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Di(2-ethylhexyl) phthalate induces apoptosis through mitochondrial pathway in GC-2spd cells

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Keywords:	Di(2-ethylhexyl) phthalate, GC-2spd cells, oxidative stress, apoptosis, mitochondrial pathway

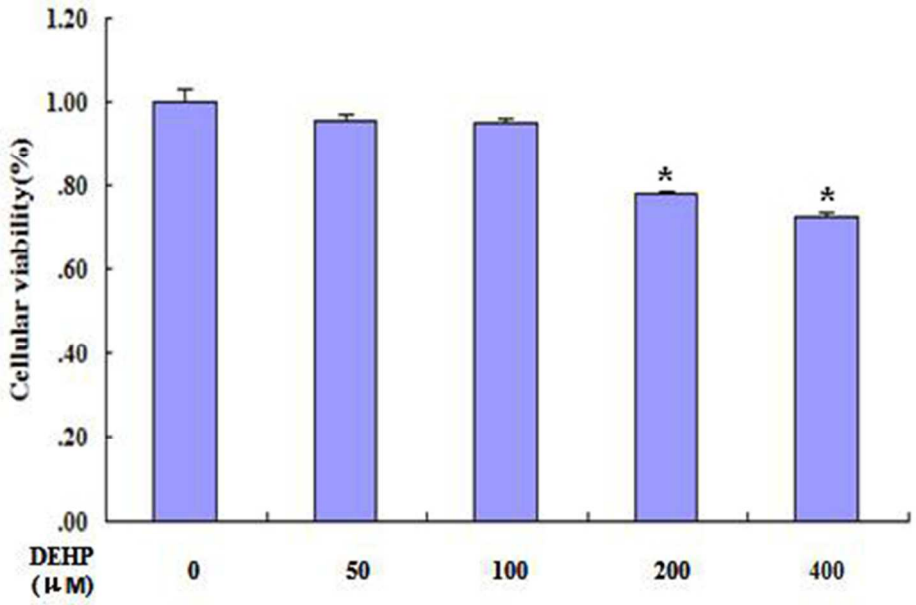

Fig. 1. Cell viability of GC-2spd cells after treatment with 0.1% DMSO, 50, 100, 200 or 400μM DEHP for 24h. Data are obtained from MTT assay (mean±SD) and are normalized to DMSO group. Statistical significance was analyzed by one-way ANOVA. Significant difference: (*) $P < 0.05$.
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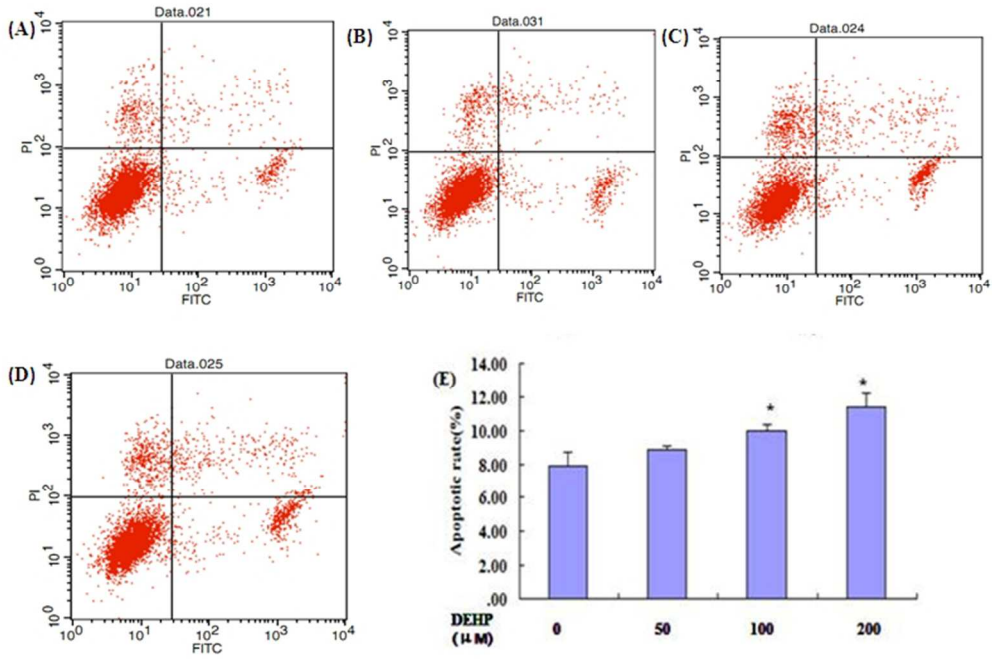

Fig. 2. Flow cytometric analysis of GC-2spd cells after treatment with DEHP. Cellular apoptosis was tested by apoptosis detection kit. A–D represented as treatment of 0.1% DMSO (A), 50μM DEHP (B), 100μM DEHP (C), 200μM DEHP (D). The comparison of apoptotic rate(%) after treatment with different concentration of DEHP is presented in E. Data were presented as mean±SD. There are three independent experiments performed in triplicate. Significant difference: (*) $P < 0.05$, compared with the control group.
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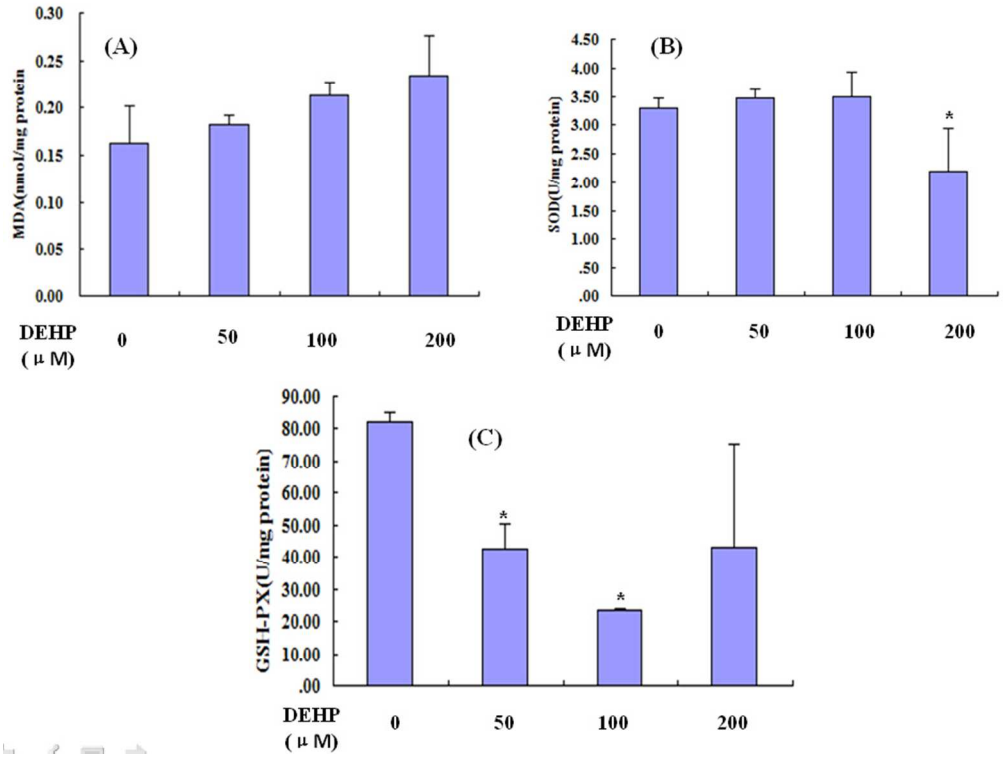

Fig. 3. A-C represent the oxidative stress levels after treatment with DEHP. Effect of different concentration of DEHP on Malondialdehyde (MDA) Level(A), Superoxide Dismutase (SOD) Activity(B) and Glutathione Peroxidase (GSH-Px) Activity(C) in GC-2spd cells. Significant difference: (*) $P < 0.05$, compared with the control group.
248x189mm (96 x 96 DPI)

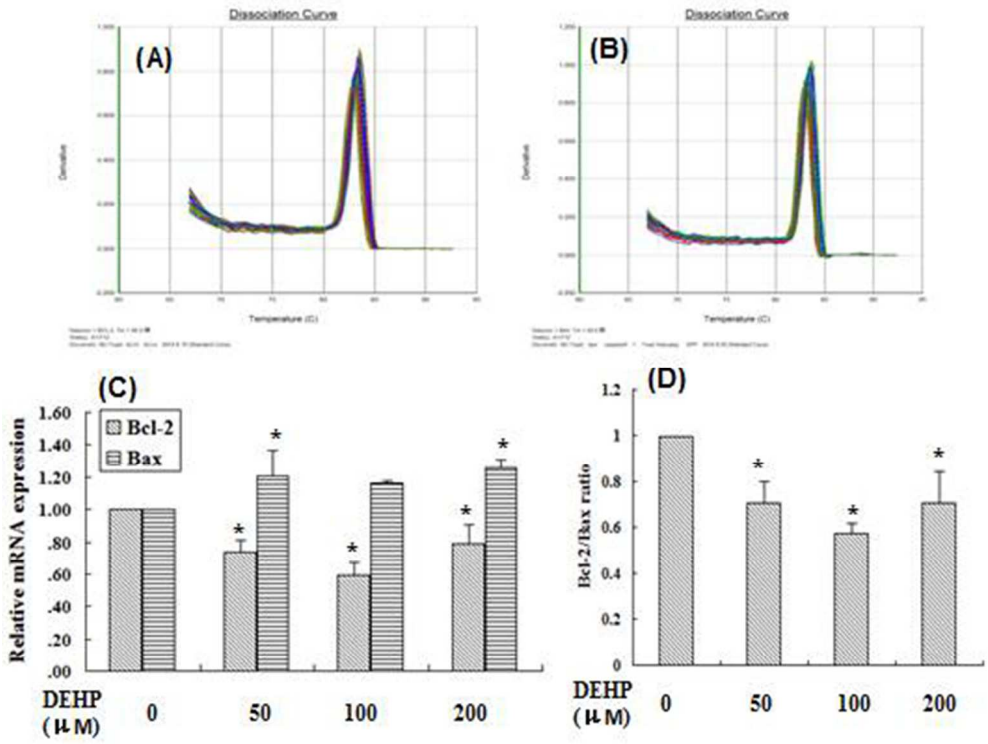

Fig. 4. A-B represent the real time quantitative PCR dissociation curve of Bcl-2(A) and Bax(B). C-D is the effect of different concentration of DEHP on Bcl-2 and Bax relative mRNA expression（C）, and the ratio of Bcl-2 and Bax(D), Significant difference: (*) P<0.05, compared with control.
142x109mm (96 x 96 DPI)

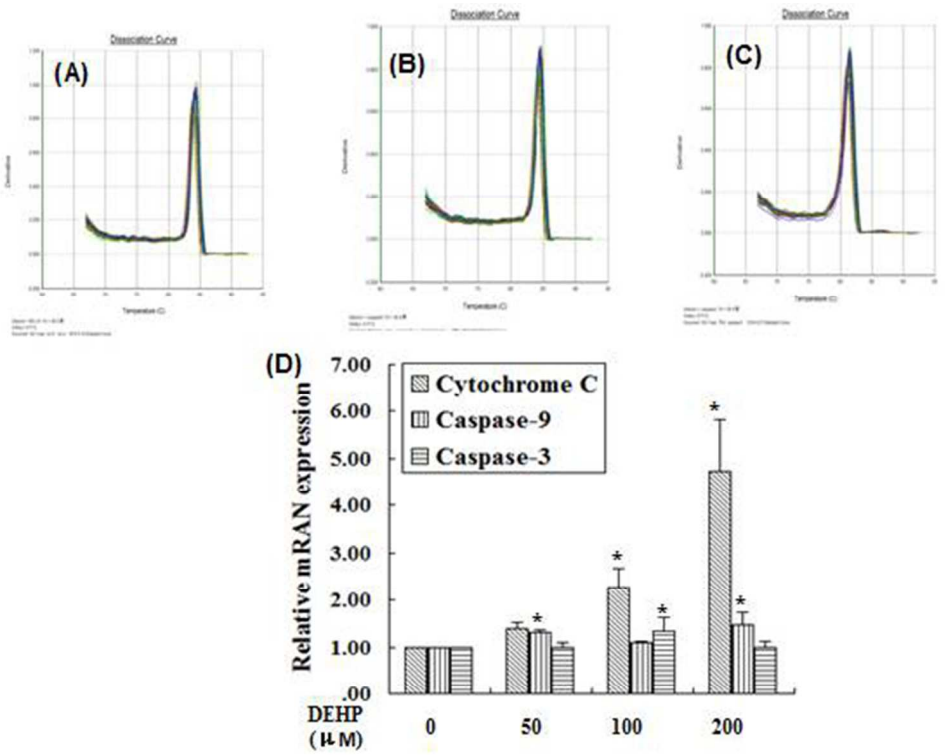

Fig. 5. A-C represent the real time quantitative PCR dissociation curve of cytochrome c (A) caspase-9(B) and caspase-3(C). D is the effect of different concentration of DEHP on cytochrome c, caspase-9 and caspase-3 relative mRNA expression(D). Significant difference: (*) P<0.05, compared with control.
152x118mm (96 x 96 DPI)

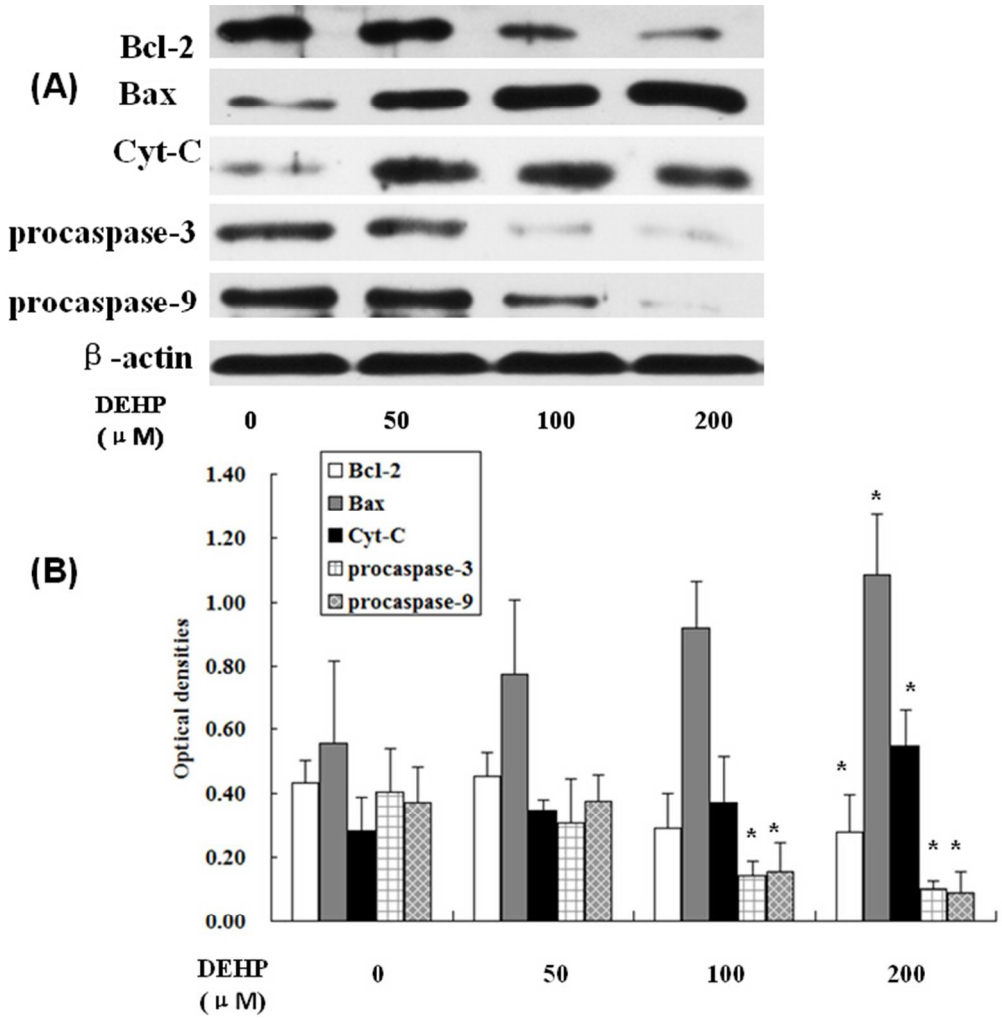

Fig. 6. Effects of DEHP exposure on the expression levels of Bcl-2, Bax, cytochrome c, procaspase-9 and procaspase-3 in GC-2spd cells(A).The level of proteins was measured using immunoblotting(B). Values represent the mean±SD. Significant difference: (*) $P<0.05$, compared with control.
199x201mm (96 x 96 DPI)

Di(2-ethylhexyl) phthalate Induces Apoptosis through Mitochondrial Pathway in GC-2spd Cells

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ABSTRACT: Di(2-ethylhexyl) phthalate (DEHP), a plasticizer of synthetic polymers, is a well-known endocrine disrupting chemical (EDC) and reproductive toxicant. Addressing the unclear mechanism of DEHP-induced reproductive dysfunction, this study used GC-2spd cells to investigate the molecular mechanism involved in the DEHP-induced toxicity in the male reproductive system. The results indicated that the apoptotic cell death was significantly induced by DEHP exposure over 100 μM. Furthermore, DEHP treatment could induce oxidative stress in GC-2spd cells involving in the decrease of superoxide dismutase (SOD) activity (200 μM) and glutathione peroxidase (GSH-Px) activity (50 and 100 μM). In addition, DEHP induction also caused the elevated ratios of Bax/Bcl-2, release of cytochrome c and decomposition of procaspase-3 and procaspase-9 in GC-2spd cells. Taken together, our work provided the evidence that DEHP exposure might induce apoptosis of GC-2spd cells via mitochondria pathway mediated by oxidative stress.

Keywords: Di(2-ethylhexyl) phthalate (DEHP), GC-2spd cells, oxidative stress, apoptosis, mitochondrial pathway

INTRODUCTION

Endocrine disrupting chemicals (EDCs) are a group of exogenous agents acting as endogenous hormones to alter the functions of endocrine system of both vertebrates and invertebrates, and thereby interfere with their survival, development, sexual differentiation and reproduction (Zoeller et al., 2012). These environmental xenobiotics may lead to dysgenic development of reproductive organ (Borch et al., 2004; Parks et al., 2000) and abnormal reproductive functions in adulthood (Daxenberger, 2002)

Di(2-ethylhexyl) phthalate (DEHP), a phthalate derivative and a well-recognized EDC, is widely used as a plasticizer and solvent in polyvinyl chloride (PVC) products, cosmetics, children's toys, shampoos, medical tubing, and other products (Cho et al., 2015; Erkekoglu et al., 2011). Not covalently bound to the plastic matrix or other chemicals in the formulations, DEHP can readily leach out of the plastic products and cause contamination in the external environment (Li et al., 2012a; Li et al., 2012b). Thus, increasing populations face the exposure risks to DEHP through contaminated foods, food packaging, or medical products (Koo and Lee, 2004; Silva et al., 2006). Many previous studies have reported the adverse effects of DEHP on male reproductive system. In rats, DEHP exposure resulted in testis weight loss, delayed preputial separation, and decreased serum testosterone (Botelho et al., 2009; Helal, 2014). For marine medaka, a reduced number of spermatozoa were induced by DEHP (Ye et al., 2014). In human, an occupational exposure model demonstrated that maternal exposure to phthalates increased the risks of hypospadias in offspring (Ormond et al., 2009). Most of these studies addressed the alteration of reproductive system in histomorphology caused by DEHP exposure, however neglecting the detailed mechanisms of DEHP on reproductive system.

Spermatogenic cells apoptosis is reported as a sensitive marker for testicular histopathology (Park et al., 2002; Wang et al., 2010). Apoptosis is a process of spontaneous and programmed cell death, with the roles in maintaining the evolution and homeostasis as well as the development of several system (Hotchkiss et al., 2009). Spermatogenic cells include spermatogonia, spermatocytes, spermatids, and

spermatozoa (Wang et al., 2014). Spermatogenesis represents the development process of immature spermatogonia into spermatozoa in seminiferous tubules (RAO and SHAHA, 2000). It is well known that normal spermatogenesis is a complex and coordinated process of cell differentiation which depends on a balance of cell apoptosis and proliferation (Qu et al., 2014), and the alteration in apoptosis rate might lead to the disruption in spermatogenesis (Shukla et al., 2012; Tripathi et al., 2009). The exposure to DEHP or MEHP was reported to increase germ cells apoptosis in rat testis (Giammona et al., 2002; Kijima et al., 2004; Park et al., 2002). Pachytene spermatocytes have been suggested to be vulnerable to DEHP-induced apoptosis in the testis (Zoeller et al., 2012). To date, few studies investigated the apoptosis pathway induced by DEHP in pachytene spermatocytes.

The present study aims to reveal the effects of DEHP concentration on apoptosis of GC-2spd cells, a mouse pachytene spermatocyte-derived cell line, and discuss the mitochondria-mediated apoptotic pathway. By quantifying the expressions of Bax, Bcl-2 and cytochrome c in DEHP-induced apoptosis, we identified that caspase family members play an important role in spermatogenesis and apoptosis. It is also of interest to determine the regulation of caspase-3 and caspase-3-9 in DEHP-induced apoptosis.

MATERIAS AND METHODS

Subculture of GC-2spd Cells and Cell Treatment

The GC-2spd cells were presented by Nanjing Medical University. The cells were cultivated with modified RPMI-1640 medium (HyClone, Beijing, China), containing 10% fetal bovine serum (Invitrogen, USA) and supplemented with 1% penicillin and streptomycin (Genom, China), in a moist atmosphere (95% air and 5% carbon dioxide, v/v) at 37°C. The culture medium was renewed every other day.

DEHP (Sigma-Aldrich, USA) was dissolved to different concentrations with dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) as stock solution, followed by serious dilution with culture medium to different final concentrations (50, 100, 200, 400 μM) which were chosen according to our preliminary results. In the control group,

the cells were exposed to culture medium with 0.1% DMSO only. The final concentration of DMSO in all the treatments was 0.1%, which did not affect the viability of GC-2spd cells.

MTT Assay

The assay is based on the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Beyotime, China) which is restored by mitochondria succinodehydrogenase of viable cells to blue-violet formazan detected by spectrophotometer. MTT was dissolved with phosphate-buffered saline (PBS) solution, added to each well with the final concentration of 5 mg/mL, and cultivated for 4 hours. Subsequently, the mediums were discarded and replaced by 200 μL DMSO to dissolve the residual formazan crystals. The culture plate was further incubated at 37°C for 15 min with shaking. The optical density (Zoeller et al.) of each well was detected at 570 nm with an ELISA Reader (Bio-Rad, USA). Cellular viability (%) was calculated according to the following equation: Cellular viability(%)= $(OD_{treatment}/OD_{control})\times100\%$.

Apoptosis Assay with Flow Cytometric Analysis

Translocation of phosphatidylserine from the inner cellular membrane to the outer leaflet in the early stage of apoptosis is an important characteristic of apoptosis (Clewell et al., 2010). After the GC-2spd cells were seeded in 6-well plate and treated with different concentrations of DEHP, the apoptosis was measured by AnnexinV/PI apoptosis kit (Multi Science, China) according to manufacturer's instruction. The treated GC-2spd cells were trypsinized, collected by 1000 rpm centrifugation for 5 min and washed with PBS. The cell pellets were further resuspended in 1 mL 1×buffer solution, 5 μL AnnexinV-FiTc and 10 μL propidium iodide (Gavathiotis et al.), and incubated in the dark at room temperature for 5 min. Finally, the stained cells were tested by a flow cytometer (Becton Dickinson, USA) and the data were analyzed by Cellquest software (Becton Dickinson, USA).

Measurement of Malondialdehyde (MDA) Level, Superoxide Dismutase (SOD) Activity and Glutathione Peroxidase (GSH-Px) Activity

The treated GC-2spd cell pellets were lysed in 0.5 mL cell lysis solution (containing 1

mM Na_2EDTA, 150 mM NaCl, 10 mM PMSF, 10 mM Tris, 1 mM aprotin) to evaluate the lipid peroxidation with MDA, SOD and GSH-Px assay (Nanjing Jiancheng Bioengineering Institute, China) according to manufacturer's instruction. As the final thermal decomposition products of cytomembrane lipid peroxidation, MDA reflects the level of oxidative damage indirectly and was detected by the absorbance of the products of MDA and thiobarbituric acida (TBA). SOD activity was measured by the reduction rate of nitro blue tetrazolium (NBT) to O_2^- produced by the xanthine-xanthineoxiase system. The one unit of SOD activity was defined as the inhibition ratio of NBT reduced by 50 percent. GSH-Px is the key enzyme in the process of glutathione (GSH) to oxidized glutathione, and its activity was assayed by the reduction of GSH. The one unit of GSH-Px activity was defined as the 1μM reduction of GSH in 1 min per mg protein.

Real time quantitative PCR

Real time quantitative PCR (RT-qPCR) was performed to determine the mRNA levels of cytochrome c, caspase-9, caspase-3, Bcl-2, Bax, and β-actin. The total RNA was extracted from the treated GC-2spd cells using Trizol reagent (Invitrogen, USA) according to the methods of Shi, et al (Shi et al., 2009). RNA purity was tested by BioPhotometer (Eppendorf, Germany), which showed a satisfactory optical density ratio (OD_{260}/OD_{280}) between 1.8 and 2.0. The cDNAs were synthesized from 1.0 μg total RNA with RevertAid First Strand Synthesis Kit (Thermo Scientific, Lithuania) in accordance with manufacturer's instructions. Then 1 μL incubation mixture was diluted to a final concentration of 1:20 and added to the individual capillary tube with reagents in Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, USA). Some primer pairs (Bioasia Corp, China) were used to amplify the targeting cytochrome c (5'-AGACAGGACAAGCACCAGGA-3'; 5'-TCACTCTTCTTTTTGATACC-3'), caspase-9 (5'-GCAAAGGAGCAGAGAGTAGT-3'; 5'-TCCCTGGAACACAGACATCA-3'), caspase-3 (5'-GTCTGACTGGAAAGCCGAAA-3'; 5'-GCAAAGGGACTGGATGAACC-3'), Bcl-2 (5'-ACTTCTCTCGTCGCTACCGT-3'; 5'-ACAATCCTCCCCCAGTTCAC-3'), Bax

(5'-GCTGATGGCAACTTCAACTG-3'; 5'-CCCGAAGTAGGAGAGGAGGC-3') and β-actin (5'-GTGACGTTGACATCCGTAAAGA-3'; 5'-GTAACAGTCCGCCTAGAAGCAC-3'), respectively.

The cDNAs of cytochrome c, caspase-9, caspase-3, Bcl-2, Bax and β-actin were individually amplified with an ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems, USA). The 10 μL PCR reaction system contained 5 μL SuperMix, 0.2 μL ROX Reference Dye, 0.2 μL of each primer (10 μM), 2 μL diluted cDNA template, and 2.4 μL PCR-grade water. The amplification program was: initial denaturation at 50°C for 2 min and 95°C for 10 min; 40 cycles of 95°C for 15 s, 60°C for 1 min. PCR products were collected 40 cycles after reaching the log-linear phase. Dissociation curves were obtained by an additional cycle (95°C for 15 s, 60°C for 15 s and 95°C for 15 s). The relative expression of target genes was calculated using $2^{-\Delta\Delta Ct}$.

Western Blotting

About 5×10^6 treated GC-2spd cells were lysed in 100 μL lysis buffer (20 mM Tris-HCl (pH 7.4), 10 mM EDTA, 2 mM EGTA, 250 mM sucrose, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl chloride, and 100 mM PMSF) and scraped from the plate to detect cytochrome c, procaspase-9, procaspase-3, Bcl-2, Bax proteins. Each protein sample was measured by a DC protein assay (Bio-Rad, USA). Cells extracts were separated in SDS-polyacrylamide gel and transferred electrophoretically onto a PVDF membrane. The membranes were blocked in PBS containing 5% (w/v) nonfat dry milk and then incubated at 4°C overnight with anti-Bcl-2 (Cell Signaling Technology, USA), anti-Bax (Epitomics, China), anti-cytochrome c (Epitomics, China), anti-procaspase-9 (Santa Cruz Biotechnology, USA) and anti-procaspase-3 (Bioworld Technology, USA) at 1:1000 dilution. Then the membranes were incubated at 37°C for 2 h with the secondary antibody combined with horseradish peroxidase (1:5000 dilution, Amersham Pharacia, UK). Finally, the immune-reactive proteins were detected using an ECL western blotting detection system (Pierce Biotechnology, USA), and the densitometric analysis of immunoblots was performed with Gel pro 3.0 software.

Statistical Analysis

Results were represented as mean ± standard deviation (SD). Significance was assessed by One Way ANOVA following variance normalization and equalization where necessary. Mean values were compared by subsequent student-Newman-Keuls (SNK) using the SPSS statistical package 20.0 (SPSS, USA). A difference at $p < 0.05$ was considered statistically significant.

RESULTS

DEHP Inhibited the Viability of GC-2spd Cells

Treated with 50, 100, 200 and 400 μM DEHP for 24h, the viability of GC-2spd cells were analyzed by MTT assay and the results were illustrated in Figure 1. Compared to the control, the viability of GC-2spd cells was significantly reduced ($P<0.05$) in the treatments with 200 or 400 μM DEHP. Particularly in 400 μM DEHP treatment, the cellular viability was less than 70% of that in the control. Accordingly, 50, 100 and 200 μM was the optimal concentration to study the impacts of DEHP on apoptosis and was used in the following experiments.

DEHP-induced Apoptosis of GC-2spd Cells

After exposing GC-2spd cells to different concentrations of DEHP (50, 100 and 200 μM) for 24h, the results of Annexin/PI double staining characterized the phosphatidylserine exposure and revealed the DEHP-induced apoptosis of GC-2spd cells. From Figure 2, the dose-effect relationship was identified between DEHP exposure and apoptotic cell death. A significantly increasing apoptotic ratios of GC-2spd cells were found in the treatments with 100 and 200 μM DEHP ($P<0.05$).

Effects of DEHP on MDA Level, SOD Activity and GSH-Px Activity in GC-2spd Cells

To assess the influence of oxidative stress, MDA level, SOD activity and GSH-Px activity were measured in GC-2spd cells. As illustrated in Figure 3, DEHP induction showed slightly positive dosage effect on MDA level, but without significant difference compared to the control ($P>0.05$). The SOD activity was limited affected after exposure to 50 and 100 μM DEHP, but significantly declined in 200 μM DEHP treatment ($P<0.05$). As for GSH-Px activity, a remarkable decrease was found in 50

and 100 μM DEHP treatments compared to that in the control (P<0.05).

DEHP Affected Expression of Cytochrome c, Caspase-3, Caspase-9, Bcl-2 and Bax in GC-2spd cells at mRNA LeveL

The mRNA levels were detected by RT-qPCR in the GC-2spd cells exposed to different concentrations of DEHP. The transcriptional changes of cytochrome c, caspase-3 and caspase-9 were shown in Figure 4(D). Cytochrome c mRNA levels in 100 and 200 μM treatments were significantly higher than that of the control (P<0.05). Compared to the control, caspase-9 was remarkably higher transcribed when exposed to 50 μM and 200 μM DEHP (P<0.05), whereas the caspase-3 mRNA level in 100 μM DEHP treatment was statistically higher than that of the control (P<0.05). From the mRNA levels of Bcl-2 and Bax illustrated in Figure 5(C), the transcription of Bcl-2 was significantly declined in all the DEHP treatments (P<0.05), and remarkably higher Bax mRNA level was observed in 50 μM and 200 μM DEHP treatments (P<0.05). Meanwhile, Figure 5(D) showed that the transcriptional ratio of Bcl-2 and Bax in different DEHP treated groups was statistically lower than that of the control (P<0.05).

DEHP Altered Expression of Cytochrome c, Procaspase-3, Procaspase-9, Bcl-2 and Bax in GC-2spd cells at Protein Level

From the results of Western blot (Figure 6), Procaspase-3 and Procaspase-9 were significantly decreased after treated with 100 μM or 200 μM DEHP, suggesting the activation of proapoptotic caspase-3 and caspase-9 genes. DEHP also led to markedly declining expression of Bcl-2 in 200 μM DEHP treatment but induced the expressions of Bax and cytochrome c.

DISCUSSION

The present study revealed that DEHP was toxic to GC-2spd cells, resulting in their decreasing viability and the induction of apoptosis. Furthermore, mitochondria-related proteins (cytochrome c, Bax and Bcl-2) might play critical roles in DEHP-induced apoptosis.

Apoptosis is an important process to eliminate unwanted or defective cells

through an orderly process of cellular disintegration (Ameisen, 1996) and the alteration in apoptosis rate may result in disruption in spermatogenesis (Shukla et al., 2012; Tripathi et al., 2009). Previous studies have already reported that some known endocrine disruptors could induce spermatogenic cell apoptosis, such as MEHP (an active metabolite of DEHP) (Awal et al., 2004), bisphenol A (BpA) (Wang et al., 2010) and aroclor 1254 (Qu et al., 2014). In the present study, we revealed the effects of DEHP exposure on GC-2spd cell apoptosis using flow cytometric assessment. Our results suggested that a significant apoptosis of GC-2spd cells was induced after exposure to 100 and 200 μM DEHP. The findings were compatible with recent report by Erkekoglu (Erkekoglu et al., 2012) who suggested that 1000 mg/kg DEHP treatment caused an approximately 8-fold increase of apoptosis in rat germ cells. The excess apoptosis of spermatogenic cells therefore might be one of the major molecular mechanisms of endocrine disruptors in male reproductive toxicity.

Oxidative stress is often caused by various environmental pollutes. Constant oxidative stress can produce an imbalance between endogenous and exogenous reactive oxygen species (ROS) levels, which subsequently lead to lipid peroxidation, antioxidant defenses and even oxidative damage in organisms (Khan et al., 2015; Ott et al., 2007; Valavanidis et al., 2006). There is a general agreement that male reproductive organs are particularly susceptible to oxidative stress, which ultimately lead to impaired fertility (Jacobson, 1996). Song found that p,p'-DDE exposure induced the apoptosis of rat sertoli cell via oxidative stress with the elevation of ROS, decrease in SOD activity, and increase in the leakage rate of lactate dehydrogenase (LDH) and MDA levels (Song et al., 2008). Madhubabu reported that oral exposure to allethrin had increasing lipid peroxidation and declining activities of catalase, glutathione peroxidase, glutathione-S-transferase and superoxide dismutase, consequently affecting fertility (Madhubabu and Yenugu, 2014). In the present study, SOD and GSH-Px (ROS scavengers) were depleted and MDA was accumulated as a product of lipid peroxidation in GC-2spd cells, consistent with previous findings that DEHP exposure induced spermatogenic disturbance mediated by oxidative stress (Hirai et al., 2015).

From many previous studies, cell apoptosis could be induced via the activation of oxidative stress (Abdullah et al., 2015; Kannan and Jain, 2000). Zoeller (Zoeller et al., 2012) suggested that the MEHP-induced apoptosis of spermatocytes follows the enhancing generation of ROS by testicular cells. The mitochondrial pathway of apoptosis is under the control of Bcl-2 family (Maire et al., 2005) which can be categorized into three subfamilies, namely anti-apoptotic Bcl-2 members (Bcl-2, Bcl-XL and Mcl-1), pro-apoptotic Bax members (Bax, Bak and Bok), and BH3-only members (Bad, Bid, Bim, Bik and Puma) (Cory and Adams, 2002). It is well-established that the major mechanism of mitochondria-mediated apoptosis is the regulation of the mitochondrial outer membrane permeabilization (MOMP) and the release of mitochondrial intermembrane space (IMS) proteins, such as cytochrome c (Renault and Chipuk, 2014). Bax and Bcl-2, the positive and negative regulator respectively, are the representative members of Bcl-2 family (Borner, 2003). Under a normal state, Bax might be either on outer mitochondrial membrane or in the cytosol. Once triggered by apoptotic signals, the BH3-only proteins such as Bid (Desagher et al., 1999; Wei et al., 2000) and Bim (Gavathiotis et al., 2008; Kim et al., 2009) act as direct activators to induce oligomerization and enhance pore-forming activity of Bax. Then the homodimer Bax/Bax with the structure of micropore will induce MOMP and allow the release of mitochondrial IMS proteins (e.g. cytochrome c) (Kluck et al., 1997; Liu et al., 1996) to activate the cell death proteases called caspases (Cory and Adams, 2002). Bcl-2 is located on outer mitochondrial membrane. It can combine the Bax competitively to form more stable heterodimer Bax/Bcl-2 and therefore inhibit the apoptosis induced by the Bax/Bax (Rogerio et al., 2006). Thus, Bcl-2/Bax ratio is very important in apoptosis regulation. The down-regulation of Bcl-2 protein and the up-regulation of Bax protein can cause MOMP and induce cell apoptosis eventually (Kim et al., 2015). The present study showed an increasing expression of Bax at both mRNA and protein levels in GC-2spd cells after exposure to DEHP, accompanying with the decreasing expression of Bcl-2. Thus, a declining Bcl-2/Bax ratio was observed in DEHP treatments, compatible with the findings that the exposure of mice to BPA during puberty resulted in a significant increase in Bax/Bcl-2 ratio in testes

and a consequent increase in germ cells apoptosis(Wang et al., 2010).

Cytochrome c is a key factor in mitochondrion-mediated apoptosis and presents in the mitochondrial intermembrane serving as a transducer of electrons in the respiratory chain under normal conditions. Once mitochondrial dysfunction causing the collapse of mitochondria membrane potential(MMP) and intracellular imbalance between Bcl-2 and Bax proteins, the mitochondrial permeability transition pore (MPTP) will open (Fulda and Debatin, 2006; Wang et al., 2013; Zhang et al., 2015), leading to the release of cytochrome c from the mitochondrial intermembrane space into the cytoplasm. Then cytochrome c can bound with Apaf-1 and procaspase-9 (Zhivotovsky et al., 1998), causing the formation of apoptosome (Adrain and Martin, 2001).The apoptosome activates caspase-9, and then cleaves and activates other caspases, such as caspase-3 (Zhivotovsky et al., 1998). Caspase-3 serves as the final executor of apoptosis responsible for the cleavage of key cellular proteins and contributable to apoptosis (Budihardjo et al., 1999). In agreement with the theory, our study showed that the cytochrome c was upregulated after 200μM DEHP treatment at both mRNA and protein levels. Procaspase-9 and procaspase-3 are the inactive precursor forms of caspase-9 and caspase-3. Our results illustrated the increasing mRNA levels of caspase-9 and caspase-3, intrinsically linked to the decreasing protein levels of procaspase-9 and procaspase-3 in DEHP-treated GC-2spd cells. These data is consistent with previous findings that BPA caused the release of cytochrome c and subsequently increased the expression of caspase-3 significantly in GC-2spd cells confirming the involvement of mitochondria-dependent pathway in BPA-induced apoptotic events in GC-2spd cells(Qian et al., 2015). However, Wójtowicz et al (Wojtowicz et al., 2007) reported decreasing caspase-3 in DDE-treated JEG-3 cells. The different mechanism for DEHP and DDE in different cell lines may be the best explanation.

Frankly, there are some limitations in the present study. Firstly, the ROS inhibitor such as *N*-acetyl-l-cysteine (NAC) might affect the apoptosis induced by DEHP and should be tested here to get more convictive conclusions. Additionally, it is well known that apoptosis occurs via two primary pathways: the extrinsic pathway, which

is associated with cell death receptors and their ligands on the cellular surface, and the intrinsic pathway, which is dependent on mitochondria. This study only investigated the roles of mitochondria pathway on the DEHP-induced apoptosis of GC-2spd cells, and some more studies should be included and will be further investigated on another pathway.

In conclusion, DEHP induced the apoptotic rate in GC-2spd cells, possibly explained by the mechanism involving mitochondria-mediated pathway. *In vitro* exposure to DEHP can enhance oxidative stress, induce an increasing expression of Bax, cytochrome c, caspase-9 and caspase-3 at both mRNA and protein levels, and suppress the expression of Bcl-2 in GC-2spd cells. Mitochondrial pathway is an extrinsic program of apoptotic death, which is characterized in this study by the disruption of Bcl-2/Bax balance and the activation of cytochrome c and caspase-9. Finally, the apoptosis of GC-2spd cells is regulated by a final executioner, Caspase-3, thereby disturbing the spermatogenic process. The results in the present study provide preliminary but valuable evidence on the mechanisms of DEHP-induced apoptosis of GC-2spd cells and suggestions for further study of reproductive endocrine disorder resulting from environmental EDCs.

Acknowledgments

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