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·*Original Article*·

Effects of estradiol-17β and bisphenol A administered chronically to mice throughout pregnancy and lactation on the male pups' reproductive system

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Abstract

Aim: To assess the effect of estradiol-17β (E_2) and bisphenol A (BPA) administered chronically by implanting a silicone tube throughout pregnancy and lactation on male pups' reproductive system in ICR mice. **Methods:** Female mice were implanted with a tube filled with 10 ng, 500 ng, 1 μg, or 10 μg of E_2, or 100 μg or 5 mg of BPA, before mating. The tube was kept in the mice throughout pregnancy and lactation, until the pups had weaned at 4 weeks of age. During the period, E_2 was released from the tube at 120 pg or 6, 12 or 120 ng/day, and BPA at 1.2 or 60 μg/day. **Results:** Most of the mice given 1 μg and 10 μg of E_2 did not maintain their pregnancy. However, the other groups showed high rates of birth, more than 70%. At age of 4 weeks, the male pups were killed. Body weight and reproductive organ weights (testes, epididymides and accessory reproductive glands) in the treated groups did not differ from the control values, whereas the percentage of seminiferous tubules in the testis with mature spermatids was significantly lower in the groups given 10 ng and 500 ng of E_2 and 5 mg of BPA than that in the control. **Conclusion:** Chronic exposure to E_2 and BPA might disrupt spermatogenesis in male pups. *(Asian J Androl 2008 Mar; 10: 271–276)*

Keywords: estradiol-17β; bisphenol A; chronic administration; silicone tube; spermatogenesis; ICR mice

1 Introduction

The endocrine disruptive chemicals continuously being released into the environment might disrupt endocrine function in wild animals and humans. Reproductive abnormalities induced by exposure to these compounds early in postnatal life have been found in experimental animals as well as in humans [1]. Bisphenol A (BPA) is an important industrial compound used principally as a monomer in polycarbonate plastics and as a constituent of the epoxy resins used extensively in food and drink cans [2]. BPA has been reported to be weakly estrogenic both *in vivo* and *in vitro*, showing at least 10 000-fold [3] less relative affinity for the estrogen receptor than estradiol-17β (E_2). BPA has an anti-androgenic effect on binding activity in *in vitro* yeast-based assays [4] and on spermatogenesis in male mice [5]. Several *in vivo* studies have found that when given to pregnant animals, BPA significantly affected the reproductive system of their pups: BPA was given to pregnant mice on day 11 through day 17 of gestation [6] and to pregnant/lactating mice [7]. Some experiments *in vitro*

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have also shown the effects of BPA on embryos during the preimplantation period [8]. In contrast, other studies have found that a low dose of BPA had no effect on male and female pups of mice and rats exposed during pregnancy [9, 10] and during pregnancy and lactation [11]. Therefore, the effect of a low dose of BPA during pregnancy and lactation has been controversial.

We developed a slow-release silicone tube for chronic administration of steroid-related materials, E_2 and BPA [12]. The tube is suitable for sustained-release, delivering a controlled concentration over a certain period and removing it whenever required. The present study was designed to expose female mice to E_2 or BPA throughout pregnancy and lactation using the silicone tube. Birth rate, postnatal growth and male reproductive organs of pups were examined for changes following exposure throughout embryonic, fetal and postnatal periods.

2 Materials and methods

2.1 Animals

Female ICR mice were purchased from SLC Japan (Shizuoka, Japan) at 7 weeks of age. The animals were acclimated to the room to be used for the experiments for 1 week prior to the start of the study. This room was maintained at 22 ± 3ºC with 14 h of artificial light and 10 h of darkness daily (lights on 5:00 to 19:00) and the animals were provided with food pellets (LABO MR STOCK; Nihon Nosan Kogyo, Yokohama, Japan) and water *ad libitum*. Type B tubes prepared as described in our previous paper [12] were implanted s.c. into 46 female mice. The tubes were medical silicon tubes (inner diameter 2.0 mm, outer diameter 3.0 mm, length 20 mm; KANEKA MEDEX, Osaka, Japan) covered with a polyethylene tube (inner diameter 3.0 mm, outer diameter 4.0 mm, length 8.0 mm; IMAMURA, Tokyo, Japan) at each end. E_2 (β-ESTRADIOL, E-8875; Sigma Chemical, St. Louis, MO, USA) was dissolved in sesame oil (Sigma-Aldrich Japan, Tokyo, Japan) at a concentration of 0.2 μg/mL, 10 μg/mL, 20 μg/mL or 200 μg/mL and bisphenol A (BPA, Tokyo Kasei Kogyo, Tokyo, Japan) was dissolved in sesame oil at a concentration of 2 mg/mL or 100 mg/mL. Each compound (50 μL) was added to a tube (final volume: 10 ng, 500 ng, 1 μg, or 10 μg for E_2; 100 μg or 5 mg for BPA) and implants were sealed with a special glue for silicone (Shin-Etu Silicon, Shin-Etu Chemical, Tokyo, Japan). Each implant was incubated in 6 mL of physiological saline at room temperature for one night before use. The control group was implanted with a tube filled with 50 μL of sesame oil in a similar manner. For 3 days, and 3 days after the implantation, female mice were placed individually in mating cages that contained a male mouse. All mice judged to be pregnant based on the presence of a vaginal plug were allowed to give birth. On postnatal day 0, the number of pups was randomly adjusted to eight. At age of 4 weeks, all mice were weighed and killed. After blood collection, the testes, epididymides and accessory reproductive glands (seminal vesicles with coagulating glands) were dissected out from the male pups and weighed. All experimental procedures were conducted in accordance with the guidelines for animal experiments, College of Bioresource Science, Nihon University.

2.2 Histological examination

The reproductive organs from the male pups of all treatment groups were fixed in Bouin's solution. The fixed tissues were embedded in paraffin. Tissue sections of 5 μm were cut and stained with azan for histological examination. Four hundred seminiferous tubules were counted per animal from four tissue sections. The number of seminiferous tubules with steps 9–16 elongate spermatids [13] was calculated.

2.3 Enzyme immunoassay for testosterone

The concentration of testosterone (T) in the male pups' serum was measured with a commercially available kit for enzyme immunoassay (EIA) (Cayman Chemical, Ann Arbor, MI, USA). This method is based on the competition between serum T and the T-acetyl cholinesterase conjugate for binding sites on an antibody-coated plate. The serum samples were diluted with EIA buffer 5-fold to 640-fold and assayed in duplicate. The intraassay and interassay coefficients of variation were 7.4% and 11.5%, respectively. The detection limit (80% B/B_0) of the assay is 6.0 pg/mL according to the information supplied by the company. The concentrations were calculated from a calibration curve established using authentic T.

2.4 Statistical analysis

The data were expressed as the mean ± SEM after the elimination of some data among experimental groups using Smirnoff's elimination test. The infant mortality rate was evaluated using Fisher's exact probability test.

The significance of differences between the control and E_2 or BPA-treated groups was evaluated using analysis of variance and Duncan's new multiple range test. $P < 0.05$ was considered statistically significant.

3 Results

3.1 Birth and infant mortality rates and body and relative reproductive organ weights

Birth and infant mortality rates and body and relative reproductive organ weights of the male pups at 4 weeks of age are summarized in Table 1. None of the pregnant mice treated with 10 μg of E_2 gave birth. Treatment with 1 μg of E_2 caused a marked decline in the rate of birth (16.7%; 1 of 6 dams) and number of pups (6 pups). However, the groups treated with 500 ng of E_2 and 100 μg and 5 mg of BPA showed a high birth rate (77.8%, 80.0% and 71.4%, respectively). The dams treated with 10 ng of E_2 and the controls had no abortions (6 and 7 dams, respectively). The group treated with 500 ng of E_2 had high birth rates (77.8%), although the rate of infant mortality (19.64%; 11 of 56 pups) during the first week of life was significantly higher than in the other groups (3.57% for control, 4.17% for 10 ng E_2, 6.25% for 100 μg BPA, 2.50% for 5 mg BPA).

Neither E_2 nor BPA treatment had a significant effect on the body weight of male pups as compared to the control. However, treatment with 100 μg of BPA resulted in significantly higher body weight as compared to that with 5 mg of BPA (29.4 g *vs.* 25.5 g, respectively). There were no differences in the relative weights of male reproductive organs (testes, epididymides and accessory reproductive glands) in any of the treated groups.

3.2 Histological change in the testis

The histology of the testes of male pups treated with E_2 and BPA is shown in Figures 1 and 2. Exfoliation of a few germ cells and a reduction in the number of elongate spermatids were observed in the seminiferous tubules of the testis in the treated groups (Figure 1). The percentage of seminiferous tubules having elongate spermatids decreased significantly in male pups treated with the two doses of E_2 and 5 mg of BPA as compared to the control (32.9%, 39.8% and 40.3% *vs.* 53.8%, Figure 2). Notably, the lowest rate was obtained in the group given 10 ng of E_2 (32.9%).

3.3 Hormone levels

Serum T concentrations of the male pups are shown in Figure 3. Treatment with 10 ng of E_2 or 5 mg of BPA resulted in T concentrations that were slightly but not significantly reduced compared to controls. Treatment with 500 ng of E_2 or 100 μg of BPA tended to increase the concentration relative to the other groups and mice treated with 100 μg of BPA showed a significantly higher level than the controls and the mice treated with 5 mg of BPA (4.99 ng for 100 μg of BPA *vs.* 1.93 ng and 1.45 ng, respectively).

Table 1. Long-term effect of estradiol-17β (E_2) and bisphenol A (BPA) on birth rate, pup number, infant death rate and body and relative reproductive organ weights (mg/BW) at various concentrations. [a]Birth rate (%): No. of parturient females/No. of females that copulated; [b]One datum missing; [c]Infant mortality rate (%): No. of dead pups / total No. of pups on postnatal day 7; [d]Significantly different from control ($P < 0.01$) and 10 ng of E_2 ($P < 0.05$); [e]Significantly different from 5 mg of BPA ($P < 0.01$).

Variable	Control	E_2				BPA	
		10 ng	500 ng	1 μg	10 μg	100 μg	5 mg
Birth rate[a] (%)	100 (7/7)	100 (6/6)	77.8 (7/9)	16.7 (1/6)	0 (0/6)	80 (4/5)	71.4 (5/7)
Pup number	13.50 ± 0.96[b]	12.80 ± 2.22[b]	13.50 ± 2.06[b]	6.0	–	12.00 ± 0.58	12.50 ± 0.65[b]
Infant mortality rate[c] (%)	3.57 (2/56)	4.17 (2/48)	19.64 (11/56)[d]	0 (0/6)	–	6.25 (2/32)	2.50 (1/40)
No. of infant sample	25	22	16	–	–	10	19
Body weight (g)	27.78 ± 0.87	26.91 ± 0.58	27.39 ± 1.22	–	–	29.43 ± 0.79[e]	25.54 ± 1.00
Relative testis weight	5.10 ± 0.12	5.21 ± 0.13	5.30 ± 0.16	–	–	5.21 ± 0.26	5.41 ± 0.11
Relative epididymis weight	1.53 ± 0.04	1.60 ± 0.05	1.62 ± 0.04	–	–	1.58 ± 0.07	1.60 ± 0.04
Relative accessory reproductive glands weight	2.19 ± 0.14	2.26 ± 0.15	2.27 ± 0.12	–	–	2.47 ± 0.17	2.17 ± 0.19

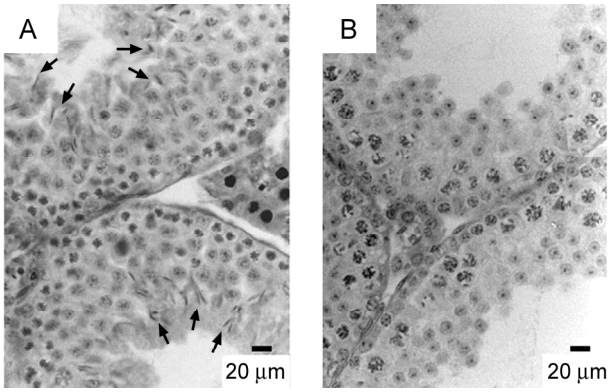

Figure 1. Histopathological changes in male ICR pups from dams exposed to estradiol-17β (E_2) throughout pregnancy and lactation. Photomicrographs of azan-stained sections. (A): Control male testis. No abnormalities were observed. Arrows indicate elongate spermatids. (B): Testis from the 10 ng E_2 group. There are few elongate spermatids in the seminiferous tubules.

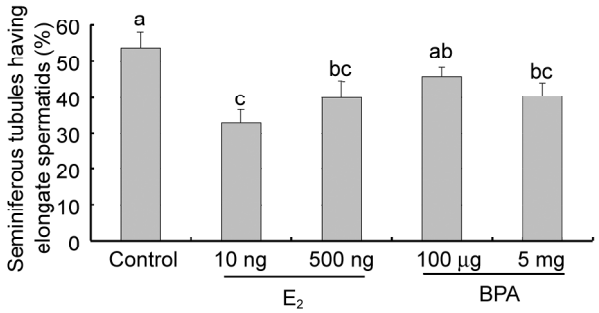

Figure 2. Long-term effect in male ICR pups from dams exposed to estradiol-17β (E_2) or bisphenol A (BPA) throughout pregnancy and lactation on the proportion of seminiferous tubules with elongate spermatids. Mean ± SEM values with different letters differ significantly ($P < 0.01$).

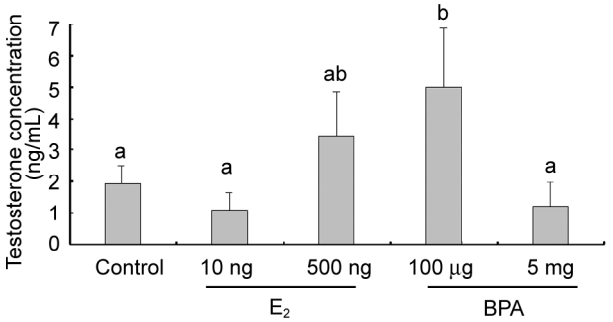

Figure 3. Long-term effect in male ICR pups from dams exposed to estradiol-17β (E_2) or bisphenol A (BPA) throughout pregnancy and lactation on serum total testosterone concentrations. Mean ± SEM values with diffe-rent letters differ significantly ($P < 0.01$).

4 Discussion

We developed a slow-release silicone tube for chronic administration of steroid related materials [12]. The tube is suitable for sustained-release, delivering a controlled concentration over a certain period and removing it whenever required. In the present study, the tube was implanted s.c. and left throughout pregnancy and lactation in female mice. The present results clearly show the effects on the birth and infant mortality rates of the pups of dams treated with E_2 or BPA. The dams implanted before mating with a tube filled with 10 μg of E_2 and five of six dams with 1 μg of E_2 did not show any signs of pregnancy. These female mice did not increase in body weight despite having a vaginal plug. Because the type B tube releases E_2 and BPA at a rate of 1.2%/day [12], a tube filled with 1 μg and 10 μg of E_2 would release 12 ng/day and 120 ng/day, respectively. The present results suggest that E_2 treatment at a dose of more than 12 ng/day practically stopped all implantation in mice. However, exposure to 10 ng and 500 ng of E_2 and 100 μg and 5 mg of BPA resulted in a high birth rate comparable to the control, although several pups in the group given 500 ng of E_2 died during the first week after birth. Therefore, these results suggest that the abortions and infant mortality in the groups treated with higher concentrations of E_2 were not a result of the operation for implantation of the tube but dependent on the concentration of E_2.

Male pups from dams exposed to E_2 showed a significant decrease in the proportion of seminiferous tubules having elongate spermatids at 4 weeks of age. Interestingly, the disruptive effects on spermatogenesis and the serum T concentration were more remarkable at a lower dose of E_2 of 10 ng than at 500 ng at 4 weeks of age. Ten nanograms in the tube in this experiment would be released at 120 pg/day based on a calculation using our previous result [12]. In the published literature, concentrations higher than this dose of E_2 resulted in detrimental effects on male pups administered during pregnancy and the lactation period. The male pups from pregnant ICR mice receiving s.c. injections of 1 μg of E_2 on day 2 of gestation had reduced testosterone levels and heavier seminal vesicles at 6 months of age [14]. However, the testicular weight and structure of the male offspring showed no significant effects on the day of birth and at 2, 4, 8 and 14 weeks of age after a single s.c. injection of 1 mg or 5 mg of E_2 given to female mice on

day 14 of pregnancy [15]. In newborn ICR mice treated with E_2 at 1, 3, 5, 7, 9 and 11 days of age, the lowest dosage, 6.25 ng was regarded as the no observed adverse effect level (NOAEL) for the respective parameters [16]. By contrast, the same strain of mice did not show a significant difference in the percentage of elongate spermatids in seminiferous tubules compared to the control when the animals were administered with a silastic tube filled with 40 μg of E_2 from 22 to 43 days of age [17]. In the present study, some significant differences from results reported in the published literature were revealed in male pups of ICR mice treated with a lower dose of E_2. Therefore, the results suggest that continuous exposure throughout pregnancy and lactation might be highly detrimental to the male reproductive system.

In the present experiment using BPA, however, a lower dose, 100 μg, had no significant effect on spermatogenesis compared to the control. In contrast, a higher dose, 5 mg, clearly disrupted spermatogenesis. Several other studies have found no effect on the reproductive systems of mouse or rat pups when a low concentration of BPA was given p.o. during pregnancy [9–11, 18]. In contrast, some studies have reported that sperm production, body weight [6] and the weight of the testes [7] in pups were significantly changed compared with the controls when treated with a lower concentration of BPA than that used in the present study. Therefore, reports on the effects of a low dose of BPA on the male reproductive system have been inconsistent.

The route of administration of endocrine disruptive chemicals is quite important. There were differences in the bioavailability and metabolism of BPA depending on whether rats were administered p.o., i.p. or s.c. [19]. The maximal level of BPA in blood was approximately 40 times higher on i.p. administration than p.o administration in male rats. A subcutaneous injection of BPA is approximately 20 times stronger than a p.o. administration in terms of the uterotropic activity in immature rats [20].

The present study has provided an easy and sensitive method, using a silicone implant, for exposing experimental animals to endocrine disruptive chemicals long term. The proposed method also has the clear advantage that the animals are handled only once, therefore reducing stress and facilitating investigation. Finally, the method has potential practical applications for examining the effects of exposure to endocrine disruptive chemicals on embryos and pups throughout pregnancy and lactation.

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