Perinatal Exposure to Low Doses of Bisphenol A Affects Body Weight, Patterns of Estrous Cyclicity, and Plasma LH Levels

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The nonsteroidal estrogenic compound bisphenol A (BPA) is a monomer used in the manufacture of polycarbonate plastics and resins. BPA may be ingested by humans as it reportedly leaches from the lining of tin cans into foods, from dental sealants into saliva, and from polycarbonate bottles into their contents. Because BPA is weakly estrogenic—approximately 10,000-fold less potent than 17β-estradiol—current environmental exposure levels have been considered orders of magnitude below the dose required for adverse effects on health. Herein we demonstrate measurable effects on the offspring of Sprague-Dawley female rats that were exposed, via their drinking water, to approximately 0.1 mg BPA/kg body weight (bw)/day (low dose) or 1.2 mg BPA/kg bw/day (high dose) from day 6 of pregnancy through the period of lactation. Offspring exposed to BPA exhibited an increased in body weight that was apparent soon after birth and continued into adulthood. In addition, female offspring exposed perinatally to the high dose of BPA exhibited altered patterns of estrous cyclicity and decreased levels of plasma luteinizing hormone (LH) in adulthood. Administration of neither the dose of BPA that caused effects during perinatal exposure nor a 10-fold higher dose was able to evoke a uterotropic response in ovariectomized postpubertal females. These data indicate an increased sensitivity to BPA during the perinatal period and suggest the need for careful evaluation of the current levels of exposure to this compound. Key words: Bisphenol A, body weight, BPA, development, endocrine disruptors, environmental estrogens, estrous cycles, reproductive function, xenoestrogen.

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The route and time of exposure can also affect the potency of BPA. Results of a recent study indicated that the relative bioavailability of BPA is lower after oral administration than after subcutaneous or intraperitoneal delivery (15). Consistent with those data, Laws and colleagues (16) demonstrated a greater increase in uterine wet weight in response to subcutaneous delivery relative to oral administration of equivalent levels of BPA to immature female rats. With regard to the time of exposure, oral administration of very low levels of BPA (0.002–0.020 mg/kg) to pregnant (CF-1) mice during days 11–17 of gestation produced measurable effects in their offspring (12,13). Significant alterations have also been reported in the behavior of the offspring of female Sprague-Dawley rats that were treated with 0.040–0.40 mg/kg BPA during pregnancy and lactation (19). To date, there is no evidence that these same levels of BPA exposure would exert measurable effects in adults of the same species and strain.

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The particular consequences of estrogenic exposure and the mechanisms involved undoubtedly differ depending on the time of exposure. Limited exposure of adult animals to estrogenic compounds can produce effects that are mostly reversible when exposure ceases. In contrast, perinatal exposure to exogenous estrogenic compounds is likely to produce organizational effects that are irreversible (20,21). To date, the limited number of studies that have examined the effects of perinatal BPA exposure have used different species, strains, doses, and routes of administration, and they have measured different end points (13,14,19,22). Hence, integrating the available data into a coherent picture has proven difficult. The aim of the present study was to assess the effects of BPA during development and to compare the sensitivity of the developing organism with that of the adult animal. An oral route of BPA...
Materials and Methods

Animals. We purchased female Sprague-Dawley rats from Taconic Farms (Germantown, N. Y.). We purchased one group of 2- to 3-month-old females as timed pregnant females (n = 18). They were shipped to our animal facility on day 5 of pregnancy for the study of BPA exposure during the perinatal period. We purchased a second group of animals (n = 30) as ovariectomized young adult females for use in a uterotropic assay to assess the estrogenicity of BPA. Animals were maintained in the Division of Laboratory Animal Medicine with food (Purina Rodent Chow, St. Louis, M O) and water available ad libitum. We used glass water bottles in these studies to ensure that related compounds did not leach from plastic water bottles and potentially confound the results of our studies. We used plastic cages to house the animals; beforehand, however, we evaluated ethanol extracts from the cages using the E-Screen assay (23,24) to confirm that measurable levels of estrogenic compounds did not leach from the cages. Animals were maintained on a light:dark cycle (14:10) with lights on at 0500 hr and off at 1900 hr. The care of the animals was in accordance with the Guidelines for the Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committee on our campus.

Exposure of pregnant and lactating females to BPA in the drinking water. Upon arrival in the facility, the timed pregnant females were weighed and randomly assigned to 1 of 3 groups. We exposed experimental females to BPA in their drinking water at concentrations of 1 mg/L (low dose BPA, n = 6) or 10 mg/L (high dose BPA, n = 6) beginning on day 6 of pregnancy. BPA exposure was continued throughout the period of lactation. We gave control females (n = 6) water containing only the concentration of ethanol (1%) used as a diluent for the BPA solutions. Beginning on the day of weaning, pups were supplied with unadulterated drinking water.

We estimated the mean levels of BPA consumed daily by pregnant females from the drinking water at approximately 0.1 mg/kg bw/day (low dose) and 1.2 mg/kg bw/day (high dose). These estimates were based on the measurements of the difference in the amount of water placed in the water bottle each day and the amount remaining on the following day. The assessments assume that all the water lost from the bottle was consumed. They do not account for possible leakage, evaporation, or spillage of the water or for potential loss of BPA activity during the 24-hr period. Therefore, the estimates of the level of BPA exposure may be somewhat higher than actual exposure levels. The actual levels of BPA that reached the fetuses during gestation or that were ingested postnatally by the offspring during the period of lactation were not estimated in the present study.

Assessment of the effects of BPA exposure. We examined the pregnant females daily and weighed them several times during pregnancy. On the day of delivery, we observed the females continuously and recorded the time of delivery of litters for each individual. The sex of the offspring was initially recorded on postnatal day 2. We weighed the pups several times during the neonatal period and at frequent intervals through 110 days after birth. The body weight data collected before weaning were not segregated by sex. To facilitate body weight measurements with the least amount of disruption to the litters, each pup was removed and rapidly weighed without first assessing its sex.

Male and female offspring from each of the three groups were killed at various times after birth, and the genital tracts were examined for evidence of macroscopic abnormalities at each time point. We examined the males neonatally (n = 12), at 3 months (n = 12), and at 5 months (n = 18) and the females neonatally (n = 12), at 8 months (n = 24), and at 12–16 months (n = 34). At all time points, animals were selected from as many different litters from each group as possible. For example, we assessed anogenital distances in animals killed during the neonatal period: 12 males and 12 females, including one male and one female from four different litters from each of the three experimental groups.

We checked female offspring daily beginning on day 28 to assess the day of vaginal opening. We examined vaginal cytology daily in the female offspring for 18 consecutive days at 4 months of age and then again at 6 months of age to determine the pattern of estrous cyclicity in adulthood (n = 69). Females were considered to show clear evidence of estrous cyclicity if a minimum of two, and in many cases three, consecutive 4–5 day estrous cycles were confirmed during the daily assessments of vaginal cytology. Eight female offspring in each of the three groups were ovariectomized and killed 3 months later to assess circulating lutetinizing hormone (LH) levels. We collected trunk blood and assayed the serum for LH titers using the rat LH assay kit obtained through the National Hormone and Pituitary Program, the National Institute for Diabetes and Digestive and Kidney Disease (Bethesda, M D) and A.F. Parlow (LH antiserum S-11, lot number AFP-C697071P). All plasma samples were run in a single assay. The intra-assay variability was 7%.

Uterotropic assay. Two weeks after ovariectomy, young adult females were exposed for 3 days to the same two concentrations of BPA in their drinking water as the pregnant and lactating females (1 mg/L or 10 mg/L)—or a concentration 10 times greater than the high dose given pregnant females (100 mg/L). Other groups of identically treated animals were exposed to estrone (1 mg/L or 0.1 mg/L) or to water containing 1% ethanol (diluent) for 3 days. We examined vaginal cytology before treatment began and then daily during treatment. After 3 days, animals were decapitated and the uterine wet weights were determined. We examined the animals carefully to confirm that no ovarian tissue remnants were present.

Statistical analysis. Overall differences in body weights, LH levels and uterine wet weight were analyzed by ANOVA. Once significance was established, post hoc tests (t-tests, Tukey, or LSD) were performed to make comparisons between groups. The incidence of mammary tumors was analyzed by the chi-square test. Overall comparisons of the proportion of cycling females at 4 and 6 months were made using the Kruskall-Wallis test, and Mann-Whitney U tests were used to compare between groups where appropriate.

Results

The first significant findings we noted were increased body weights of the pups born to BPA-treated females relative to those born to control females. On postnatal days 4, 7, and 11 (Figure 1), animals exposed to both low- and high-dose BPA treatments weighed more than animals born to control females (p < 0.0001). On days 11 (p < 0.025, Figure 1) and 22 (p < 0.005, Figure 2A and C), animals exposed to the low dose of BPA were heavier than those exposed to the high dose of BPA. On day 28, we observed a significant difference in mean body weight between high- and low-dose animals in the females (p < 0.005; Figure 2A) but not in their male littersmates (Figure 2C), although the male offspring did exhibit significant body weight effects of BPA through postnatal day 54 (Figure 2D). On days 87 and 110, low-dose BPA females retained higher body weights than both control and high-dose females (p < 0.05; Figure 2B).

We observed evidence of functional alterations of the reproductive system in female offspring exposed to the high-dose regimen of BPA. Most females exposed perinatally to high-dose BPA failed to exhibit evidence of regular estrous cycles when examined at 4 months (only 21% exhibited regular estrous cycles) and at 6 months (only
23% exhibited regular estrous cycles (Figure 3A). The def- fered for offspring of the high-dose animals differed significantly from the mean number confirmed in offspring of control and low-dose females (Figure 3B).

Besides altered patterns of estrous cyclicity, the offspring of the high-dose BPA females also revealed significantly lower levels of plasma LH relative to control females after treatment. The mean number of regular 4–5 day estrous cycles confirmed for offspring of the high-dose animals differed significantly from the mean number confirmed in offspring of control and low-dose females (Figure 3B).

During observations of the female offspring, which extended through 16 months of age when the last group of animals was sacrificed, 10% of the female offspring of controls (2/19), 20% of female offspring exposed to the low dose (3/15), and 28% exposed to the high dose (7/25) of BPA developed mammary tumors. Although the data are provocative, the differences in the incidence of mammary tumors were not statistically significant. The precise time that each tumor was first palpable is not known because regular examination for mammary tumor development had not been planned. Rather, the tumors were detected during routine handling of the animals when they had reached a diameter >1 cm.

Many parameters examined in this study were not significantly affected by perinatal exposure to BPA. The mean number of pups per litter did not differ (control 12.0 ± 0.7; low dose 11 ± 1.3; high dose 11.6 ± 1.0), and the sex ratios of the litters were comparable in the three groups. The day of vaginal opening did not differ among groups (34.8 ± 0.22, control; 34.7 ± 0.28 low dose; and 34.8 ± 0.30 high dose), nor did measurements of anogenital distance taken during the neonatal period. Furthermore, no macroscopic abnormalities were observed in genital tract tissues examined at any time during the study. Exposure to the same levels of BPA given to pregnant and lactating mothers was not able to stimulate a uterotrophic response in ovariec- tomized postpubertal females (Table 2). Moreover, females that received a dose of BPA 10 times higher than the high dose administered to pregnant and lactating females did not exhibit an increase in uterine wet weight (Table 2). In contrast, a 4-fold increase in uterine wet weight occurred in animals that received levels of the potent natural estrogen estrone comparable to levels of BPA received in the low-dose treatment. Consistent with the increase in uterine wet weights, all females in this estrone-treated group exhibited cornified vaginal smears on the day that they were killed. In contrast, the vaginal cytology of females in the other groups examined revealed the predominance of leukocytes.

Discussion

Several recent studies have demonstrated subtle effects rather than gross macroscopic abnormalities in rodents that were exposed prenatally and/or neonatally to low doses of BPA (12,13,19). Our findings are consistent with this trend. The increase in body weight...
observed in the offspring of BPA-treated female rats in our study confirm and extend recently published findings in mice. Daily oral administration of 0.0024 mg BPA/kg to pregnant mice on days 11–17 of gestation increased the body weight of female offspring (13). Our data demonstrate that BPA increased body weight in both male and female rats born to exposed mothers, although the increase persisted longer in females. Moreover, in the female offspring, the lower of the two BPA doses produced a larger and more persistent effect on body weight relative to the higher dose. This intriguing finding is consistent with the nonmonotonic or inverted-U-shaped dose-response curve that has been reported with this compound for prostate wet weight (12).

In the experiments reported here, the effects of BPA on body weight were observed during the period of exposure, and they persisted after BPA administration ceased. The increase in body weight reported in mice by Hovdeshell et al. (13) was also observed after in utero exposure to BPA. Both sets of data suggest that the effect of BPA on body weight does not require the constant presence of the stimulus. Similarly, the altered estrous cycle patterns observed in the female offspring of dams exposed during gestation and lactation to the high dose of BPA occurred long after BPA exposure ceased. Altered patterns of estrous cyclicity have recently been observed in adult females examined during the period of daily exposure to 100 mg BPA/kg body weight administered by oral gavage (16). However, these apparently comparable effects on estrous cyclicity are likely to be mediated through different pathways, because the latter occurred during exposure to BPA and the former occurred long after the exposure had ended. The permanent alterations in estrous cyclicity we observed in females exposed to BPA perinatally might be expected to limit reproductive fertility and decrease overall reproductive success.

In a recent study of Sprague-Dawley female rats, Kwun and colleagues failed to observe significant differences in body weight or in patterns of estrous cyclicity after perinatal exposure to much higher levels of BPA than those used in the present study (25). Doses up to 320 mg/kg/day BPA were administered to dams from day 11 of gestation through day 20 of lactation. Although an oral route of administration was used in that study, BPA was administered daily as a single bolus by oral gavage and not via continuous low-level exposure in the drinking water, as in our study. It is not clear whether the different modes of oral administration might have influenced the results observed in the two studies. It is possible that the physiologic response to a large bolus of the compound is to activate pathways that will ensure its rapid elimination.

Differential processing of high doses relative to low doses of BPA may contribute to the inverted-U-shaped nonmonotonic dose-response curve that has been described for this and other compounds (12,26). A recent study demonstrated that oral administration of a bolus of BPA (10 mg/kg or 100 mg/kg) resulted in the conversion of BPA to the monoglucuronide conjugate, the major urinary metabolite of the compound (15). Moreover, after oral gavage, the levels of BPA detected in plasma were low and the period of time that detectable levels of BPA were present in the plasma was abbreviated relative to subcutaneous or intraperitoneal routes of administration (15).

Although the observed disruption of regular estrous cycles indicates that perinatal exposure to the high dose of BPA altered the function of the hypothalamic-pituitary-gonadal axis, the site(s) of the disturbance is (are) unclear. The decrease in circulating LH levels observed in females exposed perinatally to the high dose of BPA is consistent with an effect at the hypothalamic and/or pituitary level. Mean LH levels in the low-dose animals did not differ significantly from those in either the control or high-dose females perhaps because of a higher variability. Hovdeshell et al. (13) demonstrated that the sensitivity of mice to BPA can vary markedly in individual animals. Exposure to endogenous levels of gonadal steroids in utero may differ in rodents depending on the position of the developing embryo and the sex of proximal littermates in the uterine horn (27–31). These subtle differences in exposure to endogenous gonadal steroids during development may influence the sensitivity of individual animals to low levels of exposure to exogenous estrogenic compounds. Evidence for such additive effects were reported in turtles in vivo exposure to exogenous estrogens (32).

The mechanisms by which perinatal exposure to BPA elicits the effects observed in our study remain to be determined. It is interesting to note that the perinatal BPA exposure here spanned the classically defined critical period of brain sexual differentiation, which extends from several days before birth through approximately postnatal day 10 in

![Figure 3. Patterns of estrous cyclicity in female offspring of animals exposed to BPA and vehicle. (A) The proportion of animals exhibiting evidence of regular 4- or 5-day estrous cycles (± SEM) recorded for animals in each group. Vehicle, n = 23. Low-dose BPA, n = 18. High-dose BPA, n = 28.

| Age   | Number of estrous cycles |
|-------|--------------------------|
| 4 months old | 4 |
| 6 months old | 1 |

Table 1. Plasma LH levels (mean ± SEM) in ovariectomized females.

| Group            | No. of rats | LH levels (ng/mL) |
|------------------|-------------|-------------------|
| Control females  | 7           | 14.97 ± 0.51      |
| Low-dose females | 6           | 12.17 ± 1.54      |
| High-dose females| 8           | 12.14 ± 0.46*     |

*F < 0.001, high-dose vs. control females.

Table 2. Results of uterotropic assay in ovariectomized young adults.

| Group            | No. | Estimated chemical exposure/day | Mean uterine wet weight (mg ± SEM) |
|------------------|-----|---------------------------------|-----------------------------------|
| Control          | 6   | 16.90 mg/kg bw                  | 78.2 ± 5.0                        |
| Estrone (1 mg/L) | 5   | 0.17 mg/kg bw                   | 326.4 ± 14.9**                    |
| Estrone (0.1 mg/L)| 5 | 0.02 mg/kg bw                   | 97.6 ± 5.6                        |
| BPA (100 mg/L)   | 4   | 16.90 mg/kg bw                  | 82.9 ± 8.9                        |
| BPA (10 mg/L)    | 4   | 1.30 mg/kg bw                   | 88.9 ± 7.8                        |
| BPA (1 mg/L)     | 4   | 0.15 mg/kg bw                   | 78.2 ± 5.0                        |

Chemicals were delivered in the drinking water. Controls received vehicle (1% ethanol).

** Estrone (1mg/L) differs from all other groups, p < 0.0001.
Perinatal exposure to Bisphenol A in rats

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Perinatal exposure to Bisphenol A in rats (33). During this time, circulating levels of testosterone produced by the testes can affect nervous system development and cause a male-typical pattern of neuroendocrine regulation and behavior (33–36). Increased body weight, premature loss of estrous cyclicity, and a decrease in postcastration LH levels have all been documented in females that were treated with exogenous testosterone during development (37,38). Many of the actions of testosterone during brain sexual differentiation occur via its local aromatization to estradiol within specific sites in the developing brain (33–35), and the importance of estrogen in the differentiation of male behavioral and neuroendocrine regulation is supported by many lines of investigation (reviewed in 35). Therefore, it is tempting to postulate that the estrogenic compound BPA, which binds estrogen receptors (33,39,40), alters the expression of estrogen and androgen receptors (41), and possesses antiandrogenic activities (42), may have interfered with central processes involved in brain sexual differentiation in animals exposed perinatally. As a nonsteroidal estrogen, BPA could potentially bypass the protective mechanisms that limit exposure to circulating estrogens during fetal and early neonatal development (33,43,44). In developing mice and rats, high concentrations of the liver-secreted protein alpha fetoprotein bind estradiol and reduce the availability of this steroid from the circulation during the critical period of sexual differentiation (33–35). In contrast, testosterone in the circulation is free to enter the brain, where it can be converted to estradiol and interact with estrogen receptors. Behavioral effects consistent with central actions of BPA during development have recently been reported in rats after oral exposure to BPA at a level and time frame similar to that used in the present study (19).

Perinatal exposure to BPA appeared to result in a slight but not statistically significant increase in the incidence of mammary tumor development in the female offspring during the course of our study. These data are provocative and suggest that a possible relationship between perinatal exposure to BPA and mammary tumor development warrants further investigation. In this regard, recent epidemiological data suggest that increased estrogen exposure in utero increases the risk of breast cancer (45,46). Although not a result of perinatal exposure, BPA administration has been reported to stimulate lobular maturation and epithelial cell proliferation in young adult mice (47).

To date, the relative sensitivity to a specific regimen of BPA exposure has not been assessed in a single animal strain during development and in adulthood, and the marked strain differences in BPA sensitivity (10,11) have hindered the integration of currently available data. Therefore, we evaluated the sensitivity of ovariectomized adult Sprague-Dawley rats to BPA, using the uterotrophic assay as a measure of estrogenicity. As shown, exposure to the same levels of BPA provided to the pregnant and lactating mothers, as well as a dose 10 times higher than the high dose, was unable to stimulate a uterotrophic response in ovariectomized postpubertal females. Moreover, Gould and colleagues (48) demonstrated that administration of even higher doses of BPA (150 mg BPA/kg bw) failed to increase uterine wet weight in immature Sprague-Dawley females. The data from the present study therefore suggest that the sensitivity to BPA exposure is significantly increased during early development.

BPA exposure may be particularly deleterious during the perinatal period because of its long binding affinity to serum proteins (49) and the potential for nonsteroidal estrogenic compounds to bypass mechanisms that limit exposure to the fetus and the fetal brain to circulating estrogens (33,45,46). In addition, developmental exposure to BPA produces effects that persist long after the causal agent is removed. Moreover, the body weight data in this study suggest that low doses of BPA may be more effective than high doses in altering some physiologic parameters. Data from other studies have demonstrated that low-dose exposure to this compound may in fact be more detrimental than high doses (12) and that persistent exposure to low doses of the compound over long periods may be as effective as shorter exposures to somewhat higher levels (19). These findings indicate the compelling need for reevaluation of the end points used for the toxicologic assessment of BPA, of the acceptable levels of exposure to this compound, and of other xenoestrogens present in the environment.

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