Pubertal exposure to bisphenol A affects the reproduction of male mice and sex ratio of offspring

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Objective To study the effects of pubertal exposure to bisphenol A (BPA) on the reproduction of male mice in adulthood and subsequent generation mice.

Methods Male mice aged 21 d were exposed to BPA at a dose of 50 mg/kg per day for 7 d by intraperitoneal injection. Sperm count, sperm deformity rate and testis histology were evaluated 35 d after exposure. Male fertility index and newborns were further observed by mating with the normal female mice.

Results The epididymal sperm number was decreased by 20.6% in BPA exposure group compared with the control (P<0.01). Sperm deformity rate in BPA group was increased by 9.65% compared with the control (P<0.05). Testis seminiferous tubules were abnormal with sloughing of germ cells; BPA exposure had no significant effects on the fertility of male mice in adulthood; sex ratio of male to female offspring was increased.

Conclusion Pubertal exposure to BPA disrupted spermatogenesis in adult mice, and the proportion of male offspring was increased compared with the control.

Key words: bisphenol A (BPA); spermatogenesis; sex ratio

Bisphenol A (BPA) is a kind of chemical, also known as environmental endocrine disruptor, widely used to manufacture a variety of polymer materials including plastics. BPA could be leached from food packaging in the high temperature, high fat, acid, alkaline conditions due to its fat-soluble trait, which enters the human body. The human BPA exposure is very
universal\cite{1}. BPA is a low toxic substance because its oral LD$_{50}$ is 2 400 mg/kg body weight in mouse, however, BPA was also linked with many diseases reported recently\cite{2}. However, effects of BPA on the reproduction and offspring are still inconsistency due to many conflict research data existed\cite{3}. Most research has focused on BPA effects of the perinatal exposure rather than male puberty exposure on the adulthood reproduction. This study aims to investigate the effects of pubertal BPA exposure on reproduction in adulthood of male mice and offspring, and provide animal experimental data for understanding the effects of BPA on reproduction and development of human beings.

**Materials & Methods**

**Animals and reagents**

C57BL/6J mice aged 21-day-old with 12 ± 2 g body weight were purchased from Shanghai laboratory animal Co. Ltd. Mice were free access to water and food and housed at animal rooms (temperature: 24 ± 2 °C, relative humidity: 40%–50%) with a 12 h light/dark cycle. BPA was purchased from Sigma, Bouin fixative was purchased from Shanghai Shengzhe Co. Ltd., HE solution was purchased from Shanghai Hongqiaoalexion Co. Ltd.

**BPA exposure**

Sixty 21-day-old male mice were labeled and randomly divided into 2 groups (control group and BPA group, 30 mice per group) according to the body weight. BPA dose of 50 mg/kg per day was given by intraperitoneal injection once daily for 7 d. The mice in control group were received solvent corn oil only.

**Sperm parameters analysis**

After BPA administration for 35 d, 15 mice from BPA group and control group were taken randomly for epididymal sperm count and sperm morphology analysis, respectively. The mice were sacrificed by cervical dislocation and the epididymis was clipped, which were then put into a dish filled with 2 ml of saline. The epididymis was cut once vertically, followed by cutting transversely for 3–4 times and floating for 10 min at 37 °C in an incubator, making to sperm suspension. An aliquot of 5 µl sperm suspension was put into count plate for number counting. Another aliquot was made into smears and then dried naturally at room temperature (RT), followed by fixation with 75% ethanol for 2 min and dried at RT. The air-dried smears were stained using 20 g/L Eosin for 15 min, rinsed with water and dried at RT. Sperm morphology was checked by using a high magnification microscope and the type of sperm abnormalities were determined according to a previously described method\cite{4}. For each sample, at least 200 sperms were checked to calculate the sperm deformity rate.

**Testis histology analysis**

Testis were fixed in Bouin for 24 h, dehydrated in gradient ethanol, transparentized by
xylene, embedded in paraffin and cut to 4 µm tissue sections. Then sections were stained routinely with hematoxylin and eosin for testis histological examination under microscope. Thirty seminiferous tubules in each section were observed and recorded the number of normal and abnormal seminiferous tubules. Histopathology of testis includes germ cells arrangement disorder, exfoliation, vacuolization, etc. The proportion of abnormal seminiferous tubule was calculated in each group.

**Male fertility and offspring**

Five weeks after BPA exposure, the other mice in the control and BPA group mated with untreated female mice respectively. Pregnancy and parturition were observed and the number of the alive and the death per litter, the number of male and female, body weight and body appearance were also recorded. Fertility index = the number of fertility mice/the number of total mice × 100%, sex ratio = the number of male mice/the number of female mice×100.

**Data analysis**

All data were statistically analyzed using SPSS20.0 software. Enumeration data were presented as mean±standard deviation (x ±s). Two groups were compared using Student’s t-tests. P<0.05 was considered statistically significant.

**Results**

**Pubertal BPA exposure on the sperm quality in adulthood**

Epididymal sperm count results showed that the BPA group was 20.6% lower than the control, P<0.01 (Figure 1A). Sperm morphology analysis showed sperm abnormality mainly occurred in the head, less tail malformation. Sperm abnormality rate in BPA group was increased 9.65% compared with the control, P<0.05 (Figure 1B), indicating that puterbal BPA exposure severely disturbed the sperm quality of male mice in adulthood.

**Effects of pubertal BPA exposure on mouse testis histomorphology in adulthood**

HE staining of testis showed that, most of the seminiferous tubules arranged closely, in
which germ cells in different spermatogenic stage arranged regularly, a few seminiferous tubules with exfoliation of germ cells occasionally were observed in the control (Figure 2A). However, in BPA treatment group, there were many seminiferous tubules in which spermatogenic cells detached from basement membrane, sloughing of germ cells into the lumen, even completely blockage of lumen (Figure 2B, 2C). Results of quantitative analysis showed that the proportion of abnormal seminiferous tubules in BPA group was significantly higher than the control ($P<0.01$) (Figure 2D).

**Pubertal BPA exposure on the fertility of male mice on adulthood and offspring**

Effects of BPA on male fertility and offspring are shown in Table 1. The fertility index of adult mice, pup numbers of delivered per litter, birth body weight, survial index of offspring had no statistical difference between BPA group and control group ($P>0.05$); the appearance of newborns was normal and there was no detectable malformation. However, the sex ratio of male to female was 118.8 in BPA group and 101.4 in the control, with a statistically significant difference between the two groups ($P<0.05$).
Discussion

BPA, which is an endocrine disruptor structurally similar to estrogen, exerts a weak estrogen-like effect as well as an anti-androgenic effect. Studies showed that BPA can cause a wide range of harmful effects in the immune system, cardiovascular system and reproductive system of animals (including humans)\[^5\]. Although many studies have been centered on the toxicity of BPA on the male reproductive system, there are many inconsistencies in these reports\[^6\]. This may be mainly ascribed to the different reproductive toxicity, which was caused by different exposure doses, routes and timing of BPA administration, etc.

Most of the current BPA researches focus on the effects of adult reproductive system after perinatal BPA exposure, but relatively few studies on the pubertal male BPA exposure. In this study, transient exposure to BPA at puberty led to significantly decline in the number of sperm and increased percentage of abnormal sperm in adulthood mice. The results are consistent with those of perinatal BPA exposure reported previously\[^7\]. Testis histomorphology analysis revealed that BPA treatment resulted in pathological changes, including larger space between basement membrane and germ cells, sloughing of germ cells into the lumen, suggesting the functions of Sertoli cells may be damaged. Sertoli cells provide spermatogenic cells with hormones and nutrients, etc., and are fused into tight junction with adjacent Sertoli cells, forming a part of the blood-testis barrier. It has been reported that BPA can penetrate the blood-testis barrier and may directly influence the Sertoli cells and, thereby affect the activities of lactate dehydrogenase and glucose-6-phosphate dehydrogenase, resulting in spermatogenic cells detached from Sertoli cells and influencing spermatogenesis development\[^8\]. In addition, in this study, we found that the sperm deformity caused by BPA accured mainly in the sperm head, inferring that BPA could interact with the sperm DNA directly or indirectly. Moreover, the increment of the sperm deformity rate, to some extent, reflected the genotoxicity of BPA.

Another starting point of this study was to investigate whether abnormal spermatogenesis

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<th>Table 1 Effects of BPA on male mouse reproduction data and offspring ((\bar{x} \pm s))</th>
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<td><strong>Index</strong></td>
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<td>Fertility index (%)</td>
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<td>Pups delivered per litter (n)</td>
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<td>Survial rate (%)</td>
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<td>Birth body weight (g)</td>
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caused by BPA exposure had an impact on fertility and offspring of mice. To eliminate the influence of the female parent, we chose the normal female mice (without exposure to BPA) to breed offspring. We found that although BPA can lead to decreased spermatogenesis in mice, no significant difference in male fertility was observed between the experimental group and the control, suggesting that upon BPA exposure to the pubertal mice at the level of 50 mg/kg per day, no obvious harmful effects on male fertility in adulthood. BPA has little effects on average number of pups per litter as well as offspring birth weight. In addition, although no statistical significance in the survival rate of the mice offspring of these two groups was detected, but the BPA exposure appeared to lead to the tendency towards a decline in the survival rate of offspring. Thus, brief exposure to BPA has no obvious embryo toxicity for the male mice. In fact, currently there is no clear conclusion regarding the effects of BPA on the male offspring. Oishi and his colleagues reported that BPA exposure at a dose more than 235 mg/kg per day can reduce birth weight\(^9\). However, Tyl et al.\(^{10}\) reported that BPA exposure at a dose less than 5 mg/kg per day had little effects on a variety of reproductive-related indicators (e.g. organ index, sperm number, etc.), and only exposure at a high-dose of the 50 mg/kg and 500 mg/kg per day can lead to reduced offspring quantity per litter and survival rate.

More BPA researches focused on the reproductive toxicology, but usually did not care about birth sex ratio. There are some results showing that sex ratio at birth is within the normal range\(^{11}\). We found an interesting phenomenon when we analyzed offspring sex ratios, that was BPA-treated mice produced more male offspring and sex ratio was biased. At present sex ratio bias induced by BPA is rarely reported. Izumi et al.\(^{12}\) reported the sex ratio of the imago shifted in favor of males when eggs and larvae were exposed to BPA, in a study of BPA effects on the life cycle of the housefly. The researchers also found the offspring sex ratio bias in a study of BPA effects on deer mice sexually selected traits\(^{13}\). The female deer mouse aged 8–12 weeks feeding food supplemented with 50 mg of BPA/kg feed weight, 2 weeks prior to mating until weaning, born in a high proportion of male, reached 65%, but the authors did not do further research.

Male mice aged 7–12-week old were exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and mated with the normal female mice to breed progeny, the proportion of female offspring was increased and only relevant with paternal exposure\(^{14}\). The researchers detected the Y type sperm-specific SRY gene and X type sperm-specific androgen receptor (AR) gene using quantitative PCR, to evaluate X/Y ratio of gametes and sex ratio of 2-cell embryos, the results showed that there was no difference in the proportion of Y sperm and X sperm. But sex ratio of 2-cell stage embryos in TCDD group was significantly lower than in the control; therefore sex ratio decline occurred during fertilization instead of in the spermatozoa stage. Another researcher suggested the decisive reason was that TCDD caused a decrease in testosterone concentration of father, leading to reduced fertilization.
ability of Y-type sperm, but its mechanism was still not clear\cite{15}.

In this study, why BPA can cause an increase in the proportion of male offspring? We examined whether BPA induced a bias in the proportion of X/Y type gametes of the F0 generation. However, the results of real-time PCR detection of Y-gamete-specific SRY gene showed that the ratio of X/Y gametes were basically the same in BPA group and the control\cite{14}, therefore, we excluded the reason of the X/Y type gamete imbalance. In addition, there are still other possible causes, such as the relative increase of the Y-sperm fertilizing capacity. The mechanism of BPA increasing the proportion of male offspring needs further research.

The effects of endocrine disruptors on sex ratio at birth has been concerned by the researchers. Epidemiological survey data indicated that pregnant women in early pregnancy exposure to diethylstilbestrol (DES), as a representative of endocrine disruptors, the proportion of male offspring was significantly increased\cite{16}. Another investigation revealed the possibility to give birth to a girl increased with an increase of the serum TCDD concentrations in father, when the parents exposure to TCDD between 1977 and 1996. Fathers exposed at younger than 19 years of age born significantly more girls than boys, sex ratio of male to female was 0.38\cite{17}. This study showed that offspring sex ratio imbalance was relevant with male pubertal TCDD exposure. Therefore, the impact of environmental endocrine disruptors on human is worthy of more attention. The reason for nearly 30 years of sustained high sex ratio in China, in addition to subjective factors (human factors), we can not ignore the objective factors, for example, the natural environment changed and the population widely exposed to a variety of environmental endocrine disruptors, which can cause human physiology changes that may lead to bias of birth sex ratio.

In summary, our current study demonstrated that treatment of BPA of adolescence mice raised ratio of male/female of their offspring. It is significant to study whether a similar effect exist in human beings.

References


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