journal)

Apomorphine-induced rotational behavior (left) and the number of TH-immunopositive neurons in the substantia nigra pars compacta (SNc) (right) of vehicle-fed sham-lesioned (A), vehicle-fed 6-hydroxydopamine (6-OHDA)-lesioned (B), green tea polyphenol (GTP) 150 mg/kg/day-fed 6-OHDA-lesioned (C), and GTP 450 mg/kg/day-fed 6-OHDA-lesioned (D) rats. n = 6 animals per group for each measurement. * Indicates significantly different from vehicle-fed sham-lesioned group, p < .05; # p < .05 compared with vehicle-fed 6-OHDA-lesioned group.
PBN and DETC-Fe complex in midbrain and striatum homogenates are shown on Figures 3A and 3B. Quantitative analysis revealed a significant effect in both the midbrain (Figure 3C) and striatum (Figure 3D) of treatment [midbrain: ROS: $F_{(23,71)} = 7.9$, $p < .0001$; NO: $F_{(23,71)} = 1.9$, $p < .05$; striatum: ROS: $F_{(23,71)} = 5.7$, $p < .0001$; NO: $F_{(23,71)} = 6.3$, $p < .0001$], of the side of the brain [midbrain: ROS: $F_{(11,35)} = 4.7$, $p < .001$; NO: $F_{(11,35)} = 2.3$, $p < .05$; striatum: ROS: $F_{(11,35)} = 6.3$, $p < .0001$; NO: $F_{(11,35)} = 6.3$, $p < .0001$] and of their interaction [midbrain: ROS: $F_{(11,35)} = 5.4$, $p < .001$; NO: $F_{(11,35)} = 4.6$, $p < .001$; striatum: ROS: $F_{(11,35)} = 2.3$, $p < .05$; NO: $F_{(11,35)} = 2.3$, $p < .05$]. Post hoc analysis showed that whereas the unlesioned left sides were identical among the four groups for both the ROS and NO in both the midbrain and striatum (Figure 3), the midbrain and striatal levels of ROS and NO in the lesioned right sides of the GTP 150 mg/kg/day-fed 6-OHDA-lesioned rats and GTP 450 mg/kg/day-fed 6-OHDA-lesioned rats were significantly lower than that in the vehicle-fed 6-OHDA-lesioned animals ($p < .05$) and not different from the

Figure 2. Examples of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining in the right substantia nigra 3 weeks after sham or 6-hydroxydopamine (6-OHDA) injection of vehicle-fed sham-lesioned (A), vehicle-fed 6-OHDA-lesioned (B), green tea polyphenol (GTP) 150 mg/kg/day-fed 6-OHDA-lesioned (C), and GTP 450 mg/kg/day-fed 6-OHDA-lesioned (D) rats. Scale bar in panels A–D is of 250 μm (E). Higher magnification of the area showed by arrows in panel B that reveals characteristic TUNEL nuclear staining. Scale bar: 30μm.
vehicle-fed sham-lesioned animals (Figure 3). For the unlesioned control groups, both GTP 150 mg/kg/day and GTP 450 mg/kg/day fed can decrease the generation of NO and ROS (p < .05, Figure 3). Therefore, GTP treatment would prevent 6-OHDA-induced ROS and NO generation.

**GTP Treatment Prevents 6-OHDA-Induced Lipid Peroxidation**

As shown by increases in TBARS in various brain areas (Figure 4), 6-OHDA-induced ROS may increase lipid peroxidation. Quantitative measurement of TBARS in the right brain revealed a significant effect of treatments in the midbrain \( F(11,35) = 45.3, p < .0001 \), striatum \( F(11,35) = 36.8, p < .0001 \), cortex \( F(11,35) = 15.8, p < .0001 \), and hippocampus \( F(11,35) = 9.2, p < .05 \). Whereas 6-OHDA alone induced increased TBARS in the four areas, GTP treatment significantly reduced it \( p < .05 \), particularly in the GTP 450 mg/kg/day-fed 6-OHDA-lesioned group (Figure 4). Therefore, GTP (150 mg/kg/day and 450 mg/kg/day) can decrease the level of lipids peroxidation in midbrain and striatum of unlesioned groups \( p < .05 \; \text{Figure 4} \).

**GTP Treatment Preserves Free Radical Scavenging Capability of the Brain**

Antioxidant ability was measured by the scavenging effects of the brain tissues on both superoxide (Figure 5, top) and hydroxyl (Figure 5, bottom) radicals using the EPR spin trapping technique. Quantitative analysis revealed a significant effect in both the midbrain (Figure 5, left) and striatum (Figure 5, right) of treatment [midbrain: superoxyde: \( F_{(25,71)} = 18.4, p < .0001 \); hydroxyl: \( F_{(25,71)} = 12.9, p < .0001 \); striatum: superoxyde: \( F_{(25,71)} = 10.2, p < .0001 \); hydroxyl: \( F_{(25,71)} = 25.6, p < .0001 \)], of the side of the brain [midbrain: superoxyde: \( F_{(11,35)} = 12.3, p < .0001 \); hydroxyl: \( F_{(11,35)} = 8.7, p < .0001 \); striatum: superoxyde: \( F_{(11,35)} = 9.7, p < .0001 \); hydroxyl: \( F_{(11,35)} = 17.5, p < .0001 \)], and of their interaction [midbrain: superoxyde: \( F_{(11,35)} = 11.2, p < .0001 \); hydroxyl: \( F_{(11,35)} = 11.5, p < .0001 \); striatum: superoxyde: \( F_{(11,35)} = 1.3, p = .27 \); hydroxyl: \( F_{(11,35)} = 10.1, p < .0001 \)]. The midbrain and striatal levels of superoxyde and hydroxyl radicals in the GTP 150 mg/kg/day-fed 6-OHDA-lesioned rats and GTP 450 mg/kg/day-fed 6-OHDA-lesioned rats were significantly higher than in the vehicle-fed 6-OHDA-lesioned animals \( p < .05 \); they were not different from the vehicle-fed sham-lesioned animals (Figure 5). In addition, GTP (150 mg/kg/day and 450 mg/kg/day) can increase the scavenging effects of superoxyde \( p < .05 \) and hydroxyl radicals \( p < .05 \) in midbrain and striatum of unlesioned groups (Figure 5). Therefore, GTP treatment may prevent 6-OHDA-induced decrease in the brain’s scavenging capability.

**GTP Treatment Prevents 6-OHDA-Induced Increase in Nitrite/Nitrate Content**

The nitrite/nitrate content in the midbrain and striatum was determined using the Griess method (25). Quantitative analysis revealed a significant effect in both the midbrain (Figure 6, left) and striatum (Figure 6, right) of treatment [midbrain: \( F_{(25,71)} = 75.4, p < .0001 \); striatum: \( F_{(25,71)} = 57.3, p < .0001 \)], of the side.

**Figure 3.** Reactive oxygen species (ROS) and nitric oxide (NO) levels detected by electron spin resonance (ESR) in midbrain (left) and striatum (right). The ESR spectra of ROS trapped by N-tert-butyl-a-phenylnitrone (PBN) and NO trapped by diethyldithiocarbamate-ferrous complex are shown both for midbrain (top left) and striatum (top right) homogenates. (A) left side of group A at 1 week, (B) right side of group B at 1 week, (C) right side of group B at 2 weeks, (D) right side of group C at 3 weeks, (E) right side of group C at 3 weeks, (F) right side of group D at 3 weeks, (G) left side of group C at 3 weeks, (H) left side of group D at 3 weeks. Quantitative data are shown for both ROS and NO in midbrain (bottom left) and striatum (bottom right). Groups are designated with capital letters: A, vehicle-fed sham-lesioned; B, vehicle-fed 6-hydroxydopamine (6-OHDA) lesioned; C, green tea polyphenol (GTP) 150 mg/kg/day-fed 6-OHDA-lesioned; D, GTP 450 mg/kg/day-fed 6-OHDA-lesioned; E, vehicle-fed unlesioned; F, GTP 150 mg/kg/day-fed unlesioned; G, GTP 450 mg/kg/day-fed unlesioned. n = 6 animals per group for each measurement. * Indicates significant differences from the vehicle-fed 6-OHDA-lesioned group and # indicates significant differences from the vehicle-fed 6-OHDA-lesioned and with GTP group (p < .05). L, left side of brain; R right side of brain.
of the brain [midbrain: F_{11,35} = 90.5, p < .0001; striatum: F_{11,35} = 69.1, p < .0001], and of their interaction [midbrain: F_{11,35} = 35.2, p < .0001; striatum: F_{11,35} = 8.1, p < .0001]. The midbrain and striatal levels of nitrite/nitrate in the GTP 150 mg/kg/day-fed 6-OHDA-lesioned rats and GTP 450 mg/kg/day-fed 6-OHDA-lesioned rats were significantly lower than in the vehicle-fed 6-OHDA-lesioned animals (p < .05) and either not different or significantly lower than in the vehicle-fed sham-lesioned animals (p < .05; Figure 6). In the unlesioned groups, GTP (150 mg/kg/day and 450 mg/kg/day) decreased the nitrite/nitrate content in midbrain and striatum (p < .05; Figure 6). Therefore, GTP treatment appears to prevent 6-OHDA-induced increase in nitrite/nitrate content in the brain.

**GTP Treatment Prevents 6-OHDA-Induced Increase in nNOS and iNOS Levels**

In the midbrain and striatum, NO produced by both nNOS and iNOS was detected by Western blot (Figure 7). Quantitative analysis revealed a significant effect in both the midbrain (Figure 7, left) and striatum (Figure 7, right) of treatment [midbrain: nNOS: F_{25,71} = 70.2, p < .0001; iNOS: F_{25,71} = 45.2, p < .0001; striatum: nNOS: F_{25,71} = 51.8, p < .0001; iNOS: F_{25,71} = 13.2, p < .0001], of the side of the brain [midbrain: nNOS: F_{11,35} = 25.2, p < .0001; iNOS: F_{11,35} = 19.6, p < .0001; striatum: nNOS: F_{11,35} = 20.9, p < .0001; iNOS: F_{11,35} = 6.1, p < .0001], and of their interaction [midbrain: nNOS: F_{11,35} = 16.2, p < .0001; iNOS: F_{11,35} = 8.1, p < .0001; striatum: nNOS: F_{11,35} = 17.3, p < .0001; iNOS: F_{11,35} = 6.0, p < .0001]. The midbrain and striatal levels of nNOS and iNOS in the GTP 450 mg/kg/day-fed 6-OHDA-lesioned rats were significantly lower than those in the vehicle-fed 6-OHDA-lesioned animals (p < .05) and not different from the vehicle-fed sham-lesioned animals (Figure 7). Therefore, GTP treatment appears to prevent 6-OHDA-induced increase in nNOS and iNOS, in keeping with data on NO (Figure 3) and nitrite/nitrate (Figure 6) generation.

**GTP Treatment Prevents 6-OHDA-Induced Increase in Protein-Bound 3-NT**

Ultimately, increase in NO, nitrite/nitrate, and peroxynitrite should result into production of protein-bound 3-NT in the brain (Figure 8). Quantitative analysis revealed a significant effect in both the midbrain (Figure 8, left) and striatum (Figure 8, right) of treatment [midbrain: F_{11,35} = 154.7, p < .0001; striatum: F_{11,35} = 15.6, p < .0001], of the side of the brain [midbrain: F_{5,17} = 309.6, p < .0001; striatum: F_{5,17} = 17.7, p < .0001], and of their interaction [midbrain: F_{5,17} = 38.1, p < .0001; striatum: F_{5,17} = 3.8, p < .05]. The midbrain and striatal levels of protein-bound 3-NT in the GTP 150 mg/kg/day-fed 6-OHDA-lesioned and GTP 450 mg/kg/day-fed 6-OHDA-lesioned rats were significantly lower than in the vehicle-fed 6-OHDA-lesioned animals (p < .05) and not different from the vehicle-fed sham-lesioned animals (Figure 8). Therefore, GTP treatment appears to prevent 6-OHDA-induced increase in protein-bound 3-NT, in keeping with the data on generation of NO (Figure 3), nitrite/nitrate (Figure 6), and nNOS and iNOS levels (Figure 7).

**Discussion**

This study shows that, in vivo, GTP partially protected TH-positive dopaminergic neurons (3.7 times more remaining neuron in GTP-treated than vehicle-treated animals) from 6-OHDA-induced cell death through ROS-NO pathway by 1) modulating ROS and NO levels, 2) preserving the free radical scavenging capability of the nigrostriatal pathway, 3) preventing increase in nitrite/nitrate levels, and 4) preventing increase in nOS and iNOS levels. These in vivo effects further extend previous in vitro observations from our lab (8) and others (7,10).

The 6-OHDA treated rats in our study showed a 95% decrease in TH-positive neurons, and GTP provided dose-dependent partial protection culminating at 81% of the lesion. In 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice, symptoms have been shown to appear when SNC neuronal death is of 43% and DA nerve terminal death in the striatum is 80%, as deduced from known kinetics of nigrostriatal degeneration in this animal.
model (32). From a behavioral point of view, a difference of 14% in TH-positive neurons corresponded to the difference between severe parkinsonian (i.e., similar to end-stage patients) and moderately impaired monkeys (i.e., similar to de novo patients) and other pharmacologic strategies with comparable efficacy on the number of neurons displayed clinically relevant behavioral efficacy in the MPTP-treated monkey model (33). Furthermore, such dramatic differences caused by relatively small differences in the absolute number of DA neurons are thought to be a consequence of the sparing of specific subset of SNc neurons that innervates more abundantly the motor striatum, suggesting that lesion of these neurons would precipitate the appearance and worsening of symptoms (34,35).

6-OHDA-derived free radicals damage cellular enzymes, induce the oxidation of membrane component, which may be responsible for the disruption of Ca$^{2+}$ homeostasis caused by 6-OHDA (36). Reactive oxygen species induced by 6-OHDA react with biological target molecules, induce the lipid peroxidation, and damage the mitochondrial membrane, resulting eventually in the collapse of mitochondrial membrane potential and thus leading to the disruption of Ca$^{2+}$ homeostasis. Several lines of evidences suggest that ROS-mediated 6-OHDA toxicity is due to formation of highly reactive quinone compounds and superoxide radicals via a nonenzymatic auto-oxidation process (37).

Both NO and NOS are known to be involved in the pathogenesis of PD (38,39). Moreover, elevated levels of iNOS have been reported in PD brain (40). Interestingly, GTP dose-dependently counteracts 6-OHDA-induced increase in NO and nitrite/nitrate levels and expression of nNOS and iNOS, as also reported in an MPTP-treated mice model of PD (6,41). The production of NO is a calmodulin-dependent process that is preceded by an elevation in intracellular Ca$^{2+}$ concentration (42), and we have shown in vitro that GTP inhibits such process (7). The expression and activity of iNOS are dependent on the activity of NF-κB. GTP are known to inhibit both 6-OHDA-induced NF-κB translocation and binding in SH-SY5Y cells (7). Together these data suggest

**Figure 5.** Scavenging capabilities of midbrain (left side) and striatum (right side) on superoxide generated from xanthine/xanthine oxidase (top half) and hydroxyl free radicals generated by the Fenton reaction (bottom half). Groups are named with capital letters: A, vehicle-fed sham-lesioned; B, vehicle-fed 6-hydroxydopamine (6-OHDA) lesioned; C, green tea polyphenols (GTP) 150 mg/kg/day-fed 6-OHDA-lesioned; D, GTP 450 mg/kg/day-fed 6-OHDA-lesioned; E, vehicle-fed unleesioned; F, GTP 150 mg/kg/day-fed unleesioned; G, GTP 450 mg/kg/day-fed unleesioned. n = 6 animals per group for each measurement. * indicates a significant difference with vehicle-fed sham-lesioned group and # with vehicle-fed 6-OHDA-lesioned group (p < .05). L, left side of brain; R right side of brain.
that GTP inhibits the elevation of NO by stabilizing 6-OHDA-impaired Ca$^{2+}$ homeostasis and reducing the activity of NF-/B.

Ultimately, increase in NO, nitrite/nitrate, and peroxynitrite should result in production of protein-bound 3-NT in the brain. Our results confirm this hypothesis because protein-bound 3-NT increased following 6-OHDA at both ends of the nigrostriatal pathway, further suggesting that peroxynitrite is produced in the nigra and the striatum after 6-OHDA administration, as shown in vitro (7). The production of peroxynitrite in vivo is highly dependent of metabolic pathways of NO and superoxide. Under normal conditions, the concentrations of NO and O$_2$- in brain cells are low, and it is difficult for them to react. Under pathological conditions, however, the production of ROS and NO increases, and they can thus form peroxynitrite (43). Miller et al. (44) showed that NOS could produce superoxide as well as NO in the presence of low arginine levels and that mtNOS could be a peroxynitrite synthase, thereby constituting an alternative source of peroxynitrite in vivo. Peroxynitrite can react with and damage many biological target molecules by oxidizing or nitrat- ing proteins, lipids, and DNA (43). Unfortunately, the cell membrane offers no significant barrier to diffusion of peroxynitrite within or between cells (45), thus allowing peroxynitrite to induce DNA damage (46) and to mediate oxidative stress activated p38 MAPK pathway (47).

Together our data and similar evidence in MPTP-treated mouse models of PD (6,41) further support the protective potential of GTP in PD. Treatment of animals with GTP alone also appears to have protective effects in our model but was not dose dependent. The experiment showed that GTP could penetrate the blood–brain barrier and remain for more than 24 hours (49), suggesting that GTP may be a safe and easily administrable drug (48).
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