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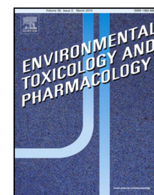

Effect of dibutyl phthalate on expression of connexin 43 and testosterone production of leydig cells in adult rats

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ABSTRACT

To investigate the adverse effect of dibutyl phthalate (DBP) on Leydig cells and its mechanism related to gap junction, Leydig cells isolated from adult rats were treated with 0.1% dimethylsulfoxide (DMSO), 50 mg/L DBP, 50 mg/L DBP + 10 μM prostaglandin E2 (PGE2) and 40 μM flutamide respectively. Radioimmunoassay, semi-quantitative RT-PCR, immunofluorescence and Western blot were applied to determine the expression of testosterone and Connexin 43 (Cx43) in Leydig cells. The expression of testosterone and Cx43 were both decreased in DBP group ($P < 0.05$). While Cx43 was up-regulated after administered to PGE2, there was no significant change in testosterone. However, testosterone was down-regulated with a significant decrease of Cx43 in flutamide group. The results indicated that the inhibitory effect of DBP on testosterone production was not through the down-regulation of Cx43. On the contrary, the change of testosterone can influence the expression of Cx43 in Leydig cells.

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1. Introduction

Dibutyl phthalate (DBP), used primarily as plasticizer, is one of environmental endocrine disruptors (EEDs) that exists in various products such as food packages, building materials, polyvinyl chloride, cosmetics, medical equipments and toys (Adibi et al., 2008; Bao et al., 2011). DBP can easily migrate from plastics to the environment as it is not irreversibly bonded to the polymer matrix, which leads to its ubiquity in the environment (Bosnir et al., 2003).

A lot of studies have showed the adverse effects of DBP on male reproductive system, which can disrupt the normal functions of androgens and lead to testicular atrophy, genital tract malformation, spermatocyte and sperm cell loss and even infertility (Foster et al., 2001; Kavlock et al., 2002). However, compared with other anti-androgen products, DBP displays male reproductive toxicity by disrupting testosterone biosynthesis instead of directly contacting with androgen receptors (Gray et al., 2006; Kabir et al., 2015). Some studies have indicated that DBP can inhibit the steroidogenic acute regulatory protein (StAR) which is critical for cholesterol uptake, suppress the marker enzymes for steroidogenesis (i.e., P450scc, 3β-HSD and P450c17, etc.) and alter the expression of steroidogenesis-related genes like *Hsd3b, Cyp11b1, Insl3* and so on (Hannas et al., 2012; Li et al., 2016a). Leydig cells which exist in the interstitial of testes are the main source of androgens, and also the principal target cells of reproductive toxicants (Hu et al., 2009). However, the molecular mechanism of DBP-induced Leydig cell functional disorder still remains obscure.

Gap junctions (GJ) are essential structural components in the plasma membranes that act important roles in many cellular processes such as the control of cell growth, hormone responsiveness, embryogenesis, intercellular homeostasis, metabolic support and so on (Pointis et al., 2010). The connexins proteins that make up connexon hemichannels are crucial to the assembly of gap junctions. Connexin 43 (Cx43), the most widely studied connexin, is of great significance to testis which is critical to the maintenance of spermatogenesis, the homeostasis of blood-testis barrier (BTB) and is possibly involved in steroidogenesis (Goldenberg et al., 2003; Kidder and Cyr, 2016). Mice lacking Cx43 gene could manifest a massive losses of germ cells and spermatogenesis would be greatly impaired (Giese et al., 2012; Roscoe et al., 2001). Hence, the normal expression of Cx43 is required in testis.

Abbreviations: 3β-HSD, 3β-hydroxy steroid dehydrogenase; ANOVA, analysis of variance; AR, androgen receptor; BTB, blood-testis barrier; Cx43, connexin 43; DBP, dibutyl phthalate; DMSO, dimethylsulfoxide; EED, environmental endocrinal disruptor; FBS, fetal bovine serum; GJ, gap junction; GJIC, gap junctional intercellular communication; HBSS, Hank's balanced salt solution; PGE2, prostaglandin E2; RT-PCR, reverse transcription polymerase chain reaction; S.E.M, standard error of the mean; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; StAR, steroidogenic acute regulatory protein.

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Current studies showed that testicular connexins could be the target of environmental toxicants on male reproductive system (Fiorini et al., 2008; Li et al., 2016b; Pointis et al., 2011). Toxicants like DBP can disturb the membrane structures by altering the process of junctional proteins localization and affect the gap junction intercellular communication which can lead to cell dysfunction. It was previously considered that Cx43 is the only connexin expressed in Leydig cells (Pointis et al., 2010; Risley et al., 2002). Given the predominance of Cx43 in testis and its potential effect on hormone regulation, we hypothesized that DBP may inhibit the production of testosterone via its effect on Cx43 in Leydig cell.

Although a variety of studies have been concentrating on the reproductive toxicity of DBP, the adverse effects of DBP on Cx43 in Leydig cells have not been elucidated. In this study, radioimmunoassay, semi-quantitative reverse transcription polymerase chain reaction (RT-PCR), immunofluorescent assay and Western blot were applied to determine whether DBP disturb testosterone production and to assess the relationship between testosterone and Cx43 in Leydig cells.

2. Materials and methods

2.1. Isolation and culture of leydig cells

The Ethics Committee for Animal Experiments of Jilin University approved all animal work (Permit number: SCXK Ji 2010-0005) and the experimental protocols complied with the principles and guidelines in the "Guide for Care and Use of Laboratory Animals".

Leydig cells were obtained from male Sprague-Dawley rats (3 months old) after killed by inhalation of diethyl ether, which involved enzymatic digestion and Percoll gradient centrifugation, according to the procedure described previously with slight modification (Shan and Hardy, 1992). First, decapsulated testes were digested in 0.25 mg/mL type I collagenase (Sigma, USA) by a shaking water bath at 34 °C. Stop digesting by 10% FBS (Gibco, USA) in DMEM/F12 (Gibco, USA) when seminiferous tubules were largely detached but still intact. Keep still for 3 min, filter the supernatant with a 200 mesh sieve and then centrifuge at $200 \times g$ for 5 min. The pellet was washed twice using Hank's balanced salt solution (HBSS) by centrifugation followed by resuspending the pellet in DMEM/F12. Discontinuous gradient centrifugation was applied to purify the cells. The cell suspension was loaded onto the Percoll centrifuge system composited with 70%, 60%, 30% and 5% Percoll (Pharmacia, USA) from bottom up and centrifuged at $500 \times g$ for 30 min. Keep still for 5 min, cells between 30% Percoll (1.064 g/mL) layer and 60% Percoll (1.070 g/mL) layer were collected and washed twice. The cells were resuspended in DMEM/F12 with 10% FBS and cultured at a density of 10^6 cells per milliliter in six-well plates in 37 °C, 5% CO_2. Non-adherent cells were removed after cultured for 24 h. Typan blue exclusion test and histochemical staining for 3β-hydroxy steroid dehydrogenase (3β-HSD) were applied to detect the viability and purity of Leydig cells. The Leydig cells isolated by this method could be with viability of more than 98% and purity of more than 95%.

2.2. Cell treatments

Leydig cells were cultured for 48 to 72 h and then removed non-adherent cells. The cultured Leydig cells were incubated for 24 h in the following treatments: 0.1% DMSO (Solarbio, China) solvent control, 50 mg/L DBP (Sigma, USA), 50 mg/L DBP + 10 μM prostaglandin E2 (PGE2, Sigma, USA) or 40 μM flutamide (Sigma, USA). Flutamide is a specific testosterone blocker and PGE2 can strengthen Cx43 expression. Cell viability was detected by MTT method. The cells were used for semi-quantitative RT-PCR, immunofluorescent assay and Western blot.

2.3. Radioimmunoassay of testosterone

After incubated in different groups for 24 h, the culture media were collected and the production of testosterone was detected by a radioimmunoassay kit (Sigma, USA), according to the manufacturer's instructions. All experiments were repeated three times respectively.

2.4. Semi-quantitative reverse transcription polymerase chain reaction

Total RNA was extracted from Leydig cells in different groups using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. The cDNA was reverse-transcribed from 1 μg total RNA using the oligo (dT) primer and Superscript II reverse transcriptase (Invitrogen, USA). Then 3 μL cDNA was subjected to PCR using Taq polymerase (Invitrogen, USA) with the following procedure: denaturation at 94 °C for 5 min followed by 30 cycles consisted of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, and final cycle at 72 °C for 7 min. The sequences of Cx43 primers were as follows: forward 5′-TACCACGCCACCACCGGCCCA-3′ and reverse 5′-GGCATTTTGGCTGTCGTCAGGGAA-3′. Primers for β-actin were used as internal control which sequences were forward 5′-TCAGGTCATCACTATCGGCAAT-3′ and reverse 5′–AAAGAAAGGGTGTAAAACGCA-3′. The PCR products were separated by electrophoresis on 2% agarose gels and visualized using ethidium bromide. The signals were determined using the GelDoc XRS imaging system (Bio-Rad, USA) and Quantity One software (Bio-Rad, USA).

2.5. Immunofluorescence microscopy analysis

Leydig cells were incubated in different groups for 24 h, and then fixed with 4% paraformaldehyde for 30 min after removing the culture media. The cells were washed in PBS and permeabilized with 0.1% v/v Triton at room temperature. Cells were then incubated with mouse anti-Cx43 antibody (diluted 1:100; Solarbio, China) containing 5% BSA overnight at 4 °C. After washed three times in PBS, cells were incubated for 1 h with goat anti-mouse TRITC-conjugated antibody (diluted 1:100; Sigma, USA) at room temperature. Eventually, cells were mounted with DAPI (Solarbio, China) to label nuclei. As regards to negative controls, cells were treated without the primary antibody. Images were taken immediately with a fluorescence microscope to measure the expression of Cx43 in Leydig cells.

2.6. Western blot analysis

Total protein in difference groups was extract in the lysis buffer (1% Triton X-100; 50 mM Tris-HCl, pH 6.8; 0.1% SDS; 50 mM dithiothreitol; 1X Sigma protease inhibitor cocktail) by sonication and centrifuged at $1000 \times g$ for 10 min, 4 °C to remove cellular debris. The protein concentration was then measured by bicinchoninic acid protein assay. Mixed with the 5X SDS loading buffer (50% v/v glycerol; 2.5% v/v 2-mercaptoethanol; 10% SDS; 250 mM Tris–HCl, pH 6.8; 0.5% w/v bromophenol blue), the protein aliquots was loaded on a 10% polyacrylamide gel with a 4% stacking gel for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein was transferred onto nitrocellulose membranes and followed by incubating the membranes in a blocking buffer containing 5% nonfat dry milk for 4 h at room temperature. The membranes were then incubated with primary antibody (mouse anti-Cx43, diluted 1:1000; Solarbio, China) overnight at 4 °C. After washed three times with 0.05% Tween-20 in TBS, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (goat anti-mouse, diluted 1:1000; Sigma, USA) for 45 min at room

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