Different Effects of Five Catechins on 6-Hydroxydopamine-Induced Apoptosis in PC12 Cells

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Five catechins [(−)-epigallocatechins gallate (EGCG), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC), (−)-epicatechin (EC), and (+)-catechin (C)] were compared with regard to their effects on 6-hydroxydopamine (OHDA)-induced apoptosis in PC12 cells—the vitro model of Parkinson's disease. Measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, 6-OHDA inhibited cell viability in a time- and concentration-dependent manner. When PC12 cells were pretreated with the five catechins for 30 min before exposure to 250 μM 6-OHDA, MTT results showed that the five catechins had different effects: EGCG and ECG had obvious concentration-dependent protective effects at 50−400 μM; EC and (+)-C had almost no effects; and EGC especially decreased cell viability. Catechins also had different effects on apoptotic morphology. Only 200−400 μM EGCG and ECG kept cells adhering well. When pretreated with other catechins at any concentration, PC12 cells became round and some of them were detached as when treated with 6-OHDA. In addition, typical apoptotic characteristics of PC12 cells were determined by fluorescence microscopy, flow cytometry, and DNA fragment electrophoresis after the cells were treated with 250 μM 6-OHDA for 24 h or pretreated with catechins before it. Preincubation with 200−400 μM EGCG and ECG led to significant inhibitory effects against PC12 cell apoptosis, as shown by flow cytometry. The other catechins have little protective effect. Therefore, at 200−400 μM, the classified protective effects of the five catechins were in the order ECG > EGCG ≫ EC > (+)-C > EGC. The data also indicated that EGCG and ECG might be potent neuroprotective agents for Parkinson's disease. The results of fluorescence microscopy and DNA fragment analysis supported the conclusion.

Keywords: *Apoptosis; catechins; PC12 cells; 6-OHDA; Parkinson's disease; antioxidant*

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the degeneration of dopaminergic neurons in the substantia nigra zona compacta and the decrease of the neurotransmitter dopamine content in striatum (*1*, *2*). The incidence of PD is ∼10% in aged people over 65 by epidemiology investigation. At present, there are no therapy approaches to the treatment of PD that halt or retard the progression of nigral cell pathology. Drugs currently used for PD therapy can only improve clinical symptoms and, moreover, severe side effects will appear in the long-term treatment. Therefore, other approaches are critically needed for PD patiens. Very recently, neuroprotective strategy, a new method for pharmacotherapy of PD, has been proposed (*3*−*5*). On the basis of the understanding that the causes of PD are mainly oxidative stress and mitochondrial dysfunction (*6*, *7*), antioxidants, free radical scavengers, monoamine oxidase inhibitors, and other such drugs are expected to be used.

The study of antioxidants is becoming one of the most important and popular subjects for the research. Green tea polyphenols (GTPs) and their major catechin components, natural antioxidants and efficient free radical scavengers (*8*, *9*), have been shown to have diverse pharmacological activities, such as antimutagenic and anticarcingenic effects (*10*−*12*). In the central nervous system (CNS), there is also some evidence to show that oral administration of GTPs and flavonoid-related compounds has preventive effects on iron-induced lipid peroxide accumulation and age-related accumulation of neurotoxic lipid peroxides in rat brain (*13*, *14*). (−)-Epigallocatechins gallate (EGCG), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC), and (−)-epicatechin (EC), four components of GTPs, have been compared in their antioxidant activities and their protective effects against lipid peroxidation in synaptosomes (*8*, *9*). Salah et al. (*15*) also investigated the antioxidant activities of catechins.

To study the effects of catechins on PD, an in vitro model of PD was established as 6-hydroxydopamine (6-OHDA)-induced apoptosis in rat pheochromocytoma PC12 cells. 6-OHDA is a selective catecholaminergic neurotoxin (*16*), and the cytotoxicity of this neurotoxin has been thought to be based on the damage of dopaminergic neurons in two ways: it easily forms free radicals, and it is a potent inhibitor of the mitochondria respiratory chain complexes I and IV (*17*). Under physiological conditions, 6-OHDA is rapidly and nonenzymatically oxidized by molecular oxygen to form hydrogen peroxide and the corresponding *p*-quinone (*18*). The former can react with iron(II) to form the reactive and damaging hydroxyl free radical. In view

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of this autoxidation process and formation of ROS by 6-OHDA, EGCG and other catechins are expected to have great effects on the oxidative stress mediated pathogenesis process. The present study was initiated to evaluate the different effects of five catechins on 6-OHDA-induced apoptosis in PC12 cells.

MATERIALS AND METHODS

Materials. PC12 cells were supplied by the Medical School of Beijing University in China. RPMI 1640 cell culture medium, newborn calf serum, donor horse serum, HEPES, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from GIBCO Life Technologies. 6-OHDA, trypsin, sodium pyruvate, DMSO, PI, EB, agarose, proteiase K, DNA marker, Fluo-3.AM, BECEF.AM, Rodamine 123, Hoechst 33342, poly(D-lysine) (30000−70000), and EGCG, ECG, EGC, EC, and (+)-C were obtained from Sigma. All other chemicals were made in China and were of analytical grade.

Cell Culture. The rat pheochromacytoma PC12 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated donor horse serum, 5% heat-inactivated newborn calf serum, 100 units/mL pencillin, 100 μg/mL streptomycin, 10 mM HEPES, 1.0 mM sodium pyruvate, and 1.5 g/L $NaHCO_3$. All experiments were performed on the cells plated at a density of 2×10^5 cells/mL on 96-well plates and 2×10^6 cells/mL in poly(D-lysine)-coated 35 mm dishes or 25 cm^2 flasks. Cells were grown at 37 °C in 5% CO_2/95% air with 100% relative humidity. After 36−48 h, cells were exposed to 6-OHDA alone or pretreated with different concentrations of five catechins for 30 min, respectively.

Measure of Cell Viability by MTT Assay. MTT assay was performed as described previously with modification (*19*). Cells were processed with the MTT assay after 36 h of incubation. In the MTT assay, viable cells convert the soluble dye MTT to insoluble blue formazan crystals. Briefly, at the indicated time after the treatment, 1 mg of MTT [200 μL of a 5 mg/mL stock solution in phosphate-buffered saline (PBS)] was added per milliliter of medium and incubation continued at 37 °C for 3 h. The MTT was removed, and colored formazan was dissolved in dimethyl sulfoxide (DMSO). The absorbance (Abs) at 590 nm of each aliquot was determined with a Bio-Rad 3350 microplate reader. The reduction of PC12 cells in each well was expressed at the percentage of control cells.

Morphological Changes. *Morphological Changes of PC12 Cells under Phase Contrast Microscope.* Cells (2×10^6) were cultured in flasks for 36 h. After exposure to 6-OHDA in 250 μM or pretreatment with five catechins for 30 min before 6-OHDA addition, cells were observed under a phase contrast microscope.

Fluorescence Microscopy Assay. The changes of nuclear morphology of apoptosis were investigated by labeling the cells with the nuclear stain Hoechst 33258 (HO33258) and were visualized under fluorscence microscopy. Briefly, PC12 cells preplated in 35 mm dishes (5×10^5 cells/dish) were treated with 6-OHDA of different concentrations. Catechins were added 30 min before 6-OHDA addition if necessary. Treatments continued for 24 h, and then the cells were fixed and stained with Hoechst 33258 (3 μg/mL). Hoechst staining was observed under fluorescence microscopy. Apoptotic cells were distinguished from control cells by the presence of a fragmented or highly condensed nucleus.

Flow Cytometry Analysis. A flow cytometry analysis assay was performed as described previously with minor modification (*20*). Cells were trypsinized as a single cell suspension, harvested by centrifugation, and washed with PBS. After fixture in ice-cold 70% ethanol at −20 °C overnight, the cells were harvested by 300*g* centrifugation for 5 min, washed twice with PBS, resuspended in PBS supplemented with RNase A (100 μg/mL), and incubated at 37 °C for 30 min. Then the cells were stained with propidium iodide (PI; 3 μg/mL) at 4 °C for 30 min and then analyzed by flow cytometry (Coulter EPICS XL).

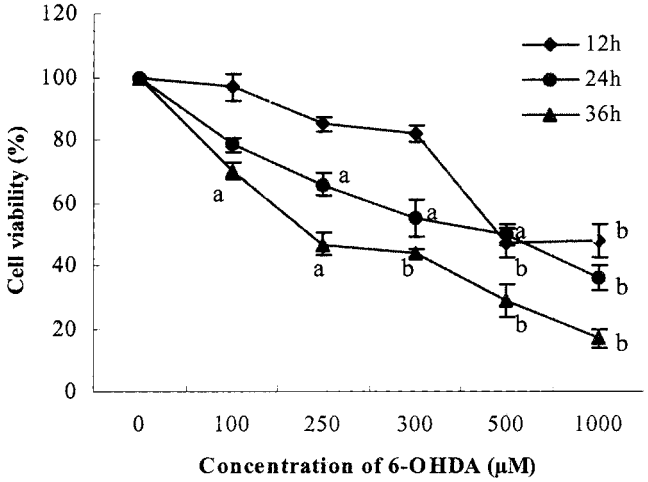

Figure 1. Effect of 6-OHDA on PC12 cell viability. PC12 cells were incubated in drug-free medium or medium containing different concentrations of 6-OHDA (0, 100, 250, 500, and 1000 μM). Cell viability was estimated by MTT assay after the cells had been treated with 6-OHDA for 12, 24, and 36 h: (a) $P < 0.05$ in comparison with normal cells; (b) $P < 0.01$ in comparison with normal cells. Data are mean ± SD, $n = 7$.

Analysis of DNA Fragmentation. After the treatment, cells were precipitated by centrifugation at 300*g*, washed twice with PBS, and then incubated with lysis buffer (100 mM NaCl; 10 mM Tris-HCl, pH 8.0; 25 mM EDTA, pH 8.0; 0.5% SDS) with added proteinase K (0.1 mg/mL) at 50 °C for 10 h. The nuclear lysates were extracted twice with phenol/chloroform/isoamyl ethanol (25:24:1). DNA was then precipitated with 0.1 volume of 10 mM ammonium acetate and 2 volumes of ice-cold ethanol at −20 °C overnight. DNA was pelleted by centrifugation at 12000*g* for 20 min at 4 °C, washed twice with 70% ethanol, and dried in air. After DNA was resuspended in 30 μL of TE (10 mM Tris-HCl/1 mM EDTA, pH 8.0) and 4 μL of RNase A for another 1.5 h at 37 °C, DNA was electrophoresed in a 1.2% agarose gel in TAE buffer (40 mM Tris-HCl/20 mM acetic acid/1 mM Na_2EDTA, pH 8.0). The gel was stained with 0.5 μg of ethidium bromiole (EB) and photographed by UV transillumination.

RESULTS

Different Effects of Five Catechins on Cell Viability. PC12 cell viability was greatly reduced when cells were exposed to 6-OHDA, and the cytotoxicity of 6-OHDA was concentration- and time-dependent (Figure 1). When PC12 cells were preincubated with catechins for 30 min before they were exposed to 250 μM 6-OHDA, the cell viability was very different. In 50−100 μM, no catechins had any obvious effects, but in 200−400 μM, their effects were very different. The cells pretreated with EGCG and ECG survived well (the cell viability was >90%), and cells preincubated with EC and (+)-C had ∼70−80% cell viability; EGC had no protective effects, but at 400 μM, it decreased cell viability (∼50%) (Figure 2). Therefore, at 200−400 μM, the five catechins were classified in the order ECG > EGCG ≫ EC> (+)-C > EGC.

Changes of Cell Morphology. After treatment with 250 μM 6-OHDA for 24 h, the cells had great changes in cell shape. Observed under a phase contrast microscope, the cells became round. Some of them (undergoing apoptosis) lost their ability to adhere to the plate surface and floated in the medium (Figure 3B). Pretreated with EGCG and ECG at 200−400 μM, the cells kept adhering on the plate surface well. The cells preincubated with EC, (+)-C, and EGC were little

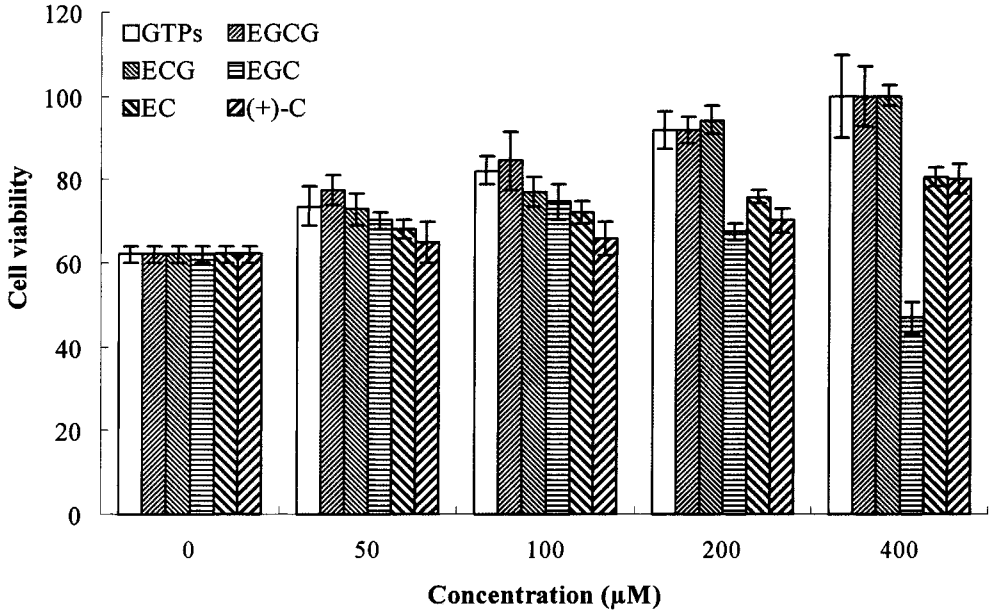

Figure 2. Effect of five catechins on 6-OHDA-induced decrease in PC12 cell viability. Cell viability was estimated by MTT assay after 24 h. Different concentrations of five catechins were added 30 min before 250 μM 6-OHDA treatments. Data are mean ± SD, $n = 7$.

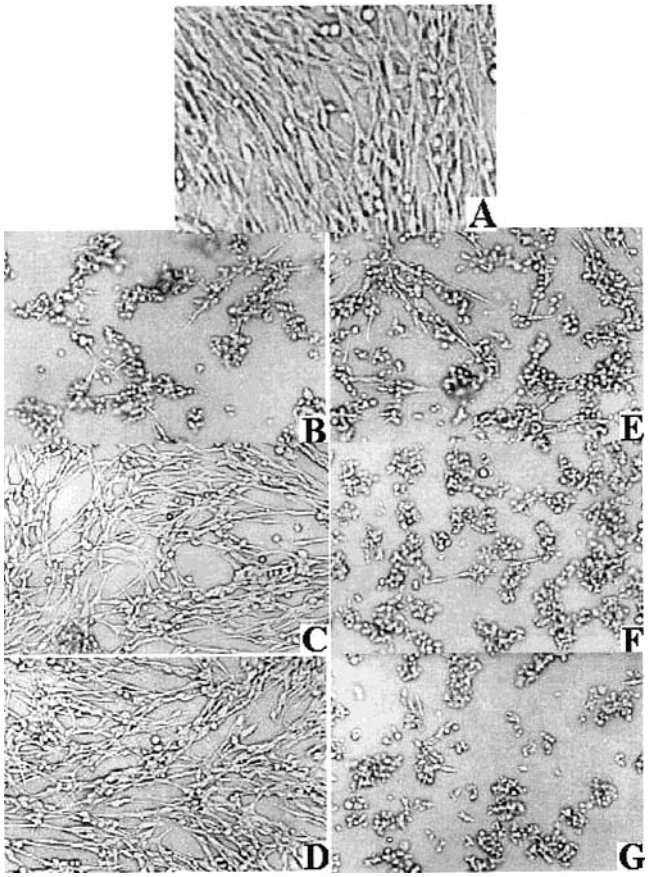

Figure 3. Effects of five catechins on morphology of PC12 cells poisoned with 250 μM 6-OHDA for 24 h observed under phase contrast microscope: (A) normal cells; (B) 6-OHDA-poisoned cells; (C) preincubated with 200 μM EGCG, (D) ECG, (E), EC, (F), (+)-C, or (G) EGC.

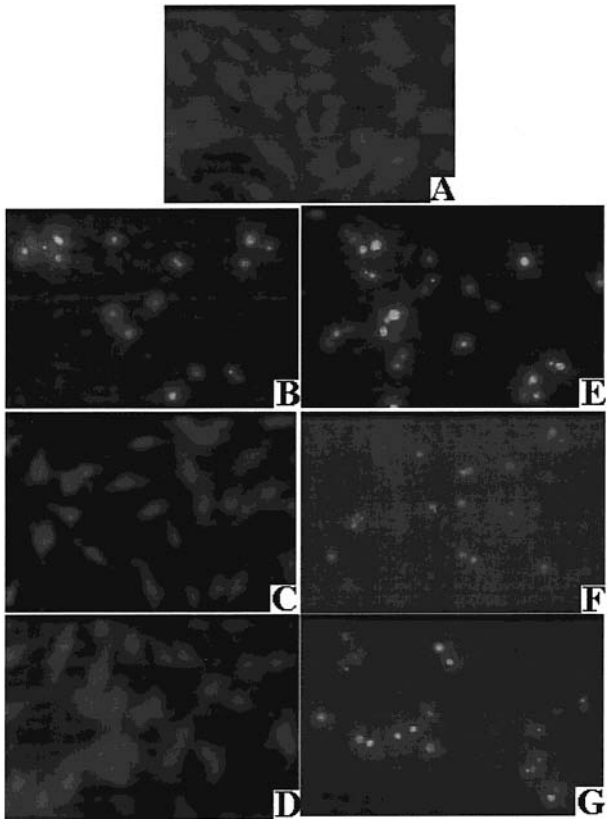

Figure 4. Fluorescence micrographs of PC12 cell nuclei from untreated cultures (A) and from cultures exposed to 250 μM 6-OHDA for 24 h (B) or preincubated with 200 μM EGCG (C), ECG (D), EC (E), (+)-C (F), or EGC (G). Cells were stained with the DNA-binding fluorochrome Hoechst 33258.

different from cells poisoned by 6-OHDA (Figure 3). The results showed EGCG and ECG had obviously protective effects.

Different Effects Shown by Changes of Nuclear Morphology. Apoptotic nuclei were apparent after exposure to 250 μM 6-OHDA for 24 h (Figure 4B). The nuclei condensed or the fragments appeared. These changes of nuclear characteristic of apoptosis disappeared or decreased in percentage in the cells pretreated with 200 μM EGCG and ECG (Figure 4C,D). Other catechins at 200 μM had no obvious effects (Figure 4).

Table 1. Effects of Five Catechins on the Apoptic Ratio of PC12 Cells Induced by 6-OHDA Detected by Flow Cytometry

group	apoptotic ratio[a] (%)
control cells	0.95 ± 0.63**
200 μM ECG	3.34 ± 0.68**
200 μM EGCG	4.59 ± 0.9**
200 μM EC	28.9 ± 3.6
200 μM (+)-C	32.7 ± 1.6
250 μM 6-OHDA-treated cells	32.8 ± 1.77
200 μM EGC	33.8 ± 4.04

[a] **, $P < 0.01$ in comparison with 6-OHDA-poisoned cells.

Percentage of Apoptosis. Flow cytometry can identify and determine the apoptotic cells based on DNA content analysis. In the cells undergoing apoptosis, DNA is degraded to low molecular weight DNA fragment with subsequent leakage from the cell. DNA content is decreased. When a number of cells containing apoptotic cells are stained with a DNA specific fluorochrome PI, a special DNA peak (usually called the sub-G_1 peak or apoptotic peak) appeared. This peak was thought to be one of the three characteristics of apoptosis. Table 1 and Figure 5 show the ratio of apoptotic cells. From the data of flow cytometry, after treatment of 250 μM 6-OHDA for 24 h, 32.5% cells underwent apoptosis. Preincubation with different concentrations of different catechins gave very different percentages of apoptotic cells: 200−400 μM EGCG and ECG inhibited the cytotoxicity of 6-OHDA significantly (the inhibitory ratios of EGCG were 88.3 and 90.3%; those of ECG were 91.9 and 90.5%, respectively). The inhibitory ratios of other catechins were <40%. EGC had little damaging effect, contrary to the protective effects of EGCG and ECG. The classified protective effects of five catechins were in the order ECG > EGCG ≫ EC > (+)-C > EGC. The result was the same as the result of cell viability.

Results of Agarose Gel Electrophoresis of DNA. One of the important hallmarks of apoptosis is the DNA fragmentation into multiples of 180−200 bp in length, which appear in the typical "DNA laddering" pattern on DNA electrophoresis gel. The obvious DNA ladder was observed in the cells treated with 250 μM 6-OHDA (Figure 6, lane 1). After pretreatment with the five catechins, respectively, the DNA ladder disappeared in the pattern of 200 and 400 μM EGCG and ECG but not in the pattern of any concentrations of other catechins. Figure 6 shows the result of DNA electrophoresis in cells pretreated with 200 μM concentrations of the five catechins.

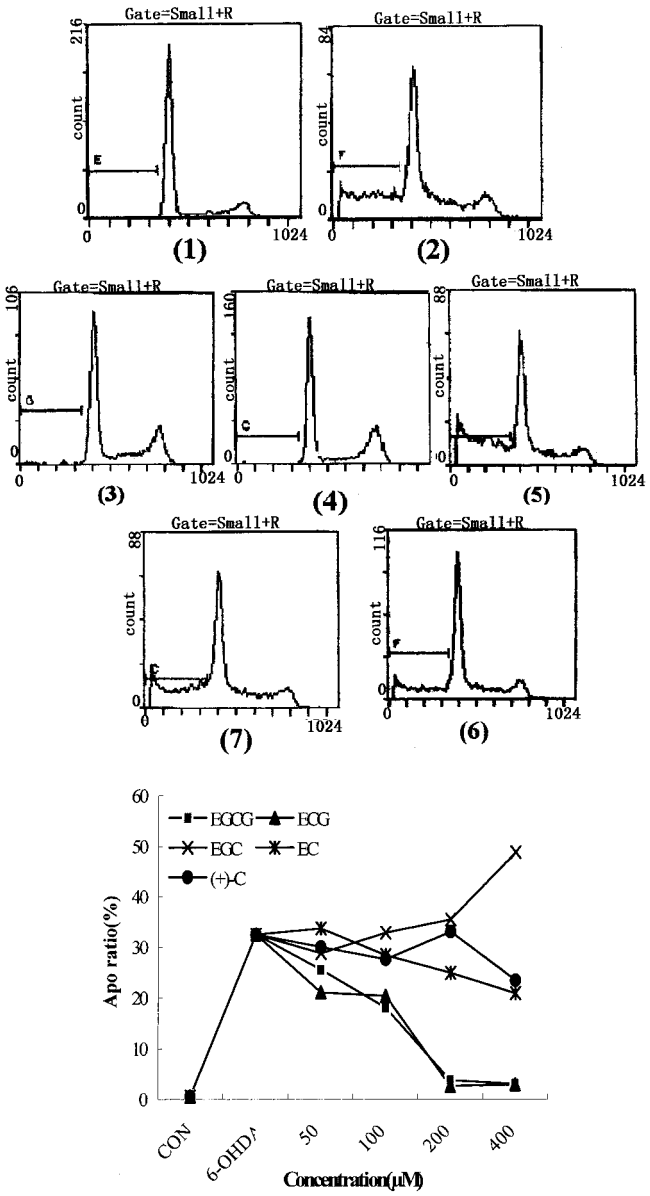

Figure 5. Flow cytometric analysis of PC12 cells after 24 h of treatment: (1) normal cells; (2) after exposure to 250 μM 6-OHDA only; and before 250 μM 6-OHDA treatment, with addition of (3) 200 μM EGCG, (4) ECG, (5), EGC, (6), EC, and (7), (+)-C. The *x*-axis is DNA content, and the *y*-axis is the number of cells. (Bottom panel) Flow cytometric analysis of the percentage of apoptotic PC12 cells after 24 h of treatment. Cells were exposed to 250 μM 6-OHDA only or preincubated with 50−400 μM EGCG, ECG, EGC, EC, or (+)-C for 30 min before 6-OHDA treatment.

DISCUSSION

Although the direct causes of the selective neurodegeneration in PD are not entirely clear, apoptosis has been suggested to be involved in this process (*21*, *22*). In vitro, 6-OHDA-induced apoptosis in PC12 cells (they secrete dopamine and possess a dopamine transporter) is frequently used to model PD-like cell loss. 6-OHDA is a mitochondrial complex I inhibitor and can reproduce PD-like cell loss in vivo (*23*). The mechanism of 6-OHDA toxicity has been investigated with positive and negative results (*17*, *18*, *24*−*27*). The possible mechanism of 6-OHDA-induced apoptosis includes two pathways. One is via extracellular toxicity of 6-OHDA. 6-OHDA uptake by PC12 cells via the dopamine transporter is unlikely, and its effects on PC12 cells are related neither to direct mitochondrial inhibition nor to enzymatic determination by monoamine oxidase but to generation of H_2O_2 through autoxidation (*26*, *27*). H_2O_2 and other ROS seem to play an essential role in 6-OHDA-induced apoptosis (*28*−*30*). The other pathway is that 6-OHDA crosses the cell membrane through a dopamine transporter (*23*) to inhibit the mitochondrial respiratory chain complexes I and IV and to generate intracellular ROS (*31*). Our result of the present study indicates that catechins might have effects on the toxicity of 6-OHDA either outside or inside the cell or both. A simple explanation is that catechins are antioxidants.

Catechins [EGCG, EGC, EC, (+)-C, and their corresponding epimers] have been studied with regard to their structure−antioxidant activity relationship (*8*). The results show that EGCG has the most efficient

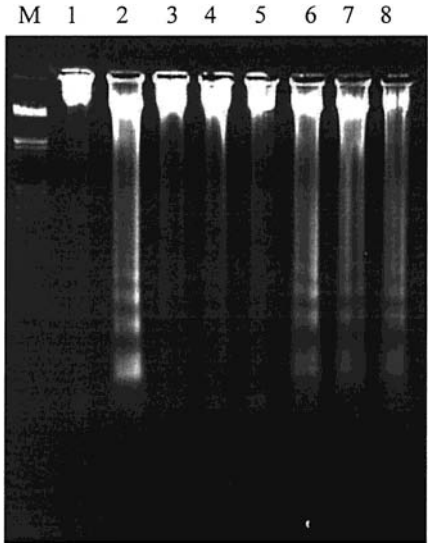

Figure 6. Agarose gel electrophoresis of DNA extracted from cells exposed to 250 μM 6-OHDA for 24 h: (lane 1) DNA marker; (lane 2) normal cells; (lane 3) cells damaged with 250 μM 6-OHDA only; (lanes 4−8) cells pretreated with 200 μM EGCG, ECG, EGC, EC, or (+)-C, respectively.

scavenging effects on ROS. The study of four catechins against lipid peroxidation showed that ECG and EGCG have more scavenging effects than EC and EGC on lipid free radicals: the order is ECG >EGCG > EC > EGC (*9*). The investigation into the antioxidant activities of catechins also showed that their order of effectiveness as radical scavengers was ECG > EGCG > EGC (*15*). Therefore, the antioxidation of catechins contributes to their protective effects against 6-OHDA-induced apoptosis in PC12 cells. Their different antioxidant activities give rise to their different effects on PC12 cells.

In this study, the results indicate that EGCG and ECG may possess neuroprotective property and ECG is more efficient than EGCG. Considering EGCG is the main component of GTPs (9−10% EGCG, only 3−6% ECG) and the contribution of EGCG to the total antioxidant activity of GTPs is almost one-third of all the antioxidant activities (ECG takes 7%) (*15*), EGCG is worth investigating for its neuroprotective property.

In conclusion, the results of this study show that different catechins have different effects on 6-OHDA-induced apoptosis in PC12 cells. The protective effects of catechins is in the order ECG > EGCG ≫ EC > (+)-C > EGC. The mechanism is related with their antioxidant activities. The results also show that EGCG and ECG may have value as neuroprotective agents.

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