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Bisphenol-A Affects Spermatogenesis in the Adult Rat Even at a Low Dose

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Abstract: Bisphenol-A Affects Spermatogenesis in the Adult Rat Even at a Low Dose: Motoharu SAKAUE, *et al.* Environmental Health Sciences Division, National Institute for Environmental Studies—Bisphenol-A (BPA), a xenobiotic estrogenic compound widely used as a plastics monomer, has been suspected to have a so-called low dose effect on the reproductive system when administered transplacentally. In the present study, we investigated possible low-dose effects of BPA on spermatogenesis in adult rats. Male rats (13 weeks old; W13) were administrated a daily oral dose of BPA, ranging from 2 ng to 200 mg/kg, for 6 days and examined for testicular weight (TW) and daily sperm production (DSP) at W14 and W18. A BPA dose as low as 20 μg/kg tended to decrease TW and significantly reduced both DSP and the efficiency of spermatogenesis (DSP per gram testis) at W18, showing that BPA suppressed a normal increase in DSP and TW from W13 to W18. A single administration of 20 μg BPA/kg to W13 rats affected the intensity or mobility of several protein spots in the testicular cytosol fraction as shown by two-dimensional gel electrophoresis analysis. The present study showed that BPA at a low dose affects spermatogenesis in the adult rat.
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Key words: Bisphenol-A, Low dose, Adult rat, Testis, Spermatogenesis, Daily sperm production, Testicular weight, 2D-PAGE

Bisphenol-A (BPA) is a monomer of some plastics (polycarbonate and epoxy-resin) that are widely used in the lining of food and beverage cans, and in dental sealant, dishes, laboratory flasks and tubes, and non-negligible quantities of BPA have been reported to leach from these plastic products[1, 2]. Acceptable daily intake in Japan is 50 μg/kg body weight (bw). Net BPA-production in Japan has been increasing every year, from about 250,000 tons in 1996 to 360,000 tons in 1999. Therefore it is conceivable that industry workers who are engaged in the production of bisphenol-A may have an increasing chance of exposure to this compound. Up to now, exposure to a low dose of BPA during gestation has been reported to alter the male reproductive system in adulthood, for example, an increase in the prostate weight[3] and reduction in the efficiency of spermatogenesis[4], and accelerated period of puberty in female offspring[5]. In contrast, other workers claimed that they failed to find these effects on male offspring caused by BPA under an identical protocol[6], so that it has been an extraordinarily controversial issue how BPA can possibly affect the reproductive tissues in both male and female offspring.

Estrogen receptors, α and β, (ERs) are expressed in the testis. ERα has been found in the Leydig cells of the rat[7] and man[8]. The presence of ERβ, in turn, has been described in Sertoli cells, pachytene spermatocytes, and round spermatids of the adult rat testis[9]. The ERs have been shown to be expressed in other tissues of the male reproductive tract[10]. In addition, recent studies revealed that 17β-estradiol plays an indispensable role in spermatogenesis[8, 11–14]. In combination with the earlier reports on BPA bioactivity, in which BPA has the capacity to stimulate the proliferation of cultured human breast cancer cells[15] and interact with the ERs[16, 17], one cannot rule out the possibility that BPA could affect spermatogenesis in adult males. Nevertheless, it is generally thought that endocrine effects of BPA on adults are few, because these effects may be transient, i.e. only while the chemical is present or until it is completely

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metabolized[4], and that its disruption of the development of fetuses and pups makes the functioning of cells and organs irreversibly changed in adulthood[4, 18]. Here we show for the first time that a very low dose of BPA orally administrated to adult male rats suppresses an increase in the number of germ cells in their testes and that two-dimensional gel electrophoresis (2D-PAGE) analysis of testicular cytosol protein comfirmed the effects of BPA on adult testis.

Materials and Methods

Animal and treatment

Male Sprague-Dawley rats (11 weeks old) were purchased from CLEA Japan, Inc. (Tokyo, Japan) and housed in stainless wire cages until 13 weeks old (W13) for acclimatization. Rodent feed (CE-2, CLEA Japan) and water were provided *ad libitum*. BPA (99.6%, Wako Pure Chem., Osaka, Japan) was dissolved in ethanol and then diluted with corn oil (Sigma, St. Louis, MO, USA). The final concentration of ethanol was 6.5% in corn oil. In experiment 1 (Exp1), a specified dose of BPA, ranging from 20 μg/kg bw to 200 mg/kg bw was administered by gavage daily to W13 male rats (n=5) for 6 days. Control rats received corn oil containing 6.5% ethanol as vehicle. The rats were dissected on day 8 (14 wk old, W14) or day 36 (18 wk old, W18) after the first administration. Paired testes were weighed, and then the left testis was homogenized in 150 mM NaCl containing 0.05% (v/v) Triton X-100. After a 10-fold series of dilution, the number of elongated spermatid nuclei resistant to homogenization was counted with a hemocytometer. Daily sperm production (DSP) and DSP per gram testis (DSP/gt) were calculated according to the method described by Robb *et al*[19]. The right testis was removed, immersed in Bouin's fixative overnight, and embedded in paraffin. Sections (5 μm in thickness) were sliced at 200 μm intervals from the top to the end of the testis and then stained with hematoxylin and eosin. All sections were examined histopathologically. In experiment 2 (Exp2), the testes were obtained from rats (n=8) dosed with BPA (2 ng/kg bw to 2 mg/kg bw), and DSP and DSP/gt were determined by the same protocol as was used in Exp1.

Two-dimensional gel electrophoresis

The right testis was taken from the W13 male rats at 6 hours after a single oral dose of 20 μg BPA/kg bw and homogenized in a Teflon-glass homogenizer in 5 ml of 50 mM phosphate buffer, pH 7.4, containing 0.25 M sucrose and 0.15 M NaCl. After centrifugation at 20,000 $\times$ g for 10 min, the supernatant was centrifuged again at 100,000 $\times$ g for 60 min. Then the resulting testicular cytosol fraction was analyzed by two-dimensional gel electrophoresis. The protein concentration of the cytosol was determined by Lowry's method, and the cytosol specimen was diluted with sample buffer (9.9 M urea, 4% (v/v) NP-40, 5.5% (v/v) ampholytes, and 100 nM dithiothreitol) to make a protein concentration of 80 μg/20 μl. The cytosol preparations were analyzed according to the method described previously[20, 21], and the gels were fixed in 40% methanol and 10% acetic acid solution, followed by staining with a silver staining kit (Wako Pure Chem.).

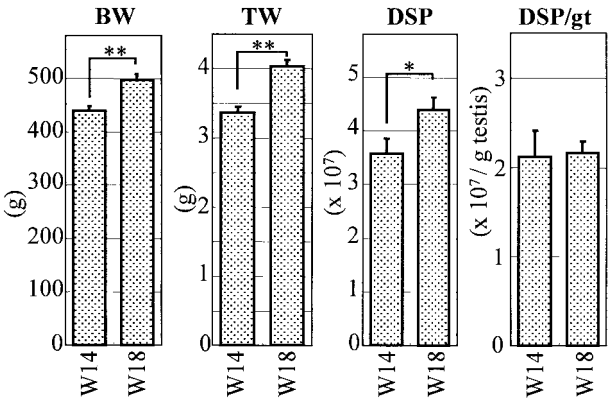

Fig. 1. Changes in body weight (BW), paired testicular weight (TW), daily sperm production (DSP), and DSP per gram testis (DSP/gt) in vehicle-treated control rats from W14 through W18. BW, TW, and DSP significantly increased from W14 to W18, but DSP/gt did not. The results are expressed as the mean ± S.E. for 5 rats. Differences between means of each parameter at W14 and W18 were statistically analyzed by unpaired Student's *t*-test, *: P<0.05, **: P<0.005.

Statistical analysis

For statistical analysis, StatView for Windows version 5.0 (SAS Institute Inc., Cary, NC, USA) was used. Statistical differences between groups were determined by the unpaired Student's *t*-test or by one-way analysis of variance (ANOVA). Fisher's PLSD test was employed to compare individual means as a *post-hoc* test.

Results and Discussion

We first studied the possible effects of a large dose of BPA on spermatogenesis in adult male Holtzman rats (W13) by administering BPA at a daily dose of 200 mg BPA/kg bw for 6 d. In this experiment, we found a significant reduction in DSP at W18 (data not shown). To investigate whether a much lower dose of BPA would affect spermatogenesis, we carried out an experiment (Exp1) with the same protocol in Sprague-Dawley rats at doses of BPA (20 μg/kg bw to 200 mg/kg bw). In vehicle-treated control rats, the mean values of paired testicular weight (4.03 g) and DSP (4.40×10^7/testis) significantly increased from W14 through W18, but the level of DSP/gt (2.16×10^7/g testis) was not changed (Fig. 1). This observation indicates that the W13 rat testis

is still in the growing phase and that the capacity of sperm production per testis continuously increases. The testis is constituted of germ cells, Sertoli cells, interstitial cells including Leydig cells and macrophages, and an extracellular matrix. There was no difference in DSP/gt between at W14 and W18, which indicates that the ratio of the number of spermatids to the volume of the other testicular components such as Sertoli cells, interstitial cells and the extracellular matrix is constant. It is reported that the Sertoli cells never proliferate after puberty[22, 23]. Taken together, these observations suggest that the number of Sertoli cells is not responsible for an increase in testicular weight from W14 to W18 and that the numbers of germ cells nourished by each Sertoli cell continues to increase even after puberty.

When BPA was administered at a dose of 20 μg/kg bw or greater to W13 rats, there were significant decreases in the DSP (3.20×10^7/testis) and DSP/gt (1.72×10^7/g testis) of W18 rats compared to those of the vehicle-treated control rats (Fig. 2A). Nevertheless the level of the DSP was comparable to that of the W14 controls. Interestingly, the degree of these BPA effects was similar in all doses used in Exp1. The right testes of the all BPA-treated rats at W18 were cut into a series of sections every 200 μm and all the sections were histopathologically examined, but there was neither disruption of spermatogenesis nor atrophy in any seminiferous tubule (data not shown). Since the ratio of the number of germ cell to the volume of the other testicular components is constant in control animals, the reduction in this ratio in BPA-treated groups suggests that BPA suppressed the increase in germ cells per Sertoli cell from W14 through W18. Although no statistically significant difference was observed, there also seems to be a decrease in testicular weight in response to BPA administration (Fig. 2A).

Since the so-called low-dose effects of endocrine disrupters are a key issue not only for risk assessment/management but also for clarifying the mechanism of these compounds, it is extremely important to study the dose-response relationship as well as to confirm the reproducibility of the experiment. Accordingly, we performed Exp2 to determine the lowest observed effect level (LOEL) of BPA by utilizing lower doses (2 ng/kg bw to 2 mg/kg bw) under exactly the same condition as Exp1. Mean values of paired testicular weight (3.91 g) and DSP (4.93×10^7/testis) of the control at W18 were once again significantly higher than those at W14 (3.67 g, 2.55×10^7/testis, respectively). The body weight of rats in BPA-treated groups was not different from that of vehicle-treated control rats at W18. BPA treatment as low as 2 ng/kg bw caused a downward tendency in TW, DSP, and DSP/gt but the BPA dose at 20 μg/kg bw or greater resulted in a statistically significant decrease in DSP and DSP/gt at W18 when compared to vehicle-treated rats (Fig. 2B). Furthermore, although the numbers of germ cells per testis and Sertoli cell are normally increased from W14 though W18, these results suggest that BPA suppresses the normal increase in these numbers.

By using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), we next studied whether protein expression was affected by a low dose of BPA, and analyzed the cytosol fraction of testis obtained 6 h after a single dose of 20 μg BPA/kg bw. The electrophoresis pattern showed changes in intensity or relative mobility of protein spots in at least three areas (Fig. 3). BPA treatment resulted in a decrease in the intensity of two protein spots in areas A and B, as well as a shift from isoelectric point (pI) 5.8 to pI 5.7 of a protein spot in area C (Fig. 3). We have not succeeded in identifying the proteins, but phosphorylation is probably responsible for this shift of the spot in the area C. These changes in the protein spot pattern were repeatedly observed in different rats, indicating that administration of 20 μg BPA/kg bw could affect protein expression in the adult testis.

In mature male animals, estrogen has been shown in several studies to play an indispensable role in normal spermatogenesis. For example, ERα knockout mice exhibited abnormal spermatogenesis, including male infertility[11]. This abnormality is thought to be due to the disruption of normal functions in the epithelium of the reproductive tract, since ERα was detected in the epithelial cells[7, 10] and was involved in an estrogen-mediated reabsorption of luminal fluid[12]. It has also been determined that Sertoli cells, spermatocytes and spermatids themselves express ERβ[9]. 17β-Estradiol inhibits male germ cell apoptosis *in vitro*[8]. In another study, adult mice lacking a functional aromatase, which converts testosterone to 17β-estradiol, exhibited incomplete differentiation of spermatids on a part of the seminiferous epithelium[13]. BPA is considered an estrogen agonistic chemical because it transactivated the estrogen-responsive element of ERs *in vivo* and *in vitro*[15, 24] and also affected preimplantation mouse embryos by counteracting with tamoxifen[25]. On the other hand, BPA functioned as an estrogen antagonist when rats were treated with 17β-estradiol together *in vivo*[26], which suggests that BPA might act as an estrogen antagonist during spermatogenesis by preventing 17β-estradiol from inhibiting germ cell apoptosis[8], and decrease the level of sperm production. We do not have an explanation for the mechanism(s) behind this BPA's apparently contradictory actions on spermatogenesis, and it should be elucidated whether BPA acts as an estrogen agonist or antagonist on spermatogenesis in rats.

Estrogens are endogenously present not only in the ovary but also in the testis. We have measured the concentration of 17β-estradiol in the testis of 5- and 13-week-old (W5 and W13) control SD rats by enzyme immunoassay and detected 35 ± 4 pg/g testis and 814 ± 67 pg/g testis, respectively (unpublished data). This result indicates that

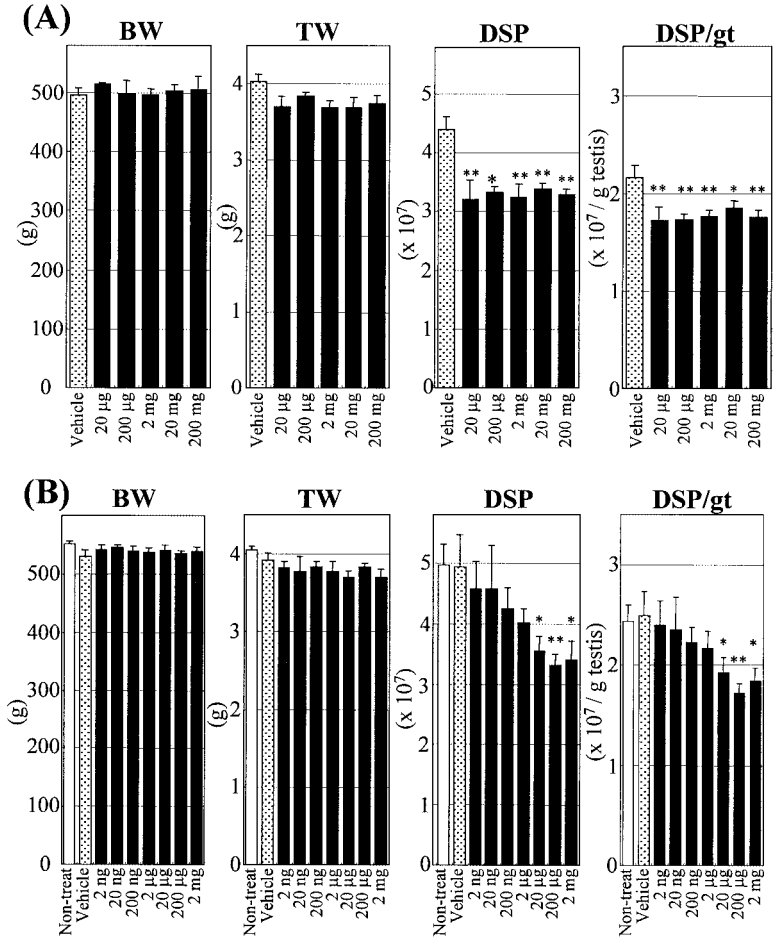

Fig. 2. Effects of BPA on body weight (BW), paired testicular weight (TW), daily sperm production (DSP), and DSP per gram testis (DSP/gt) in adult male rats. BPA was orally administered at W13 with a specified daily dose for 6 days, and its effects were examined at W14 (not shown) and W18. A, In all the BPA-dosed groups, DSP and DSP/gt were significantly decreased compared to the vehicle-treated group, but no difference in any of these parameters was observed among different dose groups of BPA-treated rats (n=5). B, Changes in BPA-treated groups in a lower dose range in Exp2 at W18 (n=8). The treatment with BPA at a dose of 20 μg BPA/kg bw or more exerted a statistically significant decrease in DSP and DSP/gt, but showed a dose-dependent decline from vehicle-treatment to 20 μg BPA/kg bw. TW also showed a tendency to decline compared to that of the vehicle control group. These data were analyzed by analysis of variance with a *post-hoc* comparison of difference in means by Fisher's PLSD test, *: $P<0.05$; **: $P<0.005$, compared with data from the vehicle control group.

the animals used in the present study had a relatively high concentration of 17β-estradiol in the testis, and suggests that this endogenous 17β-estradiol should play an essential physiological role in the testis at this age. In our preliminary study, BPA was detected at as high as 245 ± 40 ng/g testis in W13 male SD rats at 1 h after an oral administration at 20 mg BPA/kg bw (unpublished data). The estrogenic activity of the 200 ng BPA can be estimated to be comparable to that of 40 pg 17β-estradiol according to the report of Krishnan *et al*[15]. Combining the content of endogenous estrogens (814 ± 67 pg/g testis) with the estimated estrogenic activity of BPA (40 pg/g testis), we anticipate that total estrogenic activity in the testis could be regarded essentially at the same level as the activity of endogenous estrogen alone. A previous report has also shown that the laboratory animal chow contains large

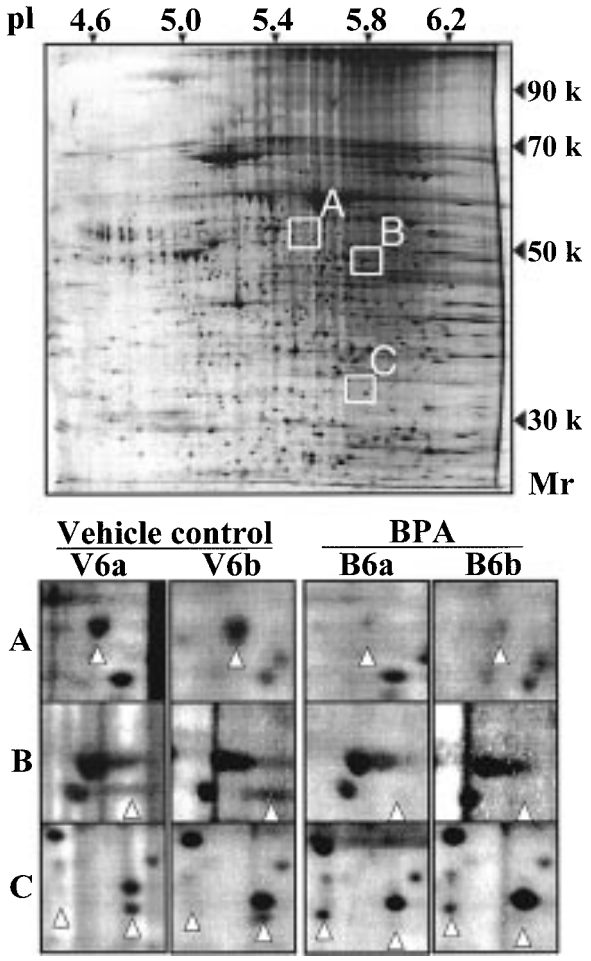

Fig. 3. Two-dimensional electrophoretic analysis of testicular cytosol specimens obtained from vehicle- or BPA (20 μg BPA/kg bw)-treated rats at 6 h after a single administration. Representative data for two individual rats treated with either vehicle (V6a and V6b) or BPA (B6a and B6b) are shown. The lower panel is a magnified portion from the open squares A, B, and C in the upper panel. The staining intensity of protein spots in areas A and B was reduced in BPA-treated rats. A protein spot was observed in area C in each BPA-treated rat. The isoelectric point (pI) and relative molecular mass (Mr) of these protein spots were calculated: pI 5.5 and 53 kDa in area A, pI 5.8 and 47 kDa in area B, pI 5.8 to 5.7 and 34 kDa in area C, respectively.

amounts of phytoestrogens[27]. In fact, the animal chow used in the present study contained phytoestrogen-containing ingredients such as soy cake, alfalfa, yeast and rice bran according to the manufacturer's gradient regimens, and is thought to include substantial amounts of phytoestrogens as reported by Boettger-Tong *et al*. Therefore, we estimate that the total amounts of estrogenic compounds which can be distributed in the rat testis would be much higher than 814 ± 67 pg 17β-estradiol/g testis. It is therefore very difficult to explain why BPA acts as an estrogen agonist in the testis and then decreases the efficiency of sperm production. The remaining possibility for the action of a low dose of BPA would be that it acts as an estrogen antagonist, as in the report by Gaido and coworkers[26].

The present study clearly shows that a very low dose of BPA affects spermatogenesis in the adult rat in a monotonous fashion rather than an inverted U-shape manner. Up to now, vom Saal and coworkers have clearly indicated the presence of the inverted U-shape relationship of either diethylstilbestrol or 17β-estradiol with prostate weight[28], but no inverted U-shape curve for so-called low-dose effects of BPA on prostate weight has been reported. Although we failed to find an inverted U-shape response, we here show for the first time that environmental endocrine disrupters such as BPA alter spermatogenesis in a linear manner in a dose range which is perhaps relevant to the daily level of exposure in man.

A decline in the number of sperm in human populations during the last 50 yr has been suspected[29]. Sharpe and Skakkebaek have hypothesized that environmental estrogens interfere with spermatogenesis by affecting proliferation and differentiation of Sertoli cells during fetal life[18]. It is not clear whether a low dose of BPA exerts adverse effects on the offspring of mice that are transplacentally exposed to this compound. vom Saal and associates showed that maternal exposure to a low dose of BPA caused a reduction in daily sperm production, seminal vesicle weight and an increase in the prostate weight of male mouse offspring[3, 4], but Cagen and coworkers were unable to reproduce these findings under the same experimental protocol[6]. The present results are consistent with the earlier observation of vom Saal and coworkers, but the mechanisms of BPA effects on fetuses and adult males may differ. Regarding the implications of the present results, one has to bear in mind the following points: the impact of BPA on spermatogenesis and the testis growth rate was not aggravated in a dose-dependant manner over a BPA dose of 20 μg/kg bw. It should therefore be pointed out that sperm numbers do not decrease further with an increase in the BPA dose. In addition, it is not clear from the current study whether there is a more sensitive age than 13 weeks old in terms of a reaction to BPA, whether the effect of BPA on the testis is reversible or not, whether repeated exposure to BPA results in chronic testicular effects, or how BPA exerted subtle alterations in the spermatogenesis. These questions are open to further investigation, and answering them could provide useful information in the risk assessment of this environmental estrogenic compound.

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