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ORIGINAL ARTICLE

Effects of di-n-butyl phthalate on male rat reproduction following pubertal exposure

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Di-n-butyl phthalate (DBP) is an endocrine-disrupting chemical that has the potential to affect male reproduction. However, the reproductive effects of low-dose DBP are still not well known, especially at the molecular level. In the present study, pubertal male Sprague–Dawley rats were orally administered DBP at a wide range of doses (0.1, 1.0, 10, 100 and 500 mg kg^{-1} day^{-1}) for 30 days. The selected end points included reproductive organ weights, testicular histopathology and serum hormonal levels. Additionally, proteomic analysis was performed to identify proteins that are differentially expressed as a result of exposure to DBP at low doses (0.1, 1.0 and 10 mg kg^{-1} day^{-1}). Toxic effects were observed in the high-dose groups, including anomalous development of testes and epididymides, severe atrophy of seminiferous tubules, loss of spermatogenesis and abnormal levels of serum hormones. Treatment with low doses of DBP seemed to exert a 'stimulative effect' on the serum hormones. Proteomics analysis of rat testes showed 20 differentially expressed proteins. Among these proteins, alterations in the expression of HnRNPA2/B1, vimentin and superoxide dismutase 1 (SOD1) were further confirmed by Western blot and immunohistochemistry. Taken together, we conclude that high doses of DBP led to testicular toxicity, and low doses of DBP led to changes in the expression of proteins involved in spermatogenesis as well as changes in the number and function of Sertoli and Leydig cells, although no obvious morphological changes appeared. The identification of these differentially expressed proteins provides important information about the mechanisms underlying the effects of DBP on male rat reproduction.
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INTRODUCTION

Phthalic acid esters (PAEs) are industrial chemicals used primarily as plasticizers. They are present in a wide variety of products, including building materials, food packaging and medical devices. PAEs are not irreversibly bound to the polymer matrix, and they can migrate from the plastic to the external environment.[1] Generally, humans can be exposed to these chemicals through ingestion of contaminated food and water, indoor air or dermal contact.[2]

PAEs are usually absorbed from the gastrointestinal tract after oral administration, and they are catalytically converted to toxicologically active monoesters by esterases.[3] Previous studies have found that the reproductive system is particularly susceptible to PAEs, and some specific PAEs exhibited age-dependent toxicities towards the male reproductive system. Male rats exposed *in utero* to PAEs exhibited diverse profiles of reproductive malformations, including hypospadias, cryptorchidism, agenesis of the prostate and epididymis, and degeneration of testicular seminiferous epithelium.[4–6] Unlike other anti-androgens, PAEs did not directly interact with the androgen receptor, but they exerted their anti-androgenic effects by disrupting testosterone (T) biosynthesis.[7–9] The inhibition of T synthesis was due to the downregulated expression of genes involved in cholesterol transport and the testosterone synthesis pathway in male rats.[10,11]

In many studies, the typical dose of PAEs used in animal models is three to four orders of magnitude greater than the estimated daily exposure of humans. It is impossible for humans to be exposed to such high concentrations of these chemicals (e.g., 500 mg kg^{-1} day^{-1} or above). Therefore, both *in vivo* and *in vitro* studies should be focused on the effects of low doses of PAEs. Recently, some scholars have concerned about the biological effects of PAEs at doses that are similar to the estimated exposure of the general human population. For example, aromatase activity in the brain was inhibited by 0.135 mg kg^{-1} day^{-1} di-(2-ethylhexyl)-phthalate (DEHP),[12] and serum T levels were elevated after exposure to 0.045 mg kg^{-1} day^{-1} DEHP.[13] Changes in the expression of genes related to T production in the foetal rat testis were induced by 0.1 mg kg^{-1} day^{-1} di-n-butyl phthalate (DBP).[14] Our previous study also indicated that low concentrations of mono-butyl phthalate affected the steroidogenesis in mouse Leydig tumour cells.[15] Furthermore, epidemiological studies have revealed that PAEs could affect pubertal development, decrease the anogenital distance, reduce sperm concentration and disrupt sperm

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motility in humans.[16–18] All these studies indicated that low doses of PAEs have the potential to affect male reproduction.

The present study was designed to explore the reproductive effects of low-dose DBP on pubertal Sprague–Dawley rats. The estimated levels of human exposure to DBP ranged from 0.84 to 113 μg kg^{-1} day^{-1}.[19] Therefore, we exposed pubertal animals to relatively low doses of DBP (0.1, 1.0 and 10 mg kg^{-1} day^{-1}) in addition to high doses (100 and 500 mg kg^{-1} day^{-1}). Proteomic analysis was performed to evaluate changes in the expression of proteins in the three low-dose treatment groups (0.1, 1.0 and 10 mg kg^{-1} day^{-1}) compared with the control group.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats approximately 4 weeks old were purchased from Zhejiang Laboratory Animal Center (certification No. 0006505) and housed under controlled temperature (22±2 °C), lighting (12-h light and 12-h dark cycle) and relative humidity (40%–70%). As the soybean-derived diet has the potential to influence the results of studies aimed at measuring endocrine-related effects,[20] a soy-free breeding diet and reverse-osmosis water were provided *ad libitum*.

Study design

All animals were acclimated for 1 week before dosing. They were distributed randomly to one vehicle control group and five treatment groups. Each group comprised 20 rats. Vehicle control animals were treated with corn oil by gavage (5 ml kg^{-1}) daily for 30 days. Five treatment groups were treated by gavage with 0.1, 1.0, 10, 100 or 500 mg kg^{-1} day^{-1} of DBP (Guanghua Chemical Co., Shantou, China) for 30 days. Food intake, body weight and clinical signs were monitored throughout the dosing period. Food was suspended 12 h before the end of the experiment, and water was supplied until killing. Animals were killed humanely with carbon dioxide. Blood samples were obtained by cardiac puncture. The total body weight, the weight of the testes and the weight of the epididymis were measured. One testis from each rat was fixed in Bouin's solution for histopathological evaluation, and the other one was snap-frozen in liquid nitrogen and stored at −70 °C until use.

Hormone measurement

Serum was prepared by centrifuging at 2000*g* at 4 °C for 10 min and was stored at −20 °C prior to analysis. The serum concentrations of T, estradiol (E_2), luteinising hormone (LH) and follicle-stimulating hormone (FSH) were determined by a radioimmunoassay technique using radioimmunoassay kits (Beijing North Institute of Biological Technology, Beijing, China).

Histological evaluations

After sacrifice, testes were fixed in Bouin's solution and dehydrated with 70% ethanol. The tissues were embedded in paraffin, and then 5-μm sections were cut and mounted onto slides. The slide sections were stained with haematoxylin and eosin Y. The histopathological features of the testes were determined by optical microscopy.

Determination of testicular cell numbers

Germ cell numbers were determined using paraffin sections of testes that had been stained by haematoxylin and eosin Y. Sertoli cell numbers were determined using sections immunostained with a vimentin antibody and haematoxylin. Five testicular sections per animal were used, and 10 microscopic fields were chosen randomly from these five sections. Circular lumens were selected for the calculation of the numbers of individual cell types. The analyses were conducted using Image-Pro Plus 4.5.1 with Stereologer-Pro 5 plug-in software (Media Cybernetics UK, Wokingham, Berkshire, UK). Germ cells were distinguished as being spermatogonia, spermatocytes and round spermatids based on their morphology and localisation; elongate spermatids were not counted because of the elongated shape of their nucleus.

Protein extraction

Three testes from control and each low-dose group (0.1, 1.0 or 10 mg kg^{-1} day^{-1}) were randomly selected for protein extraction. Testes were homogenized in lysis buffer (7 mol l^{-1} urea, 2 mol l^{-1} thiourea, 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 2% (w/v) dithiothreitol and 2% (v/v) immobilized pH gradient buffer (pH 3–10)) in the presence of 1% (v/w) protease inhibitor cocktail (Pierce Biotechnology, Rockford, IL, USA). The mixture was placed on a shaker at 4 °C for 1 h, and the insoluble matter was subsequently removed by centrifugation at 40 000*g* at 4 °C for 1 h. The protein concentration in each sample was determined by the Bradford method using bovine serum albumin as the standard.

Two-dimensional electrophoresis (2-DE), gel image analysis and protein identification

Proteins from testes were separated by 2-DE as previously reported.[21,22] Gels were silver stained, scanned (300 dpi resolution) and analysed using ImageMaster 2D platinum software (version 5.0; GE Healthcare, San Francisco, CA, USA). The expression level was determined by the relative volume of each spot in the gel and expressed as %Vol (%Vol=spot volume/Σ volumes of all spots resolved in the gel). The values from three independent experiments in each group were averaged, and the statistical difference was assessed with the ANOVA test using ImageMasterTM 2D platinum software. A spot was regarded as differentially expressed between groups if the average change in the value of the spot intensity was greater than twofold and the *P* value by the ANOVA test less than 0.05.

Protein spots with differential expression between the DBP treatment and control groups were excised. Gel pieces were denatured, alkylated, trypsin-digested and analysed by Ultraflex II MALDI-TOF/TOFTM mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) under the control of FlexControl 2.4 software (Bruker Daltonics GmbH). The analysis and search conditions used for protein identification were the same as those previously described,[23] except that the resulting peptide mass lists were used to search the IPI rat database (41251 sequences) with Mascot (version 2.1.03; Matrix Science, London, UK) in automated mode.

Bioinformatics analysis

Pathway Studio software (v5.00; Ariadne Genomics Inc., Rockville, MD, USA) was used to find the relationship between proteins and cellular processes. Pathway Studio includes an automated text-mining tool that enables the software to generate pathways from the PubMed database and other public sources. The protein list was imported into Pathway Studio to identify the cellular processes that were influenced by the treatment. In the analysis, each identified cellular process was confirmed manually using the relevant PubMed/Medline hyperlinked abstracts.

Western blot analysis

Among the proteins that were found to be differentially expressed between the groups, heterogeneous nuclear ribonucleoprotein A2/B1 (HnRNPA2/B1), vimentin and superoxide dismutase 1 (SOD1) were

selected for further validation. Proteins were separated on 12% polyacrylamide gels and transferred to polyvinylidenefluoride membranes (GE Healthcare). These membranes were incubated for 1.5 h at room temperature in Tris-buffered saline containing 5% non-fat milk powder. Antibodies against HnRNPA2/B1 (diluted 1 : 200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), vimentin (diluted 1 : 200; Sigma-Aldrich, St Louis, MO, USA) and SOD1 (diluted 1 : 5000; Novus Biologicals, Littleton, CO, USA) were used. An antibody against GAPDH (diluted 1 : 1000; Sigma-Aldrich) was used as the positive control. After incubation with primary antibodies overnight at 4 °C, the membranes were washed three times in Tris-buffered saline. Then the blots were incubated with horseradish peroxidase-conjugated secondary antibody (1 : 1000; Beijing Zhongshan Biotechnology Co., Beijing, China) for 1 h. Specific signals were detected using ECL reagents (GE Healthcare) and AlphaImager FluorChem5500 (Alpha Innotech, San Leandro, CA, USA). The protein expression levels were analysed by AlphaEaseFC software (Alpha Innotech).

Immunohistochemistry

Immunohistochemistry was performed to study the localisation of the four abovementioned proteins in normal rat testes. Sections were dewaxed and dehydrated through descending grades of alcohol to distilled water, followed by blocking of the endogenous peroxidase with 3% (v/v) hydrogen peroxidase in phosphate-buffered saline; the sections were then subjected to microwave antigen retrieval in 0.02 mol l^{-1} EDTA. Thereafter, they were washed in phosphate-buffered saline and blocked with goat serum (Beijing Zhongshan Biotechnology Co.) or bovine serum albumin for 2 h. They were subsequently incubated overnight at 4 °C with primary antibodies against HnRNPA2/B1 (1 : 100), vimentin (1 : 50) and SOD1 (1 : 100). After washing four times in phosphate-buffered saline, the sections were incubated with an horseradish peroxidase-conjugated secondary antibody (1 : 1000; Beijing Zhongshan Biotechnology Co.) for 1 h at 37 °C. Immunoreactivity was revealed with diaminobenzadine (Sigma-Aldrich) for increased sensitivity, resulting in the formation of a brown insoluble precipitate at immunopositive sites. Sections were counterstained with haematoxylin and mounted onto a cover glass. The negative controls were incubated with a solution containing IgG.

Statistical analysis

All values tested in this study were expressed as mean±s.e. The one-way ANOVA test was performed to compare each treatment group with the control group. In all cases, differential expression was considered significant if the P value was less than 0.05 by statistical analysis.

RESULTS

Basic state of rat development

After exposure to DBP, the weights of the testes and epididymides were decreased in the group of rats treated with 500 mg kg^{-1} day^{-1} compared with the control group ($P<0.01$, $P<0.05$). In addition, the ratio of testis/body weight was also decreased in the group treated with 500 mg kg^{-1} day^{-1} ($P<0.01$). No apparent differences in these indexes in testes and epididymides were found in the groups treated with DBP doses less than 500 mg kg^{-1} day^{-1} compared with the control group (**Table 1**).

Serum hormonal changes

The serum T levels decreased in rats treated with 500 mg kg^{-1} day^{-1} DBP ($P<0.01$), but no difference was observed in groups treated with lower dosage compared with the control group. The levels of E_2 increased in the group treated with 0.1 mg kg^{-1} day^{-1} compared with the control group ($P<0.05$), but they did not change in the group treated with 1.0, 10 and 100 mg kg^{-1} day^{-1}. Unexpectedly, the serum E2 level increased sharply in the group treated with 500 mg kg^{-1} day^{-1} $P<0.01$) . Serum LH levels

Table 1 The effects of DBP on the organ weight and organ/body weight ratios

DBP (mg kg^{-1} day^{-1})	*Organ weight (g)*		*Organ/body weight ratios ($\times 10^{-2}$)*	
	Testis	*Epididymis*	*Testis*	*Epididymis*
0 (control)	3.40±0.06	0.78±0.03	0.97±0.02	0.22±0.01
0.1	3.30±0.05	0.79±0.03	0.98±0.02	0.24±0.01
1.0	3.33±0.07	0.76±0.03	0.95±0.02	0.22±0.01
10	3.28±0.06	0.79±0.02	0.94±0.02	0.23±0.01
100	3.33±0.07	0.80±0.02	0.94±0.02	0.22±0.01
500	2.54±0.17**	0.67±0.03*	0.75±0.05**	0.20±0.01

Abbreviation: DBP, di-n-butyl phthalate.
All values are expressed as mean±s.e.
* $P<0.05$, versus control by ANOVA.
** $P<0.01$, versus control by ANOVA.

Table 2 The effects of DBP treatment on the serum hormones

DBP (mg kg^{-1} day^{-1})	*T (ng ml^{-1})*	*E_2 (pg ml^{-1})*	*LH (mIU ml^{-1})*	*FSH (mIU ml^{-1})*
0 (control)	3.01±0.29	127.92±10.23	6.36±0.21	1.72±0.15
0.1	3.26±0.40	197.52±18.33*	7.50±0.33*	2.06±0.10
1.0	3.93±0.35	175.17±16.65	7.04±0.23	2.73±0.19**
10	2.71±0.31	140.36±13.23	8.27±0.42**	2.67±0.12**
100	2.43±0.28	94.83±8.56	3.18±0.28**	2.33±0.21*
500	1.55±0.17**	235.99±18.92**	2.53±0.28**	2.77±0.17**

Abbreviations: DBP, di-n-butyl phthalate; E_2, estradiol; FSH, follicle stimulating hormone; LH, luteinising hormone; T, testosterone.
All values are expressed as mean±s.e.
* $P<0.05$, versus control by ANOVA.
** $P<0.01$, versus control by ANOVA.

increased in the groups treated with 0.1 and 10 mg kg^{-1} day^{-1} ($P<0.05$, $P<0.01$), and they decreased in the groups treated with 100 and 500 mg kg^{-1} day^{-1} ($P<0.01$, $P<0.05$). Serum FSH levels increased in all treatment groups except the group treated with 0.1 mg kg^{-1} day^{-1} (**Table 2**).

Histological changes

No detectable histological changes were found in the testes of rats treated with DBP doses of 10 mg kg^{-1} day^{-1} and below. There was relatively slight damage to the testicular tissue in the group treated with 100 mg kg^{-1} day^{-1}. Obvious injury of the testicular tissue, characterized by severe atrophy and vacuoles of the seminiferous tubules, Leydig cell hyperplasia and loss of spermatogenesis, was observed in the group treated with 500 mg kg^{-1} day^{-1} (**Figure 1**). The numbers of individual testicular cell types, including Sertoli cells, spermatogonia, spermatocytes and round spermatids, were calculated. At a dosage of 100 mg kg^{-1} day^{-1} or more, the number of Sertoli cells, spermatogonia, spermatocytes and round spermatids decreased. These data are shown in **Figures 2** and **3**.

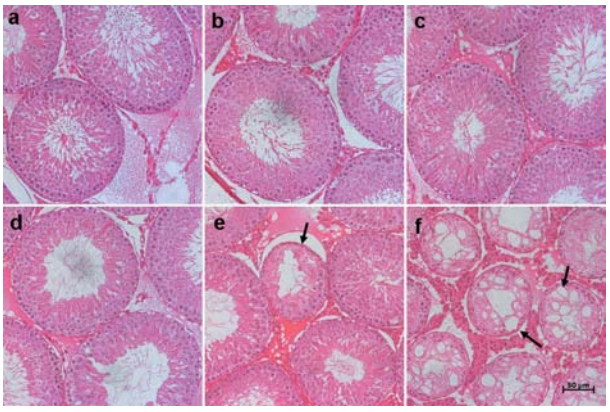

Figure 1 Dose-dependent changes in testicular histopathology following di-n-butyl phthalate (DBP) exposure. Testis cross-sections from rats are shown following exposure to corn oil (control) or DBP at 0.1, 1.0, 10, 100 or 500 mg kg^{-1} day^{-1}. (**a**–**d**) Control, 0.1, 1.0 and 10 mg kg^{-1} day^{-1}, respectively. No obvious abnormalities in the seminiferous tubular structure and no loss of spermatogenesis; (**e**) 100 mg kg^{-1} day^{-1} resulted in slight damage to the seminiferous tubules (arrow); (**f**) 500 mg kg^{-1} day^{-1} resulted in severe damage to the seminiferous tubules, atrophy of the seminiferous tubules, vacuoles in Sertoli cells and loss of spermatogenesis (arrows). Scale bar=50 μm.

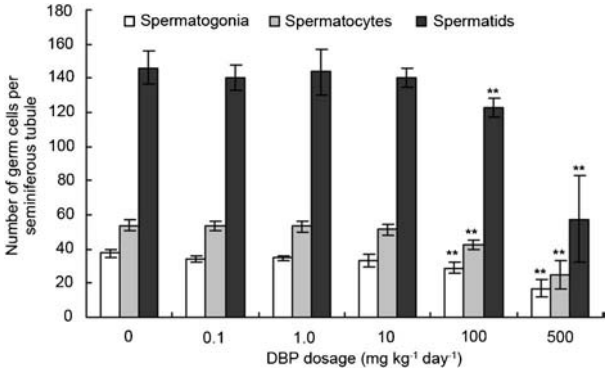

Figure 2 Germ cell numbers in postnatal rats 9 weeks after exposure to either corn oil (control) or five different doses of di-n-butyl phthalate (DBP). Values are expressed as mean±s.e. $^{**}P<0.01$, in comparison to the respective control value (DBP, 0 mg kg^{-1} day^{-1}).

Quantitative comparison and identification of protein spots on 2-DE gels

Testes protein samples of the control and DBP-treated groups were analysed individually. **Figure 4** showed representative 2-DE gel images of the protein expression pattern in testes. Using Image-Master 2D Platinum software and MALDI-TOF/TOF, 20 proteins were successfully identified as being differentially expressed ($P<0.05$) in the selected four groups (**Table 3**).

Bioinformatics analysis

Bioinformatics analysis showed that 12 of the 20 proteins participated in cellular process of spermatogenesis, including spermatogenesis and the cell cycle (**Figure 5**). In addition, we also found seven proteins involved in cell proliferation and two proteins involved in steroid biosynthesis.

Protein expression and location

We focused on three spots on the 2-DE gels, which were identified as HnRNPA2/B1 (spot 1156), vimentin (spot 1145) and SOD1 (spot

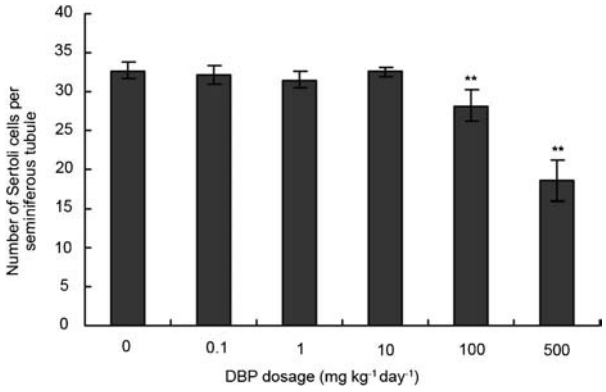

Figure 3 Sertoli cell numbers in postnatal rats 9 weeks after exposure to either corn oil (control) or five different doses of di-n-butyl phthalate (DBP). Values are expressed as mean±s.e. $^{**}P<0.01$ in comparison to the respective control value (DBP, 0 mg kg^{-1} day^{-1}).

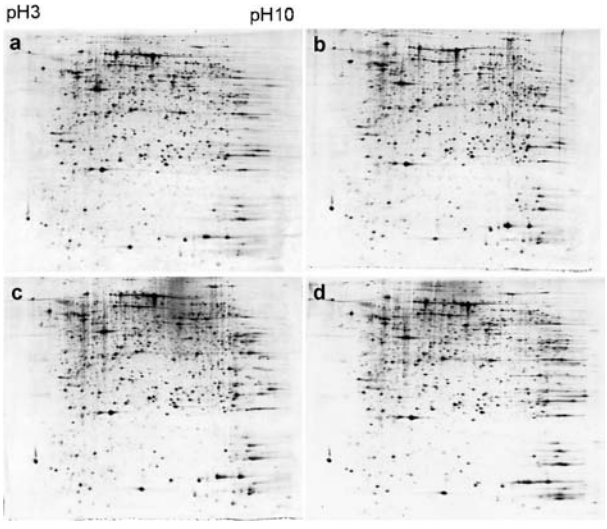

Figure 4 Representative two-dimensional electrophoresis (2-DE) images of testes from control and di-n-butyl phthalate (DBP)-treated rats. Proteins were extracted from rat testes, separated by 2D-PAGE, and visualized by silver staining. (**a**) Control; (**b**) 0.1 mg kg^{-1} day^{-1}; (**c**) 1.0 mg kg^{-1} day^{-1}; (**d**) 10 mg kg^{-1} day^{-1}.

Table 3 Twenty proteins differentially expressed after DBP treatment

Spot ID	*International protein index accession No.*	*Protein name*	*Score*	*Sequence coverage (%)*	*Molecular Weight/ Isoelectric Point*
403	IPI00327830	Fatty acid-binding protein, epidermal	60	61	15335/6.73
437	IPI00231643	**Superoxide dismutase 1**	148	51	16073/5.88
505	IPI00763735	Similar to Putative GTP-binding protein RAY-like	77	32	21219/5.42
570	IPI00191749	Proteasome subunit beta type 1 precursor	93	55	26690/6.90
686	IPI00208205	Heat shock cognate 71-kDa protein	86	29	71055/5.37
695	IPI00364976	Integrin-β4-binding protein	83	25	27067/4.63
754	IPI00331856	Carbonyl reductase (NADPH) 1	94	35	30844/8.22
875	IPI00212969	Heterogeneous nuclear ribonucleoprotein	226	67	32506/8.94
883	IPI00364093	ZH14 protein	110	44	27515/4.93
1145	IPI00230941	**Vimentin**	63	19	53757/5.06
1156	IPI00212723	**Heterogeneous nuclear ribonucleoprotein A2/B1**	75	29	30948/7.68
1423	IPI00464648	Coiled-coil domain-containing protein 91	68	27	50306/5.02
1429	IPI00332042	Retinal dehydrogenase 1	75	26	54994/7.94
1509	IPI00204772	Pre-mRNA-processing factor 19	88	27	57723/6.14
1525	IPI00199305	Vacuolar ATP synthase subunit B, brain isoform	78	27	56857/5.57
1644	IPI00206624	78-kDa glucose-regulated protein precursor	123	32	72473/5.07
1669	IPI00202154	Similar to myo-inositol 1-phosphate synthase A1	220	43	61472/5.73
1699	IPI00200847	T-complex protein 1 subunit alpha	179	37	60835/5.86
1707	IPI00198468	Outer dense fibre protein ODF84	152	40	74081/6.95
1876	IPI00210566	Heat shock protein HSP 90-alpha	72	25	85161/4.93

A spot was regarded as differentially expressed between groups if the average change in the value of the spot intensity was greater than twofold and the *P* value by the ANOVA test was <0.05. Proteins which would be further confirmed by Western blot and immunohistochemistry are shown in bold.
Abbreviation: DBP, di-n-butyl phthalate.

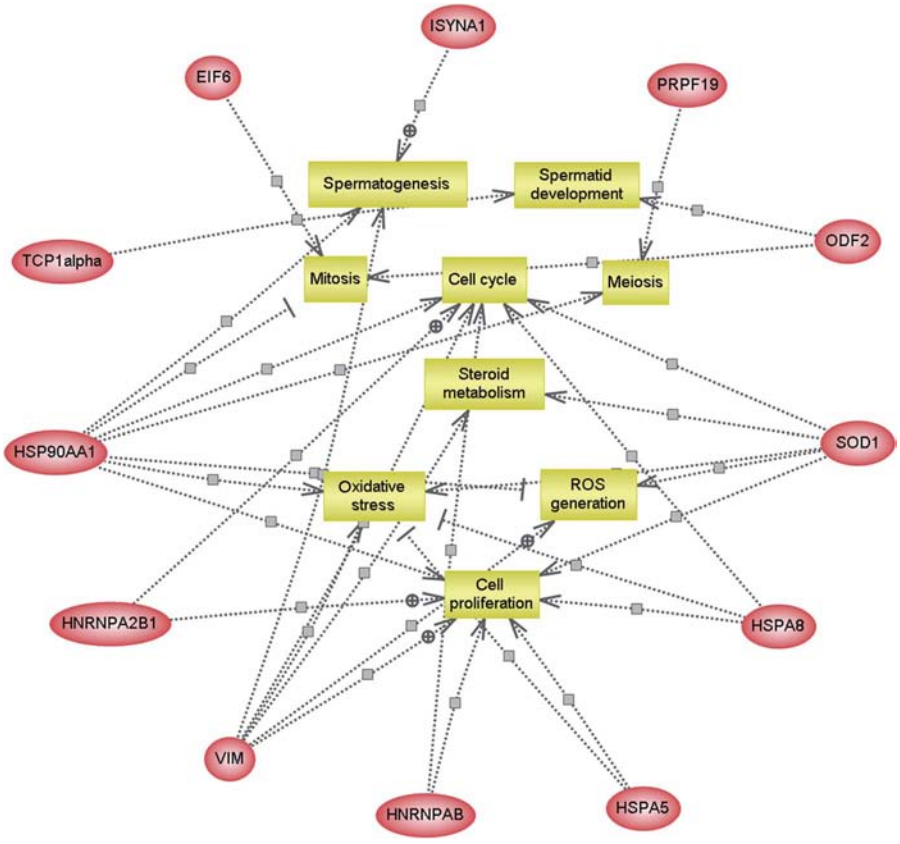

Figure 5 Cellular processes regulated by differentially expressed proteins. The involved cellular processes included spermatogenesis, spermatid development and the cell cycle. Proteins are shown as ovals; regulated processes are indicated by squares. ⊕represents positive-regulated, and ⊣ represents negative-regulated.

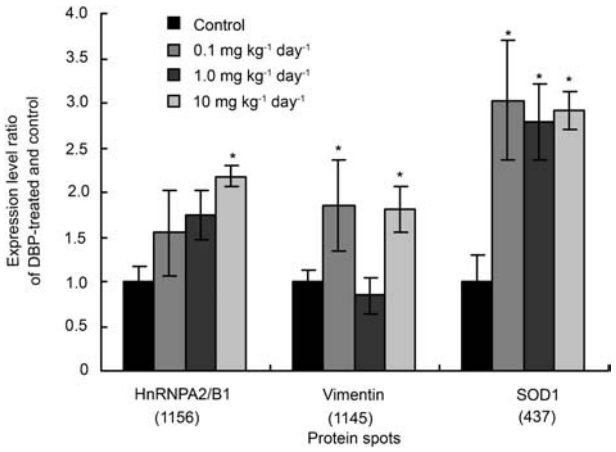

Figure 6 The effects of di-n-butyl phthalate (DBP) exposure on the expression level of proteins on two-dimensional electrophoresis (2-DE) gels. Data are presented as the ratio of the spot intensity in the treated group to that in the control group, and the results are expressed as mean±s.e. The bars with an asterisk are significantly different (*$P<0.05$), with an average change in spot intensity greater than twofold compared to the control group.

437). The expression levels of these three protein spots are shown in **Figure 6**. The expression of HnRNPA2/B1 increased with DBP dosage, and the expression of SOD1 increased in the three DBP treatment groups. The expression of vimentin increased in the group treated with 0.1 mg kg^{-1} day^{-1}, decreased in the group treated with 1.0 mg kg^{-1} day^{-1} and subsequently rebounded in the group treated with 10 mg kg^{-1} day^{-1} (all $P<0.05$).

The expression trends in Western blots of these three selected proteins were consistent with the 2-DE results (**Figure 7**). To explore their localisation in normal rat testes, immunohistochemical staining was performed. As shown in **Figure 8**, HnRNPA2/B1 was expressed in the nuclei from spermatogonia to spermatocytes. The vimentin protein was expressed in the cytoplasm of Sertoli cells, as expected, and SOD1 was present in Leydig cells.

DISCUSSION

Exposure to external chemicals during adolescence could cause direct or indirect injuries to structures and functions of reproductive system because this period is the key point for gonad development, endocrine hormone synthesis and metabolic regulation. Therefore, we selected the pubertal rats as a research model to study the effects of exposure to DBP. Our findings demonstrated that treatment with 500 mg kg^{-1} day^{-1} resulted in apparent toxic effects, including anomalous development of testes and epididymides, severe atrophy of seminiferous tubules and histological disruption of spermatogenesis, which is consistent with previous studies.[24]

Exposure to high doses of DBP decreased the levels of the serum hormones T and LH, while E_2 and FSH levels were increased in this study. The changes in serum LH and FSH levels indicate that pituitary function may be affected by DBP exposure as DBP was reported to affect pituitary hormone-producing cells at both prepubertal and adult stages in males.[25] Because LH secreted by the pituitary is the primary regulator of T synthesis in testes, one possible explanation for the changes in the levels of serum T is that DBP may decrease T production in the testes by decreasing the LH secretion in the pituitary gland. FSH is an important factor for the initiation of spermatogenesis and the regulation of the functions of Sertoli cells. A possible mechanism for the increase of FSH might be that DBP causes severe damaged to the structure and function of Sertoli cells, resulting in a decrease in the number of Sertoli cells and decreased secretion of inhibin, which represses the secretion of FSH. In combination with decreased T, the dysfunction and decreased number of Sertoli cells may affect spermatogenesis and lead to the reduction of spermatogenic cells.

Low doses of DBP exerted a stimulatory effect on serum E_2, LH and FSH (**Table 2**). The serum E_2 levels in the group of rats treated with 0.1 mg kg^{-1} day^{-1} were elevated compared with the control group. Accordingly, the concentrations of serum LH and FSH increased in rats treated with a low dose of DBP in the present study. A possible reason might be the multiple levels of cross-talk between T, E_2, LH and FSH. It was known that E_2 is converted to T by aromatase in testis. In immature rats, the main site of aromatisation is located in the Sertoli cells, and aromatase activity is regulated by FSH.[26,27] In adult rats, aromatisation occurred dominantly in Leydig cells, and LH was the key regulatory factor.[28,29] Therefore, the increase of E_2 levels may be due to the increased expression of LH or FSH induced by DBP. A similar 'stimulatory effect' was reported in a previous study where exposure to 10 mg kg^{-1} day^{-1} of DEHP during puberty increased the secretion of LH and the serum concentrations of T and E_2.[30] In another study, serum T levels were markedly raised in 4-week-old Wistar rats that inhaled DEHP at doses of 5 or 25 mg m^{-3}.[31] Therefore, close attention should be paid to the effects of low doses of PAEs on reproduction.

The testes exhibited no obvious morphologic abnormalities, although their functions may have been disrupted in the three low-dose groups. It is reasonable to expect that some biological processes would be affected by DBP at low doses. Proteomic analysis was performed to elucidate cellular responses to DBP treatment.

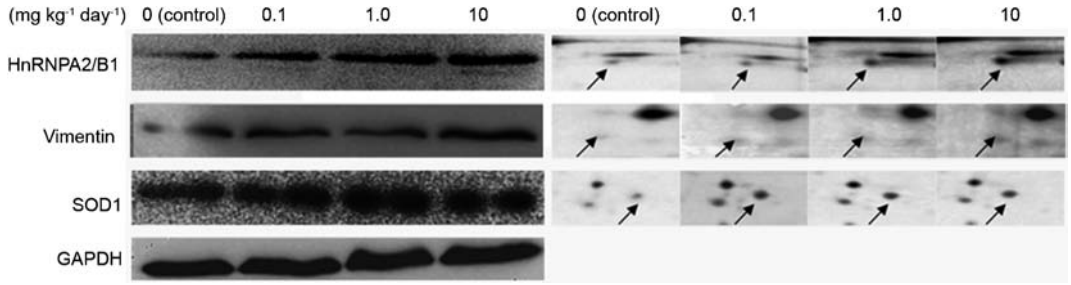

Figure 7 Western blot analysis of HnRNPA2/B1, vimentin and superoxide dismutase 1 (SOD1) protein expression in control and di-n-butyl phthalate (DBP)-treated rat testes. The left panel shows the results of western blot analysis, and the expression of GAPDH in corresponding tissues is displayed at the bottom as a loading control. The right panel shows the corresponding spots with the same molecular weight distributed in the two-dimensional electrophoresis (2-DE) gels. The expression tendencies were almost identical.

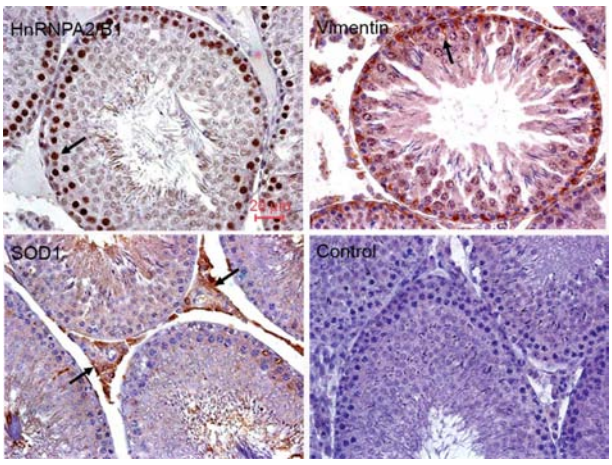

Figure 8 Immunolocalisation of HnRNPA2/B1, vimentin and superoxide dismutase 1 (SOD1) in normal rat testes. HnRNPA2/B1 was expressed in the nuclei of cells from spermatogonia to spermatocytes, vimentin was expressed in the cytoplasm of Sertoli cells and SOD1 was expressed in Leydig cells. Immunopositive sites are visualized as brown staining, and the positive signals are shown with arrows.

Proteomic analysis of individual cell types can provide critical information regarding the cell types affected, and this approach can avoid the artefacts that can result from the presence of other types of somatic cells. However, because of some limitations of the experimental methods for purifying individual cell types, we performed the proteomic analysis with the whole testes. Twenty differentially expressed proteins were identified by 2-DE and mass spectrometry. These proteins were closely related to spermatogenesis and the structure and function of Sertoli and Leydig cells.

Among the 20 proteins, we found that 12 of these participated in cellular process of spermatogenesis, including spermatogenesis, the cell cycle and oxidative stress. Different expressions of these proteins after DBP-exposure may further affect spermatogenesis. We also found seven proteins involved in cell proliferation. Changes in the expression of these proteins may affect testicular cell proliferation, causing, for example, Leydig cell hyperplasia. Two proteins were identified that participate in steroid biosynthesis, and abnormal expression of these may affect the biosynthesis of steroid hormones such as T in the testes, further affecting spermatogenesis.

Vimentin is an important Sertoli cell cytoskeleton component, and it plays an important role in positioning the Sertoli cell nucleus and anchoring spermatogenic cells to the seminiferous epithelium.[32,33] Damaged vimentin filaments were associated with seminiferous epithelium disintegration, which was reversed during the recovery of spermatogenesis after the unfavourable conditions subsided.[34] It is interesting that vimentin expression levels were improved by low doses of DBP in the present study, and this is a novel result because the collapse of Sertoli cell vimentin filaments had been previously reported in rats treated with 500 mg kg^{-1} day^{-1} DBP.[35]

HnRNPA2/B1 is one of the most important RNA binding proteins in HnRNP family, and it is involved in biogenesis and transport of mRNA. It was differentially expressed at different stages of spermatogenesis, and researchers have suggested that HnRNPA2/B1 might play an important role as a chaperone during spermatocyte meiosis.[36] According to the results of our immunohistochemistry, HnRNPA2/B1 was expressed in nuclei of spermatogonia to spermatocytes, which is consistent with the research of Kamma *et al.*[37] The increased expression of HnRNPA2/B1 following DBP exposure observed in the present study suggests that low doses of DBP could affect spermatogenesis-related molecular events, though there were no obvious morphologic abnormalities.

A correlation has also been established between reproductive dysfunction and oxidative stress induced by many agents.[38–40] Oxidative stress is one of the possible mechanisms through which PAEs may affect the structure and function of the testes.[41,42] The equilibrium between reactive oxygen species and antioxidant enzymes is crucial for cell health and survival. In the present study, several differentially expressed proteins were found to be related to oxidative stress. One of them was SOD1 (Cu/Zn SOD), which catalyses the dismutation of superoxide radicals into hydrogen peroxide and oxygen.[43] Immunohistochemistry revealed that SOD1 is located in rat Leydig cells, which are particularly susceptible to extracellular sources of reactive oxygen species because of their close proximity to testicular interstitial macrophages.[44] The increased expression of SOD1 that we observed in rats after exposure to low doses of DBP reflects the relatively mild oxidative stress that Leydig cells typically experience, and consequently, the cellular response to the increased reactive oxygen species to protect Leydig cells against free radical damage. Similar research showed that the gene expression of SOD1 was increased after MEHP treatment in oocytes *in vitro*.[45] Additionally, SOD1 may play a key role in cell proliferation[46,47] and steroid biosynthesis.[48] In our study, interstitial cell hyperplasia was observed in rats treated with 500 mg kg^{-1} day^{-1}. It has been suggested that the upregulation of SOD1 expression may be associated with cell hyperplasia, although no obvious morphological changes were observed in the Leydig cells of rats exposed to lower doses. In short, adolescent exposure to low doses of DBP affects oxidative stress and the number and the function of Leydig cells, which might further affect the synthesis of steroids in Leydig cells (e.g., testosterone).

Taken together, low doses of DBP exerted a 'stimulatory effect' on pubertal rat, while high doses of DBP showed testicular toxicity. In addition, low doses of DBP for 30 days induced changes in the expression of 20 proteins that are involved in spermatogenesis as well as changes in the numbers and functions of Sertoli and Leydig cells. In humans, continuous exposure to environmental DBP, the level of which was similar to the lowest dosage used in this study, may cause suppressed spermatogenesis and a decreased number of sperm in seminal plasma. Analyses of sperm count data suggest a global downward trend by about 50% over the past decades due to exposure to increasing levels of toxic factors, such as drugs (e.g., clomiphene and tamoxifen)[49] and endocrine disruptors.[50,51] It has been proposed that DBP may play a role in this phenomenon. Further functional analyses of the proteins identified in this study would improve our understanding of the mechanisms underlying the effects of DBP on male rat reproduction.

AUTHOR CONTRIBUTIONS

YBW, ZMZ and JHS designed the experiments. AMB, XMM, XJG, HBD and FQW performed the research. XJG analysed the data and performed the statistical study. AMB wrote the manuscript, which was read and approved by all authors.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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