

Protective and reversal effects of conserved dopamine neurotrophic factor on PC12 cells following 6-hydroxydopamine administration

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Abstract. Conserved dopamine neurotrophic factor (CDNF), a member of the mammalian mesencephalic astrocyte-derived neurotrophic factor family of conserved secreted factors, has been reported to protect and rescue dopaminergic neurons *in vivo*. PC12 pheochromocytoma cells are widely used as a cell model for Parkinson's disease (PD) for experimental studies. In the present study, PC12 cells were induced using 6-hydroxydopamine (6-OHDA) to mimic PD, which was used to investigate the protective and reversal effects of CDFN against PD *in vitro*. Cell growth was assessed using an MTT assay, the rate of cell apoptosis was detected using flow cytometry and the apoptotic morphology of cells was observed using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining. Pre-treatment of PC12 cells with CDFN (50, 100 and 200 nM) prior to exposure to 100 μ M 6-OHDA for 24 h, resulted in a significant increase in cell viability compared with that of 6-OHDA only-treated cells, with cell survival rates of 46.6, 54.7 and 69.6%, respectively. In addition, PC12 cells were treated with CDFN (50, 100 and 200 nM) following 6-OHDA administration, which resulted in cell survival rates of 47.7, 57.6 and 57.5%, respectively. Flow cytometric and TUNEL staining analyses revealed that CDFN exhibited significant dose-dependent protective and reversal effects on the apoptotic rate of PC12 cells following 6-OHDA treatment. In conclusion, the results of the present study showed that CDFN exhibited neuroprotective and reversal effects on the 6-OHDA-induced apoptosis of PC12 cells in a dose-dependent manner.

Introduction

Parkinson's disease (PD) is a prevalent neurodegenerative disease, which is neuropathologically characterized by the loss of substantia nigra pars compacta (SNpc) dopaminergic neurons, resulting in disabling motor abnormalities (1,2). Numerous studies have explored the pathogenesis of PD; however, the etiology of PD remains to be fully elucidated. Previous studies have indicated that mitochondrial dysfunction upregulates the production of reactive oxygen species (ROS), which initiates the neurodegeneration of dopaminergic cells *in vivo* and *in vitro* (3-6). 6-Hydroxydopamine (6-OHDA), a hydroxylated analogue of dopamine, was reported to induce ROS degeneration, therefore resulting in models of PD *in vivo* and *in vitro* (1,7-9). PC12 rat pheochromocytoma cells, derived from rat pheochromocytoma tumors, have numerous properties that are similar to those of dopaminergic neurons and are therefore widely used for studies investigating the pathogenesis and treatment of PD (10-12). In the present study, PC12 cells exposed to 6-OHDA served as a typical experimental model for the investigation of PD *in vitro*.

Conserved dopamine neurotrophic factor (CDNF), a member of the mammalian mesencephalic astrocyte-derived neurotrophic factor family, has been reported to significantly protect and reverse the loss of dopaminergic neurons due to 6-OHDA treatment *in vivo*, through the direct infusion of CDFN proteins or CDFN-engineered vectors into the brain (2,13-16). The present study aimed to investigate whether CDFN exhibited comparable neuroprotective and reversal effects in 6-OHDA-treated PC12 cells *in vitro*.

Materials and methods

Harvesting and identification of CDFN protein production. Total RNA was isolated from mouse heart and skeletal muscle using TRIzol[®] reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). First-strand cDNA was synthesized using SuperscriptII reverse transcriptase (Invitrogen Life Technologies) and then amplified through polymerase chain reaction (PCR). Recombinant mouse CDFN (pFastBacHTb-mCDFN) labeled with Flag and 6X His tags (Invitrogen Life Technologies) was produced through

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baculoviral expression in Sf9 insect cells (Invitrogen Life Technologies), as described previously (12). Secreted CDNF was purified from culture supernatants using nickel affinity chromatography followed by anion exchange chromatography. Rats were kept at the Anhui Province Key Laboratory of Brain Function & Brain Disease (Anhui, China) and all experimental procedures were performed in accordance with the Guidelines for Animal Care and Use of the National Institutes of Health. The present study was approved by the Ethical Committee of Anhui Provincial Hospital (Anhui, China).

Cell culture and drug treatment. PC12 cells were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China), and maintained in Dulbecco's modified Eagle's medium (DMEM; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Cells were treated with 6-OHDA (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) at concentrations of 1, 2.5, 5, 25, 50, 100 and 150 μ M and then cultured for 24 h in order to determine the optimal concentration of 6-OHDA for inducing PD model cells. PC12 cells were pre- or post-incubated with 50, 100 and 200 nM CDNF protein in order to investigate the protective and reversal effects of CDNF protein on 6-OHDA-induced neurotoxicity. The groups used in the present study were as follows: A, control; B, 6-OHDA treatment only; C, 6-OHDA + CDNF pre-treatment; and D, 6-OHDA + CDNF post-treatment.

MTT assay. Cell viability was measured using a colorimetric MTT assay. This method involves the cleavage of a yellow tetrazolium salt into a purple formazan compound through the dehydrogenase activity of intact mitochondria. In brief, cells were washed once with phosphate-buffered saline (PBS) prior to the addition of 0.1 ml serum-free medium containing MTT (1 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) to each well. Following incubation for 3 h, the supernatant was removed and the formazan product obtained was dissolved in 1 ml dimethylsulfoxide (Sigma-Aldrich) and incubated for 15 min with agitation on a microtiter plate shaker (CFJ-II; Shanghai Lei Yun Test Instrument Manufacturing Co., Ltd., Shanghai, China). The absorbance was then measured at 570 nm using an enzyme immunoassay instrument (DG5031; Shanghai Kehuai Instrument Co., Ltd, Shanghai, China). Cell viability was expressed as a percentage of that of the untreated cells, which served as the control group.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-mediated nick end labeling (TUNEL) staining. A TUNEL kit was purchased from Roche Diagnostics (Basel, Switzerland). Following 24 h of culture under the experimental conditions described above, PC12 cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h. Cells were then washed with PBS, treated with 0.3% H_2O_2 in methanol for 10 min and then washed with 0.1% sodium citrate containing 0.1% Triton X-100 (Santa Cruz Biotechnology, Inc.). In order to detect nuclear DNA fragmentation, the TUNEL reagent was applied to the fixed cells. In brief, cells were incubated with 50 μ l terminal deoxynucleotidyl transferase (TdT) solution (45 μ l equilibration buffer, 1 μ l biotin-11-deoxyuridine triphosphate and 4 μ l TdT enzyme; Beijing Zhongshan Golden

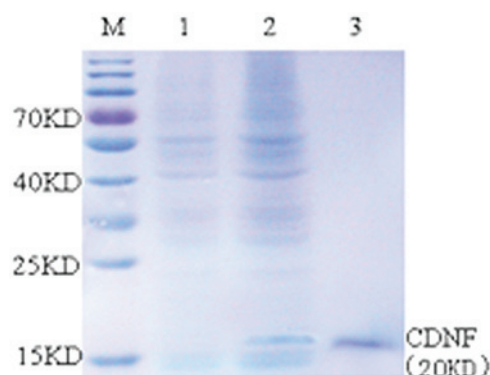


Figure 1. Reverse transcription-polymerase chain reaction was performed on conserved dopamine neurotrophic factor. Lane M, molecular weight protein markers; lane 1, control Sf9 supernatant; lane 2, supernatant from CDNF-expressing Sf9 cells; and lane 3, CDNF protein (20 kDa) eluted following nickel exchange chromatography.

Bridge Biotechnology Co., Ltd, Beijing, China) in the dark, in a humid atmosphere at 37°C for 60 min. Samples were then further incubated with 50 μ l streptavidin-horseradish peroxidase (HRP) solution (0.5 μ l streptavidin-HRP and 99.5 μ l PBS; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd) in the dark for 30 min at room temperature. In order to determine the rate of cell death, staining for peroxidase was performed using 100 μ l diaminobenzidine (DAB) solution (5 μ l of 20X DAB, 1 μ l of 30% H_2O_2 and 94 μ l PBS; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd) for 10 min. All samples were washed three times with PBS and then observed under a microscope (BX51; Olympus, Tokyo, Japan) in order to count the positive-stained cells. A minimum of ten fields were observed and >1,000 cells were counted in order to determine a statistically significant percentage of apoptotic cells. Data were expressed as a percentage of the control group, which was designated as 100%.

Apoptosis by flow cytometry analysis. In order to detect 6-OHDA-induced early apoptosis and late apoptosis/necrosis in the presence or absence of CDNF treatment. The collected media was centrifuged at 1,500 x g for 5 min and then supernatants placed at -80°C until further use for *in vitro* assays. PC12 cells (2×10^5 /well) were seeded onto six-well plates and cells in the corresponding groups were treated with CDNF. Cells were then harvested through centrifugation at 1,500 x g for 5 min, fixed with 70% ethanol (in PBS) at 4°C overnight. Cells were then resuspended in PBS, containing 40 μ g/ml propidium iodide (Invitrogen Life Technologies), 0.1 mg/ml RNase (Invitrogen Life Technologies) and 0.1% Triton X-100, and incubated in the dark for 30 min at 37°C. Cells were analyzed using a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA) with an argon ion laser at 488 nm. Data were collected using Cell Quest software (Becton-Dickinson).

Statistical analysis. Values are expressed as the mean \pm standard error of the mean. Statistical analysis between two groups was performed using Student's t-test with SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference between values.

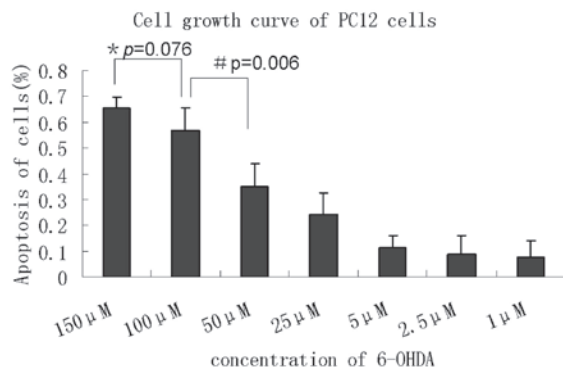


Figure 2. 6-OHDA-induced apoptosis in PC12 cells. * $P=0.076$ vs. $150\mu\text{M}$ 6-OHDA and # $P=0.006$ vs. $50\mu\text{M}$ 6-OHDA. 6-OHDA, 6-Hydroxydopamine.

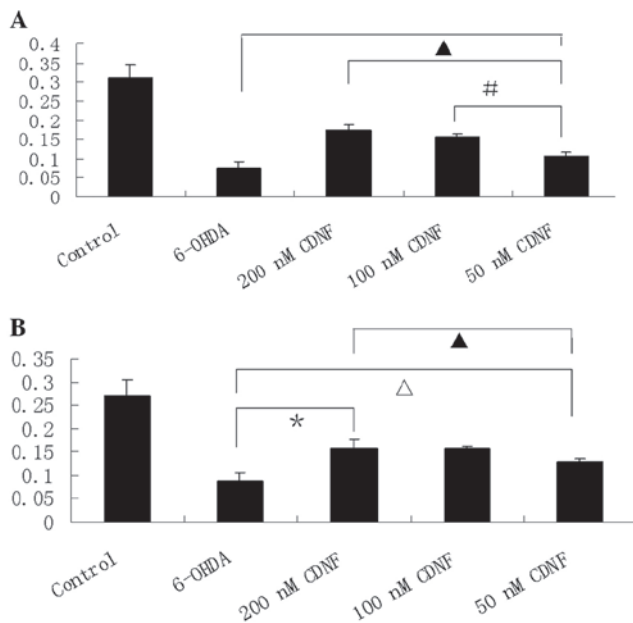


Figure 3. Pre- and post-treatment with CDNF protects and reverses the effect of 6-OHDA on PC12 cell viability. Cell viability of PC12 cells following treatment with CDNF (A) prior to 6-OHDA exposure for 24 h (* $P=0.000$ vs. 6-OHDA only; $\Delta P=0.006$ vs. 200 nM CDNF; $\#P=0.008$ vs. 100 nM CDNF) or (B) following 6-OHDA exposure for 24 h (* $P=0.015$ or $\Delta P=0.032$ vs. 6-OHDA; $\#P=0.103$ vs. 200 nM CDNF). CDNF, conserved dopamine neurotrophic factor; 6-OHDA, 6-hydroxydopamine.

Results

Harvesting and identifying CDNF. Total RNA was extracted from mouse heart and skeletal muscle, mouse CDNF cDNA was then reverse transcribed and amplified using PCR. Recombinant CDNF was produced using a baculovirus expression system in Sf9 cells, where CDNF was secreted into the culture medium and then purified (Fig. 1).

Cell viability of 6-OHDA-induced PC12 cells. In order to investigate the effect of 6-OHDA on cell viability in PC12 cells, the cells were incubated with 6-OHDA at concentrations of 1, 2.5, 5, 25, 50, 100 and $150\mu\text{M}$ and then cultured for 24 h. The viability of PC12 cells was then measured using an MTT assay. As shown in Fig. 2, at concentrations $<25\mu\text{M}$, 6-OHDA exhibited no significant effect on apoptosis in PC12

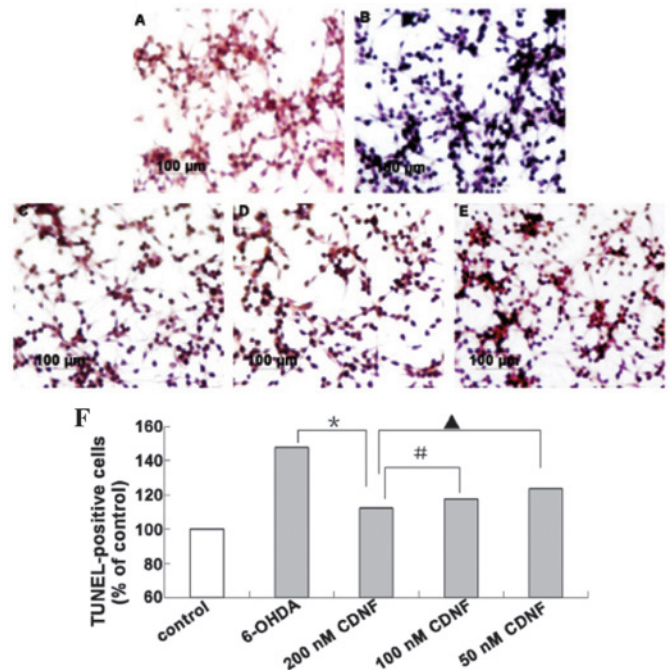


Figure 4. Protective effects of CDNF on 6-OHDA-induced apoptosis in PC12 cells. In order to detect apoptosis, TUNEL-staining was performed in PC12 cells treated as follows: (A) Control; (B) $100\mu\text{M}$ 6-OHDA; and (C) 200, (D) 100 and (E) 50 nM CDNF pre-treatment, respectively, followed by $100\mu\text{M}$ 6-OHDA. (F) Percentage of TUNEL-positive cells compared with that of the control cultures. Values are presented as the mean \pm standard error of them mean. * $P<0.05$ vs. 6-OHDA only; $\#P>0.05$ and $\Delta P<0.05$ vs. 200 nM CDNF. CDNF, conserved dopamine neurotrophic factor; 6-OHDA, 6-hydroxydopamine; TUNEL, terminal deoxynucleotidyl transferase deoxyuridine triphosphate-mediated nick end labeling.

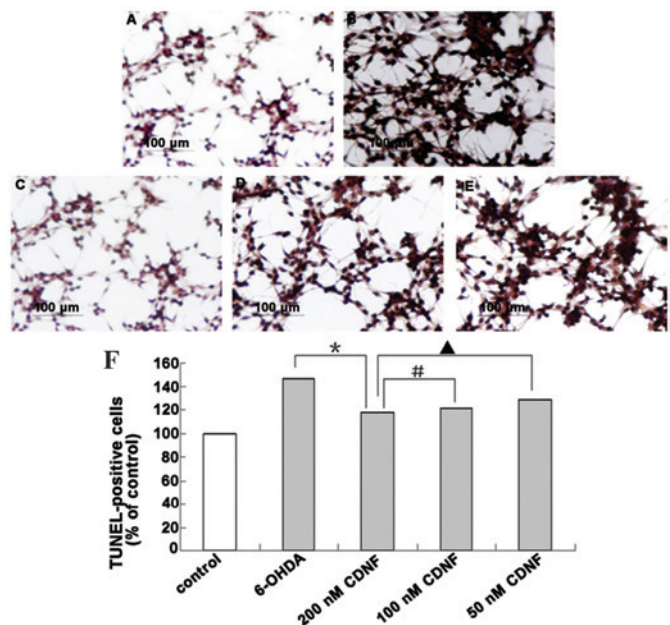


Figure 5. Reversal effects of CDNF on 6-OHDA-induced apoptosis in PC12 cells. In order to detect apoptosis, TUNEL-staining was performed in PC12 cells treated as follows: (A) Control; (B) $100\mu\text{M}$ 6-OHDA; (C-E) $100\mu\text{M}$ 6-OHDA followed by 200, 100 and 50 nM CDNF post-treatment, respectively. (F) Percentage of TUNEL-positive cells compared with that of the control cultures. Values are presented as the mean \pm standard error of them mean. * $P<0.05$ vs. 6-OHDA only; $\#P>0.05$ and $\Delta P<0.05$ vs. 200 nM CDNF. CDNF, conserved dopamine neurotrophic factor; 6-OHDA, 6-hydroxydopamine; TUNEL, terminal deoxynucleotidyl-mediated transferase deoxyuridine triphosphate nick end labeling.

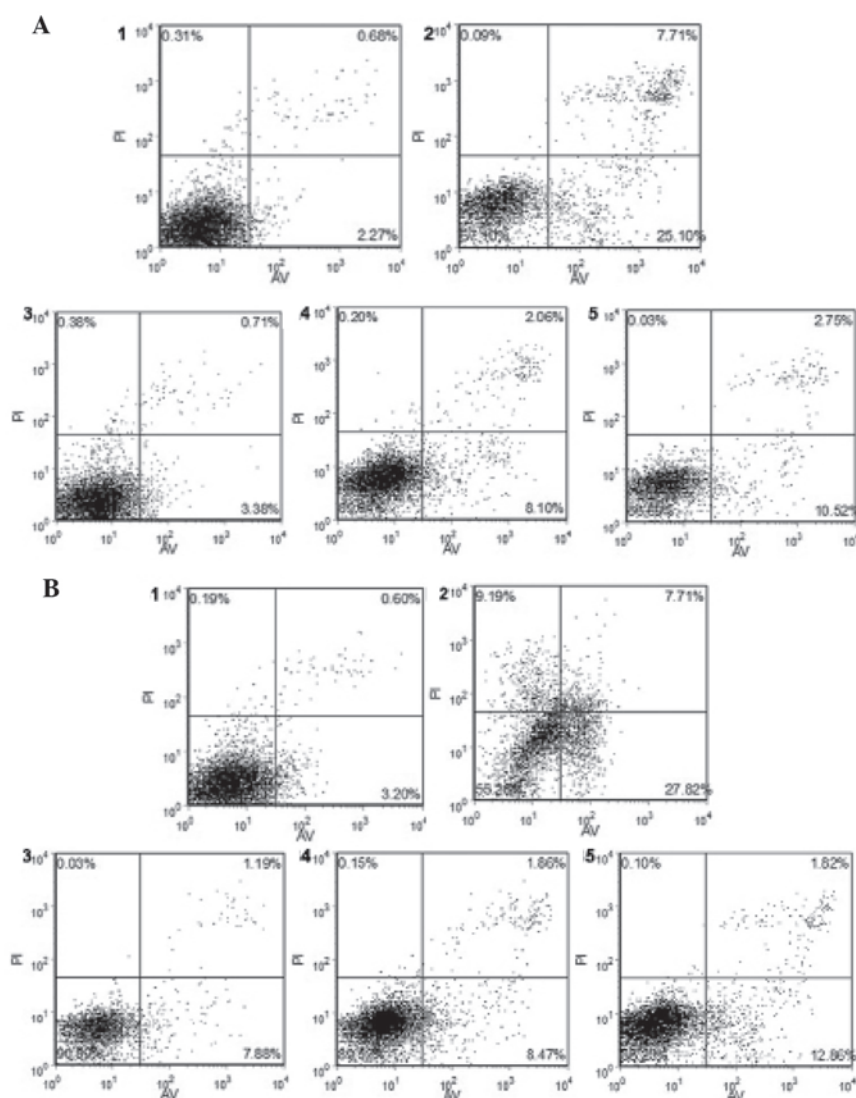


Figure 6. Flow cytometric analysis of PC12 cell apoptosis. PC12 cells were treated with CDFN (A) prior to and (B) following culture with 6-OHDA for 24 h. Cell groups were as follows: 1, control; 2, 100 μ M 6-OHDA; 3-5, 100 μ M 6-OHDA with pre- or post-treatment with 200, 100 and 50 nM CDFN, respectively. CDFN, conserved dopamine neurotrophic factor; 6-OHDA, 6-hydroxydopamine.

cells. However, significant increases in 6-OHDA-induced apoptosis occurred in a dose-dependent manner at concentrations $\geq 25 \mu$ M in PC12 cells. In addition, there was a marked increase in apoptosis between 50 and 100 μ M ($P=0.006$), which resulted in the apoptotic rate exceeding 50% at 100 μ M. Although no significant increase was observed between 100 and 150 μ M ($P=0.076$), the number of necrotic cells in the 150 μ M group was observably increased (Data not shown), which would interfere with the investigation of the reversal effect of CDFN protein. Therefore, in order to reduce the toxicity of 6-OHDA, 100 μ M was deemed the optimal experimental concentration (Fig. 2).

CDFN exerts protective and reversal effects on 6-OHDA-induced PC12 cell viability. PC12 cell viability was determined using an MTT assay. Following treatment with 6-OHDA alone (100 μ M), cell viability was significantly reduced (21.8%) compared with that of the control group. CDFN treatment (50, 100 and 200 nM) for 30 min prior to 6-OHDA (100 μ M) exposure for 24 h resulted in the

significant increase of PC12 cell viability compared with that of the 6-OHDA alone treatment, with survival rates of 46.6, 54.7 and 69.6% of control, respectively (Fig. 3A). In addition, PC12 cells incubated with CDFN following exposure to 6-OHDA for 24 h demonstrated a significant increase in cell viability compared with that of those treated with 6-OHDA alone, with survival rates of 47.7, 57.6 and 57.5% of control, respectively (Fig. 3B). These data therefore indicated that CDFN prevented and reversed the effects of 6-OHDA on cell viability.

Attenuation of 6-OHDA-induced apoptosis by CDFN. A distinctive biochemical characteristic of apoptotic cell death is the occurrence of internucleosomal DNA fragmentation. In the present study, apoptotic cells were assessed using TUNEL staining, a widely used method for detecting DNA fragmentation *in situ*. As shown in Fig. 4 and 5, following exposure to 100 μ M 6-OHDA, the number of TUNEL-positive PC12 cells was significantly increased compared with that of the control group ($P<0.05$). In addition, the anti-apoptotic effects

of CDNF (200, 100 and 50 nM) were examined in PC12 cells prior to and following 6-OHDA-induced apoptosis. As shown in Fig. 4, treatment of PC12 cells with CDNF (200, 100 and 50 nM) for 30 min prior to 6-OHDA exposure for 24 h resulted in a significant decrease in the number of TUNEL-positive cells compared with that of the 6-OHDA only group, with apoptotic rates of 112.47%, 117.87% and 123.65% of control, respectively. Furthermore, when incubated with CDNF following exposure to 6-OHDA for 24 h, the number of TUNEL-positive cells was also markedly decreased compared with that of the 6-OHDA only group, with apoptotic rates of 116.98%, 121.65 and 128.12% of control, respectively (Fig. 5).

Flow cytometric analysis of the effects of CDNF on 6-OHDA-induced apoptosis in PC12 cells. In order to examine the effects of 6-OHDA and CDNF on apoptosis in PC12 cells, flow cytometric analysis was performed. The results showed that following treatment with 6-OHDA alone, ~39.79% of cells were apoptotic at 24 h; however, pre-treatment of 6-OHDA-induced PC12 cells with CDNF (200, 100 and 50 nM) significantly reduced the apoptotic rates to 5.62, 10.36 and 13.31%, respectively (Fig. 6A). In addition, following incubation with CDNF protein (200, 100 and 50 nM) post-exposure to 6-OHDA for 24 h resulted in significantly decreased apoptotic rates of 9.1%, 10.48% and 14.78%, respectively (Fig. 6B).

Discussion

The present study demonstrated that 6-OHDA induced the decrease of PC12 cell viability to 21.8%, which was in accordance with a previous study by Gorman *et al* (17). In addition, the present study demonstrated that CDNF prevented and reversed the effect of 6-OHDA on PC12 cell apoptosis in a dose-dependent manner, suggesting a neuroprotective function in neuronal cells *in vitro*.

Previous studies have elucidated that neurotrophic factors, including BDNF and GDNF, may prevent neurodegeneration (18,19). In addition, GDNF was previously reported to improve symptoms in patients with PD; however, this potential treatment was found to have serious adverse effects and low clinical benefit (20-22). These findings encouraged novel studies into potential neurotrophic factors that may slow down or reverse the progression of neuronal degeneration. Lindholm *et al* (13) reported that CDNF exerted neuroprotective and reversed the neurodegenerative effects of dopaminergic neurons *in vivo*, which significantly decreased the loss of dopaminergic neurons in rat models induced by 6-OHDA and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, at the pathological and behavioral levels (2,13,14). The aim of the present study was to evaluate the neuroprotective effects of CDNF *in vitro*. PC12 cells pre-cultured with CDNF prior to 6-OHDA treatment exhibited increased cell viability, in a dose-dependent manner, as detected using an MTT assay. The maximum protective effect on cell viability was observed at a pre-culture concentration of 200 nM CDNF, which increased cell viability to 69.6%. In addition, pre-treatment with CDNF decreased the number of TUNEL-positive cells and the apoptotic rate of PC12 cells in a dose-dependent manner. Furthermore, in the present study, PC12 cells were

cultured with 6-OHDA for 24 h and then post-treated with various concentrations of CDNF. These results showed that cell viability was also increased compared with that of the control group. In addition, the reversal effects of CDNF were detected using TUNEL-staining and flow cytometry; however, the protective effects were marginally inferior to those of the CDNF pre-treatment. By contrast, the neuroprotective effect on cell viability of CDNF *in vitro* (200 nM, 69.6%) was markedly less potent than the reported effect of CDNF *in vivo* (96%) (13). The explanation for this may be due to the fact that the PC12 cell model induced using 6-OHDA is an acute model of PD, which is not completely consistent with the chronic progress of neurodegeneration *in vivo* (23-25). In addition, single cell models do not replicate the survival microenvironment of dopaminergic neurons *in vivo*. Another explanation may be that CDNF proteins stimulate other cytokines or signal pathways, which may be involved in neuroprotection *in vivo*. Notably, in the present study, the reversal effect of post-treatment with CDNF on cell viability (200 nM, 57.5%) was comparable to that of the reported effect *in vivo* (58%) (13). This may be attributed to the fact that 6-OHDA treatment led to later stages of apoptosis and necrosis in the PC12 cells pre-cultured with 6-OHDA, as shown in Fig. 5, whereas the earlier stages of apoptosis were more prominent following pre-treatment with CDNF. This therefore indicated that CDNF was unable to reverse the late stages of apoptosis and necrosis in PC12 cells.

Furthermore, the results of the present study also demonstrated that the preventive and reversal effects of CDNF occurred in a dose-dependent manner, regardless of pre- and post-incubation, which was consistent with those of other neurotrophic factors (26,27). In addition, the cell morphology observed by TUNEL-staining and cell apoptosis as determined using flow cytometry also demonstrated the dose-dependent neuroprotective and reversal effects of CDNF; in particular, the effects of CDNF on the protection against early apoptosis.

In conclusion, the results of the present study showed that CDNF exerted neuroprotective and reversal effects on 6-OHDA-induced PC12 cells in a dose-dependent manner. Therefore CDNF may have therapeutic potential for PD.

Acknowledgements

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