

Original Article

PGE2 regulating Cx43 to affect testosterone synthesis in rats leydig cell exposed at DBP

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Abstract: To observe the effect of dibutyl phthalate (DBP) on connexin 43 (Cx43) proteins in rat testicular Leydig cells and testosterone secretion induced by Cx43, and to investigate whether Cx43 is involved in the regulation of testosterone by DBP. Purified Leydig cells of the primary culture were divided into 3 groups, 50 µg/ml DBP treatment group, 50 µg/ml DBP+10 µmol/L PGE2 group and DMSO control group, which were processed for 24 hours, respectively. Expression of Cx43 on cell membrane was detected by immunofluorescence, and total protein level of Cx43 was evaluated by Western blot. Testosterone production was detected by testosterone kit. Compared with control group, Cx43 expression and testosterone levels were both significantly reduced after DBP processing for 24 hours ($P<0.05$). Compared with DBP group, the addition of Cx43 enhancer (PGE2) enabled Cx34 expression recovery, but the change of testosterone level was insignificant ($P>0.05$). Both testosterone and Cx43 can be regulated by DBP. However, the inhibitory effect of DBP on testosterone synthesis in Leydig cells may not be necessarily achieved through the regulation of Cx43 expression.

Keywords: Dibutyl phthalate, Cx43, leydig cells

Introduction

DBP is a plasticizer widely present in every aspect of human life. Because there is no chemical bond between DBP and the polymer matrix, DBP is able to migrate out of the gross product, such as plastics, thus causing environmental pollutions [1]. A number of studies have revealed the significant reproductive toxicity to the male induced by DBP. But different from other androgen antagonists, instead of working on androgen receptors (AR) directly, the reproductive toxicity of DBP is induced by interference with the biosynthesis of testosterone in testicular Leydig cells. However, the detailed mechanism of the action still remains unclear [2, 3].

Gap junction intercellular communication (GJIC) is a way of communication between adjacent cells to achieve signal transduction. Extensively present in the testes, GJIC is essential in testicular development, as well as the initiation and maintenance of spermatogenesis [4]. As the predominant GJIC protein expressed in the testes, Cx43 has been reported to play a key

role in spermatogenesis and the regulation of GJIC [5, 6]. Cx43 is also the only protein identified so far expressed in Leydig cells [7], yet its function in testicular Leydig cells has not been extensively studied.

Thought its reproductive toxicity has been broadly reported, investigations of the effect of DBP on testosterone synthesis in testicle Leydig cells remain blank. By using Western blot and immunofluorescence, this study observed the influence of DBP on Cx43 and testosterone, and investigated the involvement of Cx43 change in DBP's effect on testosterone.

Materials and methods

Materials

DMEM/F12 and fetal bovine serum were purchased from Gibco (US). DBP, collagenase I, PGE2, dehydroepiandrosterone (DHEA), nicotinamide, nitroblue tetrazolium (NBT), coenzyme nicotinamide adenine dinucleotide (NAD), tetramethylrhodamine isothiocyanate (TRITC) conjugated goat anti-mouse IgG antibody, and tes-

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tosterone kit were purchased from Sigma Co. (US). Percoll cell separation media was bought from Pharmacia. 4',6-diamidino-2-phenylindole (DAPI), mouse anti-rat Cx43 polyclonal antibody and rabbit anti-rat β -actin polyclonal antibody were purchased from Solarbio Co. The rest materials were all domestic analytical reagents.

Isolation, identification and treatment exposure of leydig cells

The methods reported by Genissel C. et al. [8] were used and improved. One adult male rat, weighted around 300 g, was anesthetized with ether, euthanized by cervical dislocation and disinfected using alcohol. Under sterile conditions, the testes were removed and decapsulated. After flushed with D-Hanks buffer, the testes were added with 0.5% collagen I and digested in a shaking water bath at 34°C for 5-6 min. Digestion was terminated by adding 10% fetal bovine serum. Supernatant was collected after standing 2-3 min and filtered using 200 mesh cell filters. The cells were washed with D-Hanks buffer, centrifuged at 1000 r/min for 5 min and rinsed twice. Cells were added with DMEM/F12 culture media containing 10% fetal bovine serum and pipetted repeatedly to make a 2 ml suspension. 2 ml of each Percoll gradient medium was pipetted and slowly added into a 10 ml centrifuge tube from high to low density (gradients from bottom to top were 70%, 58%, 30% and 5%, respectively), and cell suspension was added to the top. The tube was centrifuged and allowed to stand for 5 min at room temperature. The band of target cells, which was located between 30% and 58% Percoll gradients, was sucked up, centrifuged and washed twice. Cells were counted and diluted into a concentration of 10×10^5 /ml. The purity of cells was examined by 3 β -HSD staining in 6-well plates. The cells were divided into 3 groups, 2 experiment group treated with 50 μ g/ml DBP or 50 μ g/ml DBP+10 μ mol/L PGE, and control group treated with DMSO. Cells were incubated for 24 h at 37°C in a 5% CO₂ incubator.

Detection of testosterone levels in leydig cells

Cell culture in each group was collected 24 h after treatment. Testosterone levels were detected using radioimmunoassay with testosterone kits purchased from Sigma Co. (US). Assay was performed following the manufacture protocol of the kit.

Radioimmunoassay of Cx43

After culture for 24 h, old media were discarded, and Leydig cells in each group were incubated in serum free media for another 6 h. The following steps were performed: cells were fixed in 4% paraformaldehyde at room temperature for 30 min. Rabbit anti-rat Cx43 polyclonal antibody was added to cells (1:100) and incubated overnight at 4°C. Cells were added with TRITC labeled IgG and incubated at room temperature for 1 h. The nucleus was stained using DAPI (100 ng/ml), incubated at room temperature for 5 min and mounted in 50% buffered glycerol. The cellular expression of Cx43 was observed using fluorescence microscope.

Cx43 expression detected by Western blotting

Cells were collected and lysed completely by sonication in lysis buffer. Cellular protein samples were collected and protein concentration was detected using BCA assay. 50 μ g protein sample was loaded in each lane. After SDS-PAGE, samples were transferred to NC membrane and incubated with Cx43 primary antibody overnight (1:100) at 4°C. The membrane was then washed three times using TBST, for 10 min each time. After added with goat anti-rabbit secondary antibody (1:1000), the membrane was incubated in dark at room temperature for 1 h. The substrates were illuminated and imaged by infrared scanning system. Quantitative analysis of the intensity of the bands of interest was conducted using Quantity One software.

Statistical analysis

Experimental data were analyzed by SPSS13.0 software. Data were expressed by mean \pm standard deviation ($\bar{x} \pm s$). $P < 0.05$ indicated that difference was statistically significant.

Results

Cultivation and Identification of rat testicular interstitial cells

Examined by 3 β -HSD staining, rat testicular interstitial cells exhibited blue-black color under microscope, while other testicular cells were not stained. 24 h after cells attaching to the plates, old medium and non-adherent cells were discarded. The rest cells were rat testicular interstitial cells, of a high purity up to 95%. At 75% to 80% confluence, the number of the

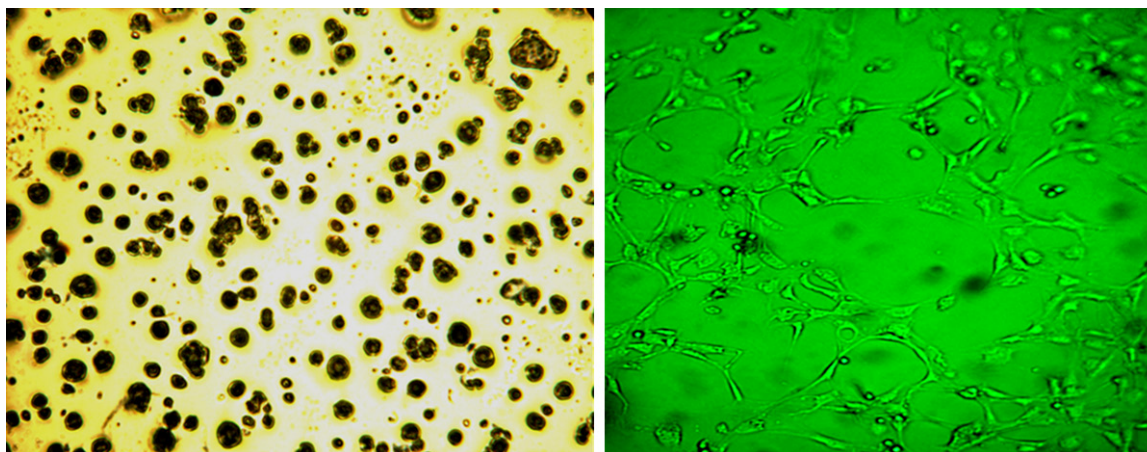


Figure 1. Identification and cultivation of testicular Leydig cells.

Table 1. Analysis of testosterone level in different groups (n=6)

Groups	Testosterone level (ng/ml)
DMSO group	16.92±1.85
DBP+PGE2 group (50 µg/ml+10 µmol/l)	10.16±1.31*
DBP group (50 µg/ml)	8.73±1.08*

Note: *compared with DMSO group (P<0.05).

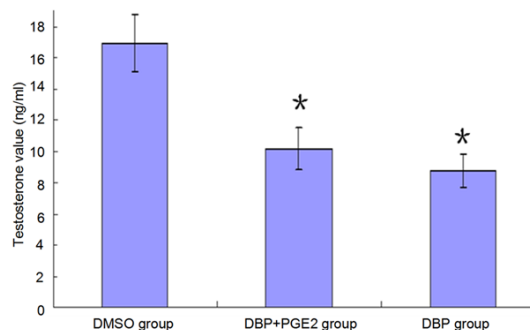


Figure 2. Results analysis of testosterone in each group.

above cells reached 3×10^6 to 4.5×10^6 (Figure 1).

Results of testosterone levels

Cells exhibited significant decreased testosterone levels 24 h after exposed to DBP, compared with the control group. However, the testosterone level in cells treated with DBP+PGE2 was not recovered, shown by statistically insignificant difference ($P > 0.05$) (Table 1 and Figure 2).

Immunofluorescence assay of Cx43 expression in testicular interstitial cell membrane

Cx43 was extensively expressed among cells, shown by immunofluorescence assay. 24 h after DBP exposure, Cx43 expression on cell membrane was significantly inhibited. In control group, Cx43 was mainly expressed on cell membrane, while the expression was significantly reduced and mainly present in cytoplasm after DBP exposure. The addition of Cx43 enhancer PGE recovered Cx43 level close to that of control group (Figure 3).

Cx43 total protein expression in testicular interstitial cells

As shown by Western blot, Cx43 total protein expression was significantly inhibited 24 h after Leydig cells exposed to DBP, compared with control group. Cx43 total protein level was gradually recovered in the treatment group of DBP+PGE2 compared with DBP alone (Figure 4).

Discussion

DBP, widely recognized as an endocrine disruptor in the environment, is extensively present in polyvinyl chloride, construction materials, food packaging fixative, detergents, lubricants and medical devices. The US NTP has confirmed that DBP has significant male reproductive toxicity which can cause rat testicular atrophy, decreased weight, loss of spermatocytes and sperms, degeneration and atrophy of seminiferous tubules, genital tract anomalies, etc. The

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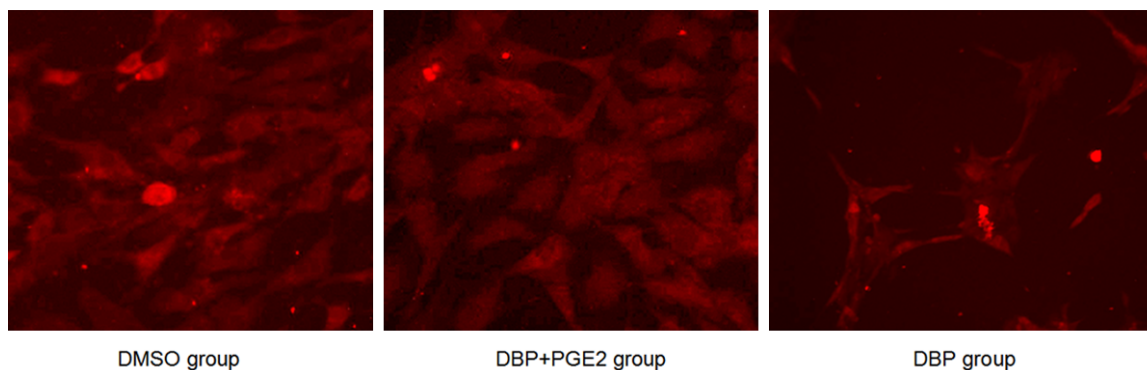


Figure 3. Cx43 expression in leydig cells detected by immunofluorescence.

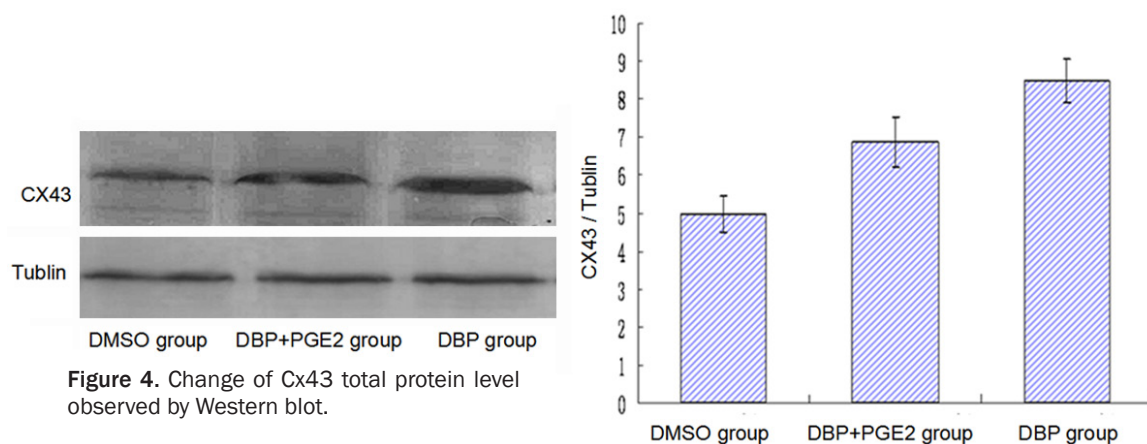


Figure 4. Change of Cx43 total protein level observed by Western blot.

cause of these changes is mainly associated with the anti-androgen effect of DBP [9, 10]. In this study, we also demonstrated that DBP could reduce testosterone levels.

GJIC is a way of signal communication achieved by gap junction structure formed by connexin proteins. Present between adjacent cells, GJIC plays a key role in the maintenance of cell signal transduction, regulation of cell proliferation and differentiation, as well as maintenance of tissue homeostasis [11]. Connexin 43 is the predominant gap junction (GJ) protein expressed in the testes. Ralph Brehm et al. using gene knock-out technology, specifically knocked out Cx43 in rat Sertoli cells. The testes of these rats after they reached sexual maturity were only weighed 50% of that in the control group. Shown by Immunohistochemistry testing, the Sertoli cells continuously proliferated and exhibited abnormal differentiation; germ cells were absent in adluninal compartment, while cells in basal compartment were normal;

Sertoli cells-only syndrome was observed [12, 13]. According to the immunofluorescence results reported by Chen et al., the location of Cx43 expression was similar to that of ZO-1. At the stage 8 of seminiferous epithelium cycle, Cx43 expression changed significantly during the process of renewal of tight junctions [14]. Cx43 is the only connexin protein expressed in Leydig cells. It was shown that the increase of number and volume of Leydig cells was accompanied by increased level of Cx43 [15].

The authors speculated that DBP might exert an influence toward Cx43 in Leydig cells, and that the regulation of testosterone level by DBP might be mediated by targeting Cx43. In this study, immunofluorescence and Western blot results showed that DBP inhibited Cx43 expression in Leydig cells and the concurrently decreased testosterone secretion. However, in the group with PGE, immunofluorescence and Western blot demonstrated that Cx43 level was recovered but not accompanied by enhanced

testosterone level, indicating that Cx43 was not involved in the testosterone regulation by DBP.

In summary, DBP is able to down-regulate Cx43 expression and testosterone level in testicular Leydig cells, but the influence of DBP on testes are is not through Cx43 expression inhibition. Since Cx43 is extensively expressed in testicular Leydig cells but not regulating testosterone secretion, further investigations are still needed to explore the function of Cx43 in Leydig cells.

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Disclosure of conflict of interest

None.

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