Mol Cell Toxicol (2015) 11:79-87
DOI 10.1007/s13273-015-0010-x

ORIGINAL PAPER

2,5-Hexanedione induces apoptosis via a mitochondria-mediated pathway in PC12 cells

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Received: 21 October 2014 / Accepted: 27 February 2015

Abstract 2,5-Hexanedione (HD) is the main active metabolite of *n*-hexane and mediates the neurotoxicity of the parent compound. Studies suggested that apoptosis involved in HD neurotoxicity. However, the mechanism of HD-induced neuronal apoptosis remains unknown. To explore its underlying mechanism, we treated PC12 cells with 5, 10 and 20 mM HD for 24 h, respectively. We found that HD induced apoptotic death in PC12 cells in a dose-dependent manner. Moreover, HD down-regulated Bcl-2 expression, up-regulated Bax expression and Bax/Bcl-2 ratio, promoted the disruption of mitochondrial transmembrane potential, induced the release of cytochrome c from mitochondria, and increased the activity of caspase-3 in PC12 cells, which were all the key regulators of intrinsic apoptotic pathway. These results indicate that HD induces apoptosis via a mitochondria-mediated pathway in PC12 cells.

Keywords 2,5-Hexanedione, Apoptosis, PC12 cells, Mitochondria-mediated pathway, Neurotoxicity

The organic solvent *n*-hexane belongs to one of the most important aliphatic compounds and is widely used in the glue industry, varnishes, paints, shoe manufacturing, printing inks, shoe repair, and the food industry. Many studies have documented that *n*-hexane is neurotoxic and can cause central-peripheral neuropathy in both humans and experimental animals[1,2]. Since *n*-hexane neurotoxicity was described firstly in 1960s[3], tens of thousands of cases have reported in Asia[4], Europe[5], North America[6] and South America[7]. Therefore, *n*-hexane neurotoxicity has been a major health concern and occupational health hazard.

Metabolism studies have demonstrated that 2,5-Hexanedione (HD) is the main active metabolite of *n*-hexane and that this metabolite mediates the neurotoxicity of the parent compound[8]. Many evidences showed that HD directly induced a spectrum of cell body modifications of neurons[9-11]. Ogawa *et al*. also reported that dorsal root ganglion cells showed significant cell loss after HD exposure in vitro[12]. Strange *et al*. evaluated the effect of HD on the neocortical neurons and discovered that the total number and size of neurons were markedly reduced[13]. Zilz *et al*. reported that apoptosis was observed in human neuroblastoma line SK-N-SH exposed to HD[14]. Furthermore, Cui *et al*. indicated that expression of regulatory proteins related to apoptosis was disturbed in nerve tissue of HD-exposed rats[15]. These studies suggested that HD could induce apoptosis in nerve cells, and the induced apoptosis seemed to involve in HD neurotoxicity. However, the mechanism of HD-induced neuronal apoptosis remains unclear.

Experimental evidence suggests that apoptosis can be mediated by several different pathways and that there are numerous regulatory molecules associated with these pathways[16,17]. Mitochondrial pathway is a major signaling leading to apoptosis. It has been documented that mitochondria-mediated pathway includes the disturbance in the expression of Bax and Bcl-2,

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loss of mitochondrial transmembrane potential (MMP), release of cytochrome *c* from the mitochondria into the cytoplasm, activation of caspase-3 and ultimately trigger apoptosis[18,19]. In vitro studies indicated that HD induced human ovarian granulosa cell apoptosis by the regulation of Bax, Bcl-2, and caspase-3[20]. Moreover, Mishra *et al*. found the loss of MMP in spermatogenic cells exposed to HD[21]. Therefore, we are interested in whether mitochondria-mediated pathway involve in HD-induced apoptosis in nervous system.

PC12 cells, a rat pheochromocytoma cell line, possesses properties of neurons and exhibits cellular responses to toxicant that are similar to those found in human primary cultures. It has been used extensively as a model for studies of neurotoxicity. In the present study, viability and apoptosis were determined in HD-exposed PC12 cells by MTT assay, flow cytometry and Hoechst 33342 staining. Mitochondrial transmembrane potential (MMP) was examined by rhodamine 123 and fluorescence spectrophotometer. We also investigated the expression of Bax and Bcl-2, cytochrome *c* translocation and caspase-3 activity by real-time PCR, western blot and immunochemistry. This study aimed at investigating the apoptotic effect of HD on PC12 cells and its underlying mechanism.

Effect of HD on viability and apoptosis of PC12 cells

The effect of HD on viability and apoptosis of PC12 cells was examined by MTT assay (Figure 1), Hoechst 33342 staining and flow cytometry (Figure 2). MTT assay showed that the survival of PC12 cells decreased at 5, 10 and 20 mM HD in a concentration-dependent manner compared to control ($P<0.05$). The results indicated that HD had significant cytotoxic effects on PC12 cells. Meanwhile, the morphological phenotype of HD-exposed cells was assessed by Hoechst 33342 staining and fluorescent microscopy detection. As shown in Figure 2A, the nuclei of control cells were of a rounded shape with homogeneous intensity, and HD-exposed cells only showed apoptotic morphology, condensation and fragmentation, with heterogeneous intensity in the nuclei. It was demonstrated that HD exerted its death effect by apoptosis. The results were confirmed with the quantified assay through flow cytometry after staining with annexin V and PI. After HD treatment, the rates of apoptotic cells increased significantly in a concentration-dependent manner at 5, 10 and 20 mM in PC12 cells (Figure 2B). The cell scatter showed that the living cells, with intact cell membranes, were negative for both annexin V and PI in the lower left quadrant; cells at early stages of apoptosis were positive for annexin V and negative for PI in the lower right quadrant; cells with late apoptotic or necrotic were positive for both annexin V and PI in the upper right quadrant; whereas non-viable cells undergoing necrosis were negative for annexin V but excluded PI in the upper left quadrant (Figure 2B).

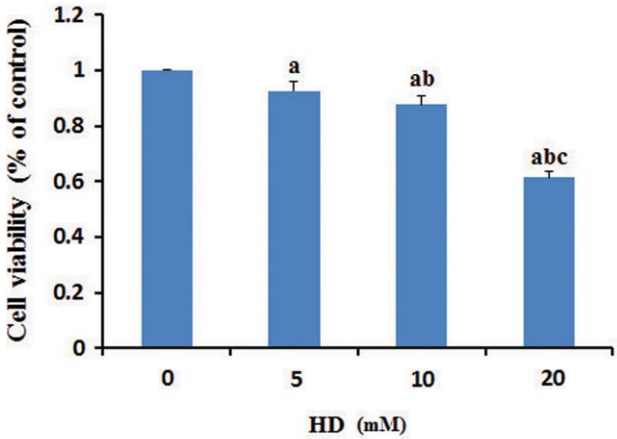

Figure 1. Viability of PC12 cells exposed to HD. PC12 cells were treated with 0, 5, 10 and 20 mM HD for 24 h. Then, MTT was used to assay the cell viability. Data were presented as mean ± SD from three independent experiments. $^{a}P<0.05$, vs. control group; $^{b}P<0.05$, vs. 5 mM HD group; $^{c}P<0.05$, vs. 10 mM HD group.

Effect of HD on the expression of Bax and Bcl-2

The effect of HD on the expression of Bax and Bcl-2 was determined by real time RT-PCR and western blot, respectively. The mRNA expression of Bax and Bcl-2 in HD-exposed PC12 cells at 0, 5, 10 and 20 mM was shown in Figure 3A. Compared with the control, HD-exposed cells showed a significant increase in mRNA expression of Bax ($P<0.05$). Moreover, mRNA expression of Bax increased in a concentration-dependent manner in the cells receiving HD with different concentrations. However, mRNA expression of Bcl-2 in HD-exposed cells was significantly lower than that in control group ($P<0.05$). As a marker for apoptosis, the alteration in Bax/Bcl-2 ratio is more sensitive and reliable than the change in individual Bax or Bcl-2. Compared with the control, the ratio of Bax/Bcl-2 gene expression significantly increased in HD-treated cells (Figure 3B).

The protein expression of Bax and Bcl-2 in HD-exposed cells is shown in Figure 4A. After treating with HD, protein level of Bax increased concentration-dependently in PC12 cells ($P<0.05$). On the contrary, the protein level of Bcl-2 markedly decreased ($P<0.05$). Compared to control cells, a significant increase in the ratio of Bax/Bcl-2 protein was observed in cells

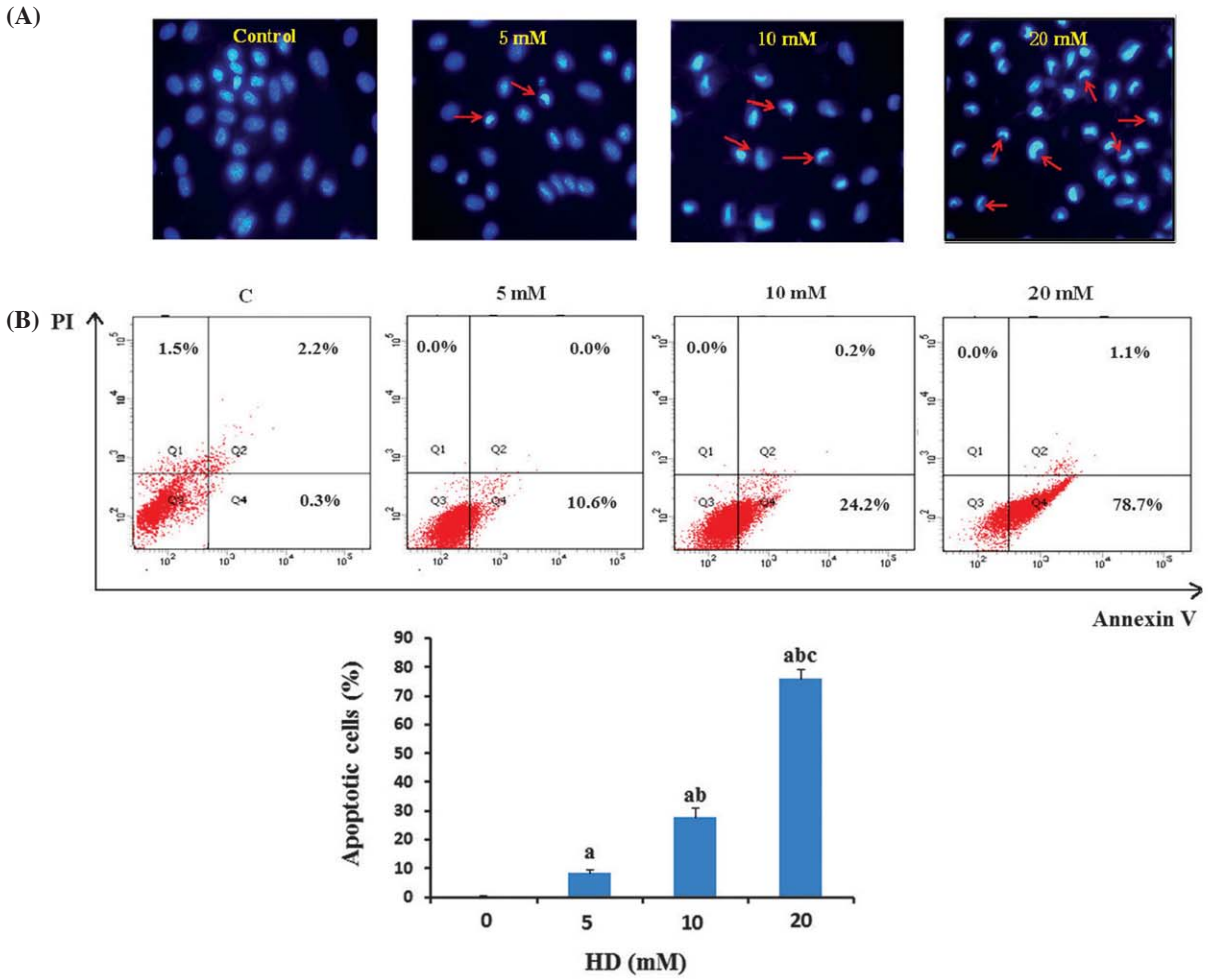

Figure 2. Apoptosis of PC12 cells exposed to HD. PC12 cells were treated with 0, 5, 10 and 20 mM HD for 24 h. A: PC12 cells were stained with the cell-permeable DNA dye Hoechst 33342 to examine the nuclear morphological changes in response to HD treatment and visualized by fluorescent microscopy (400 ×). Cells marked with arrow are apoptotic; B: Flow cytometry analyze of Annexin V/PI double-staining-labeled cells in each group. Data were presented as mean ± SD from three independent experiments. [a]$P<0.05$, vs. control group; [b]$P<0.05$, vs. 5 mM HD group; [c]$P<0.05$, vs. 10 mM HD group.

exposed to HD ($P<0.05$) (Figure 4C).

Effect of HD on MMP

Figure 5 compare MMP in control cells with cells treated with HD at various concentrations. Significant changes in MMP were observed in cells exposed to HD at all concentrations ($P<0.05$). Also, the dissipation of MMP induced by HD was concentration-dependent.

Effect of HD on cytochrome *c* release

Western blot analysis was performed to investigate the effect of HD on the changes of the expression level of cytochrome *c* in mitochondria and cytosol. Compared with the control, the protein levels of cytochrome *c* decreased in mitochondrial fraction (Figure 6A) and increased in cytosolic fraction (Figure 6B) in HD-exposed cells. Treatment of PC12 cells with HD for 24 hours facilitated cytochrome *c* release from mitochondria into cytosol as compared with the control condition. The release of cytochrome *c* from mitochondria to cytosol was significantly increased as dose-dependently.

Effect of HD on the activity of caspase-3

The activity of caspase-3 in HD-exposed cells is shown in Figure 7. The activity of caspase-3 in HD-exposed cells was significantly higher than that in control group ($P<0.05$). Moreover, the increase in the activity of

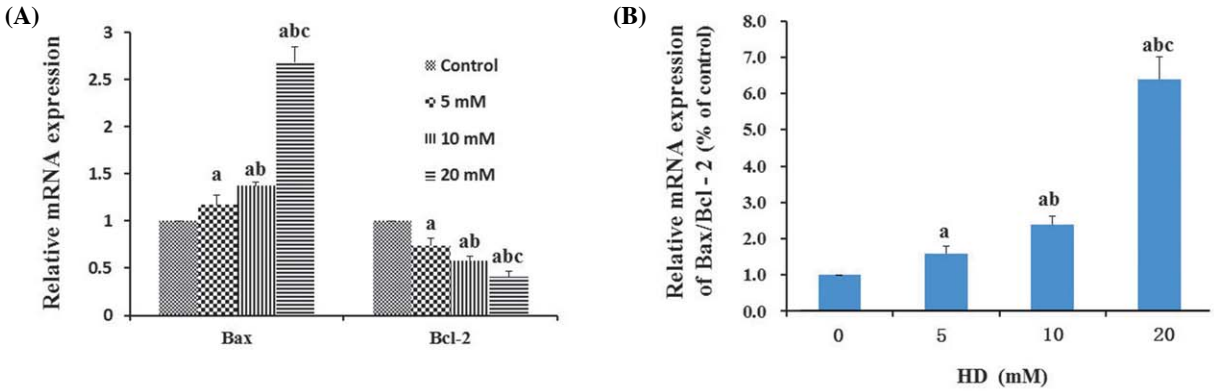

Figure 3. Expression levels of Bax and Bcl-2 mRNA in PC12 cells exposed to HD. PC12 cells were treated with 0, 5, 10 and 20 mM HD for 24 h. Then, real time RT-PCR was used to detect mRNA expression of Bax and Bcl-2. A: HD increased Bax mRNA expression and decreased Bcl-2 mRNA expression dose-dependently; B: The ratio of Bax/Bcl-2 was analyzed. Compared with the control, HD significantly increased the ratio of Bax/Bcl-2 in a dose-dependent manner. Data were presented as mean ± SD from three independent experiments. [a]$P < 0.05$, vs. control group; [b]$P < 0.05$, vs. 5 mM HD group; [c]$P < 0.05$, vs. 10 mM HD group.

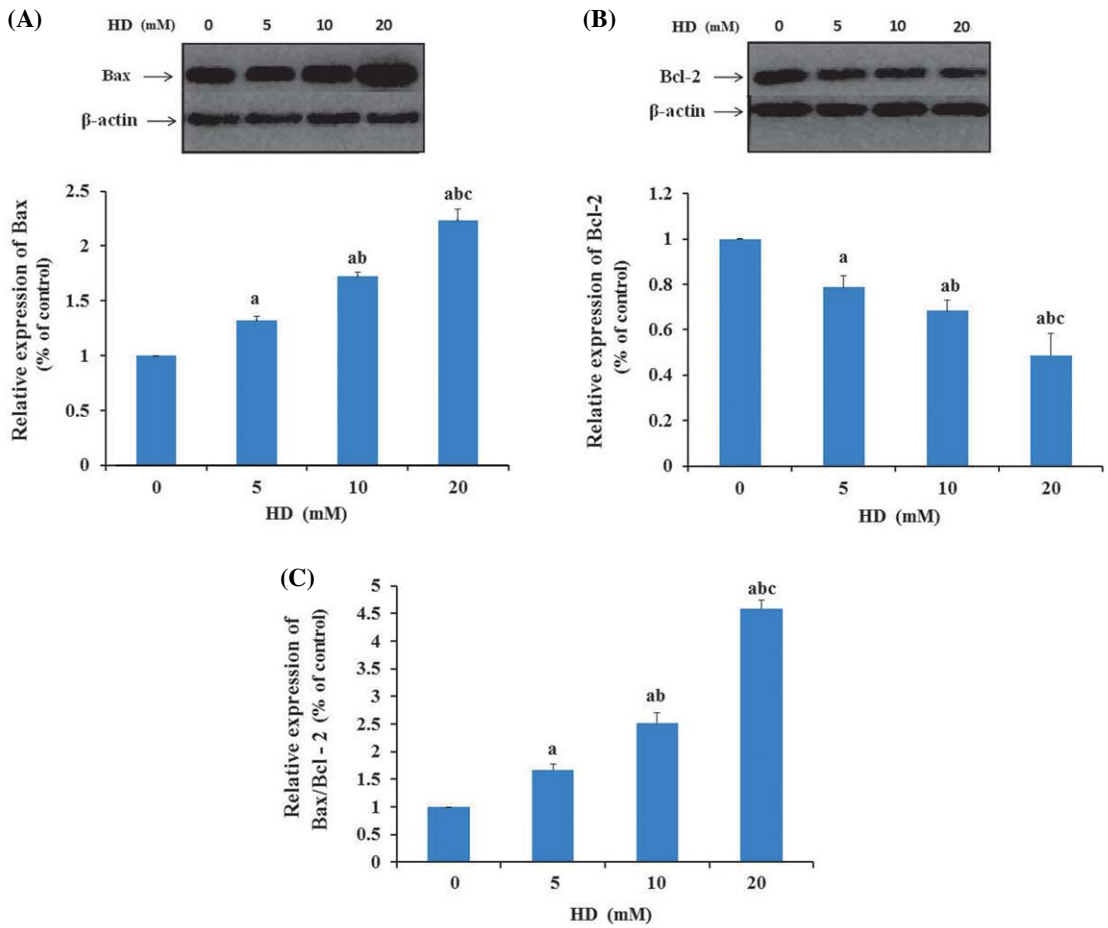

Figure 4. Expression levels of Bax and Bcl-2 protein in PC12 cells exposed to HD. PC12 cells were treated with 0, 5, 10 and 20 mM HD for 24 h. Then, Western blot was used to detect protein expression of Bax and Bcl-2. A: HD increased Bax protein expression dose-dependently; B: HD decreased Bcl-2 protein expression dose-dependently; C: The ratio of Bax/Bcl-2 protein expression significantly increased in a dose-dependent manner in HD-treated cells. Data were presented as mean ± SD from three independent experiments. [a]$P < 0.05$, vs. control group; [b]$P < 0.05$, vs. 5 mM HD group; [c]$P < 0.05$, vs. 10 mM HD group.

caspase-3 in HD-exposed cells was concentration-dependent.

Discussion

Apoptosis, also known as programmed cell death, plays an important role during neuronal development and in the homeostasis of the adult nervous system. However, an abnormal increase in apoptosis is the main form of cell death caused by various toxicants[23]. Some reports have showed that apoptosis involved in the neurotoxicity of HD[12-15]. Although the important role of apoptosis in the neurotoxic effects of HD has been indicated, the underlying mechanism of HD-induced apoptosis is not fully understood. Currently, a potential link between the mitochondrial apoptotic pathway and neuron loss is highlighted in the neurotoxic effects of several toxicants[24-26]. However, it remains unclear whether or not HD-induced neuronal apoptosis is associated with the activation of mitochondria-mediated pathway. In this study, our results showed that HD induced apoptotic death in PC12 cells in a dose-dependent manner. Moreover, we found that HD exposure disturbed the expression of apoptosis-related proteins, promoted the loss of MMP, induced the release of cytochrome *c* from mitochondria and increased the activity of caspase-3 in PC12 cells. The results demonstrated that HD could induce apoptosis via a mitochondria-dependent pathway in PC12 cells.

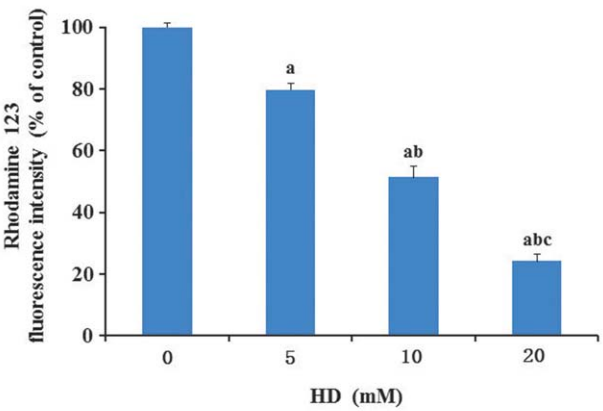

Figure 5. The MMP in PC12 cells exposed to HD. PC12 cells were treated with 0, 5, 10 and 20 mM HD for 24 h. Then, the dissipation of MMP was induced in a concentration-dependent manner after HD exposure. [a]$P<0.05$, vs. control group; [b]$P<0.05$, vs. 5 mM HD group; [c]$P<0.05$, vs. 10 mM HD group.

Mitochondria-dependent pathway is one of major signaling pathways leading to apoptosis and plays important roles in programming death induced by various toxicants[24-26]. Several proteins are known to be involved in the regulation of mitochondrial apoptosis, among them Bcl-2 family holds significant place[27,28]. The members of Bcl-2 family include both pro- and anti-apoptotic proteins, pro-apoptotic Bax and anti-apoptotic Bcl-2 belong to the most important members, which elicit opposing effects on mitochondria[29]. Under normal conditions, Bcl-2 complexes with Bax to neutralize the pro-apoptotic molecule and prevent cell death. Under apoptotic stimulation, however, it

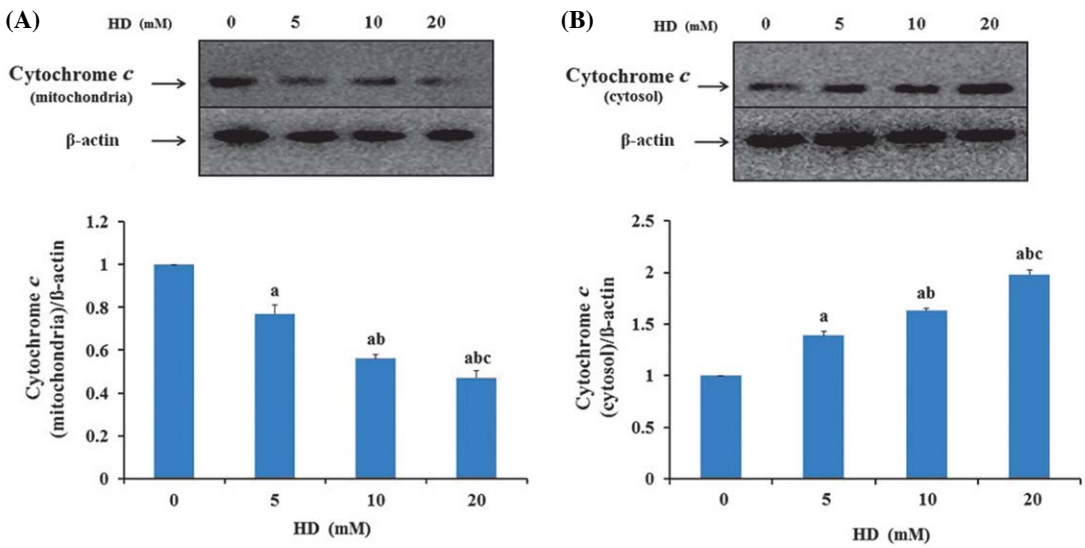

Figure 6. The release of cytochrome *c* in PC12 cells exposed to HD. Western blot was used to detect cytochrome *c* levels in mitochondrial fraction (A) and cytosolic fraction (B) in HD-exposed cells. Data were presented as mean ± SD from three independent experiments. [a]$P<0.05$, vs. control group; [b]$P<0.05$, vs. 5 mM HD group; [c]$P<0.05$, vs. 10 mM HD group.

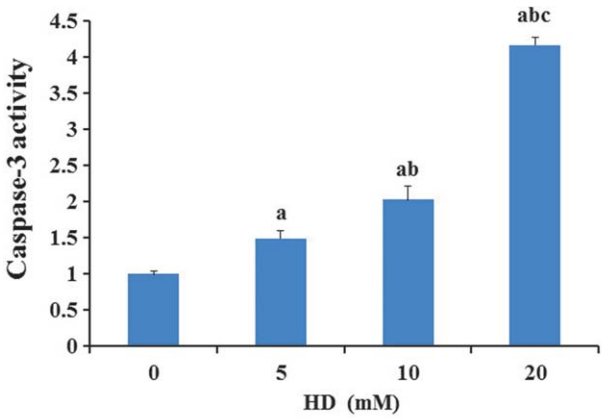

Figure 7. The activity of caspase-3 in PC12 cells exposed to HD. HD increased caspase-3 activity in PC12 cells in a concentration-dependent manner. Data were presented as mean ± SD from three independent experiments. [a]$P < 0.05$, vs. control group; [b]$P < 0.05$, vs. 5 mM HD group; [c]$P < 0.05$, vs. 10 mM HD group.

will lead to the enhancement of Bax expression level and trigger the loss of MMP. Several reports have suggested that the loss of MMP eventually causes the release of apoptogenic factors, which promotes the efflux of death-promoting cytochrome *c* from mitochondria to cytosol. Then the cytochrome *c* in cytosol in turn induces the activation of caspase cascade[30]. Caspases are a family of intracellular proteases and are responsible directly or indirectly for the morphological and biochemical events that characterize classical apoptosis.

In our study, when different concentrations of HD were applied on PC12 cells, the expression of Bax gene and protein increased with increasing HD concentration. Substantial evidences have demonstrated that up-regulation of Bax could promote apoptotic death of neuron populations in response to various toxic stimuli including cocaine[31], arsenic[24], organophosphorus esters[26] and so on. Bax-deficient mice exhibited significantly decrease in neuronal apoptosis in nervous system[32]. In contrast to Bax, Bcl-2 plays a role in controlling the integrity of the mitochondrial membrane and forms heterodimers with Bax to prevent mitochondria dysfunction. It was reported that Bcl-2 inhibited a neuronal apoptotic cascade which was induced by death-inducing neurotoxins[33-35]. The present study revealed that after HD exposure, the expression of Bcl-2 gene and protein decreased with increasing HD concentration in PC12 cells. However, Cui *et al.* reported that the level of Bcl-2 was up-regulated in the nerve tissues of HD-exposed rats. They detected no features of apoptosis in morphological studies[15]. On the contrary, we found typical apoptotic phenotype in HD-exposed PC12 cells by Hoechst 33342 staining and FACS analysis. The different degree of damage caused by HD probably led to the difference between the expression of Bcl-2. If the damage was not severe enough to make cells overt death, the increase of Bcl-2 was a protective mechanism in response to HD exposure. Along with the increased exposure of HD, the expression of Bcl-2 decreased and promoted the progress of apoptosis. As well known, Bax/Bcl-2 ratio determines the fate of cell by regulating MMP collapse, an early event in mitochondrial apoptosis[36]. In the present study, the ratio of Bax/Bcl-2 significantly increased in PC12 cells after HD exposure, which supported our hypothesis that the mechanism of apoptosis might involve mitochondria-dependent pathway.

Lots of evidences indicated that the disruption of MMP involved in various toxicant induced-apoptosis[24,25,37]. MMP collapse had been found in HD exposed- spermatogenic cells[21]. In our study, a significant decrease in MMP was also observed in HD-exposed PC12 cells in a concentration-dependent manner. It was showed that the loss of MMP eventually causes the efflux of death-promoting cytochrome *c* from mitochondria to cytosol. We also found an increase of cytochrome *c* in cytosolic fraction and a corresponding decrease in mitochondrial fraction in PC12 cells after HD exposure. The results indicate that HD exposure induces the loss of MMP and translocation of cytochrome *c* in PC12, which involved in the induction activation of caspase cascade.

Caspase-3 is the ultimate enforcer of caspases in apoptosis. Cells deficient in caspase-3 were resistant to apoptosis[38]. The activation of caspase-3 alone was sufficient to cause mitochondria-mediated apoptosis in neurons[24]. By activation caspase-3, the induction of mitochondria-mediated apoptotic death has been shown in various cells[24,37,39,40]. In our study, caspase-3 activity was significantly increased by HD in a concentration-dependent manner. It was suggested that the initiation of apoptosis was triggered by the activated caspase-3, followed by an overt apoptotic changes (showed by Hoechst 33342 staining and flow cytometry).

The current study clearly showed that HD induced apoptosis in PC12 cells. The mechanism involved with up-regulation of Bax and down-regulation of Bcl-2, followed by the loss of MMP, along with the release of cytochrome *c*, the activation of caspase-3 and finally resulted in apoptosis. This study provides a new possibility that mitochondria-mediated apoptosis involves in the neurotoxicity of HD. However, further studies still needed to explore the mechanistic details of HD-induced apoptosis, such as the change of other pro-apoptotic factors, and the expression of upstream regula-

tors mitochondria-dependent pathway, caspase-8 and -9 etc.

Materials & Methods

Cell culture and HD exposure

PC12 cells were purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM with 10% FBS, 100 U/mL penicillin and 100 U/mL streptomycin. After 48 h of incubation, PC12 cells grew into 80% confluent monolayer. The cells were treated with 5, 10 and 20 mM HD in DMEM culture media for 24 h, respectively. DMEM medium without HD was added to the control group.

Cell viability assay

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on a 96-well plate. In brief, PC12 cells were treated with 5-20 mM HD for 24 h, according to literature resources.[13,22] At the end of treatment, the cells were incubated with 0.5 mg/mL MTT (Sigma, USA) for 4 h. The medium was then removed and replaced with 750 μL DMSO. The plate was shaken for 10 min and the absorbance was measured at 570 nm using a microplate reader (SPECTRA FLUOR, Austria). Cell viability was expressed as percent of the control culture value.

Hoechst 33342 staining

After treated as described above, the cells were observed by microscope, and then fixed with 4% paraformaldehyde for 20 min at room temperature and washed with PBS thrice for 5 min. Cell nuclei were stained with Hoechst 33342 (Sigma, USA) at a final concentration of 10 μg/mL in PBS, for 20 min in a dark chamber. Then the cells were observed under converted fluorescence microscope (Olympus U-RFLT50, Japan) and photographed.

FCM with FITC-Annexin V/PI double staining

After treated as described above, the cells were gently digested with trypsin, washed thrice with PBS and 4 $\times 10^6$ cells were collected. Then resuspended in 500 μL annexin V Binding buffer and incubated with PI and annexin V-FITC (KeyGEN, China) at room temperature in dark for 30 min. Fluorescence analysis was carried out using a flow cytometer (BD FACS Calibur, USA).

Real-time RT-PCR

Total RNA was extracted from PC12 cells by using RNAiso Plus according to the manufacturer's instructions (Takara, Japan). Only RNA samples with an A260/A280 of 1.8-2.2 were used for reverse transcription. Total RNA of 100 ng/μL was reverse-transcribed using Reverse Transcription Kit (Takara, Japan). Quantitative real-time PCR was carried out with SYBR Green II PCR kit (Takara, Japan) using a TP800 Real-Time PCR Detection System (Takara, Japan). The following primer pairs were used (Takara, Dalian): Bax: 5′-CGA ATT GGC GAT GAA CTG GA-3′/5′-CAA ACA TGT CAG CTG CCA CAC-3′; Bcl-2: 5′-GAC TGA GTA CCT GAA CCG GCA TC-3′/5′- CTG AGC AGC GTC TTC AGA GAC A-3′; β-actin: 5′-GGA GAT TAC TGC CCT GGC TCC TA-3′/5′-GAC TCA TCG TAC TCC TGC TTG CTG-3′. The reaction conditions were as follows: an initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s. The β-actin mRNA was used as internal controls probe.

Western blot

Cells were homogenized in ice-cold RIPA Tissue Protein Extraction Reagent (Beyotime, China) supplemented with 1% proteinase inhibitor mix. The proteins were mixed with an equal volume of SDS-PAGE loading buffer and separated by SDS-PAGE and then electrotransferred to Hybond-PVDF membrane (Millipore, France). The membrane was incubated with appropriate primary antibodies at 4°C overnight. Antibodies used were Bcl-2, Bax (1 : 500, Cell Signaling Technology, USA) and cytochrome *c* (1 : 500, Beyotime, China). Immunoreactivity was visualized by second horseradish peroxidase-conjugated antibody (1 : 5000, Sigma, USA) and enhanced chemoluminescence. Quantified densitometry analysis was using with UVP BioSpectrum Multispectral Imaging System (Ultra-Violet Products Ltd. USA).

MMP assay

The uptake of the cationic fluorescent dye rhodamine 123 has been used for the estimation of MMP. Cells were harvested and washed twice with PBS, and the cell pellet was then resuspend in 2 mL fresh incubation medium containing 1 μM rhodamine 123 (Beyotime, China) and incubated at 37°C. Cells were then separated, and the amount of rhodamine 123 was measured using a fluorescence spectrophotometer set (Hitachi650-60, Japan) at 490 nm excitation and 520 nm emission wavelengths. Results were expressed as the fluorescence retained within the cells.

Caspase-3 activity detection

Caspase-3 activities were determined with a caspase-3 activity assay kit (Beyotime, China), which detected the production of the chromophore p-nitroanilide after its cleavage from the peptide substrate DEVD-p-nitroanilide and LEHD-p-nitroanilide. Colorimetric reaction was developed and measured at 405 nm in a BioRad plate reader.

Statistical analysis

Data were presented as mean ± standard deviation (SD) from at least three independent experiments. All data were analyzed with SPSS 11.0 for windows. Difference in mean values between groups was tested with the one-way ANOVA and LSD test. *P* values less than 0.05 were considered significant.

Acknowledgements This work was supported by the National Natural Science Foundation of China (No. 81273038, No. 81102160) and Dalian Municipal Science and Technology Plan Project (No. 2013E15SF163).

Conflict of Interest The author (s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

References

1. Abbritti, G. *et al*. Shoe-makers' polyneuropathy in Italy: the aetiological problem. *Br J Ind Med* **33**:92-99 (1976).
2. Jørgensen, N. K. & Cohr K. H. N-hexane and its toxicologic effects: a review. *Scand J Work Environ Health* **7**:157-168 (1981).
3. Herskowitz, A. *et al*. N-hexane neuropathy. A syndrome occurring as a result of industrial exposure. *N Engl J Med* **285**:82-85 (1971).
4. Neghab, M. *et al*. Electrophysiological studies of shoemakers exposed to sub-TLV levels of n-hexane. *J Occup Health* **54**:376-382 (2012).
5. Prieto, M. J. *et al*. Free and total 2,5-hexanedione in biological monitoring of workers exposed to n-hexane in the shoe industry. *Toxicol Lett* **145**:249-260 (2003).
6. Wilson, M. P. *et al*. Worker exposure to volatile organic compounds in the vehicle repair industry. *J Occup Environ Hyg* **4**:301-310 (2007).
7. dos Santos, C. R. *et al*. Evaluation of 2,5-hexanedione in urine of workers exposed to n-hexane in Brazilian shoe factories. *J Chromatogr B Analyt Technol Biomed Life Sci* **778**:237-244 (2002).
8. Couri, D. & Milks, M. Toxicity and metabolism of the neurotoxic hexacarbons n-hexane, 2-hexanone, and 2,5-hexanedione. *Ann Rev Pharmacol Toxicol* **22**:145-166 (1982).
9. Moretto, G. *et al*. Cytoskeletal changes induced by 2,5-hexanedione on developing human neurons in vitro. *Arch Toxicol* **65**:409-413 (1991).
10. Sterman, A. B. Cell body remodeling during dying-back axonopathy: DRG changes during advanced disease. *J Neuropathol Exp Neurol* **41**:400-411 (1982).
11. Sterman, A. B. & Sposito, N. 2,5-Hexanedione and acrylamide produce reorganization of motoneuron perikarya. *Neuro Pathol Appl Neurobiol* **11**:201-212 (1985).
12. Ogawa, Y. *et al*. 2,5-Hexanedione induced apoptosis in cultured mouse DRG neurons. *Int Arch Occup Environ Health* **68**:495-497 (1996).
13. Strange, P. *et al*. Total number and mean cell volume of neocortical neurons in rats exposed to 2,5-hexanedione with and without acetone. *Neurotoxicol Teratol* **13**:401-406 (1991).
14. Zilz, T. R. *et al*. Apoptotic and necrotic effects of hexanedione derivatives on the human neuroblastoma line SK-N-SH. *Toxicology* **231**:210-214 (2007).
15. Cui, N. *et al*. Expression of Bcl-2, Bax and Caspase-3 in nerve tissues of rats chronically exposed to 2,5-hexanedione. *Neurochem Res* **32**:1566-1572 (2007).
16. Gillies, L. A. & Kuwana, T. Apoptosis regulation at the mitochondrial outer membrane. *J Cell Biochem* **115**:632-640 (2014).
17. Nikolić, M. *et al*. Postnatal neuronal apoptosis in the cerebral cortex: physiological and pathophysiological mechanisms. *Neuroscience* **19**:369-378 (2013).
18. Hsia, T. C. *et al*. Cantharidin induces apoptosis of H460 human lung cancer cells through mitochondria-dependent pathways. *Int J Oncol* **45**:245-254 (2014).
19. Wang, Y. *et al*. The protective effects of selenium on cadmium-induced oxidative stress andapoptosis via mitochondria pathway in mice kidney. *Food Chem Toxicol* **58**:61-67 (2013).
20. Sun, Y. *et al*. 2,5-Hexanedione induces human ovarian granulosa cell apoptosis through BCL-2, BAX, and CASPASE-3signaling pathways. *Arch Toxicol* **86**:205-215 (2012).
21. Mishra, D. P. *et al*. Changes in cytosolic Ca^{2+} levels regulate Bcl-xS and Bcl-xL expression in spermatogenic cells during apoptotic death. *J Biol Chem* **281**: 2133-2143 (2006).
22. Coleman, M. D. *et al*. A comparison of the apoptotic and cytotoxic effects of hexanedione derivatives on human non-neuronal lines and the neuroblastoma line SH-SY5Y. *Basic Clin Pharmacol Toxicol* **102**:25-29 (2008).
23. Elmore, S. Apoptosis: a review of programmed cell death. *Toxicol Pathol* **35**:495-516 (2007).
24. Dwivedi, N. *et al*. MiADMSA reverses impaired mitochondrial energy metabolism and neuronal apoptotic cell death after arsenic exposure in rats. *Toxicol Appl Pharmacol* **256**:241-248 (2011).
25. Nie, J. S. *et al*. Involvement of mitochondrial pathway in benzo[a]pyrene-induced neuron apoptosis. *Hum Exp Toxicol* **33**:240-250 (2014).

26. Zou, C. *et al*. Activation of mitochondria-mediated apoptotic pathway in tri-ortho-cresyl phosphate-induced delayed neuropathy. *Neurochem Int* **62**:965-972 (2013).
27. Xu, X. F. *et al*. Ardipusilloside I induces apoptosis by regulating Bcl-2 family proteins in human mucoepidermoid carcinoma Mc3 cells. *BMC Complement Altern Med* **13**:322 (2013).
28. Volkmann, N. *et al*. The rheostat in the membrane: BCL-2 family proteins and apoptosis. *Cell Death Differ* **21**:206-215 (2014).
29. Anvekar, R. A. *et al*. Born to be alive: a role for the BCL-2 family in melanoma tumor cell survival, apoptosis, and treatment. *Front Oncol* **5**:34 (2011).
30. Wu, C. C. *et al*. Destruxin B isolated from entomopathogenic fungus metarhizium anisopliae induces apoptosis via a Bcl-2 family-dependent mitochondrial pathway in human nonsmall cell lung cancer cells. *Evid Based Complement Alternat Med* 548929 (2013).
31. Xiao, D. & Zhang, L. Upregulation of Bax and Bcl-2 following prenatal cocaine exposure induces apoptosis in fetal rat brain. *Int J Med Sci* **5**:295-302 (2008).
32. White, F. A. *et al*. Widespread elimination of naturally occurring neuronal death in Bax-deficient mice. *J Neurosci* **18**:1428-1439 (1998).
33. Ghosh, A. P. *et al*. The proapoptotic BH3-only, Bcl-2 family member, Puma is critical for acute ethanol-inducedneuronal apoptosis. *J Neuropathol Exp Neurol* **68**: 747-756 (2009).
34. Métrailler-Ruchonnet, I. *et al*. Bcl-2 protects against hyperoxia-induced apoptosis through inhibition of the mitochondria-dependent pathway. *Free RadicBiol Med* **42**:1062-1074 (2007).
35. Wu, J. *et al*. Lanthanum induced primary neuronal apoptosis through mitochondrial dysfunction modulated by Ca^{2+} and Bcl-2 family. *Biol Trace Elem Res* **152**: 125-134 (2013).
36. Merry, D. E & Korsmeyer, S. J. Bcl-2 gene family in the nervous system. *Annu Rev Neurosci* **20**:245-267 (1997).
37. Wu, C. C. *et al*. Involvement of oxidative stress-induced ERK/JNK activation in the Cu (2+)/pyrrolidine dithiocarbamate complex-triggered mitochondria- regulated apoptosis in pancreatic β-cells. *Toxicol Lett* **208**:275-285 (2012).
38. Yang, X. H. *et al*. Reconstitution of caspase-3 sensitizes MVF-7 breast cancer cells to doxorubicin- and etoposide-induced apoptosis. *Cancer Res* **61**:348-354 (2001).
39. Bensassi, F. *et al*. Mechanism of alternariol monomethyl ether-induced mitochondrial apoptosis in human colon carcinoma cells. *Toxicology* **290**:230-240 (2011).
40. Li, Q. *et al*. Mechanism of ziram-induced apoptosis in human T lymphocytes. *Arch Toxicol* **86**:615-623 (2012).