Environmental Toxicology and Pharmacology 44 (2016) 18–24

Contents lists available at ScienceDirect

Environmental Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/etap

Di-(2-ethylhexyl) phthalate induces apoptosis of GC-2spd cells via TR4/Bcl-2 pathway

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ARTICLE INFO

Article history:
Received 21 August 2015
Received in revised form 28 March 2016
Accepted 5 April 2016
Available online 8 April 2016

Keywords:
Di-(2-ethylhexyl) phthalate
GC-2spd cells
Cells apoptosis
Testicular orphan nuclear receptor 4
Bcl-2

ABSTRACT

Di-(2-ethylhexyl) phthalate (DEHP) is a widely used environmental endocrine disruptor. Many studies have reported that DEHP exposure causes reproductive toxicity and cells apoptosis. However, the mechanism by which DEHP exposure causes male reproductive toxicity remains unknown. This study investigated the role of the testicular orphan nuclear receptor4 (TR4)/Bcl-2 pathway in apoptosis induced by DEHP, which resulted in reproductive damage. To elucidate the mechanism underpinning the male reproductive toxicity of DEHP, we sought to investigate apoptotic effects, expression levels of TR4/Bcl-2 pathway in GC-2spd cells, including TR4, Bcl-2 and caspase-3. GC-2spd cells were exposed to various concentrations of DEHP (0, 50, 100, or 200 μM). The results indicated that, with the increase of the concentrations of DEHP, the survival rate of cell decreased gradually. DEHP exposure at over 100 μM significantly induced apoptotic cell death. DEHP decreased SOD and GSH-Px activity in 200 μM group. Compared to the control group, the mRNA levels of caspase-3 increased significantly, however, Bcl-2 mRNA decreased ($P < 0.05$). In addition, there was a significant reduction in TR4, Bcl-2 and procaspase-3 protein levels. Taken together, these results lead us to speculate that in vitro exposure to DEHP might induce apoptosis in GC-2spd cells through the TR4/Bcl-2 pathway.

1. Introduction

Phthalic acid esters (PAEs), extensively used additive, are defined as plasticizers that are always used to enhance the elasticity of chloroethylenes, and applied in the development of food packaging, plastic floor, carpet mterial, flexible plastics of roofing materials, and plastic wall materials handling, interior decoration, electric wires, electric cables, packaging materials and children's toys (Sathyanarayana, 2008). PAEs are non-covalently bonded to the product substrate, they are easily released from products and then transfer into surrounding environment to pollute the air, soil, water and even foods, and become globally environmental pollutants (Assessment, 2007). The amount of di-(2-ethylhcxyl) phthalate (DEHP) is dominant in plasticizers, accounting for about 50% of the total amount (Singh and Li, 2012). As extensively existing environmental pollutants, DEHP is detected in series, including milk, meats, fish, sea-foods, drinks, cereals and vegetables as well as breast milk. DEHP can be absorbed into human bodies through many passages containing water, foods, air fine particulates and skin-protecting products (Hauser and Calafat, 2005).

DEHP is also defined as a sort of endocrine disrupting chemicals (EDCs) characterized by anti-androgenic hormone, which can disrupt human and animal endocrine systems (Erythropel et al., 2014). EDCs have been proven to have potentially deleterious effects on development, growth, metabolism, and reproduction (Hannon et al., 2015). National Toxicology Program (NTP) makes a systematic evaluation on DEHP given that DEHP generates potential hazard on human reproductive system (Shelby, 2006). Previous studies from in vitro cultured cell and animals indicate that DEHP causes a constellation of adverse effects, hepatotoxicity, infertility, and teratogenicity (Engel and Wolff, 2013; Erkekoglu et al., 2012; Lagos-Cabre and Moreno, 2012). Testis cells are considered

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http://dx.doi.org/10.1016/j.etap.2016.04.003
1382-6689/

as a target for DEHP to affect reproductive organ development. One test showed that rats exposed to DEHP are presented with orchiatrophy, decrease in sperm count and motility (Hsu et al., 2014; Martino-Andrade and Chahoud, 2010; Yang et al., 2012).

Previous studies reported that exposure to DEHP induce germ cells apoptosis in rat testis (Giammona et al., 2002; Kijima et al., 2004; Park et al., 2002). It has been suggested that apoptosis induced by DEHP was observed primarily in pachytene spermatocytes (Kasahara et al., 2002). But few studies investigated the mechanism that how DEHP induces male germ cell apoptosis, particularly no details were known about the role of the testicular orphan nuclear receptor 4 (TR4)/Bcl-2 pathway in apoptosis in spermatocytes. The aim of the present study was to determine the effects of different concentrations of DEHP on apoptosis in GC-2spd cells (spermatocytes of mice before meiosis) and to investigate the role of TR4/Bcl-2 in apoptotic pathway.

2. Materials and methods

2.1. Cell source and culture

GC-2spd cells were provided by Nanjing Medical University.

Recovery of the GC-2spd cells. The frozen tube with GC-2spd cells was taken out from liquid nitrogen and then warmed in 37 °C water bath until cell suspension was melted. Cell suspension was transferred into 15 ml centrifuge tube containing 10 ml culture media and then centrifugation was performed for 5 min at 1000 rpm. The supernatant fluid was poured away and 1 ml culture media was added to make cell suspension. The suspended cells were transferred into culture bottle with DMEM containing 10% FBS. Culture media was changed every three times. After passaging two times, the cells were maintained in 5% CO_2, 35 °Cincubator in RPMI-1640 nutrient solution with 10% fetal calf serum (cell concentration: 1×10^5/ml, inoculated in six well culture plate).

2.2. Cell treatment

Cells cultured in monolayer were digested using 0.25% trypsin. Single cell suspension was made using DMEM culture media with 10% fetal calf serum and then seeded into a six well culture plate at 1×10^5/well and 2.0 ml/well. The culture plate was maintained in CO_2 incubator for 2 days. The cells were treated with different concentrations of DEHP (Aeresco, USA, purity: ≥99.0%) (50 μM, 100 μM and 200 μM, respectively), with DMSO as the control group. DEHP concentrations were designed, in accordance with the results of cell cytotoxicity test.

2.3. Measurement of cell activity

Cells at logarithmic phase were digested using trypsin and then cell suspension was made. Cells were seeded into a 96 well plate at 1×10^5 cells/well and 200 μl/well. Cells were maintained in CO_2 (5%) incubator at 37 °C for 24 h to achieve adherence. After culture for another day, the cells were treated with different concentrations (0, 50, 100, 200 and 400 μM, respectively) of DEHP for 24 h 50 μl MTT solution (5 mg/ml) was added into each well to culture for 4 h. After expiration of culture, supernatant fluid was removed and then 200 μl DMSO was added into each well with uniform shaker. Optical absorption value was measured using ELLSA at 570 nm. Cellular viability (%) was calculated according to the following equation: Cellular viability (%) = $(OD_{treatment}/OD_{control}) \times 100\%$.

2.4. Measurement of apoptosis rate

After cell collection, 5 μl of Annexin V-FITC and 5 μl of Propidium Iodide mixture were added. Cells were kept away from light under room temperature for 5–15 min. Fluorescence intensity was measured using flow cytometry (FCM) using 1×10^5 cells/sample. The experiments were repeated three times.

Detection of the activity of SOD and GSH-Px, and the content of MDA Cells in good growth situation were selected for digestion and centrifugation, and the supernatant was discarded. Then culture medium was added to resuspend the cells and the cells were dissociated uniformly and counted. Culture medium was added again to adjust the cell density to 4×10^5 cells/ml, and the cells were seeded into six-well culture plates as amount of 2 ml/well and cultured for 24 h. DEHP solutions with concentrations of 0, 50, 100 and 200 μM/L were applied to the cells 24 h after the treatment, the culture medium was discarded and the cells were washed with PBS. After digestion and centrifugation, the cells were collected, resuspended with PBS, and disrupted with ultrasonic cell disruption system on crushed ice. The cells were centrifuged at 3000 rpm/min under 4 °C for 10 min and the supernatant was taken for determination of protein concentration with comassie brilliant blue colorimetric method.

GSH-Px activities were assayed by quantifying the rate of oxidation of the reduced glutathione to the oxidized glutathione by H_2O_2. One unit of GSH-Px was defined as the amount that reduced the level of GSH by 1 μM in 1 min per mg protein. SOD activity in supernatant was determined by determining the reduction of nitro blue tetrazolium (NBT) by O^2- produced from the xanthine–xanthineoxiase system. One unit of SOD was defined as the amount serum inhibits the rate of NBT reduction by 50%. Results were defined as U/mg protein. The concentrations of MDA were assessed by measuring thiobarbituric-acid (TBA) reacting substances at 532 nm. The level of MDA was expressed as nmol MDA per mg protein.

2.5. Real time fluorescent quantitative PCR

Real time quantitative PCR was performed to determine the relative mRNA levels of Bcl-2 and caspase-3. Total RNA was extracted from cells with Trizol reagents (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. Trizol reagents were ready-to-use, monophasic solutions of phenol and guanidine isothiocyanate suitable for isolating total RNA, DNA, and proteins. During sample homogenization or lysis, Trizol reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. 1.0 ml of Trizol was added to each sample, and the contents were then placed in 1.5-ml Eppendorf Micro Test RNase-free Tube (EP tube). They were incubated at 0 °C for 5 min. Then, 0.2 ml chloroform was added and the tubes were shaken for 15 s, and placed on ice for 2–3 min, then they were centrifuged at 12,000 rpm for 15 min at 4 °C. The colorless upper aqueous phase containing the RNA was transferred to a new EP tube without RNase. An equal volume of isopropanol was added, and the RNA was precipitated by centrifugation. The RNA pellet was washed with 75% ethanol and dissolved in water treated with diethylene pyrocarbonate (10–20 μl). RNA purity was tested with eppendorf BioPhotometer (Eppendorf, Germany), which showed an optical density ratio (OD_{260}/OD_{280}) that was between 1.8 and 2.0. 1 μg of total RNA of was reverse transcribed to complementary DNA using Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Lithuania).

Real-time PCR was performed with an ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems, USA) using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, USA). β-actin was used in parallel for each run as internal control. A 10 μl PCR reaction system was used and included the appropriate concentration of 2 μl cDNA, 5 μl SuperMix, 0.2 μl ROX Reference Dye, 0.2 μl forward and reverse primers (10 μM) and 2.4 μl DEPC-treated H_2O. A four-step experimental run protocol was carried out and the amplification conditions were as follows: 50 °C for 2 min

Table 1
Description of primers used in this study.

Primers	Primer sequence (5′-3′)	Length (bp)
Caspase3	F: GTCTGACTGGAAAGCCGAAA R: GCAAAGGGACTGGATGAACC	212
Bcl-2	F: ACTTCTCTCGTCGCTACCGT R: ACAATCCTCCCCCAGTTCAC	190
β-actin	F: GTGACGTTGACATCCGTAAAGA R: GTAACAGTCCGCCTAGAAGCAC	287

(UDG incubation); 95 °C for 10 min (initial denaturation); 40 cycles of 15 s at 95 °C (denaturation) and 1 min at 60 °C (elongation). A melting curve was generated at the end of every run to ensure product uniformity (95 °C for 15 s, 60 °C for 15s, 95 °C for 15 s). The relative expression of target genes was calculated using $2^{-\triangle\triangle Ct}$.

The primer sequences were designed according to cDNA sequence from Genbank (Table 1). All primers were synthesized by the Bioasia Corp (Shanghai, China).

2.6. Western blotting

Cells were washed 2–3 times using PBS and proper RIPA was added into. The plate was shocked to mix reagents with cells. Cells and reagents were removed using cell scraper and then collected into 1.5 ml centrifuge tube. Cells were cooled for 30 min. Cells were blown using transferpettor to ensure cells splitting. Cells were centrifuged at 12,000g for 5 min and then supernatant fluid was collected as the total protein solution.

10% separation gels and 5% stacking gels were prepared, and the protein samples were loaded successively. The stacking gels received electrophoresis under 60 V for 30 min and the separation gels received electrophoresis under 120 V for 60 min. Proteins were transferred to PVDF membranes under a constant current of 350 mA for 60 min, then the membranes were blocked in PBS containing 5% (w/v) nonfat dry milk and then incubated at 4 °C overnight with anti-TR4(Cell Signaling Technology, Inc., Boston, USA), anti-BCI-2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-procaspase-3 (Bioworld Technology, Inc., Minnesota, USA) at 1:1000 dilution. Then the membranes were incubated at 37 °C for 2 h with the secondary antibody combined with horseradish peroxidase (Amersham Pharacia, Buckinghamshire,UK) diluted at 1:5000. At last, the immune-reactive proteins were detected using the ECL Western blotting detection system(Pierce Biotechnology Inc., Rockford, IL, USA), and the Densitometric analysis of immunoblots was performed with Gel pro 3.0 software.

2.7. Statistical analysis

Results were represented as mean ± SD. Significance was assessed by One Way ANOVA following appropriate transformation to normalize and equalize variance when necessary. Mean values were compared by subsequent student-Newman-Keuls (SNK) using the SPSS statistical package 20.0 (SPSS Inc., Chicago, IL, USA). A difference at $P < 0.05$ was considered statistically significant.

3. Results

3.1. Changes of cell activity

As shown in Fig. 1, GC-2spd cell survival rate was decreased with the increase of DEHP concentration (0, 50, 100, 200, 400 μM). Compared to the control group, the survival rate was significantly decreased in 200 μM and 400 μM groups ($P < 0.05$). And in the group treated with 400 μM of DEHP, the cellular viability was less than 70% of that in control group. On this basis, the maximum concentration of 200 μM was used in the following experiments.

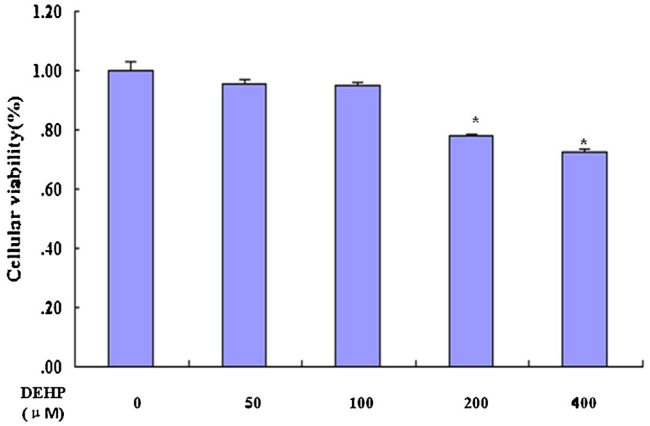

Fig. 1. Effect of DEHP on the viability of GC-2 spd Cells with MTT assay. Cells were treated with 0-, 50-, 100-, 200-, or 400-μL of DEHP for 24 h, and the optical density (OD) was measured by MTT. Data were presented as mean ± SD of three independent experiments performed in triplicate. Significant difference: $^*P < 0.05$, compared to the control group.

3.2. Effect of DEHP on GC-2spd cell apoptosis

The result was illustrated in Fig. 2. It can be observed that DEHP caused apoptotic cell death in a dose-dependent manner. And the apoptotic ratio of GC-2spd cells increased significantly after treatment with 100, 200 μM of DEHP ($P < 0.05$).

3.3. Effects of DEHP on MDA level, and SOD and GSH-Px activities in GC-2spd cells

Compared to the control group, MDA content was increased in the 50, 100, 200 μM treatment groups, but the difference between treated and control group was not significant ($P > 0.05$). SOD activity of 200 μM group was significantly lower than that in the control group ($P < 0.05$). The GSH-Px activity in 50,100 μM of the DEHP group was decreased remarkably than that in the control group ($P < 0.05$) (Fig. 3).

3.4. The effect of DEHP on Bcl-2 and caspase-3 mRNA expression in GC-2spd cells

As shown in Fig. 4, compared to the control group, caspase-3 mRNA level in 100 μM treated group was statistically higher ($P < 0.05$). In addition, Bcl-2 mRNA level of all groups was statistically lower than that of the control group ($P < 0.05$).

3.5. The effect of DEHP on TR4, Bcl-2 and proaspase-3 protein expression in GC-2spd cells

As seen in Fig. 5, compared to the control group, expressions of TR4 and Bcl-2 protein at 200 μM dose groups was decreased ($P < 0.05$). Procaspase-3 was significantly decreased after treatment with DEHP at 100 μM or 200 μM, suggesting that caspase-3 was activated.

4. Discussion

GC-2spd cells are immortalized cells derived from the preleptotene stage spermatocytes of mouse with transfections of simian virus-40 (SV-40) tumor antigenic gene and temperature sensitive

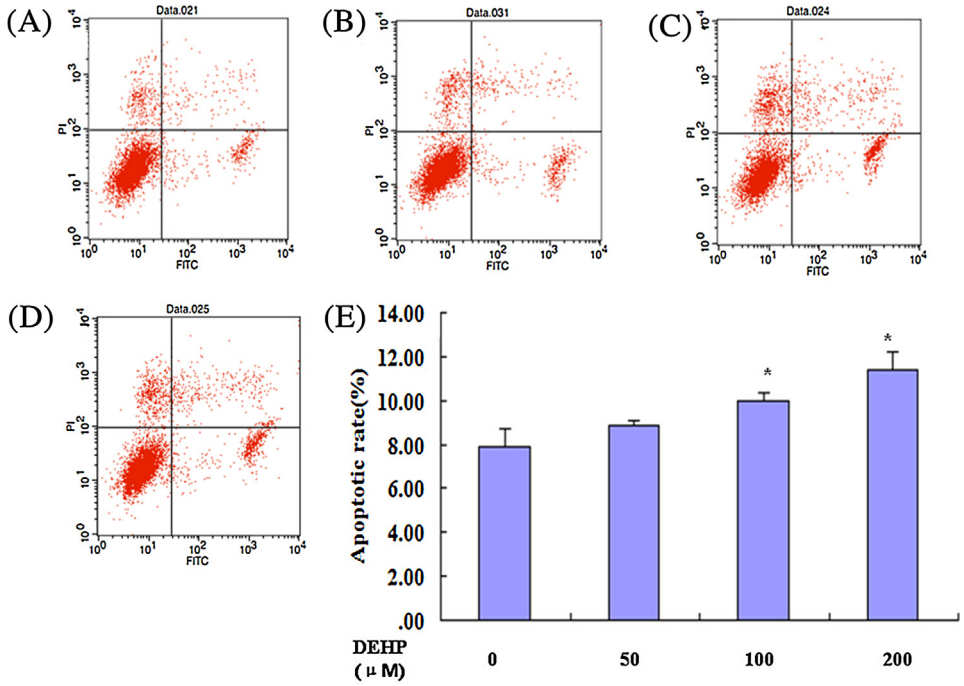

Fig. 2. The Effect of DEHP on GC-2 spd Cell Apoptosis. (A–D) Representative plots of PI-Annexin staining of GC-2 spd Cells. (E) The apoptotic rate showed that 100- or 200-μL DEHP could induce apoptotic GC-2 spd death. Data were presented as mean ± SD of three independent experiments performed in triplicate. Significant difference: $^{*}P < 0.05$, compared to the control group.

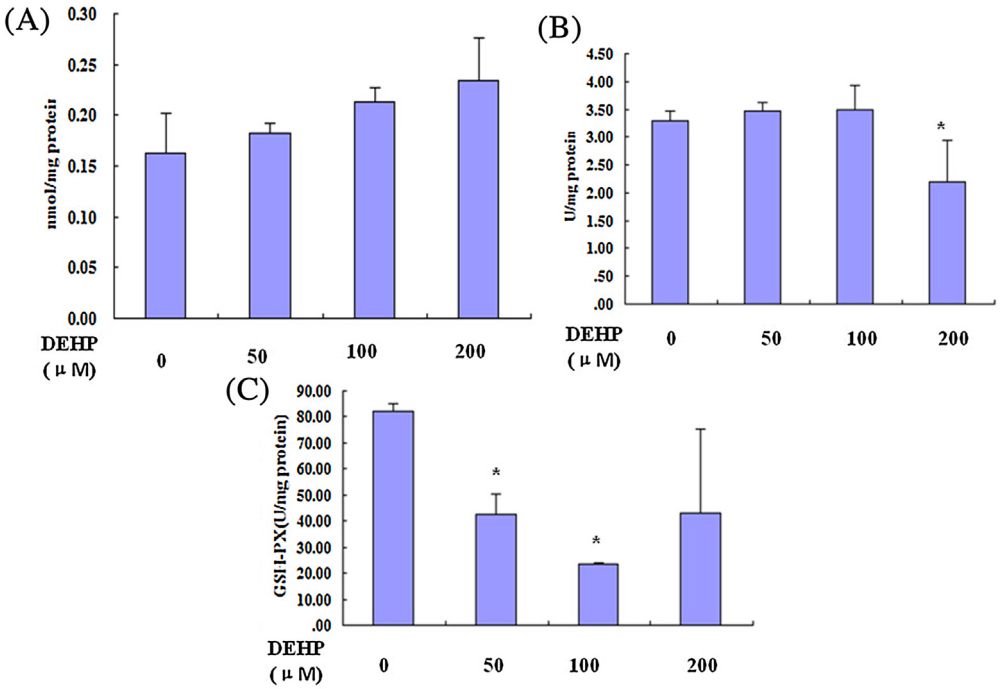

Fig. 3. Effects of different concentrations DEHP on SOD, GSH-Px Activity and MDA Content of GC-2 spd Cells. (A) Effects of DEHP on MDA Content of GC-2 spd Cells; (B) Effects of DEHP on SOD Activity of GC-2 spd Cells; (C) Effects of DEHP on GSH-Px Activity of GC-2 spd Cells. Data are indicated as mean ± SD. Significant difference: $^{*}P < 0.05$, compared to the control group.

mutant p53 gene. GC-2spd cells express the germ cell-specific lactate dehydrogenase C_4 isozyme and cytochrome c_t isoform. At the permissive temperature of 37 °C, the GC-2spd line generates cells with a haplold DNA content and morphologic and biochemical features of round spermatids, including the appearance of an acrosomic granule. The identification of a flagellar axoneme when these cells are cultured at 32 °C further indicates that these cells correspond to the early spermatid stages of spermiogenesis (Hofmann et al., 1994). GC-2spd cells have been applied in the toxicity assessment and mechanism research of reproductive toxicants (Gao et al., 2012; Lizama et al., 2011) (Fig. 6).

The normal apoptosis of germ cells can exclude malformed or damaged germ cells, ensuring genetic information accurately passes to filial generation. However, excess apoptosis decreases

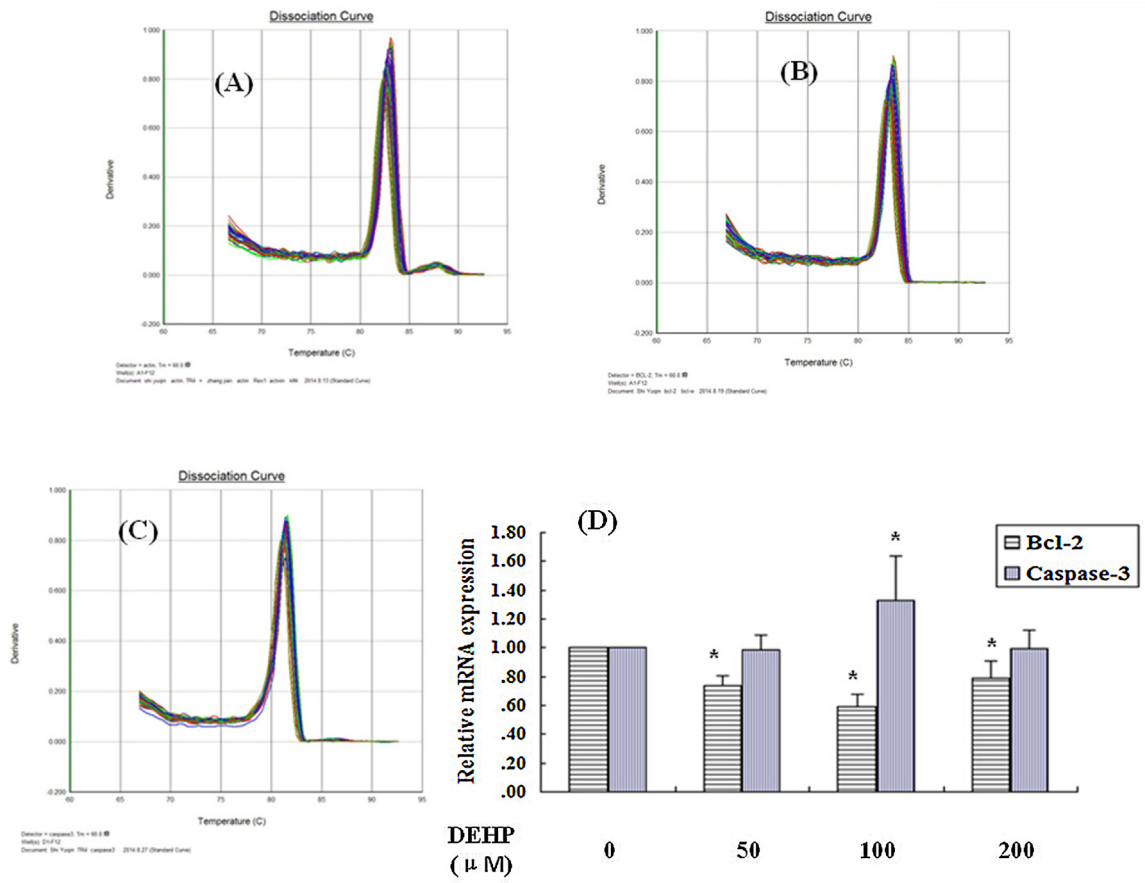

Fig. 4. Effects of DEHP on the Bcl-2 and caspase-3 mRNA expression levels in GC-2 spd Cells by real time quantitative PCR. (A–C) Dissociation curve of β-actin, Bcl-2 and caspase-3, the curves featured by a single peak at expected Tm; (D) Quantitative analysis of Bcl-2 and caspase-3 mRNA expression levels of GC-2 spd Cells exposed to different doses of DEHP. The housekeeping gene β-actin was used as an internal positive control standard for quantitative analysis. The relative expression of target genes was calculated using $2^{-\Delta\Delta Ct}$. Data were indicated as mean ± SD. Significant difference: $^{*}P < 0.05$, compared to the control group.

germ cell numbers, as a result that sperm numbers are decreased, leading to infertility (Silva et al., 2011). During the normal sperm producing process, germ cell apoptosis is common, of which the rate of apoptotic cells is different as various conditions. However, in mammals, spontaneously apoptotic cells of distinct species vary differently and spermatogenous cells and spermatocytes are dominant in apoptotic cells of rats (Silva et al., 2011). Animal experiment showed that DEHP induced obvious apoptosis of spermatogenic cells in 2-week-old male Wistar rats with a dose- and time-dependent effect (Yang et al., 2012). In this study, the apoptosis rate of GC-2spd cells was positively related to DEHP concentrations as analyzed by using flow cell technique, which was consistent with the above research.

There is a general agreement that male reproductive organs are particularly susceptible to the deleterious effects of ROS and lipid peroxidation, which ultimately lead to impaired fertility (Williams et al., 1998). Cells contain a number of antioxidant defenses to minimize fluctuations in ROS, but ROS generation often exceeds the cell's antioxidant capacity, resulting in a condition termed oxidative stress. Recent literature suggests that ROS as a signaling molecule can induce downstream apoptotic processes. SOD is the most important antioxidase, which can accelerate the disproportionation reaction rate of oxygen radicals and remove excess oxygen radicals in cells. If SOD activation is insufficient, cells can be attacked by oxygen radicals, leading to damage to the cells. As an important enzyme existing extensively in organism and catalyzing the decomposition of hydrogen peroxide, GSH-Px catalyzes the reduction reaction of reduced GSH to hydrogen peroxide specifically and thereby protecting the structure and functions of cell membrane (Koriem et al., 2014). Therefore, the activities of SOD and GSH-Px can indirectly reflect the degree of antioxidant capacity. MDA is defined as lipid peroxides formed by oxygen radicals attacked by polyunsaturated fatty acid in biomembrane, whose contents reflect cell damage and degrees indirectly (Avery, 2011). DEHP induced toxicity is associated with oxidative stress (Erkekoglu et al., 2014; Wang et al., 2012). DEHP can induce apoptosis by elevating ROS production (Hsu et al., 2014). Our study also indicates that DEHP decreases SOD and GSH-Px activities, and increases MDA contents and apoptosis in GC-2spd cells. So, DEHP induced oxidative free radicals increased, activity of antioxidase decreased, and cause the stress reaction apoptosis, which may be one of the mechanism of DEHP toxicity of spermatogenic cells.

Orphan nuclear receptor (ONR) is a nuclear receptor without ligand or not found. Testicular Orphan Nuclear Receptor 4 (TR4) is mainly expressed in the brain, spleen, testis and hematopoietic cells (Kim et al., 2010). TR4 has characterized by 615 amino acids with relative molecular mass of 67 kDa, highly homologizing with TR2. ONR subfamily composed by TR2/4 plays an important role in spermatogenesis, and lipoprotein regulation as well as central nervous system.

Studies indicate that in the mouse testis, TR4 is mainly expressed in primary spermatocytes and round spermatid, and plays an important role in meiosis prophase of spermatogenesis and successive meiosis prophase. The generation potential of TR4(-/-) mice is significantly decreased and sperm numbers at all stages is lowered obviously. Also, primary spermatocytes are degraded, some

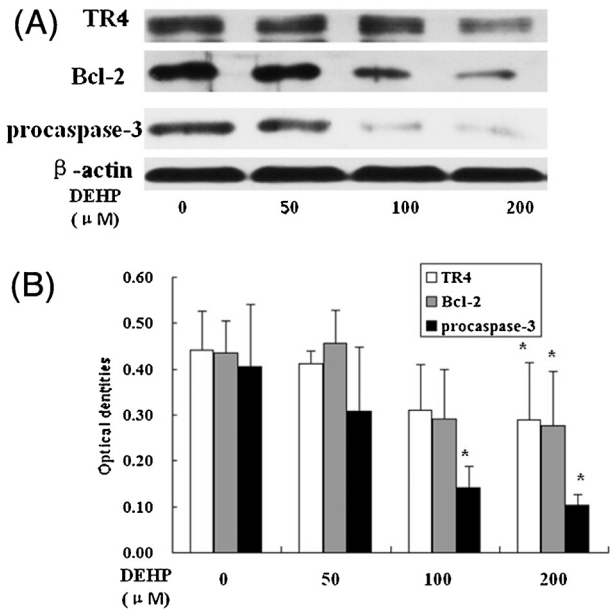

Fig. 5. (A) Effect of DEHP on the TR4, Bcl-2 and procaspase-3 protein expression levels in GC-2 spd Cells by Western blotting. (B) Quantitative analysis of the immunoreactive TR4, Bcl-2 and procaspase-3. Data are indicated as mean ± SD. Significant difference: $^{*}P < 0.05$, compared to the control group.

necrotic contorted seminiferous tubules are found, and meiosis prophase of spermatogenesis and successive reduction division phase are prolonged and disrupted, as a result that metaphase of cell division is prolonged, leading to some abnormal spermatogenic cells. The TR4(-/-) mice are lack of resistance toward oxidative stress with manifestations of cell growth arrest, DNA injury and elevation of ROS level; recovery of the TR4 expression in TR4(-/-) mice and treatment with antioxidant NAC can reduce DNA injury and recover cell growth (Lee et al., 2011), indicating that oxidative stress regulates TR4 expression.

Bcl-2 gene expression of embryo fibroblasts separated and cultured from TR4(-/-) mice is down regulated and further decreased after exposure to ultraviolet rays. Activated caspase-3 can degrade Bcl-2 to accelerate cell apoptosis, indicating that TR4 regulates cell apoptosis through inducing Bcl-2 gene expression (Kim et al., 2007). Studies have shown that the testicular tissues of 6-week-old mice showed significant cell apoptosis and reduced expression of Bcl-2 mRNA after one oral exposure to DEHP 2000 mg/kg (Kijima et al., 2004). The result of this study showed that DEHP can induce oxidative stress, and the reduced expression of Bcl-2 and TR4 in GC-2spd cells, indicating that DEHP could induce spermatogenic cell apoptosis via TR4/Bcl-2 pathway.

As an important link in the process of apoptosis, Bcl-2 acts in the downstream of caspase-3 as the direct substrate of caspase-3. Caspases are a family of cysteine proteases that are a central component of the apoptotic machinery. Caspases are synthesized as inactive precursors (procaspases) that are cleaved at specific aspartate residues to generate the active subunits. Caspase-3, as a member of caspase family, is the core component of apoptosis mechanism and one of the most important effective proleases that induces directly cell apoptosis (the performer of apoptosis), which is activated once, the apoptosis is not avoided (Larsen et al., 2010). Dalgaard et al. observed an increased activity of caspase-3 in the rat testis, 3 h and 12 h after 0.4 mg/g of MEHP-exposure (Dalgaard et al., 2001). Our study shows that DEHP induces increased expression of caspase-3 mRNA, and the reduced expression of procaspase-3 protein in GC-2spd cells. These results indicated that caspases were activated in DEHP-induced apoptosis of GC-2spd cells.

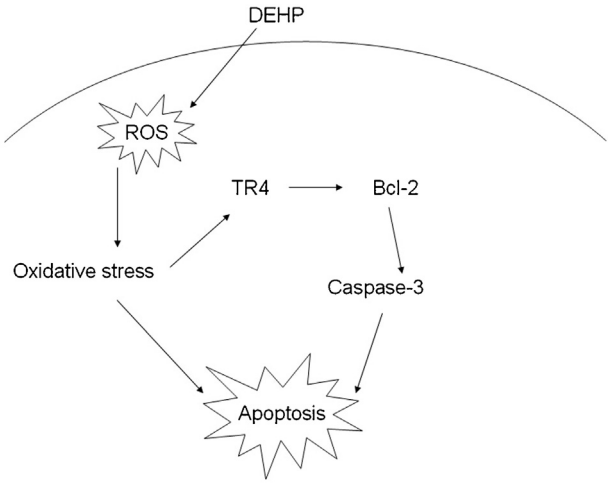

Fig. 6. Proposed model of DEHP-induced TR4/Bcl-2 pathway leading to apoptosis in GC-2 spd Cells.

This study showed that DEHP could induce an increase of caspase-3 and Bcl-2 mRNA expression and GSH-Px activity in GC-2spd cells, but did not have a significant dose response relationship. It is generally believed that the higher the dose is, the more obvious the toxic effect is, that is, the dose makes the poison. But in recent years, it was found that certain EDCs can produce biological effects at even very low dose exposure, especially it will have a long-term effect on health during critical windows period. EDCs may have more serious consequences at low dose than that at high dose, or there have different effects at different doses. Vom Saal found that intrauterine exposure to bisphenol A and diethylstilbestrol will lead to adult male mice prostatic hypertrophy, however, no hypertrophy of the prostate was found at higher doses. Moreover, the prostate gland was even smaller at high doses than that at unexposed mice. As famous toxicologist, Professor John Doull Said, in fact, these data are not entirely surprising, pharmaceutical scientists understand that drugs can produce different effects at different doses, that are equally applicable to environmental pollutants (Renner, 2004).

In conclusion, DEHP could induce an increase in apoptotic rate of GC-2spd cells by possible mechanism of the TR4/Bcl-2 pathway. In vitro exposure to DEHP induces the oxidative stress of GC-2spd cells, regulates TR4 expression, and thereby inhibits Bcl-2 gene expression, activates caspase-3 and eventually leads to the apoptosis of GC-2spd cells. Of course, the specific mechanism needs to be further researched. This study has provided a scientific basis for the prevention and treatment of male reproduction harm that resulted from DEHP.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgements

This work was supported by the grants from the National Natural Science Foundation of China (81302401) and Hubei Province health and family planning scientific research project (WJ2015MB133), and the National Institutes of Health (NIH) (HD076257).

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