Proapoptotic effects of 2,5-hexanedione on pheochromocytoma cells via oxidative injury

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Abstract. N-hexanes are prominent environmental pollutants that are able to cause neurotoxicity *in vivo* and *in vitro*. Central and peripheral neuropathies induced by n-hexane exposure are a major health concern. 2,5-Hexanedione (2,5-HD) is the most significant neurotoxic metabolite of n-hexane; however, little is known regarding the underlying mechanism of its neurotoxicity. Thus, the aim of the present study was to investigate the damaging effects of 2,5-HD on pheochromocytoma PC12 cells, and to explore the underlying mechanism. Cell viability was tested using a Cell Counting Kit-8 method, and the leakage of lactate dehydrogenase (LDH) from cells was measured using an LDH assay kit. Glutathione peroxidase (GSHPx) and superoxide dismutase (SOD) activities, and the level of malondialdehyde (MDA) were determined using corresponding assay kits. Apoptotic cells were detected using an annexin V-fluorescein isothiocyanate/propidium iodide (PI) apoptosis kit, and were subsequently observed by fluorescence microscopy. The relative expression levels of cleaved-caspase-3, Bcl-associated-X protein (Bax) and Bcl-2 were identified by western blotting. The results revealed that 2,5-HD was able to decrease the viability of PC12 cells and promoted the leakage of LDH in a concentrationdependent manner. Further analysis demonstrated that 2,5-HD decreased the activity of the antioxidative enzymes, SOD and GSHPx, and led to an increase in the levels of MDA in the supernatant of cultured PC12 cells. The annexin V/PI staining results revealed that the numbers of apoptotic cells were increased following treatment with 2,5-HD. In addition, 2,5-HD (5 and 10 mmol/l) led to significant increases in the expression levels of caspase-3 and Bax, with the concomitant downregulation of Bcl-2. The antioxidant N-acetylcysteine was identified to antagonize 2,5-HD-stimulated cleaved-caspase-3 and Bax upregulation, and Bcl-2 downregulation. Collectively, the results of the present study suggested that 2,5-HD exerts proapoptotic effects on PC12 cells via oxidative injury. These findings may be applied in the development of novel therapeutic strategies to treat neurological disorders associated with nhexane exposure.

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Introduction

n-Hexanes are organic solvents that act as environmental pollutants, and have been demonstrated to cause neurotoxicity *in vivo* and *in vitro* (1). At present, central and peripheral neuropathies caused by nhexane present a major health concern (2-4). 2,5-Hexanedione (2,5-HD), the most significant neurotoxic metabolite of n-hexane, is involved in mediating this neurotoxicity (5,6). Previously published data from experimental and clinical studies have revealed that cell apoptosis induced by 2,5-HD is the major cause of the pathophysiological changes associated with reactive oxygen species (7). It is well established that apoptosis is mediated by several different pathways, with the mitochondrial pathway, which is involved in cell apoptosis (8). Previously published data have shown that perturbations in the mitochondrial-mediated pathway lead to the dysregulated expression of proteins, including Bcl-2-associated X protein (Bax) and Bcl-2, an increased rate mitochondrial cytochrome *c* release, an increase in the loss of mitochondrial transmembrane potential and the activation of caspase-3; these processes are all associated with cell apoptosis (9,10). Zhang *et al* (11) recently demonstrated that the cell injury induced by 2,5-HD is mainly mediated via the regulation of mitochondrial-dependent apoptosis.

The clonal line PC12, originally derived from a solid rat adrenal medulla tumor, has been widely used as a dopaminergic neuronal model for *in vitro* studies, and also serves as a principal cell model for investigations in molecular neuroscience (12,13). In addition, PC12 cells present as an excellent *in vitro* model to investigate certain aspects of various neurological disorders, including glutamate excitotoxicity (14), Parkinson's disease (15), Alzheimer's disease (16), and epilepsy (17); these cells have been reported to be useful in analyzing oxidative-stress-associated effects on neuronal cell survival (18-20). The production of reactive oxygen species (ROS) induced by environmental toxicants may elicit oxidative stress, which subsequently induces a state of imbalance

between the antioxidant defense system and the generation of free radicals (21). ROS serve an important role in cell signal-transduction pathways, thereby mediating numerous pathological and physiological processes (22). A number of previously published studies have indicated that oxidative stress acts as a trigger for cell apoptosis (23,24), and pretreatment with the antioxidant, N-acetylcysteine (NAC), can effectively reverse ROS-induced cell apoptosis (25,26). Thus, the aim of the present study was to examine the proapoptotic effects of 2,5-HD on pheochromocytoma PC12 cells, and to investigate the underlying mechanism. Taken together, the results obtained suggested that 2,5-HD is able to promote the proapoptotic effects on PC12 cells via oxidative injury. Our findings may contribute towards the development of novel strategies to treat neurological disorder diseases induced by n-hexane.

Materials and methods

Reagents and cell culture. RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Gibco (Thermo Fisher Scientific, Inc.). Rabbit cleaved-caspase-3 (cat. no. AC033) and Bcl-2 (cat. no. AB112) antibodies, mouse Bax (cat. no. AF0054) and β-actin (cat. no. AA128) antibodies were obtained from Beyotime Institute of Biotechnology. Assay kits for detecting superoxide dismutase (SOD; cat. no. A001-3), malondialdehyde (MDA; cat. no. A003-1) and glutathione peroxidase (GSH-Px; cat. no. A005) were purchased from Nanjing Jiancheng Bioengineering Institute. 2,5-HD (≥99.0% purity; cat. no. 00770) was purchased from Sigma-Aldrich (Merck KGaA). All reagents for cell culture were of tissue culture grade, and all other reagents were purchased from Sigma-Aldrich (Merck KGaA), except where indicated; all were of analytical grade.

Pheochromocytoma cells (PC12 cells) were originally obtained from the American Type Culture Collection. Cells were cultured in RPMI-1640 medium supplemented with 10% FBS, and 100 U/ml penicillin and streptomycin, and maintained at 37˚C in a humidified atmosphere with 5% CO_2. Prior to the experiments, cells were cultured in RPMI-1640 medium with 1% FBS for 12 h. In the present study, the condition of the controls comprised cells that were cultured in RPMI-1640 medium with 100 U/ml penicillin and streptomycin, and maintained at 37˚C in a humidified atmosphere with 5% CO_2; durations of treatment was consistent with the 2,5-HD treatment group. To investigate whether oxidative stress is the underlying cause of 2,5-HD-induced cell apoptosis, the cells were pretreated with 10 mmol/l NAC for 1.5 h at 37˚C. Subsequently, the cells were exposed to 5 mmol/l 2,5-HD or RPMI-1640 medium, which served as control treatment, for 12 h at 37˚C in a humidified atmosphere with 5% CO_2.

Cell viability assay. Cell viability was performed using Cell Counting Kit-8 (CCK-8) assay (KA1606; Abnova Corporation). PC12 cells (1-1.5x10^5/ml) were seeded into 96-well plates in the presence of different concentrations of 2,5HD (0.1-20 mmol/l) at 37˚C in a humidified atmosphere with 5% CO_2 for the indicated time periods. Subsequently, 20-200 μl CCK8 medium per well was added, and the mixture was incubated for a further 4 h at 5% CO_2 in an incubator at 37˚C. Optical density values were then measured at 490 nm for each well with a plate reader.

Lactate dehydrogenase (LDH) assay. PC12 cells (1-1.5x10^6/ml) were exposed to the different concentrations of 2,5HD (1-10 mmol/l) for 12 h at 37˚C in a humidified atmosphere with 5% CO_2. The measurement of LDH leakage was performed using an LDH activity assay kit (Beyotime Institute of Biotechnology), according to the manufacturer's protocols. LDH leakage rates were expressed as the percentage of the total LDH activity, according to the following equation: % LDH release rate=(LDH activity in medium/total LDH activity) x100%.

Measurement of antioxidative enzymes and MDA. The PC12 cells cultured in 24-well plates were treated with 2,5-HD at the concentration of (1-10 mmol/l) for 12 h at 37˚C in a humidified atmosphere with 5% CO_2, the cells were subsequently centrifuged at 251 x g for 10 min at 4˚C. The cells were lysed in 0.3 ml lysis buffer comprising 0.5% Triton X-100 in 0.1 mol/l potassium phosphate buffer, and homogenized via two freeze/thaw cycles, using sonication (3 sec, 25˚C) between the cycles. The homogenate was centrifuged at 1,006 x g for 20 min at 4˚C, and the supernatant was subjected to spectrophotometric analysis for thiobarbituric acid reactive substances the activities of antioxidant enzymes, according to the protocols provided by assay kits. In brief, thiobarbituric acid reactive substances were assessed by measuring the MDA concentration at 532 nm. SOD activity was assayed at 550 nm on the basis of its ability to inhibit the oxidation of hydroxylamine. GSH-Px activity was measured at 412 nm on the basis of the rate of oxidation of reduced glutathione to oxidized glutathione.

Apoptosis assay. PC12 cells were exposed to the different concentrations of 2,5-HD (1-10 mmol/l) for 12 h, and the apoptotic cells were identified using an annexin V-fluorescein isothiocyanate/propidium iodide (PI) apoptosis kit (Nanjing KeyGen Biotech Co., Ltd.). In brief, the cells (1-1.5x10^5) were incubated with 5 μl annexin V-fluorescein isothiocyanate and 5 μl PI (50 mg/ml) for 20 min at 25˚C in the dark, and immediately observed under a fluorescence microscope (BX51, Olympus Corporation; magnification, x200). Three fields of view were analyzed per sample.

Western blot analysis. Total cell lysates were obtained from PC12 cells using radioimmunoprecipitation assay buffer solution (Beyotime Institute of Biotechnology) containing proteinase and phosphatase inhibitors (Beijing Sangon Biotech Co., Ltd.). The protein concentration was determined using a bicinchoninic acid protein assay kit (Sigma-Aldrich; Merck KGaA). The supernatant (30 μg protein) from the cell lysates was separated using SDS-PAGE (10%) and blotted on to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc.). The membranes were subsequently blocked with 5% non-fat milk for 1.5 h in Tris-buffered saline (TBS)/Tween 20 buffer at 25˚C and then incubated overnight with the primary antibodies against caspase-3 (1:800), Bax (1:1,000), Bcl-2 (1:900), or β-actin (1:1,500; used as the control protein for normalization of the blots) at 4˚C. After washing three times

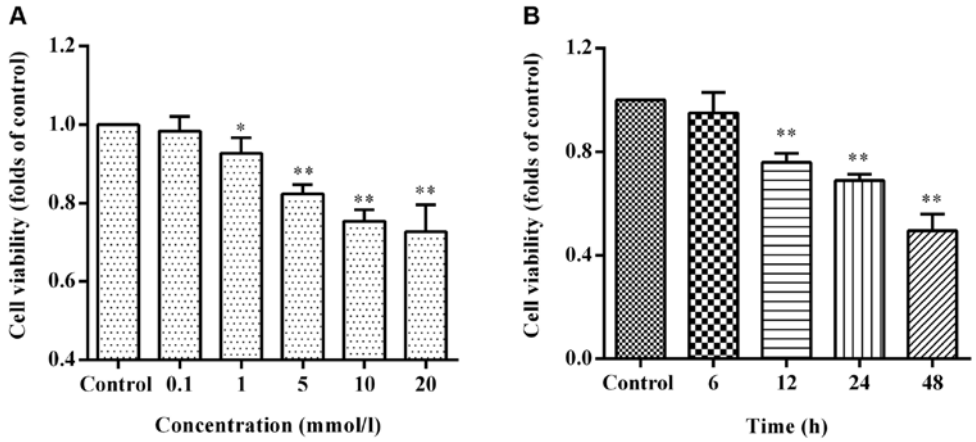

Figure 1. Effect of 2,5-hexanedione on the viability of PC12 cells. The cells were incubated with (A) different concentrations of 2,5-hexanedione for 12 h or (B) 5 mmol/l 2,5-hexanedione for the indicated durations. Cell viability was subsequently assessed using Cell Counting Kit-8 assay. The results are expressed as the mean ± standard error of the mean (n=6 independent experiments). *P<0.05; **P<0.01 vs. control.

with TBS, the membranes were incubated at 25˚C for 3 h with goat anti-mouse IgG-HRP (1:1,500; cat. no. sc-2005) or goat anti-rabbit IgG-HRP (1:1,500; cat. no. sc-2004); the second antibodies were purchased from Santa Cruz Biotechnology, Inc. Detection of the signal was performed using an enhanced chemiluminescence system (Pierce; Thermo Fisher Scientific, Inc.), and the intensity of the bands was determined via scanning and quantification using the Bio-Rad Gel Doc™ 2000 imaging system (Bio-Rad Laboratories, Inc.).

Statistical analysis. Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, Inc.). All data are presented as the mean ± standard error of the mean. Steer-Dwass or Mann-Whitney multiplecomparison tests were used to compare the differences among groups. P<0.05 was considered to indicate a statistically significant value.

Results

Effect of 2,5-HD on the viability of the PC-12 cells. To assess the effect of 2,5-HD on viability of PC12 cells, the cells were initially pretreated with the different concentrations of 2,5-HD (0-20 mol/l) for 12 h, and cell viability was subsequently detected using the CCK-8 method. As presented in Fig. 1A, from a concentration of 2,5-HD 1.0 mmol/l, the viability of PC12 cells significantly decreased in a concentrationdependent manner compared with the control. Additionally, when the cells were treated with 2,5-HD at 5 mmol/l, the viability of the cells significantly decreased from 12 h of treatment in a time-dependent manner compared with the control (Fig. 1B). Therefore, 2,5-HD may induce injury to PC12 cells.

2,5-HD increases the leakage of LDH of PC12 cells. To further verify the results of the cell viability experiments determined via the CCK-8 method, the leakage of LDH was assayed following treatment with 2,5-HD (1, 5 and 10 mmol/l) for 12 h. Our results indicated that 2,5-HD significantly promoted LDH leakage from 5 mmol/l 2,5-HD, in a concentration-dependent manner compared with the control (Fig. 2). These findings further suggested the damaging effects of 2,5-HD on PC12 cells.

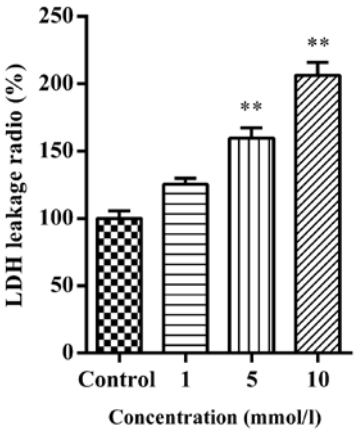

Figure 2. Effects of 2,5hexanedione on the release of LDH from PC12 cells. The cells were incubated with the different concentrations of 2,5-hexanedione for 12 h. Subsequently, the release of LDH was assayed using a spectrophotometric method. The results are expressed as the mean ± standard error of the mean (n=3 independent experiments). **P<0.05 vs. control. LDH, lactate dehydrogenase.

2,5-HD promotes the apoptosis of PC12 cells. Following treatment with 2,5-HD, the number of apoptotic cells increased (Fig. 3A). To gain insight into the mechanism of 2,5-HD-induced cell apoptosis, the protein expression levels of cleaved-caspase-3, Bax and Bcl-2 were detected. The results demonstrated that the levels of caspase-3 and Bax proteins in PC12 cells were significantly increased following treatment with 2,5-HD (5 and 10 mmol/l). Conversely, the expression levels of Bcl-2 were significantly decreased following treatment with 5 and 10 mmol/l 2,5-HD compared with the control. These findings further demonstrated that 2,5-HD promotes apoptosis of PC12 cells.

2,5-HD inhibits the activity of antioxidative enzymes and increases the level of MDA. Recent studies have shown that 2,5-HD promotes cell injury via regulating mitochondriadependent apoptosis (7,27). Oxidative stress leads to a state of imbalance between the levels of antioxidants that comprise the defense system and the production of free radicals (28). In the present study, our results demonstrated that 2,5-HD decreased

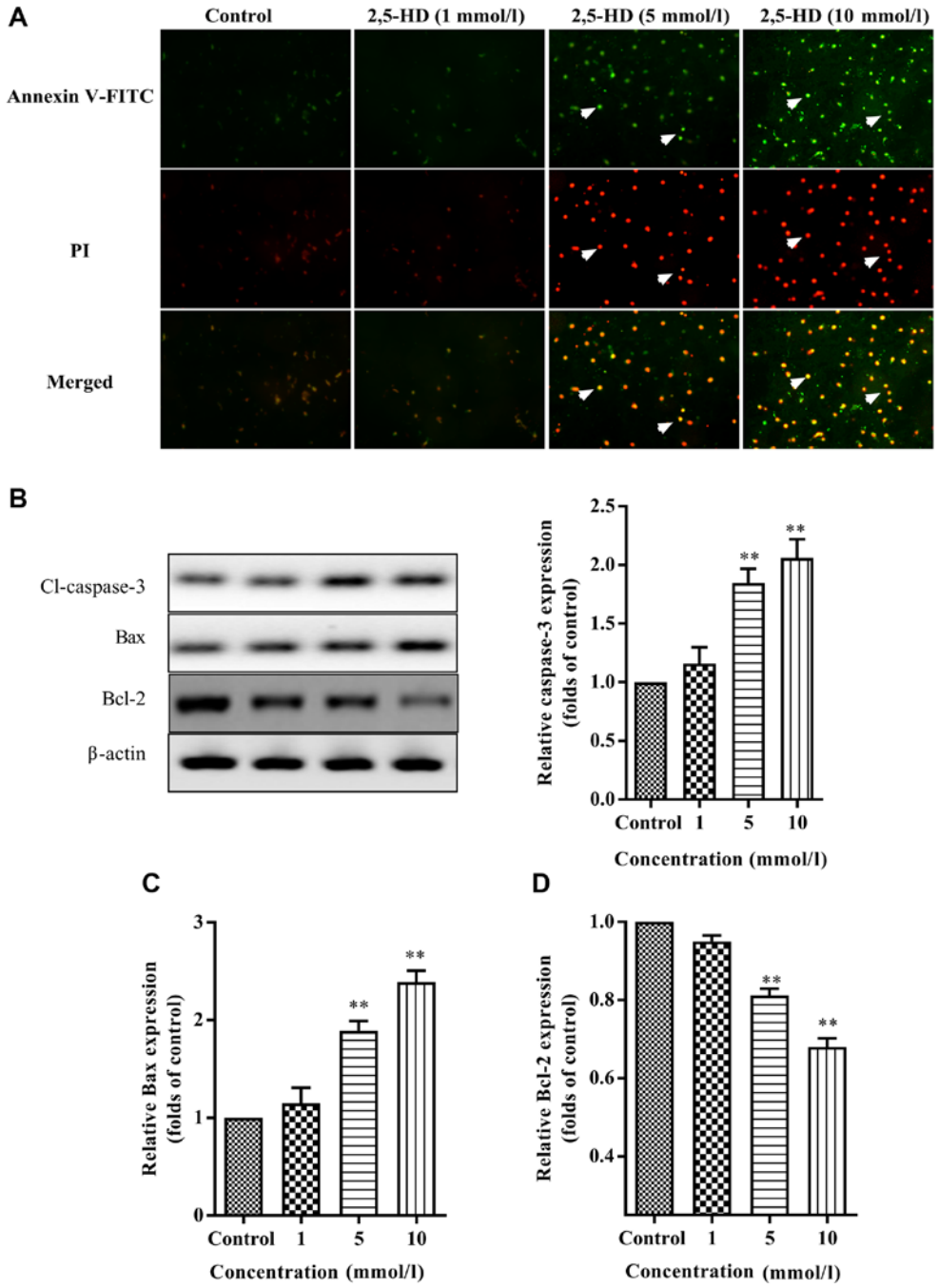

Figure 3. Effects of 2,5-hexanedione on cell apoptosis, and the levels of caspase-3, Bax and Bcl-2. The cells were incubated with the different concentrations of 2,5-hexanedione for 12 h. Subsequently, (A) cell apoptosis was identified via fluorescence microscopy (magnification, x200; green indicated early apoptotic cells and red indicated necrotic cells). The expression levels of (B) Cl-caspase-3, (C) Bax and (D) Bcl-2 were determined by western blotting. The results of three independent western blotting experiments for Cl-caspase-3, Bax and Bcl-2 are shown (expressed as the mean ± standard error of the mean). **P<0.01 vs. control. Cl-caspase-3, cleaved-caspase-3; Bax, Bcl-2-associated X protein.

the activity of the antioxidative enzymes, SOD and GSH-Px, in a concentration-dependent manner. On the contrary, 10 mmol/l 2,5-HD led to a significant increase in the levels of MDA in the supernatant compared with the control (Fig. 4). These results indicated that 2,5-HD was able to induce cell oxidative stress, and that this may account for the mechanism underlying 2,5-HD-induced cell apoptosis.

Antioxidant NAC effectively alleviates 2,5-HD-induced cell apoptosis. To further investigate whether oxidative stress is the underlying cause of 2,5-HD-induced cell apoptosis, the cells were pretreated with the antioxidant NAC 1.5 h prior to treatment with 2,5-HD. The results demonstrated that NAC was able to effectively decrease the protein levels of 2,5-HD-induced cleaved-caspase-3 and Bax, and increase the expression of Bcl-2. Of note, compared with the control group, antioxidant treatment alone did not alter the levels of cleaved-caspase-3 and Bax, and Bcl-2. These findings further supported that 2,5-HD-induced cell apoptosis could be mediated via oxidative stress (Fig. 5).

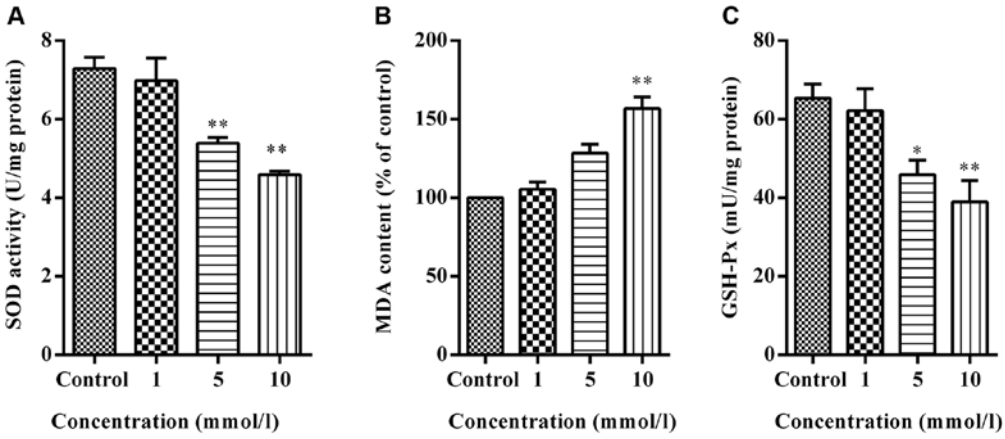

Figure 4. Effects of 2,5-hexanedione on the activity of antioxidative enzymes and MDA levels. The cells were incubated with different concentrations of 2,5-hexanedione for 12 h. Subsequently, (A-C) the activity of SOD and GSH-Px, and the concentrations of MDA were assayed using a spectrophotometric method. The results of three independent experiments are presented as the mean ± standard error of the mean. *P<0.05; **P<0.01 vs. control. SOD, superoxide dismutase; MDA, malondialdehyde; GSH-Px, glutathione peroxidase.

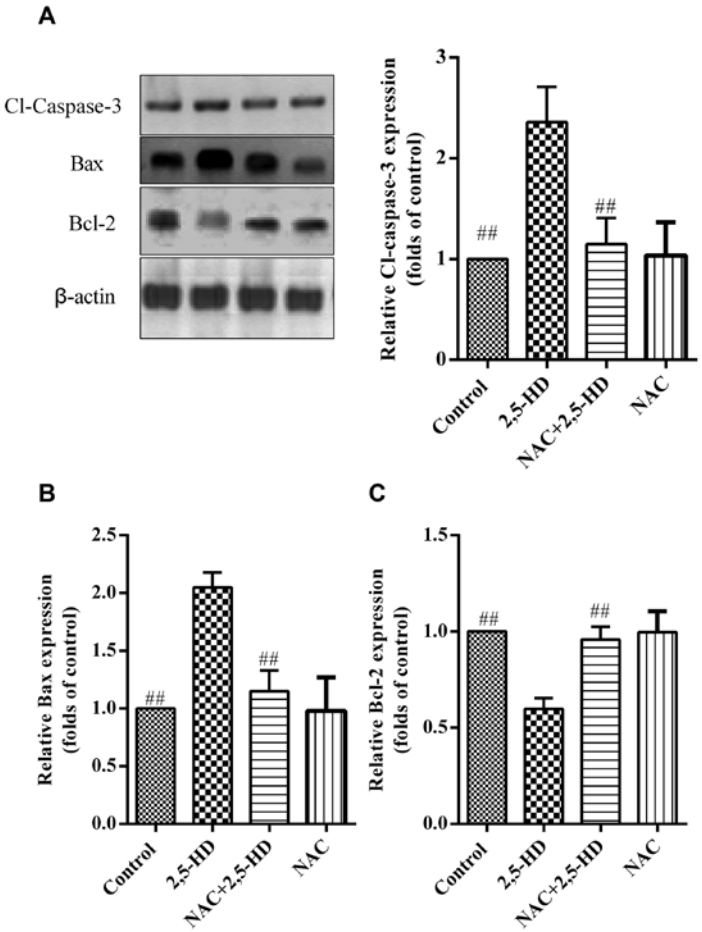

Figure 5. Antioxidant NAC effectively alleviates 2,5-HD-induced cell apoptosis. The cells were pretreated with 10 mmol/l NAC for 1.5 h. Subsequently, the cells were exposed to 5 mmol/l 2,5-HD for 12 h, and the expression levels of (A) Cl-caspase-3 (B), Bax and (C) Bcl-2 were assayed by western blot analysis. The results are expressed as the mean ± standard error of the mean (n=3 independent experiments). $^{\#\#}$P<0.01 vs. 2,5-HD. 2,5-HD, 2,5-hexanedione; Bax, Bcl-2-associated X protein; Cl-caspase-3, cleaved-caspase-3; NAC, N-acetylcysteine.

Discussion

Parkinson's disease, Alzheimer's disease and epilepsy are all neurological disorder diseases, and PC12 cells have served an excellent *in vitro* model to investigate certain aspects of various neurological disorders (29). 2,5-HD is the most important neurotoxic metabolite of n-hexane, and has elicited notable neurotoxic effects (30,31). The present study revealed that treatment with 2,5-HD led to marked decreases in the viability of PC12 cells and the activity of antioxidant enzymes;

however, increases in LDH leakage rate and the levels of MDA were noted. The subsequent experiments performed in the present study demonstrated that 2,5-HD upregulated the levels of the proapoptotic proteins, cleaved-caspase-3 and Bax, whereas the level of Bcl-2 was downregulated. Pretreatment of the cells with the antioxidant NAC appeared to have alleviated 2,5-HD-induced cell apoptosis.

Apoptosis occurs in a wide range of physiological and pathological situations (32). It is characterized by cell shrinkage, programmed DNA degradation, and the activation of caspases (33). Downregulation of Bcl-2 and upregulation of Bax is able to induce the release of cytochrome *c* from the mitochondria into the cytosol, which subsequently triggers the activation of caspase-3, resulting in cell apoptosis (34). The results of the present study demonstrated that the protein levels of proapoptotic cleaved-caspase-3 and Bax were significantly upregulated, while the expression of Bcl-2 was decreased, following treatment with 2,5-HD.

The abnormal release of LDH is an index of plasma membrane damage and cell apoptosis (35). Consistently, it has been observed that LDH leakage ratio was significantly increased following exposure to 2,5-HD (5 and 10 mmol/l) for 12 h in a concentration-dependent manner. ROS serve an important role in cell signaling, mediating numerous pathological and physiological processes, and act as a trigger for cell apoptosis (36). Glutathione serves an important role in shielding cellular macromolecules from endogenous and exogenous reactive oxygen or nitrogen species; another important antioxidative enzyme, SOD, is able to catalyze the dismutation of the superoxide radical (37,38). In the present study, GSH-Px and SOD activity were detected under *in vitro* conditions. The treatment of PC12 cells with different concentrations of 2,5-HD led to a decrease in the activity of these enzymes. MDA is a highly reactive compound that occurs naturally, and is a marker of oxidative stress (39). The present study revealed that the levels of MDA were markedly increased following cell exposure to different concentrations of 2,5-HD. In the present study, the antioxidant NAC was used to investigate whether ROS serve a role in 2,5-HD-induced cell apoptosis, according to Liu *et al* (7). Interestingly, pretreatment with the antioxidant NAC led to a marked downregulation in cleaved-caspase-3 and Bax expression, and Bcl-2 expression induced by 2,5-HD. Our findings collectively suggested that the proapoptotic effects of 2,5-HD on PC12 cells may be mediated via oxidative damage. Taken together, these results may provide be applied for the development of novel treatment strategies for neurological disorders.

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Availability of data and materials

All data sets used and/or generated during the present study are available from the corresponding author on reasonable request.

Authors' contributions

SX designed the study and performed experiments. BQ, YL and JW performed experiments and drafted the manuscript, ZZ, JL and JZ contributed to the literature search, performed experiments and interpreted the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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