

Aggressive Behavior and Serum Testosterone Concentration during the Maturation Process of Male Mice: The Effects of Fetal Exposure to Bisphenol A

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The relationship between exposure to endocrine-disrupting chemicals (EDs) and risk to reproductive organs is well documented, but the influence of EDs on behavioral development has not been studied. In this study we evaluated the effect of fetal exposure to bisphenol A, which mimics estrogenic activity, on aggressive behavior and hormonal change in male mice. On gestation days 11–17, female mice were fed bisphenol A at 2 ng/g or 20 ng/g of body weight (environmentally relevant concentration). Aggression rating and blood sampling of the offspring were done at 8, 12, and 16 weeks of age. Aggression scores increased significantly ($p < 0.01$) at 8 weeks of age in male mice exposed to bisphenol A at both the 2 ng/g and 20 ng/g concentrations compared with a control group, but no difference was found after 12 weeks. Relative testis weight (per gram of body weight) was significantly lower at 8 and 12 weeks in mice treated with 2 ng/g than in controls ($p < 0.05$) and was significantly lower at 12 weeks in mice treated with 20 ng/g than in controls ($p < 0.01$). The serum testosterone concentration in treated mice was not significantly different from that in controls. These results demonstrate that bisphenol A temporarily activated aggressive behavior in mice at 8 weeks of age and that low doses of bisphenol A interfered with the normal development of reproductive organs. The mechanism activating this aggressive behavior was not elevated testosterone concentration. **Key words:** aggression, behavior, bisphenol A, mice, sexually mature, testosterone. *Environ Health Perspect* 111:175–178 (2003). [Online 1 October 2002] doi:10.1289/ehp.5440 available via <http://dx.doi.org/>

Alterations in the normal pattern of reproductive development seen in some populations of wildlife have been linked to exposure to endocrine-disrupting chemicals (EDs), which have been shown to mimic sex steroid hormones (1). Sex steroids exert potent influences on the nervous system during the critical development periods of the neuroendocrine system and behavior (2,3).

Palanza et al. (4) reported that prenatal exposure to the estrogenic chemical diethylstilbestrol (DES) increased the frequency of both males and females that responded aggressively to a same-sex conspecific. In addition, in other studies aggressive behavior was reduced and male-typical offensive attacks were rarely displayed by transgenic estrogen-receptor-deficient mice (5,6). However, few studies have focused on the influence of EDs on behavior (4,7,8). The relationship between aggressive behavior during the maturation process and influence of bisphenol A, which is one of most common EDs, has never been examined. Bisphenol A is a monomer used in the manufacture of the resin used to line food and drink cans and from which polycarbonate plastic is made (9,10). Bisphenol A is also a component of the plastic used in dental fillings (9,10).

In social species such as mice, expression of aggression is important for determining the reproductive success and regulation of the density of animals according to ecologic conditions (11). The purpose of this study

was to investigate the influence of bisphenol A on aggression during the maturation process in male mice.

Materials and Methods

Animals. Pregnant CD-1 mice were purchased from KBT Oriental (Saga, Japan). Pregnant mothers were separated into individual, standard polypropylene mouse cages, where they remained undisturbed throughout pregnancy. The mice were maintained under standard, controlled conditions (temperature, $21 \pm 1^\circ\text{C}$; relative humidity, 60%; lights on from 800 to 2000 hr) and had free access to food and water. This experiment was approved by the Committee of Ethics in Animal Experiments of the Faculty of Medicine, Kyushu University, Fukuoka, Japan, and the law (No. 105) and notification (No. 6) of the Government of Japan.

Treatment of pregnant females with bisphenol A. The treatment protocol of this study is shown in Figure 1. We followed the protocol previously reported by vom Saal et al. (12). Briefly, bisphenol A dissolved in corn oil (catalog no. 25606-65, Nacalai Tesque, Kyoto, Japan) was fed to pregnant female mice ($n = 30$) once per day (at 1000 hr) on gestation days 11–17. Pregnant mothers were separated into individual cages on day 7 of pregnancy. The pregnant mothers were randomly selected for treatment. The dose for the two treatment groups and the controls throughout the 7 days

of treatment was 2 ng/g body weight ($n = 7$), 20 ng/g body weight (ppb) ($n = 7$), and 0 ng/g ($n = 9$) body weight, respectively, all within the range of current human exposure (12). The body weight of each mouse was measured three times a week for determination of treatment dose. A micropipette enabled delivery of an accurate volume of corn oil into the mouth.

The date of birth was designated day 0. Litters were housed with their mothers in plastic cages. Pups were weaned on day 21. Each litter had four to seven male pups. Randomly selected males from the same litter were housed in groups of four or five. The mice were maintained under standard, controlled conditions described above.

Study design. Clean plastic containers ($17 \times 28 \times 13$ cm) were used for aggression tests (13). We randomly selected 15 male mice from the nine cages of control mice to serve as opponents. These opponents were housed in three cages (five mice each). Each opponent was used for testing only once daily. The male mouse to be tested was placed alone in a neutral container for 5 min. The opponent mouse, age matched with the resident test animal, was introduced into the cage. The behavior of the resident mouse was recorded over a 7-min encounter with the opponent. This assessment included the cumulative time that the tested mouse had contact with the opponent after approaching of its own accord. This measurement included body contact by sniffing or attacking. Sniffing included exploration of the head, flanks, and anogenital region. Cumulative times were recorded by staff members trained in this method and who were not familiar with the treatment histories of the subject mice. Frequent vigorous sniffing and attacking indicated a high degree of aggression according to the report of Lagerspetz and Hautojarvi (13).

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The aggression test was performed when the mice were 8, 12, and 16 weeks of age. Mice not sacrificed until 16 weeks were tested a total of three times. Testing was done under artificial white light between 1400 and 1800 hr. The mice were maintained under standard, controlled conditions described above.

Blood collection, hormone assay, and testis weight. Randomly selected mice, 8–14 mice in each group, were sacrificed 1 week after aggression testing (ages 9, 13, and 17 weeks), and trunk blood was collected for the measurement of testosterone. Testosterone was assayed in each mouse serum sample using single commercial radioimmunoassay kits (Diagnostic Products Corporation, Los Angeles, CA, USA). Body and testis weight were recorded at the time of sacrifice.

Statistics. Data are shown as mean \pm SE. Treatment comparisons were performed by analysis of variance (StatView, version 5; SAS Institute, Cary, NC, USA). We calculated the correlation between contact time and testosterone concentration using the Spearman rank correlation test (StatView). A p -value of < 0.05 was chosen as the level of significance.

Results

Chronologic change of relative testis weight. We found no significant differences in body weight among the groups during the experiment (data not shown). Figure 2 shows the change in testis weight per body weight of male control mice and male offspring of pregnant females fed bisphenol A at 2 or 20 ng/g body weight. The relative testis weight of the 2-ng/g bisphenol A group remained low throughout the test. Relative testis weight was significantly lower in the 2-ng/g bisphenol A group than in the controls at 8 and 12 weeks (6.2 ± 0.2 vs. 6.9 ± 0.3 , 6.2 ± 0.2 vs. 7.6 ± 0.3 ; $p = 0.047$, $p < 0.0015$, respectively). Relative testis weight was significantly lower in the 20-ng/g bisphenol A group than in the controls at 12 weeks (6.8 ± 0 vs. 7.6 ± 0.8 ; $p = 0.04$).

Chronologic change of aggressive behavior. Figure 3 shows differences in contact time between the male control mice and the 2-ng/g

bisphenol A and 20-ng/g bisphenol A male offspring. Contact time in the control mice gradually increased from weeks 8 to 12. In contrast, the treated mice had high aggression scores at 8 weeks of age, after which aggression diminished.

Aggression scores (contact time) were significantly increased at 8 weeks of age in the 2-ng/g bisphenol A (43.7 ± 4.0 sec; $p = 0.003$) and 20-ng/g bisphenol A (48.0 ± 6.5 sec; $p = 0.0018$) groups compared with the control group (19.5 ± 3.3 ; $p = 0.003$), but no difference was found among these groups at 12 and 16 weeks. In this experiment, no mice showed any indication of attacking behavior.

Chronologic change of serum testosterone concentration. Figure 4 shows the change of serum testosterone concentration in male control mice and the 2-ng/g bisphenol A and 20-ng/g bisphenol A groups. The serum testosterone concentration was lower at 12 weeks of age in both the 2-ng/g bisphenol A and 20-ng/g bisphenol A groups than in the controls, but the differences were not significant.

Figure 5 shows the correlation between contact time and testosterone concentration at 8 weeks of age.

We found no significant correlation between contact time and testosterone concentration at 8 weeks of age in either of the treatment groups (2-ng/g bisphenol A and 20-ng/g bisphenol A) or the control group.

Discussion

This study shows three important features of prenatal exposure to bisphenol A in mice: *a*) behavioral change is caused by this estrogen-mimicking substance; *b*) treatment with bisphenol A at an early stage of life significantly decreased relative testis weight; and *c*) a low dose of bisphenol A apparently had a larger effect on relative testis weight than did the much higher dose of 20 ng/g.

Behavioral change. Behavioral change is caused by this estrogen-mimicking substance. Male mice exposed to bisphenol A during fetal development, a period of differentiation of organs and testosterone secretion, showed a temporary, high aggression score at 8 weeks of

age, the approximate age when mice usually reach sexual maturity. A possible weakness of this study is that we did not test younger, more sexually immature mice for comparison.

It is well documented that one of the most important factors in the induction of male patterning in the central nervous system (CNS) is estrogen converted from testosterone originating in the testis during the critical period of sexual differentiation (3,14). In females, endogenous estrogen from the ovaries cannot move to the brain because of the action of estrogen-binding α -fetoproteins (3,15). Thus, estrogen plays an important role in CNS sexual differentiation in males. The critical period of CNS sexual differentiation in mice has been reported to be postnatal (3,15). It is not clear whether bisphenol A, which has an exogenous estrogen-mimicking activity, is affected by α -fetoproteins. As the experimental results presented here show, bisphenol A might affect the development of male patterning in the CNS and aggressive behavior during the growth process. Furthermore, it is well documented that aggressive and sexual behaviors in males can be affected by testosterone, which acts not only through androgen receptors but also through estrogen receptors (ERs)

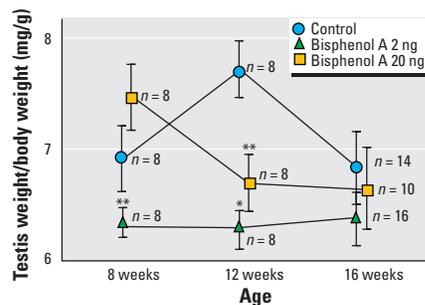


Figure 2. Chronologic change of relative testis weight per body weight (mean \pm SE) in control male mice and 2-ng/g bisphenol A and 20-ng/g bisphenol A male mice.

* $p < 0.01$ compared to control. ** $p < 0.05$ compared to control.

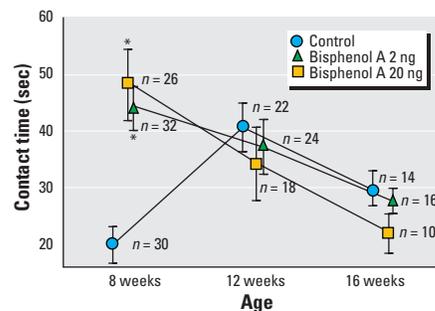


Figure 3. Chronologic change of contact time (mean \pm SE) in control male mice and 2-ng/g bisphenol A and 20-ng/g bisphenol A male mice. The number of mice in each group decreased every 2 weeks because some mice were randomly selected for sacrifice from each group after aggression testing.

* $p < 0.01$ compared to control.

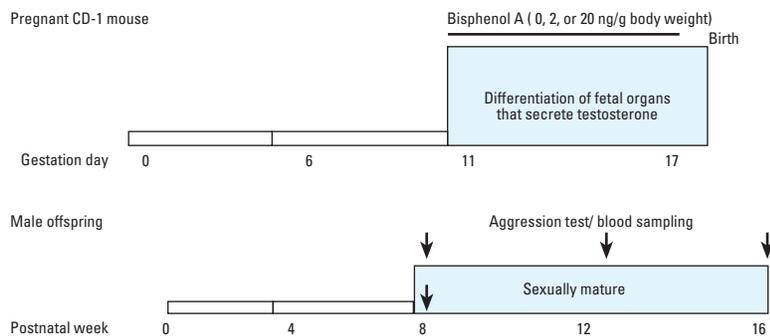


Figure 1. Experimental design indicating the period of bisphenol A treatment (2 or 20 ng/g body weight) of pregnant mice (black bar) and aggression testing (arrows) of male offspring prenatally exposed to bisphenol A.

(5,6,16). Lack of ER- α has been shown to suppress male-typical offensive attacks (5,6). However, we were unable to establish any relationship between temporary aggression in young male mice and serum testosterone concentration at 8 weeks of age. Because the surge of gonadal hormones occurs from approximately 8 weeks, along with the process of sexual maturation, we speculate that sensitivity to gonadal hormones might have been changed in our treated mice and thus caused marked changes in aggressive behavior. The first test was done at 8 weeks of age in our study, but a future study of 4–6-week-old mice will be necessary to clarify in this point.

Of question is how to explain temporary aggression in male mice. The results of this study are insufficient to explain this problem. Gorski and colleagues (3,17) reported that the volume of the sexually dimorphic nucleus of the preoptic area (SDN-POA) in the rat hypothalamus is several times larger in males than in females. They advanced the theory that gonadal steroids can stimulate SDN-POA development directly in female rats and that regulation of apoptosis by testosterone is one mechanism involved in the sexual differentiation of the SDN-POA (18,19). The histologic data of the present study will be addressed in future analyses. Other factors that were not considered, such as gonadal

hormone receptors and the postreceptor area, may have been involved. As mentioned in the “Materials and Methods,” the doses of bisphenol A used were environmentally relevant. For example, during the first hour after application of dental sealant, Olea et al. (9) detected in saliva 931 μg of bisphenol A, the monomer used to make the plastic polymer. For a person weighing 70 kg, this represents 13.3 (13.3 ppb), and for an average 8-year-old child weighing 25 kg, a dose of 37.2 $\mu\text{g}/\text{kg}$ (9). This article provides information on a new area of research into EDs. It is extremely risky to extrapolate behavior in animals to behavior in humans. However, extensive human research about the relationship between EDs and aggressive behavior by young human males will be of interest.

In our study model, the mice sniffed intruders but did not attack, in contrast with other mouse studies (4,8). Whether or not the cumulative time of contact in our study is a good measure of aggression requires further discussion. Lagerspetz and Hautajarvi (13) evaluated the degree of aggression as follows: frequent nosing is more aggressive than no interest, and wrestling is more aggressive than frequent nosing. Palanza et al. (4,8) housed mice individually for 7 days to establish a home territory before an aggression test. This methodologic difference might be related to the more gentle character of our mice. Thus, the cumulative time of contact would seem to be a useful substitute for aggression in the present study. Testing might also have been better under red light illumination during the dark phase of the light cycle because mice are most active at night.

Decreased testis weight after early exposure. As for the effects on reproductive organs, treatment with bisphenol A at an early stage of life significantly decreased relative testis weight at 8 and 12 weeks of age compared with controls. The effects of relative testis weight of the low-dose group mice were temporary, as was the aggression score. vom Saal et al. (12) have reported that a 2-ng/g dose of

bisphenol A decreased daily sperm production at 6 months of age. Recently, Cagen et al. (20) took a position against this result. They reported that a 2-ng/g dose of bisphenol A had no effects on male sexual development in mice (20), using the same experimental protocol as vom Saal et al. (12). In the present study, we did not examine this issue sufficiently, so we cannot say whether EDs had an effect on reproductive organ function. We can say that bisphenol A seems to have reduced relative testis weight temporarily in young male mice. Both high- and low-dose bisphenol A groups showed increased aggressive behavior, yet the low-dose group actually had a smaller relative testis weight than did controls. Relative testis weight seems to have had no relationship to aggressive behavior in this model, nor did testosterone concentration.

Effect of low versus high dose on testis weight. It is interesting that a low dose (2 ng/g) of bisphenol A had an apparently larger effect on relative testis weight than did the much higher dose of 20 ng/g. A nonmonotonic, inverted-U dose response of DES on prostate size and the rate of territory marking in mice has been reported (12,21). vom Saal et al. (12) have suggested that unique outcomes may occur in response to low, environmentally relevant doses of EDs that will not be observed at higher doses. We were unable to clarify the mechanisms of the low-dose effects of EDs tested in this study. One possibility is that low-dose exposure to bisphenol A during the fetal period might result in the use of a different pathway that does not go through estrogen/androgen receptors toward the target organ.

In conclusion, our mouse model to evaluate aggression showed that male mice exposed to bisphenol A were more aggressive and had a reduction in relative testis weight compared with controls at 8 weeks of age.

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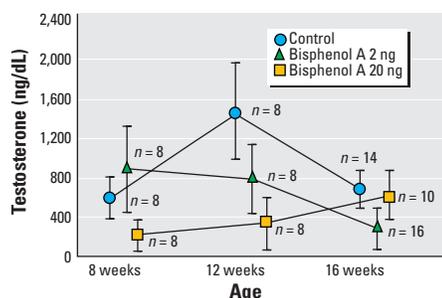


Figure 4. Chronologic change of serum testosterone concentration (mean \pm SE) in control male mice and 2-ng/g bisphenol A and 20-ng/g bisphenol A male mice.

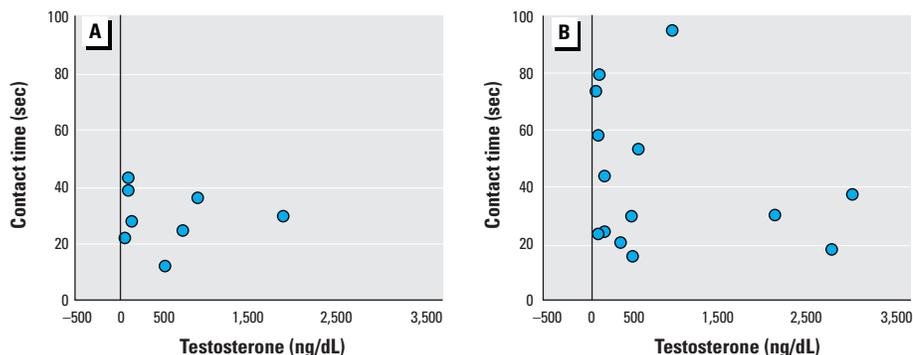


Figure 5. Correlation between contact time and testosterone concentration in 8-week-old male mice. (A) Control group ($r = 0.072$; $p = 0.82$). (B) Both treatment groups (2-ng/g bisphenol A and 20-ng/g bisphenol A) combined ($r = 0.237$; $p = 0.42$).

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