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Original Contribution

# Protective effect of green tea polyphenols on the SH-SY5Y cells against 6-OHDA induced apoptosis through ROS–NO pathway

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**Abstract**

Green tea polyphenols (GTP) are thought to help prevent oxidative stress-related diseases, such as cancer, cardiovascular disease, neurodegenerative disease, and aging. We here investigate the protective mechanisms of GTP on SH-SY5Y cells against apoptosis induced by the pro-parkinsonian neurotoxin 6-hydroxydopamine (6-OHDA). GTP rescued the changes in condensed nuclear and apoptotic bodies, attenuated 6-OHDA-induced early apoptosis, prevented the decrease in mitochondrial membrane potential, and suppressed accumulation of reactive oxygen species (ROS) and of intracellular free $Ca^{2+}$. GTP also counteracted the 6-OHDA-induced nitric oxide increase and overexpression of nNOS and iNOS, and decreased the level of protein-bound 3-nitrotyrosine (3-NT). In addition, GTP inhibited the autooxidation of 6-OHDA and scavenged oxygen free radicals in a dose- and time-dependent manner. Our results show that the protective effects of GTP on SH-SY5Y cells are mediated, at least in part, by controlling the ROS–NO pathway.


*Keywords:* Parkinson's disease; Green tea polyphenols; Neuroprotection; Apoptosis; Reactive oxygen species; Nitric oxide; Peroxynitrite

## Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder affecting ~1% of the population over 65 years of age [1]. The main pathological characteristic of PD is the loss of pigmented dopamine (DA)-containing neurons of the substantia nigra pars compacta (SNc) associated with the presence of cytoplasmic α-synuclein-positive inclusions, the so-called Lewy bodies [2,3]. At present, only symptomatic treatments of PD are of proven efficacy. Therapeutic strategies that slow or stop the neurodegenerative process of PD are expected to have a major impact for the treatment of PD. To date, however, no drug has been established to have a clinically validated neuroprotective effect and none has been approved for a neuroprotective indication [4].

The etiology of PD is still not fully understood, but animal models, human postmortem material, and genetic analyses have provided important clues [5]. For instance, data from human postmortem tissue indicate that reactive oxygen species (ROS) and decrements in mitochondrial complex I activity are important in the pathogenesis of sporadic PD [6], suggesting that compounds interfering with ROS production and impairing mitochondrial complex I activity may be protective. Green tea is a popular beverage

*Abbreviations:* 6-OHDA, 6-hydroxydopamine; DAF-2DA, 4,5-diaminofluorescein diacetate; DCF-DA, 2′,7′-dichlorofluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EGCG, (−)-epigallocatechin gallate; ECG, (−)-epicatechin gallate; EGC, (−)-epigallocatechin; EC, (−)-epicatechin; EDTA, ethylenediaminotetraacetic acid; eNOS, endothelial cell nitric oxide synthase; Fluo-3 AM, Fluo-3 acetoxymethyl ester; GSH, glutathione reduced; GTP, green tea polyphenols; iNOS, inducible nitric oxide synthase; L-NMMA, $N^G$-methy-L-arginine acetate salt; 3-NT, 3-nitro-L-tyrosine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; $ONOO^-$, peroxynitrite; PBS, phosphate-buffered saline; PD, Parkinson's disease; PI, propidium iodide; ROS, reactive oxygen species.

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used worldwide that possesses many pharmacological effects, such as antimutagenic, antiproliferative, and anticarcinogenic properties, and, more importantly here, neuroprotection in models of degenerative disorders [7–11]. These properties are thought to be mediated by the green tea polyphenols, 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 cells were in the order ECG > EGCG > EC > EGC [12]. GTP and EGCG have been proven to be neuroprotective in both cell and animal models of PD [12–16]. The involvement of different signaling pathways in mediating these protective effects has only been suggested and none has been completely elucidated.

Cell death mechanisms could be studied through the use of mitochondrial complex I neurotoxins [17], such as 6-hydroxydopamine (6-OHDA). 6-OHDA is one of the most commonly used neurotoxins in in vitro and in vivo experimental PD models, which induces apoptosis in the catecholaminergic cells specifically. 6-OHDA can produce oxidative stress in vivo [18] as well as in vitro [19,20]. Seitz et al. [21] and Soto-Otero et al. [22] found that several toxic species were generated with the nonenzymatic autooxidation of 6-OHDA, including *p*-quinones [23], superoxide radicals, hydrogen peroxide, and hydroxyl radicals. On the other hand, 6-OHDA can interact with and inhibit complex I in isolated brain mitochondria [24], and induce a ROS-related collapse in mitochondrial membrane potential [20].

Therefore, we hypothesized that GTP might protect SH-SY5Y cells against 6-OHDA-induced apoptosis through the ROS–NO pathway. We then dissected out the mechanisms of this protection by measuring the ratio of apoptotic cells, the intracellular ROS, the mitochondrial membrane potential, the intracellular calcium concentration, the intracellular NO, the protein levels of nNOS and iNOS, the level of protein-bound 3-nitrotyrosine, and the scavenging effect on the autooxidation of 6-OHDA.

## Materials and methods

### *Reagents*

Dulbecco's modified Eagle's medium (DMEM), newborn calf serum, Hepes and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from GIBCO BRL (Grand Island, NY). 6-Hydroxydopamine, 2′,7′-dichlorofluorescein diacetate (DCF-DA), 4,5-diaminofluorescein diacetate (DAF-2DA), DNase I, ethylenediaminotetraacetic acid (EDTA), glutathione reduced (GSH), Hoechst 33258, $N^G$-methy-L-arginine acetate salt (L-NMMA), trypsin, penicillin, propidium iodide (PI), and streptomycin were purchased from Sigma Chemical Co. (St. Louis, MO); Fluo-3 acetoxymethyl ester (Fluo-3 AM) was purchased from Calbiochem (San Diego, CA); fluorescein isothiocyanate (FITC)-labeled Annexin V was purchased from PharMingen (San Diego, CA); rabbit polyclonal antibody for nNOS, iNOS, and eNOS were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-3-NT antibody was presented by Prof. Tangbin Yang. GTP, with purity above 98% (analyzed by HPLC), were a generous gift from Sichuan Full-green Biology Technology Co., Ltd. (Mianyang, China). All other chemicals made in China were analytical grade.

### *Cell culture and treatment*

Human neuroblastoma SH-SY5Y cells were maintained in a medium consisting of DMEM supplemented with heat-inactivated newborn calf serum (10%, v/v), penicillin (100 IU/ml), and streptomycin (100 μg/ml) in humidified 5% $CO_2$/95% air at 37°C. Cells were cultured at a density of $2 \times 10^5$ cells/ml on 96-well plates and $2 \times 10^6$ cells/ml in 25 $cm^2$ flasks according to a previously described protocol [25].

Cells were treated with different concentrations of 6-OHDA (1-500 μM) for 24 h to investigate the neurotoxicity of 6-OHDA. GTP (50, 100, 200 μM) were added 1 h before and then incubated with 100 μM 6-OHDA for 24 h. In order to remove the reaction of GTP with 6-OHDA in the medium, the cells were treated with GTP for 1 h, washed three times, and then incubated with 6-OHDA for an additional 24 h in another group of experiments.

### *Assessment of cell viability*

Cell viability was measured by quantitative colorimetric assay with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), showing the mitochondrial activity of living cells as described in the literature [26,27]. SH-SY5Y cells in 96-well plates were incubated with 6-OHDA for 24 h, and then 500 μg/ml MTT (final concentration) was added per well and cells were incubated at 37°C for 3 h. MTT was removed, and cells were lysed with dimethyl sulfoxide (DMSO). The absorbance at 595 nm was measured using a Bio-Rad 3350 microplate reader. Control cells were treated in the same way without 6-OHDA, and the values of different absorbances were expressed as a percentage of control.

### *Morphological changes*

The changes in nuclear morphology of apoptotic cells were investigated by labeling the cells with the nuclear stain Hoechst 33258 and examining them under fluorescent microscopy. After being treated with 6-OHDA and/or GTP for 24 h, the cells were fixed with Carnoy's fixative consisting of methanol and glacial acetic acid (3:1,v/v), and incubated with Hoechst 33258 (3 μg/ml) for 30 min, and then the nuclear morphology was observed under a fluorescence microscope (Olympus, kx14e). Cells that exhibited reduced nuclear size, chromatin condensation,