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Involvement of oxidative stress in di-2-ethylhexyl phthalate (DEHP)-induced apoptosis of mouse NE-4C neural stem cells

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ABSTRACT

Di-2-ethylhexyl phthalate (DEHP) has been widely used as a plasticizer in industry and can cause neurotoxicity; however, the underlying mechanism remains unclear. In the study, we found that DEHP significantly inhibited viability of mouse NE-4C neural stem cells and caused lactate dehydrogenase (LDH) release from the cells. DEHP dramatically increased the levels of apoptosis-related proteins such as cleaved Caspase-8, cleaved Caspase-3 and Bax, as well as decreased Bcl-2 protein level. DEHP could also significantly increase the total numbers of AnnexinV-positive/PI-negative and AnnexinV-positive/PI-positive staining cells. Hoechst 33342 staining showed that marked DNA condensation and apoptotic bodies could be found in the ZnO NPs-treated cells. These results indicated that DEHP could induce apoptosis of NE-4C cells. Meanwhile, DEHP could significantly increase malondialdehyde (MDA) level, and decrease the content of glutathione (GSH) and activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX), respectively, implying that DEHP could induce oxidative stress of NE-4C cells. Furthermore, *N*-Acetyl-L-cysteine (NAC), an inhibitor of oxidative stress, could rescue the inhibition of cell viability and induction of apoptosis by DEHP. Taken together, our results showed that oxidative stress was involved in DEHP-induced apoptosis of mouse NE-4C cells.

1. Introduction

Di-2-ethylhexyl phthalate (DEHP) has been widely used as a plasticizer in industry, such as blood bags, nasogastric tubes, infusion tubing and toys (Bourdeaux et al., 2004). Due to the overuse in many products and constant release into the environment, DEHP can be found in air, ground water and soil at some amounts (Petersen and Breindahl, 2000), as well as in meat and lipid rich products like fats at higher concentrations (Serrano et al., 2014; Rowdhwal and Chen, 2018). DEHP can bind strongly to soil and dissolves very slowly when it is released, which has raised concerns pertaining to continuous exposure of human beings (Rowdhwal and Chen, 2018).

DEHP has been shown to induce reproductive toxicology (Stenz et al., 2017; Sun et al., 2018), immunotoxicity (Huang et al., 2015), and cardiotoxicity (Posnack, 2014), besides neurotoxicity (Du et al., 2017; Luu et al., 2017). DEHP is shown to cross the blood-brain barrier (BBB) and accumulate in the brain (Wu et al., 2014). DEHP exposure can dramatically decrease the brain weight of newborns of mice and reduce the mesaticephalic dopaminergic neurons (Tanida et al., 2009). DEHP exposure can also lead to neurodegeneration in the rat's brain (Dhanya et al., 2003). DEHP is also shown to affect reference memory, self-righting ability and spatial learning (Tanaka, 2005; Li et al., 2009; Cho et al., 2010). However, the underlying mechanism on DEHP-induced neurotoxicity remains unclear.

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen including superoxide ($O2^{\cdot}-$), hydroxyl radical ($OH^{\cdot}$), nitric oxide ($NO^{\cdot}$) and peroxides, which is generated as by-products of mitochondrial respiration under physiological conditions (Wen et al., 2013). Under normal conditions, reduced GSH and antioxidant enzymes such as GSH-PX and SOD can maintain the intracellular levels of ROS at low levels. However, oxidative stress will occur when there is an imbalance between the antioxidants and

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prooxidants in the cell (Jenkins and Goldfarb, 1993). Many studies show that many chemicals can induce oxidative stress (Lehnert and Iyer, 2002; Liu et al., 2016; Tiwari and Vanage, 2017); while oxidative stress is shown to be involved in DEHP-induced apoptosis (Fu et al., 2017). However, the actual effect of DEHP on mouse NE-4C neural stem cells and its potential mechanism remain unclear.

The aim of the present study is to investigate whether oxidative stress is involved in DEHP-induced apoptosis of mouse NE-4C neural stem cells. This study sets in motion our future investigation of the mechanisms underlying DEHP-induced neurotoxicity.

2. Materials and methods

2.1. Reagents

DEHP (Catalog No. 36,735) was purchased from Sigma (St. Louis, MO, USA). Mouse NE-4C neural stem cells were provided by Stem Cell Bank, Chinese Academy of Sciences (Shanghai, China). Mouse anti-Caspase-3 monoclonal antibody (sc-7272), mouse anti-Caspase-8 monoclonal antibody (sc-81656), rabbit anti-Bcl-2 polyclonal antibody (sc-492), rabbit anti-Bax polyclonal antibody (sc-493) and mouse anti-β-actin monoclonal antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The enhanced chemiluminescence (ECL) reagent was purchased from Pierce Biotechnology (Rockford, IL, USA). AnnexinV-FITC Apoptosis Detection Kit was obtained from Invitrogen Life Technologies (Oregon, USA). Oxidation-antioxidation assay kits of glutathione (GSH), malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-PX) were bought from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). *N*-acetyl-L-cysteine (NAC) and Hoechst 33342 were purchased from Sigma (St. Louis, MO, USA).

2.2. Cell culture

Mouse NE-4C cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% glutamax and 1% non-essential amino acids, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Incubations were carried out at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The cells were maintained in the logarithmic phase of growth and sub-cultured at 3 ~ 4-day intervals.

2.3. MTT reduction assay

Mouse NE-4C cells were seeded in a 96-well culture plate and treated with the indicated concentrations of DEHP in the presence or absence of 5 mM NAC for 24 h. Twenty-four hours later, cell medium containing 0.5 mg/mL MTT was replaced in each well and incubated at 37 °C in 5% CO_2/95% air for 4 h. The formazan formed was dissolved in DMSO, and the absorbance was measured in a spectrophotometer at 490 nm.

2.4. Lactate dehydrogenase (LDH) release

LDH activity in extracellular medium was assessed according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The reaction was performed in a 96-well microplate and the absorbance measured in a spectrophotometer at 450 nm. The results expressed as percentage of LDH release.

2.5. Western blotting analysis

Mouse NE-4C cells were treated with the indicated concentrations of DEHP in the presence or absence of 5 mM NAC for 24 h and then were harvested in lysis buffer containing protease inhibitor cocktail. The protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 5% stacking gel and 8% separating gel and transferred to polyvinylidene fluoride (PVDF) membrane. Following transfer, membranes were blocked with 1× Tris-buffered saline (TBS) buffer containing 0.05% Tween 20 and 5% nonfat milk for 1 h at room temperature, then incubated with primary antibodies and horseradish peroxidase-conjugated goat anti-rabbit IgG, respectively. Immunoreactive bands were detected using a ChemiDoc XRS system (Bio-Rad, Hercules, CA, USA). Relative quantification of the proteins was analyzed by using Image J software.

2.6. AnnexinV-FITC/PI apoptosis assay

Mouse NE-4C cells were treated with the indicated concentrations of DEHP in the presence or absence of 5 mM NAC for 24 h, the apoptosis assay was then analyzed by double staining the cells with FITC-labeled AnnexinV and propidium iodide (PI) according to the manufacturer's instructions. In brief, NE-4C cells were collected and washed twice with phosphate-buffered saline (PBS). Then the cells were resuspended with the AnnexinV binding buffer and transferred to test tubes containing FITC-labeled AnnexinV and PI. The cells were then incubated in the dark for 15 min at room temperature, and analyzed by flow cytometry using the FACS Calibur system (BD Biosciences, San Jose, CA, USA). The excitation wave length was 488 nm and the emission wave length was 530 nm. Flowcytometric data were analyzed using FlowJo 7.6 software. Homogeneous subpopulations of cells were identified by FSC/SSC gating according to unstained negative control. Then, FITC + and PI + cell populations were gated out according to FITC and PI single stained control, respectively. Apoptosis cells were derived from gated FITC + and/or PI + cell populations. The data displayed in dot plot of Annexin V/FITC (y-axis) against PI (x-axis). The normal healthy cells were AnnexinV/FITC and PI double-negative, whereas the late apoptotic cells were double-positive. The early apoptotic cells were only AnnexinV/FITC positive.

2.7. Hoechst staining

Mouse NE-4C cells were treated with 0–20 μM DEHP for 24 h, cells were fixed with 4% paraformaldelyde at room temperature for 30 min and washed twice with phosphate-buffered saline (PBS). The cells were stained with 2 μg/ml Hoechst 33342 at 37 °C for 10 min and then washed twice. The apoptotic nuclear morphology was observed by fluorescence microscopy (DMLB, Leica, Germany).

2.8. Oxidative stress measurement

Mouse NE-4C cells treated with 0–20 μM DEHP were homogenized, and then centrifuged at 600 *g* for 10 min at 4 °C. The supernatants were analyzed for the contents of MDA and GSH and the activities of SOD and GSH-PX according to the manufacturer's instructions. (1) MDA concentration was determined by estimating the product of the reaction between MDA and thiobarbituric acid (TBA). The supernatants of the homogenized cells were mixed with TBA and heated at boiling water bath followed by cooling on ice. N-butanol was then added and mixture was centrifuged. The reaction products were detected at 532 nm using a spectrophotometer. The concentration of MDA was expressed as μmol/mg protein. (2) GSH content was evaluated using a GSH reagent containing 5,5-dithiobes-2-ni-trobenzoic acid was mixed with 0.1 mL of the assay sample for 5 min to assess molecular clearance and oxidation resistance. The optical density value was obtained at an absorbency of 405 nm. The GSH content was expressed as μg/L. (3) SOD activity was assayed by water soluble tetrazolium salts assay (WST-1) method, which monitored the inhibition rate of SOD to the process of formazan dye formation from tetrazolium salt mediated by the superoxide anion. The absorbance was scanned at 450 nm using a microplate reader. The activity of SOD was expressed as U/mg protein. (4) GSH-PX activity was estimated by determination of reduced glutathione (GSH), the GSH

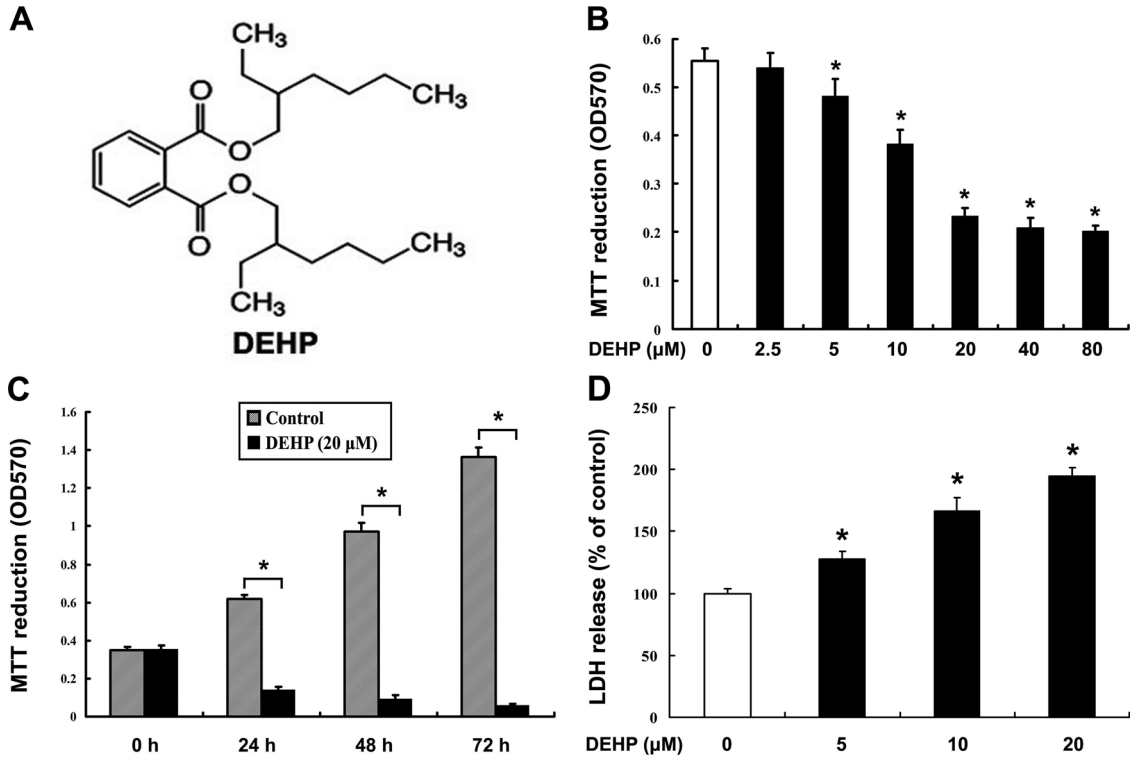

Fig. 1. DEHP inhibits viability of mouse NE-4C cells. (A) Chemical structure of di-2-ethylhexyl phthalate (DEHP). Mouse NE-4C cells were treated with 0 ~ 80 μM DEHP for 24 h (B) or treated with 20 μM DEHP for 24–72 h (C); then cell viability was observed by MTT assay. (D) NE-4C cells were treated with 0 ~ 20 μM DEHP for 24 h; LDH activity in extracellular medium was then detected. The results are expressed as mean ± SEM of three separate experiments and analyzed by one-way ANOVA. *$P < 0.05$ vs the Control group.

reacts with 5,5-dithiobis-(2-nitrobenzoic acid) and produces yellow colored compounds which were detected at 412 nm, and the final result was presented as U/mg protein.

2.9. Statistical analysis

Values are expressed as means ± SE. All data were evaluated by one-way analysis of variance (ANOVA) using SPSS software, version 15 (Chicago, USA) followed by the Newman–Keuls multiple range test for comparisons. A difference between means was considered significant at a value of $P < 0.05$.

3. Results

3.1. DEHP induces cytotoxicity of mouse NE-4C cells

To observe whether DEHP inhibited viability of mouse NE-4C cells, the cells were treated with 0, 2.5, 5, 10, 20, 40, and 80 μM DEHP for 24 h (Fig. 1B) or treated with 20 μM DEHP for 24, 48 and 72 h (Fig. 1C). We found that DEHP significantly inhibited cell viability of NE-4C cells in a dose and time-dependent manner. As shown in Fig. 1D, DEHP could also cause LDH release from the cells. These results indicated that DEHP could induce cytotoxicity of mouse NE-4C cells.

3.2. DEHP induces apoptosis of mouse NE-4C cells

To confirm whether the inhibition of viability by DEHP resulted from the induction of apoptosis, the apoptosis-related proteins cleaved Caspase-8, cleaved Caspase-3, Bcl-2 and Bax were investigated after NE-4C cells were incubated with 0, 5, 10 and 20 μM DEHP for 24 h. We found that DEHP significantly increased the protein levels of cleaved Caspase-8, cleaved Caspase-3 and Bax, as well as decreased the Bcl-2 protein level (Fig. 2). Furthermore, DEHP dramatically increased the total numbers of AnnexinV-positive/PI-negative (early apoptosis) and AnnexinV-positive/PI-positive staining cells (late apoptosis) (Fig. 3). Apoptosis was further confirmed by Hoechst 33342 staining. As shown in Fig. 4, nuclei were round and homogenously stained in the control cells; while ZnO NPs-treated cells showed marked DNA condensation and apoptotic bodies. These results indicated that DEHP could induce apoptosis of NE-4C cells.

3.3. DEHP induces oxidative stress of mouse NE-4C cells

In order to detect whether DEHP induced oxidative stress of mouse NE-4C cells, we detected the contents of MDA and GSH and the antioxidant enzyme activities of SOD and GSH-PX after the cells were treated with 0, 5, 10 and 20 μM DEHP for 24 h. As shown in Fig. 5, there was a significant increase in MDA level and dramatic decrease in the content of GSH and activities of SOD and GSH-PX in the DEHP-treated cells, respectively, indicating that DEHP could induce oxidative stress of mouse NE-4C cells.

3.4. Oxidative stress is involved in DEHP-induced apoptosis of mouse NE-4C cells

In order to investigate whether oxidative stress was involved in DEHP-induced apoptosis of NE-4C cells, cell viability and apoptosis were observed after the cells were treated with 0 ~ 20 μM DEHP in the presence or absence of 5 mM *N*-Acetyl-L-cysteine (NAC) for 24 h. As shown in Fig. 6, inhibition of cell viability by DEHP was rescued by NAC. Meanwhile, inhibition of oxidative stress could inhibit DEHP-induced apoptosis. These results indicated that oxidative stress was involved in DEHP-induced apoptosis of NE-4C cells.

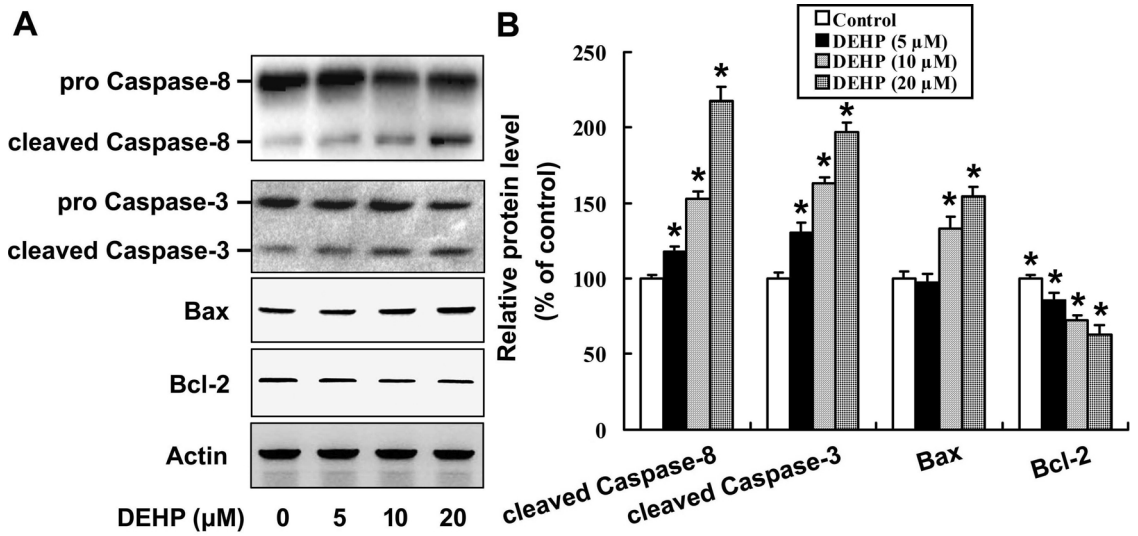

Fig. 2. Western blot analysis of apoptosis-related proteins in mouse NE-4C cells. (A) NE-4C cells were treated with 0 ~ 20 μM DEHP for 24 h; then the contents of cleaved Caspase-8, cleaved Caspase-3, Bcl-2 and Bax were detected by western blot; Actin was used as an internal control. (B) The relative protein levels were quantified by densitometry. The results are expressed as mean ± SEM of three separate experiments and analyzed by one-way ANOVA. *$P < 0.05$ vs the Control group.

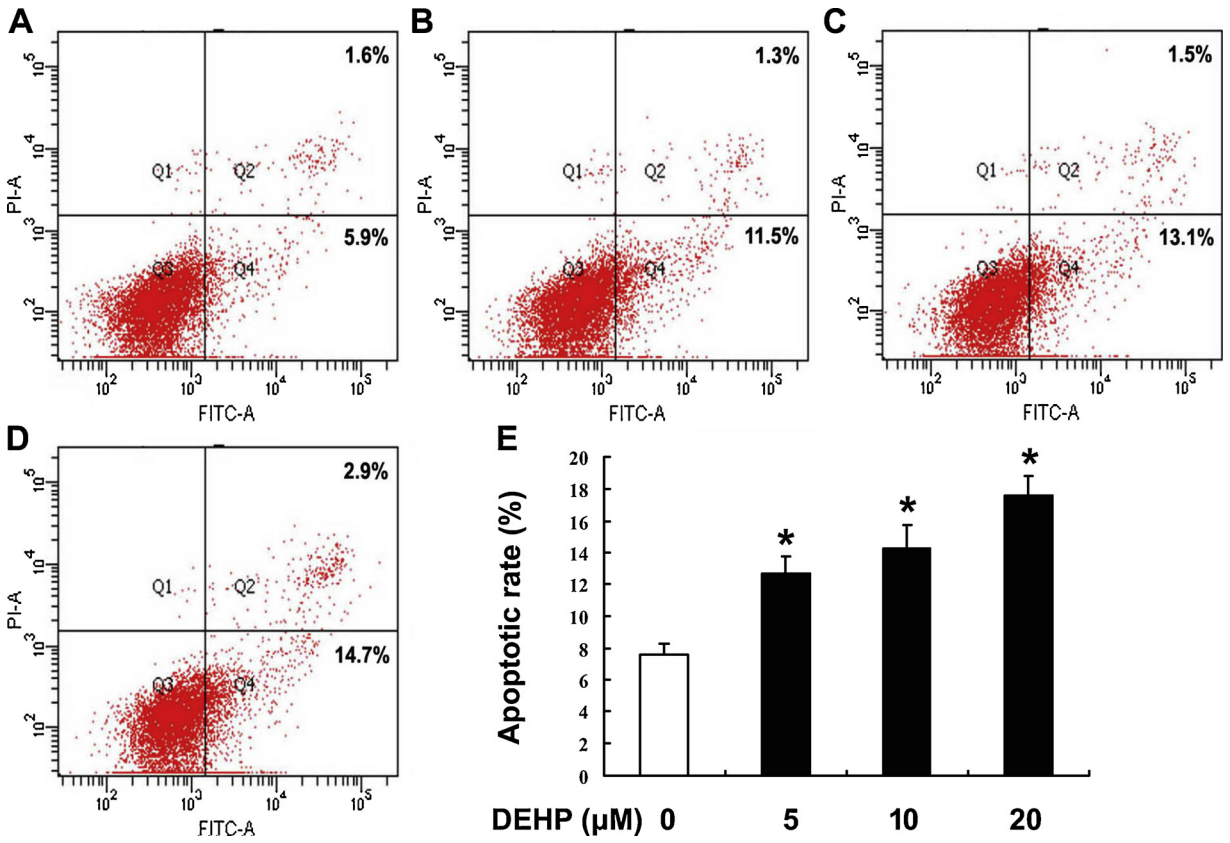

Fig. 3. AnnexinV-FITC/PI double staining detects apoptosis of mouse NE-4C cells. NE-4C cells were treated with 0 (A), 5 (B), 10 (C), and 20 (D) μM DEHP for 24 h; then the AnnexinV- positive staining cells were counted by flow cytometry. (E) Statistic results for apoptosis analysis. The results are expressed as mean ± SEM of three separate experiments and analyzed by one-way ANOVA. *$P < 0.05$ vs the Control group.

4. Discussion

DEHP is widely used in the production of polyvinyl chloride products, which causes a ubiquitous environmental contaminant. DEHP was shown to induce testicular damage (Abdel-Kawi et al., 2016). However, it is still unknown on the potential mechanism of DEHP in neurotoxicity. DEHP can cross the placenta and enter the fetal circulation, which affect neurodevelopment and lead to teratogenic anomalies by disrupting normal fetal brain development (Rowdhwal and Chen, 2018). Lin et al showed that gestational and postnatal DEHP exposure has harmful effects on rat brain development and function (Lin et al., 2015). DEHP exposure (1500 mg/kg) *in utero* led to a metabolic disturbance of the lipid metabolome of the fetal rat brain, which caused anomalous brain growth (Xu et al., 2007). To investigate the

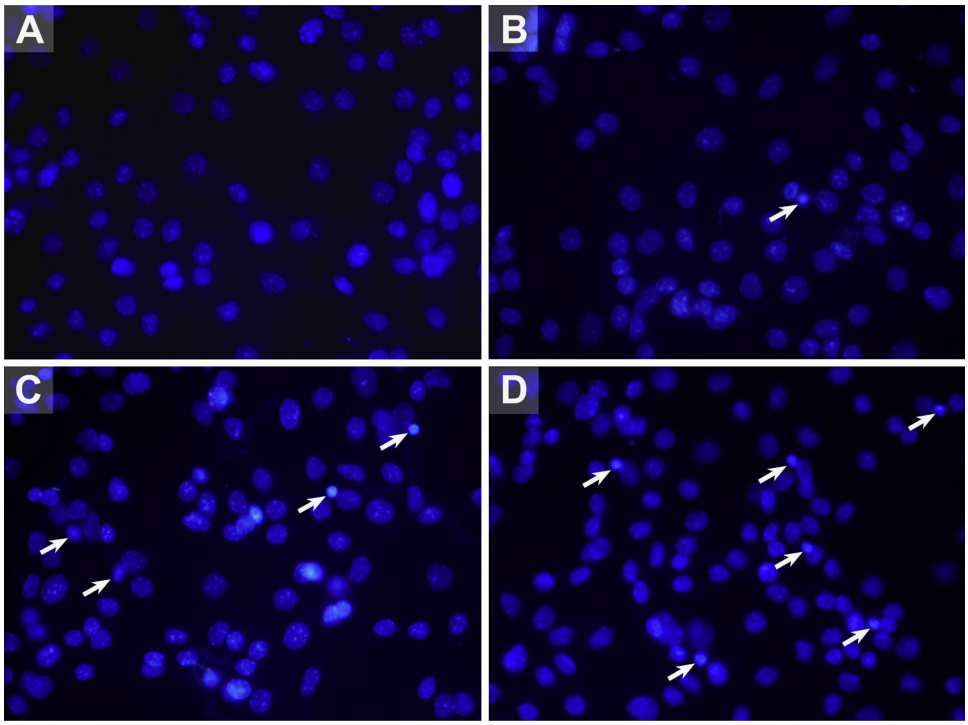

Fig. 4. Hoechst staining detects apoptosis of mouse NE-4C cells. NE-4C cells were treated with 0 (A), 5 (B), 10 (C), and 20 (D) μM DEHP for 24 h, the apoptotic nuclear morphology was stained with 2 μg/ml Hoechst 33342 and observed by fluorescence microscopy (DMLB, Leica, Germany). ZnO NP-treated cells show apoptotic cells with condensed or fragmented nuclei (indicated by arrows).

effect and the potential mechanism, mouse NE-4C cell line, a neural stem cell, was utilized as cell model in *vitro*. In the present study, we found that 0–20 μM DEHP significantly inhibited cell viability and caused LDH release from the cells, which indicated that DEHP could induce cytotoxicity of mouse NE-4C cells.

Many studies showed that inhibition of cell viability by chemicals might result from the induction of apoptosis (Lin et al., 2013; Yokoyama et al., 2003). Apoptosis is a process of programmed cell death that occurs in multicellular organisms. DEHP is also shown to induce apoptosis in many cells such as GC-2spd cells (Fu et al., 2017), ovarian granulosa cells (Li et al., 2015), hepatocyte (Ha et al., 2016), and insulinoma (INS-1) cells (Sun et al., 2015). In the present study, we found that there is a significant increase in the protein levels of cleaved Caspase-8, cleaved Caspase-3 and Bax in the DEHP-treated cells, as well as a dramatic decrease in the Bcl-2 protein level. Furthermore, DEHP dramatically increased the total numbers of AnnexinV-positive/PI-negative (early apoptosis) and AnnexinV-positive/PI-positive staining cells (late apoptosis). Hoechst 33342 staining also showed that marked

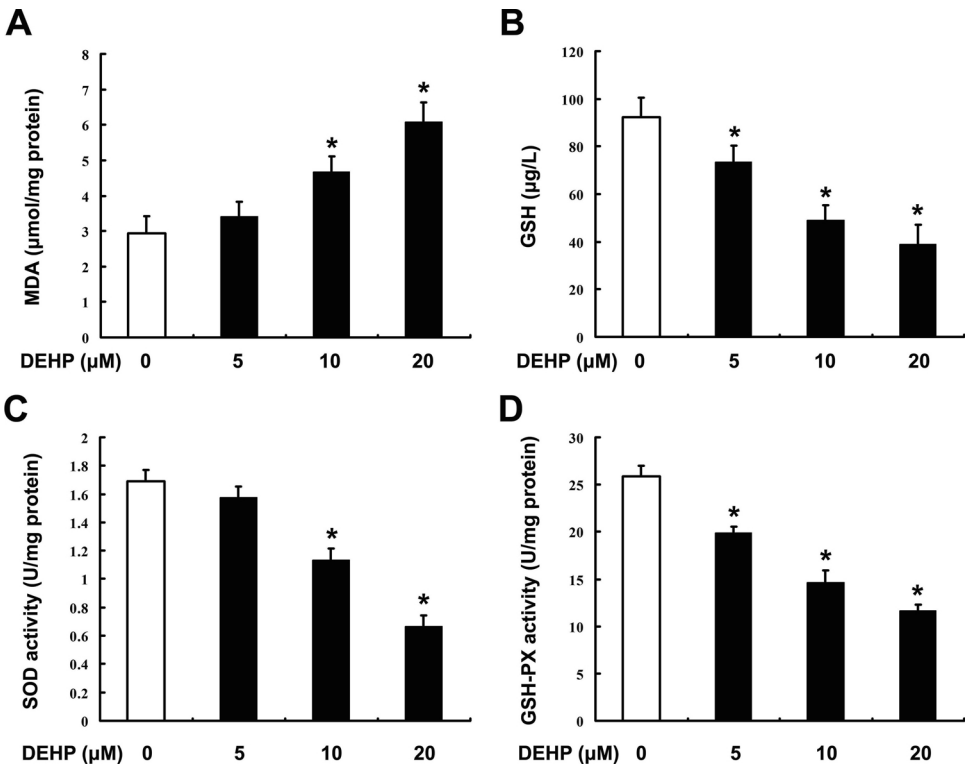

Fig. 5. DEHP induces oxidative stress of mouse NE-4C cells. NE-4C cells were treated with 0 ~ 20 μM DEHP for 24 h; then the contents of MDA (A) and GSH (B) and the enzyme activities of SOD (C) and GSH-PX (D) and were determined. The results are expressed as mean ± SEM of three separate experiments and analyzed by one-way ANOVA. $^{*}P < 0.05$ vs the Control group.

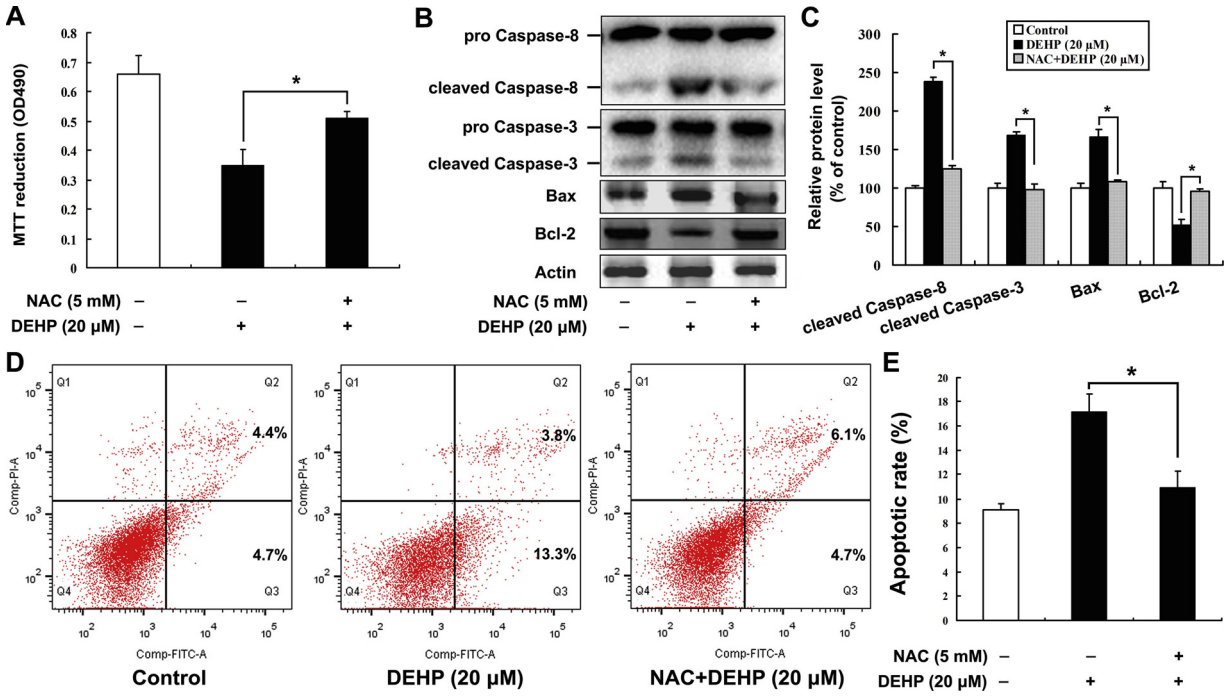

Fig. 6. Oxidative stress is involved in DEHP-induced apoptosis of mouse NE-4C cells. NE-4C cells were treated with 20 μM DEHP for 24 h in absence or presence of 5 mM NAC; then cell viability (A), the protein levels of cleaved Caspase-8, cleaved Caspase-3, Bcl-2 and Bax (B) and the AnnexinV-positive staining cells (D) were detected by MTT assay, western blot and flow cytometry, respectively. (C) The relative protein levels presented in (B) were quantified by densitometry. (E) Statistic results for apoptosis analysis. The results are expressed as mean ± SEM of three separate experiments and analyzed by one-way ANOVA. *$P < 0.05$ vs the DEHP-treated group.

DNA condensation and apoptotic bodies could be found in the ZnO NPs-treated cells. These results indicated that DEHP could induce apoptosis of NE-4C cells.

DEHP is shown to cause mitochondrial dysfunction (Li et al., 2014). Under physiological conditions, ROS is generated as by-products of mitochondrial respiration (Wen et al., 2013). In general, antioxidants and prooxidants can maintain the intracellular levels of ROS at low levels. However, oxidative stress will occur when there is an imbalance between the antioxidants and prooxidants in the cell (Jenkins and Goldfarb, 1993). It shows that many chemicals including DEHP can induce oxidative stress (Lehnert and Iyer, 2002; Liu et al., 2016; Tiwari and Vanage, 2017; She et al., 2017). To investigate whether DEHP induced oxidative stress of mouse NE-4C cells, the levels of MDA and GSH and the enzyme activities of SOD and GSH-PX were observed after the cells were treated with 0–20 μM DEHP. In the current study, DEHP could significantly increase MDA level as well as dramatically decreased the content of GSH and activities of SOD and GSH-PX, respectively, indicating that DEHP could induce oxidative stress of mouse NE-4C cells.

DEHP could induce apoptosis and oxidative stress of NE-4C cells; however, there is no evidence that oxidative stress is involved in DEHP-induced apoptosis of the cells. To investigate the potential mechanism, cell viability and apoptosis were observed after the cells were treated with the indicated concentration of DEHP in the presence or absence of NAC, a ROS scavenger. We found that inhibition of cell viability by DEHP could be rescued by NAC. Meanwhile, inhibition of oxidative stress could inhibit DEHP-induced apoptosis. These results indicated that oxidative stress was involved in DEHP-induced apoptosis of mouse NE-4C cells.

DEHP can be metabolized into the primary monoester metabolites including di-*n*-octyl phthalate (DnOP), benzyl butyl phthalate (BBzP) and diethyl phthalate (DEP) and the secondary oxidation DEHP metabolites such as mono-(2-ethyl-5-hydroxyhexyl) phthalate (5 OH-MEHP), mono-(2-ethyl-5-oxohexyl) phthalate (5 oxo-MEHP) and mono-[2-(carboxymethyl) hexyl] phthalate (2 cx-MMHP) (Huang et al., 2008; Rowdhwal and Chen, 2018). It shows that MEHP, the metabolite of DEHP can also induce oxidative stress and apoptosis (Zhang et al., 2017). It is possible that DEHP are metabolized into toxic intermediates in the NE-4C cells and then make the cells more vulnerable. So the apoptosis of NE-4C cells can be induced by DEHP itself or/and MEHP. Furthermore, it is very difficult to compare the dose between human exposure and TM3 cells in *vitro* treatment. However, it is still clinically pertinent to human health because even a dose of 50 μg/kg/day can lead to testicular toxicity (Serrano et al., 2014). This study sets in motion our future investigation of the molecular mechanisms underlying DEHP-induced neurotoxicity.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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