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Role of PI3K/AKT/mTOR signaling pathway in DBP-induced apoptosis of testicular sertoli cells in vitro

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ABSTRACT

Dibutyl phthalate (DBP) has significant male reproductive toxicity, and the Sertoli cells are the target cells of DBP. This study was to investigate the injury effect induced by DBP on rat testicular Sertoli cells in vitro. MTT results showed that DBP can significantly reduce the survival rate of Sertoli cells; Hoechst staining results showed that the Sertoli cells treated with DBP emerged with typical morphological characteristics of apoptosis, nuclear condensation and chromatin condensation; flow cytometry results showed that DBP significantly increased the apoptotic rate of Sertoli cells, and dose-dependent; Western blotting showed that the expression of PTEN protein in Sertoli cells was significantly higher than that in the control group after treated with different concentrations of DBP for 24 h, while the expression of p-PI3K1, p-AKT, p70S6K and 4E-BP1 protein in the PI3K/AKT/mTOR signal pathway were significantly decreased. It is speculated that PTEN/PI3K/AKT/mTOR signaling pathway plays an important role in DBP-induced apoptosis of testicular Sertoli cells in rats.

1. Introduction

Dibutyl phthalate (DBP) is a kind of phthalate esters, which are recognized environmental endocrine disruptors that are widely used in industrial productions. As DBP is combined with the plastic and other end products in the form of additives rather than chemical bond, so in the course of use it release continuously to the surrounding environment, causing environmental pollution (Adibi et al., 2008; Lang et al., 2008). Some studies of NTP in the United States have shown that DBP can produce male reproductive toxicity. DBP can cause male rodents to testicular atrophy, weight loss of testis, activity of testicular marker enzymes decrease, spermatogenic cell loss, atrophy of seminiferous tubules, genital tract deformity, etc (Howdeshell et al., 2007; Lehraiki et al., 2009; Mahood et al., 2007; Mylchreest et al., 2000). Scholars have found that exposure to DBP during embryonic or juvenile period can affect the formation of urethra and the differentiation and development of reproductive nodule, resulting in genotoxicity (Li et al., 2015; Shen et al., 2015). Therefore, DBP is worthy of in-depth study on human health, especially male reproductive effects.

Sertoli cells are one of the constituents of the blood-testis barrier which are the only somatic cells in the seminiferous tubules of testes. They play a decisive role in spermatogenesis which can provide spermatogenic cells with nutrition, support and protection, creating a microenvironment for spermagogenesis. In addition, Sertoli cells can also combine androgen to promote spermatogenesis, to absorb the residual body produced among sperm maturation process and to ensure the stable production of sperm with normal quantity and quality (Franca et al., 2000). Studies have found that exogenous compounds must go through the blood-testis barrier before acting on spermatogenic cells, which means that toxicants exert damage effect firstly on Sertoli cells or through Sertoli cells then influence spermatogenic cells. Therefore, the relevant morphology, functional changes, and molecular biology of Sertoli cells have become an active field of research on male reproductive physiology.

A lot of studies have shown that DBP can produce significant damage on Sertoli cells, Leydig cells and various types of spermatogenic cells. A large number of studies have shown that DBP can affect the hypothalamus- pituitary- testes gonad axis and cause reproductive endocrine hormone disorder, leading to the declination of follicle stimulating hormone (FSH), luteinizing hormone (LH) and testosterone (T) levels, etc. (Aly et al., 2016). It has been confirmed that at least 5 pathways including PI3K/AKT, MAPK signaling pathways in Sertoli cells may be activated after FSH combining with its receptor. The activation of PI3K/AKT pathway can promote the proliferation and survival of sperm. It has been researched that the PI3K/AKT pathway is linked with the growth and development of testis, and proved that AKT contributes to mediating the interaction between FSH and Sertoli cells (Chambard et al., 2007). Visibly, PI3K/AKT pathway plays an

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important role in male reproductive development and the maintenance of testicular homeostasis. So it is necessary to further study the role of PI3K/AKT signal pathway in DBP-induced Sertoli cells injury.

2. Materials and methods

2.1. Main reagents and analytical instruments

DBP (analytical reagent, purity 99.5%, Sigma, USA); Western blot kit (Wuhan Boster, China); Trizol reagent (Invitrogen, USA); DMEM high glucose medium (Hyclone, USA); fetal bovine serum (Hangzhou Sijiqing, China); Trypsin (Difco, USA); collagenase I (Sigma, USA); dimethylsulfoxide (DMSO, Amresco, USA); PI-Annexin V apoptosis kit (biosea, CHINA); MTT (Sigma, USA); rabbit anti-β-actin antibody (Sigma-Aldrich, USA); antibody of PTEN, Phospho-AKT, Phospho-p70S6 kinase (Thr389), Phospho-4E-BPl (CST, USA); Hoechst 33258 (Sigma-Aldrich, USA); FACS420 flow cytometer (Becton-Dickinson, USA); fluorescence inverted microscope (Olympus CKX71, Japan); Gel imaging analysis system (UVP, USA).

2.2. Primary culture of Sertoli cells in vitro

Primary Sertoli cells were obtained according to the protocol described previously. Male Sprague-Dawley rats (18–21 day-old) were killed through cervical dislocation. Testes were aseptically picked and then peeled the capsule, removed the blood vessels and rinsed twice by PBS. The parenchyma of testes were teared into pieces and digested in 0.25% trypsin-0.02% EDTA solution in 37 °C water bath (stop digestion when tissue was mucus-like). Add culture medium containing calf serum and centrifuge (800 r/min, 5 min × 2) to get rid of trypsin. Then 0.05% collagenase V was added and digested in 37 °C water bath for 10 min. Cell suspension was filtered through 200 mesh sieve after adding culture medium containing calf serum and centrifuged (800 r/min, 3 min × 3) to remove the collagenase. Cell suspension was prepared by adding a culture medium (DEME/F12, L-glutamine 3 mg/ml, 20% fetal bovine serum, penicillin 100 U/ml, streptomycin 100 ug/ml) and diluted to 3–3.5 × 10^5/ml and cultured in 37 °C, 5% CO_2 incubator. Culture medium was changed after 24 h to remove non-adherent cells. After 48 h, a small amount of 20 mmol/L Tris-HCl (pH 7.4) buffer was added for 3 min, and washed the cells twice with the culture medium. The cells culture was continued and prepared for follow-up experiments.

2.3. Cell viability was determined by MTT assay

Sertoli cells were seeded in 96-well plates and the cell density was about 5 × 10^4. The cells were divided into DMSO solvent control group and DBP experimental groups. The concentration of DBP was respectively 1 mg/L, 10 mg/L and 100 mg/L in the treatment groups. Each set five repeat holes, setting the null hole at the same time. After the cells were cultured for 24 h, the culture medium was exhausted, and 5 g/L MTT (20UI) was added, and then incubated for 4 h 150 μL DMSO solution was added, and the absorbance (A) was measured at 570 nm by the microplate reader. At last calculate the cell viability.

2.4. Morphological changes of nuclei were detected by hoechst 33258 staining

The cells were grouped and treated the same as above 2.3. The cells were seeded in 6-well plates and exposed to DBP for 24 h then stained with Hoechst 33258, according to the manufacture's instruction. Morphological changes of the cells were observed under fluorescence microscope, and apoptotic cells were identified by nuclear condensation, nuclear fragmentation and apoptotic bodies.

2.5. Cell apoptosis rate was detected by annexin V-FITC/PI double staining

Be treated with DBP for 24 h, cells were harvested after Trypsin digestion and washing. Cells were treated with Annexin V-FITC/PI according to the instruction. 400 μL binding buffer was mixed well, then added 4 μL PI and 4 μL Annexin V and stained for 15–20 min at room temperature in the dark. Cell apoptosis was detected by flow cytometry.

2.6. Proteins of PTEN-PI3 K/AKT/mTOR pathway were detected by western blot technique

After being treated, the cells were rinsed twice with pre-chilled PBS. Add pre-chilled cell lysis buffer and incubate on ice for 30 min and then collect the cell lysate. The centrifuged supernatant was collected by centrifugation at 15000 × *g* for 15 min at 4 °C. The protein concentration was determined by ultraviolet spectrophotometry. Add the sample buffer and boil for 5 min. According to protein content determination, add the same amount of samples. Sodium dodecyl sulfate − polyacrylamide gelelectrophoresis (SDS-PAGE) was performed and proteins were transferred to NC membranes. Then the membranes were blocked in TBS blocking solution containing 5% nonfat dry milk for 1 h. They were incubated overnight at 4 °C with primary antibodies and incubated at room temperature for 1 h with secondary antibody labeled with fluorescein. The fluorescence intensity was scanned and calculated by an infrared two-color laser imaging system.

2.7. Statistical analysis

The data were analyzed by SPSS16. The comparison between groups used one-way ANOVA followed by SNK test.

3. Results

3.1. Effect of DBP on the viability of Sertoli cells

It was found that the viability of Sertoli cells was changed after treated with different concentrations of DBP. The results of MTT showed that DBP could significantly decrease the survival rate of Sertoli cells. Viability (%) of Sertoli cells treated with 1 mg/L, 10 mg/L and 100 mg/L DBP was respectively 89.45 ± 9.87, 75.16 ± 7.24, 59.79 ± 4.66. Compared with the control group (96.34 ± 7.69), the difference was statistically significant ($P < 0.05$), and the inhibitory effect of DBP on cell proliferation was dose-dependent (Fig. 1).

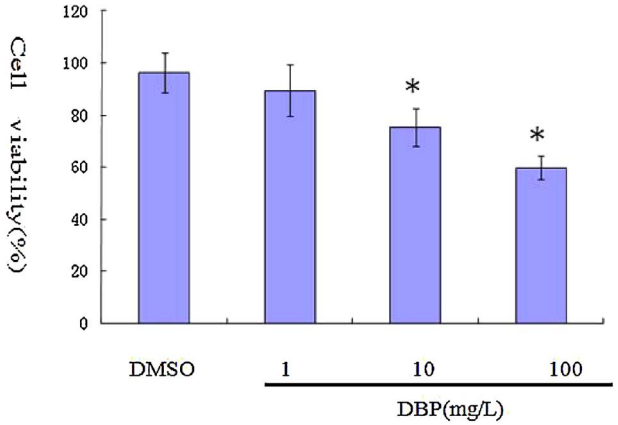

Fig. 1. DBP inhibited the viability of Sertoli cells.
Mean ± SD, n = 5, *$P < 0.05$ *vs* solvent control.
The results of MTT showed that DBP could significantly decrease the survival rate of cells. Compared with the control group, the difference was statistically significant ($P < 0.05$), and the inhibitory effect of DBP on cell proliferation was dose-dependent.