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Original Article

The toxic effects of a plasticizer, dibutyl phthalate, on rat testis

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Abstract: To study the effect of dibutyl phthalate (DBP) on testis and its potential mechanism, 80 male Sprague-Dawley (SD) rats were randomly divided into four groups and treated with three different dosages of DBP via oral gavage for 21 days. DBP was detected in testis with high performance liquid chromatography (HPLC). The expression of H3 histone was measured with immunohistochemistry and immunofluorescence. The morphological observation was executed with optical microscope or transmission electron microscope (TEM). At last, the semen quality was assessed. The results indicated that the residuary DBP between control group and each DBP group was statistical significance ($P<0.05$). The microscopic structure of seminiferous tubule, spermatogonial cell or spermatic cell in DBP group was dramatically changed. In HE staining analysis, the separation between basement membrane of seminiferous tubule and leydig was observed. In TEM analysis, karyopyknosis, mitochondrial pyknosis, enlarged vacuoles, karyorrhexis, mitochondrial decay and chromatin dissolution were found in spermatogonial cell or spermatic cell. The average optical density, positive negative area (PNA) and fluorescent intensity of H3 histone expression were significantly different between control group and each DBP group ($P<0.05$). The quality of sperm in epididymis in DBP group was significantly reduced when comparing with that in control group ($P<0.05$). These data demonstrated that DBP was toxic to male rat reproductive system and seriously influenced the semen quality. The potential mechanism of this toxic effect is most likely related to the expression level of H3 histone, however, this need to be investigated further.

Keywords: Plasticizer, dibutyl phthalate, spermatogenic cell, H3 histone, testis

Introduction

Concerns of phthalates (PAEs) on health issue increase significantly in recent years [1, 2]. PAEs are considered contaminant as environmental estrogens. It is distributed in atmosphere, industrial waste water, river, soil, food, drinking water, and so on. It has been reported that PAEs are detectable in urine, blood, amniotic fluid of fetal, and even in the urine of newborns [3, 4].

Dibutyl phthalate (DBP) belongs to PAEs family. Its molecular formula is $C_{16}H_{22}O_4$ and the molecular weight is 278.34. It is freely soluble in ethanol, ethyl ether, acetone and benzene. DBP could increase the flexibility, stability, resistance to flexure and bond-ability and waterproof properties of plastic products. Up to now, DBP is widely applied in polyvinyl chloride polymer, adhesives, printing ink additives, medical supplies, cosmetics, food packaging and is detectable in the wine, soft drink or fast food [5]. Due to its potential harm, DBP has been listed as one of the controlled pollutants with high priority in the United States and European Union.

The released data indicate that male infertility alone accounts for approximately one-fifth of all infertility cases and the unexplained male infertility is 40-50% [6]. It is necessary to reveal more factors that affect male reproductive system. Based on embryonic development or fertilized egg data, DBP mainly affects reproductive function and neural function [5, 7, 8]. Those

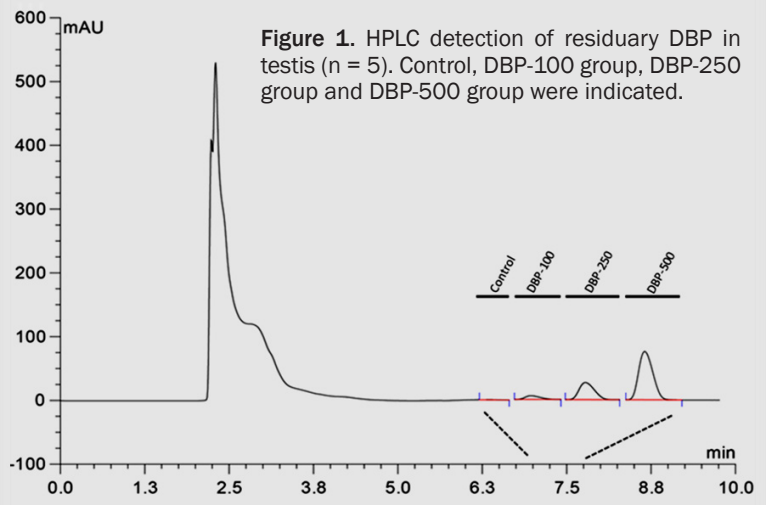

Figure 1. HPLC detection of residuary DBP in testis (n = 5). Control, DBP-100 group, DBP-250 group and DBP-500 group were indicated.

Table 1. Residuary DBP in one gram testis detected with high performance liquid chromatography (HPLC)

Group	N	Residuary DBP (μg)
Control	5	0.27±0.06
DBP-100	5	3.82±0.14*
DBP-250	5	34.15±2.63*
DBP-500	5	59.30±5.23*

Note: *Significantly different to the control group (P<0.05).

data demonstrate that DBP effect is similar to environmental estrogens, which affect cell metabolism, proliferation and active response organisms. DBP has widely effect on maintaining the normal dynamic balance, reproduction, growth and behavior of living organisms [9, 10].

H3 histone plays important role in male productive system. Zhang et al. [11] reported that the H3 histone lysine 4 methylation (H3K4me) are positively expressed in Leydig cells, type A and B spermatogonia, leptotene spermatocytes and spermatids in mouse testis. With age increasing, three demethylases of H3K4me are significantly reduced. It is suggested that H3K4me probably plays a role in male spermatogenesis and testis development. Dai et al. [12] concluded that the proper regulation of H3 histone acetylation levels is important for mouse spermatid differentiation and complex chromatin remodeling during spermatogenesis. Urahama et al. [13] found that the unstable H3.5 (a H3 histone variant) nucleosome may function in the chromatin dynamics around the transcription start sites in human testicular cells during spermatogenesis.

The toxic effect of DBP to the health of male adult reproductive system and its potential molecular mechanism in DBP induction are still obscure. To elucidate these questions, the effect of DBP on the expression of H3 histone, the change of spermatogenic cells, seminiferous tubule in morphologic structure and semen quality were investigated in the current study.

Materials and methods

Animal experiment and DBP treatment

Sprague-Dawley (SD) rats were maintained in experimental animal center of Southwest Medical University. The animals were raised under controlled temperature (20-25°C) and humidity (60-75%). Water and food were provided ad libitum. 80 rats were randomly divided into four groups (control, DBP-100, DBP-250 and DBP-500). Each group included 20 rats with an average weight at 246±17 g. Among which, five rats were used in hematoxylin and eosin (HE) staining, immunohistochemistry analysis and immunofluorescence detection, while five rats were used in transmission electron microscope (TEM) analysis. Five rats were included in high performance liquid chromatograph (HPLC) assay and five rats were executed semen quality analysis, respectively. All procedures involving the use of laboratory animals were strictly monitored in accordance with Animal Care and Use Guidelines in China. Briefly, DBP was dissolved in corn oil. The DBP dosage was followed the previously published data [14, 15]. After acclimatization for one week, rats were treated with DBP for 21 days via oral gavage at a dose of 0 mg/kg/day (control), 100 mg/kg/day (DBP-100), 250 mg/kg/day (DBP-250) and 500 mg/kg/day (DBP-500), respectively. The testis and epididymis were harvested in day 24 right after euthanasia.

Tissue preparation and observation

For slides used in HE staining, immunohistochemistry analysis and immunofluorescence detection, the method was followed the protocol of Fischer et al. [16]. Briefly, 1 cm^3 fresh testis tissue was fixed in 10% neutral formalin for 16 h. Subsequently, it was rinsed with distilled water, dehydrated in graded alcohol, rendered

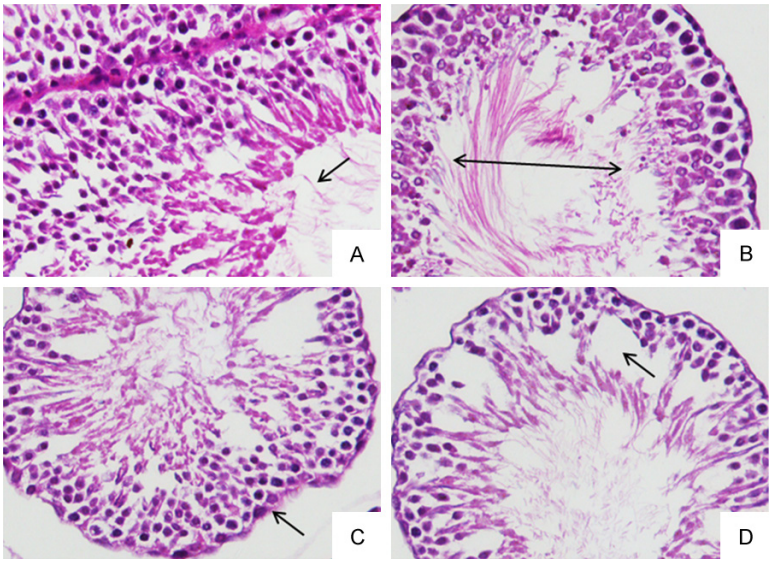

Figure 2. Seminiferous tubule and leydig in testis section with HE staining (× 400, bar = 200 μm, n = 5). A: Seminiferous tubule in control group, a smaller lumen filling with sperm (arrow) was showed; B: Seminiferous tubule in DBP-100 group, an enlarged lumen with a few sperm inside (double arrow) was indicated; C: Arrow indicated the separation between basement membrane of seminiferous tubule and leydig in DBP-250 group; D: Arrow demonstrated the obvious interstice among spermatocytes in DBP-500 group.

transparent by xylene and embedded in paraffin respectively. Paraffin section was cut at a thickness of 5 μm with Leica RM2255 rotary microtome (Leica, Germany). Finally, Sections were flattened on the surface of warm water and picked up onto microscope slides for future use. Images were captured by Olympus BX51 microscopy (Olympus, Japan) and 10 to 15 images from four slides were analyzed.

For slides observation by using TEM, 1 mm^3 testis tissue was immersed in 2.5% glutaraldehyde for 24 h, re-fixed in 1% osmic acid for 3 h and then embedded in epoxy resin. The sample was sectioned to 60 nm with Leica EM UC7 (Leica, Germany) and stained by uranium-lead double staining. Transmission electron microscopy JEOL-1400 (JEOL, Japan) was applied for observation and 10 to 15 images were analyzed.

Detection of residuary DBP in testis

Outgroup standard DBP was prepared by dissolving DBP with isopropanol at the concentration 0.17 mg/ml. For detection of residuary DBP in testis, one gram of testis tissue was comminuted in 3 ml isopropanol and the supernatant was used to analyze after centrifugation. The peak area was used to calculate the residuary DBP with HPLC (P680A quaternary gradient pump, PDA-100 diode array detector, TCC-100 temperature box, Chromeleon chromatographic work station) (Dionex Co, Ltd, USA). Chromatographic condition and system suitability test were carried out as suggested by Li et al. [17].

Expression level measurement of H3 histone

The expression level of H3 histone was analyzed by using immunohistochemistry and immunofluorescence, respectively.

Immunohistochemistry analysis: antibodies were purchased from Zhongshan Golden Bridge Biological Technology CO., Ltd (Beijing, China). Basically, the testis slide was dewaxed with dimethyl benzene and ethanol, and endogenous peroxidase was inactivated with 3% H_2O_2. Subsequently, the slide was incubated with normal goat serum and primary H3 histone antibody (1:100) at 4°C for 24 h. The secondary antibody was added and incubated at 37°C for 20 min. The slide was dehydrated with gradient ethanol. Tissue was rendered transparent by xylene and sealed with resin. Images were captured by Olympus BX51 microscopy (Olympus, Japan). By using Image-Pro Plus 5.1 software (Media Cybernetics Co., Ltd, USA), 10 to 15 images from four slides were analyzed. Following the published method [18], the average optical density (AOD) and positive negative area (PNA) of brown particle within seminiferous tubule were calculated.

Immunofluorescence detection: the first antibody is identical to that used in immunohistochemistry, while the quantum dot-conjugated secondary antibody was bought from Jiayuan Quantum Dot Technological Development Co., Ltd (Wuhan, China). The protocol of immunofluorescent staining was similar to that executed in immunohistochemistry detection. The slide was sealed with glycerol after treatment with the secondary antibody. The image was recorded under the fluorescence microscope at 605

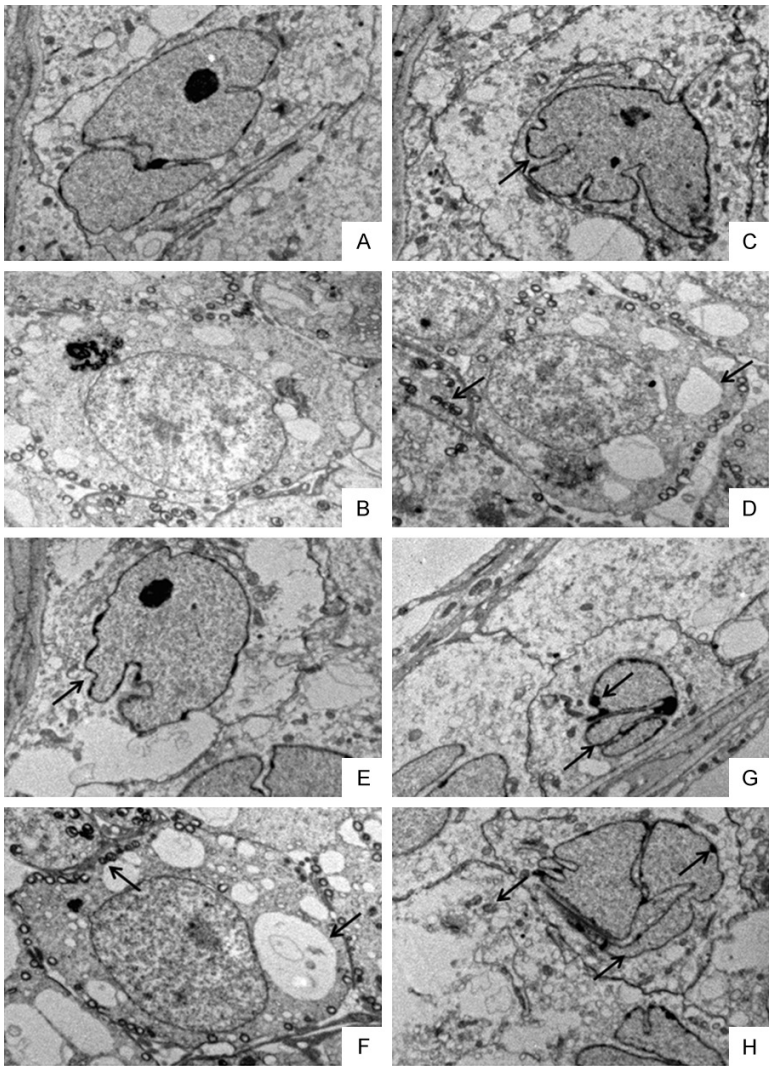

Figure 3. TEM analysis of spermatogonial cell and spermatic cell (× 8000, bar = 10 μm, n = 5). A, B: Normal spermatogonial cell and spermatic cell in control group; C-F: Spermatogonial cell and spermatic cell in DBP-100 group and DBP-250 group. Arrows indicated the karyopyknosis, mitochondrial pyknosis and enlarged vacuole, respectively; G, H: Spermatogonial cell and spermatic cell in DBP-500 group. Arrows showed the karyorrhexis, mitochondrial decay and chromatin dissolution, respectively.

nm. The calculation of fluorescent intensity within seminiferous tubule was measured with the same software and 10 to 15 images from four slides were analyzed.

Semen quality in epididymis analysis

Sperm suspension made from epididymis was incubated at 37°C. The sperm density, sperm living rate, and sperm deformity rate were tested with Sperm Quality Detection System WLJY-9000 (Weili Technological Development Co., Ltd, Beijing, China).

Statistical analysis

The data were collected via randomly blind method and analyzed with SPSS software (version 13.0 for Windows, SPSS Inc., Chicago, USA). Values of all variables were presented as mean ± standard error of mean (SEM). Least Significant Difference (LSD) test and one-way analysis of variance (ANOVA) were used to compare the statistical significance. A p value <0.05 was considered as statistical significance.

Results

Residuary DBP in testis

The residuary DBP in testis was increased as the DBP dose increasing (**Figure 1**). In four groups, the average residuary DBP in one gram testis were 0.27±0.06 μg, 3.82± 0.14 μg, 34.15±2.63 μg and 59.30±5.23 μg, respectively (**Table 1**). Comparing with control group, the residuary DBP was dramatically increased than that in DBP group (P<0.05).

Comparison of HE stained testis tissue

In control group, the stained tissue showed that the testis was consisted of evenly distributed seminiferous tubule, which was filled with sperm and thus the lumen diameter was much smaller (**Figure 2A**). Meanwhile, there is no interstice between seminiferous tubules and a few interstices between spermatocytes was demonstrated (**Figure 2A**). In DBP group, spermatocytes were reduced and the interstice among spermatocytes was enlarged, which leading to generate a big lumen in diameter (**Figure 2B**). The separation between basement membrane of seminiferous tubule and leydig was observed (**Figure 2B-D**).

Table 2. Average optical density (AOD), positive negative area (PNA) and fluorescent intensity within seminiferous tubule

Group	N	AOD	PNA	Fluorescent intensity
Control	5	0.35±0.02	26.42±2.01	36.52±4.12
DBP-100	5	0.24±0.01*	16.53±3.05*	27.16±2.76*
DBP-250	5	0.16±0.01*	13.27±2.73*	25.43±2.48*
DBP-500	5	0.12±0.01*	12.57±2.84*	24.95±1.17*

Note: *Significantly different from the control group (P<0.05).

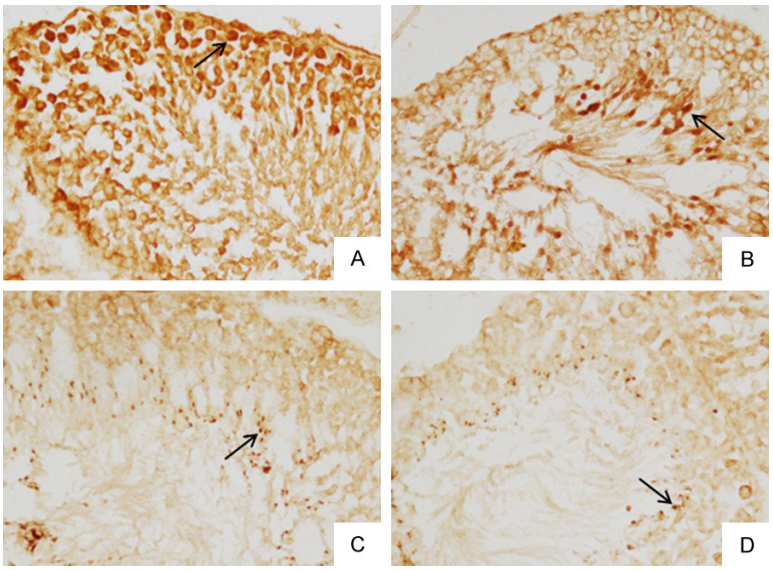

Figure 4. Immunohistochemistry analysis of H3 histone expression in seminiferous tubule (× 400, bar = 200 μm, n = 5). (A) Control group; (B) DBP-100 group; (C) DBP-250 group; (D) DBP-500 group. Arrows indicated the high level of brown signal in spermatogonial cells (A) or in spermatic cells (B-D).

TEM observation of spermatogonial cell and spermatic cell

The TEM section showed that spermatogonial cells and spermatic cells displayed normal nucleus and mitochondrion in control group (**Figure 3A** and **3B**). However, 82 percent spermatogonial cells revealed karyopyknosis (**Figure 3C**), and 85 percent spermatic cells showed mitochondrial pyknosis and enlarged vacuoles in cytoplasm in DBP-100 group (**Figure 3D**). The similar pathological data were obtained in DBP-250 group (**Figure 3E** and **3F**). Obvious karyorrhexis, mitochondrial decay and chromatin dissolution were observed in 92 percent spermatogonial cells and 90 percent spermatic cells in DBP-500 group (**Figure 3G** and **3H**).

Expression of H3 histone in seminiferous tubule

The immunohistochemistry and immunofluorescence data were listed in **Table 2**. Totally, the immunohistochemistry observation revealed that the brown signal of H3 histone in spermatogonial cells of control group was much stronger than that in spermatic cells (**Figure 4A**). Compared with spermatogonial cells in DBP group, high level of brown signal in control group was detected (**Figure 4A-D**), which showed a 0.35±0.02 average in AOD value (**Table 2**). The highest value of PNA, which was 36.52±4.12, was obtained in cells of seminiferous tubule in control group (**Table 2**). In three DBP groups, the average PNA values were 16.53±3.05, 13.27±2.73 and 12.57±2.84, respectively (**Table 2**). Besides, the brown signal of H3 histone in spermatogonial cells was much weaker than that in spermatic cells of DBP groups (**Figure 4B-D**). Statistical analysis indicated that there was a significant difference between the AOP values of control group and each DBP group (P<0.05). The similar result was also obtained in PNA analysis (P<0.05).

Immunofluorescence analysis demonstrated that the strongest fluorescent signal of H3 histone was inspected in spermatogonial cells of control group, and the average fluorescent intensity was 36.52±4.12 (**Figure 5A** and **Table 2**). In three DBP groups, the average fluorescent intensities were 27.16±2.76, 25.43±2.48 and 24.95±1.17, respectively (**Table 2**). Interestingly, the fluorescent intensity was also enhanced in spermatic cells as the DBP dose increasing (**Figure 5B-D**). Compared with control group, the average fluorescent intensity was obviously different to that in each DBP group (P<0.05).

Semen quality in epididymis analysis

The data of semen quality in epididymis analysis were indicated in **Table 3**. In control group, the sperm density and living rate were 1528±132.56 and 87.8±15.61 respectively, which were significantly increased compared with

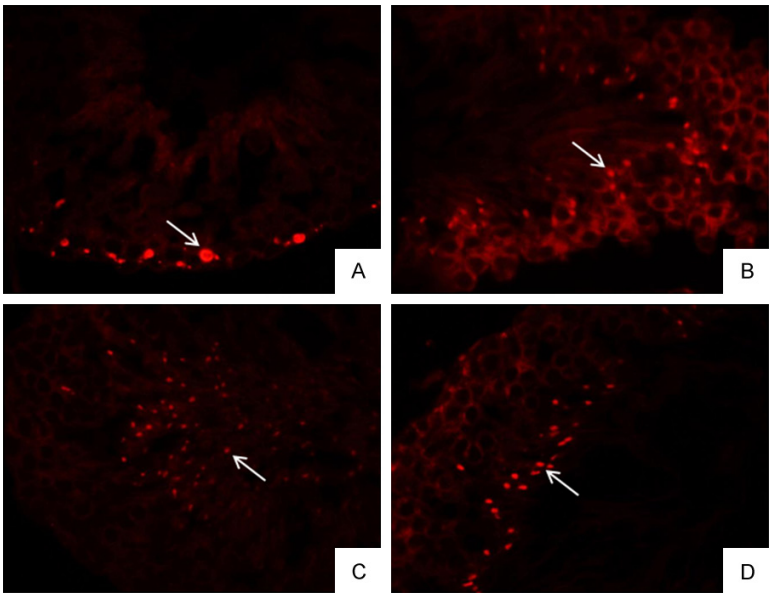

Figure 5. Immunofluorescence analysis of H3 histone expression in seminiferous tubule (× 400; bar = 300 μm, n = 5). (A) Control group; (B) DBP-100 group; (C) DBP-250 group; (D) DBP-500 group. Arrows indicated the enhanced fluorescent signal in spermatogonial cells (A) or spermatic cells (B-D).

Table 3. Semen quality in epididymis

Group	N	Density (× 10^4/ml)	Living rate (%)	Deformity rate (%)
Control	5	1528±132.56	87.8±15.61	12.2±3.16
DBP-100	5	948±75.41*	75.1±10.44*	15.6±7.28*
DBP-250	5	715±62.16*	61.3±5.79*	19.24±5.19*
DBP-500	5	627±54.73*	52.7±7.53*	24.9±4.28*

Note: *Significantly different to that in control group (P<0.05).

those in each DBP group (P<0.05). Meanwhile, the sperm deformity rate in DBP group were 15.6±7.28, 19.24±5.19 and 24.9±4.28, respectively, which were obviously elevated compared with that in control group (P<0.05).

Discussion

The reported data indicate that a slight residue of plasticizer is detectable in healthy tissues or cells due to environmental pollution [19, 20]. In the current study, a low background of DBP in rat testis was also detected in the control group by using HPLC analysis. The residuary DBP in testis was increased about 220-fold in DBP-500 group comparing with that in control group. Also, there was a higher residual in rat testis when comparing with DBP content left in other tissue reported [21, 22]. This indicated that DBP is easily remained in rat testis, which is most likely related to the binding affinity between the testis structure and the chemical property of DBP.

Due to the gathering of DBP in testis, the toxic effect on the function of the male reproductive system is generated. The published data report that chemical plasticizers lead to structural and functional changes of Sertoli cells in seminiferous tubule [23-25]. The atrophying of seminiferous tubule with degeneration of spermatogonial cell and spermatic cell is demonstrated [26-28]. Few studies are focused on spermatogonial cells. In this study, the interstice among spermatocytes, the enlarged lumen of seminiferous tubule and the obvious separation between basement membrane of seminiferous tubule and leydig also revealed the serious damage to rat testis structure in DBP group. Besides, the reduced spermatocytes and sperm demonstrated that DBP has toxic influences to male reproductive system. In TEM detection, karyopyknosis, karyorrhexis, mitochondrial pyknosis, mitochondrial decay, the enlarged vacuole and chromatin dissolution were observed in most spermatogonial cells and spermatic cells in DBP groups, which also revealed that different DBP doses could produce toxicity and led to a serious function disorder to male reproductive system. Increased cytosolic calcium, reactive oxygen species and lipid peroxidation are major causes for mitochondrial injury or dysfunction [29]. However, whether these three factors are included in the DBP induced mitochondrial injury or dysfunction in the current study needs to be verified in the future. The semen quality analysis identified significantly reduced sperm density and sperm living rate and the increased sperm deformity rate in the current study. This also indicated a great toxic effect in male reproductive function.

The previous studies suggest that DBP has neurotoxicity and regulates the expression level of H3 histone in neuron nucleus [30, 31]. It is also demonstrated that a reduction in H3 histone level leads to male infertility as well as abnormal sperm and testis morphology [19-22]. Whether DBP toxicity influences the male reproductive system via regulating H3 histone level is still unclear. In the current analysis, the dramatically decreased H3 histone level and the expression shift of H3 histone between spermatogonial cell and spermatic cell revealed that the expression of H3 histone was closely related to DBP toxicity and male reproductive function.

In general, DBP produced obvious toxic effects to male rat reproductive system in this study. The residuary DBP led to the abnormal morphology of seminiferous tubule, the reduced spermatocytes and sperm as well as the heteromorphosis of nucleus and mitochondrion in spermatogonial cell or spermatic cell. The potential mechanism of this toxic effect is most likely related to the expression level of H3 histone. In the current analysis, the chromatin dissolution was observed and it is probably due to the reduced expression level of H3 histone. However, this needs to be investigated further in the future.

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Disclosure of conflict of interest

None.

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