The Mouse Uterotrophic Assay: A Reevaluation of its Validity in Assessing the Estrogenicity of Bisphenol A

Caroline M. Markey, Cheryl L. Michaelson, Electra C. Veson, Carlos Sonnenschein, and Ana M. Soto

Department of Anatomy and Cellular Biology, Tufts University School of Medicine, Boston, Massachusetts, USA

The prevalence of synthetic chemicals in our environment that are capable of mimicking the female hormone estrogen is a growing concern. One such chemical, bisphenol A (BPA), has been shown to leach from a variety of resin-based and plastic products, including dental sealants and food and beverage containers, in concentrations that are sufficient to induce cell proliferation *in vitro*. The response to BPA *in vivo* has been varied; thus the aims of this study were to investigate *a*) whether BPA has an estrogenic effect in CD-1 mice, a strain that is useful for developmental studies; and *b*) whether the uterotrophic assay is a valid means of determining the estrogenicity of BPA by comparing it with other end points measured in the uterus. Immature female CD-1 mice were exposed to BPA in concentrations ranging from 0.1 to 100 mg/kg body weight for 3 days. Results showed that BPA induced a significant increase in the height of luminal epithelial cells within the uterus at concentrations of 5, 75, and 100 mg/kg and that BPA induced lactoferrin at concentrations of 75 and 100 mg/kg. A uterotrophic response (increase in uterine wet weight) was induced by 100 mg/kg BPA only. Further, the proportion of mice showing vaginal opening was greater after exposure to 0.1 and 100 mg/kg BPA, relative to the control animals and those receiving intermediate doses of BPA. These results demonstrate that BPA induces changes in the mouse uterus that differ depending on the exposure dose and the end point measured, and reveal that certain tissue effects show a nonmonotonic relationship with dose. These data also demonstrate that BPA induces estrogenic changes in the uterus of the CD-1 mouse, and highlight the need to reevaluate the validity of the mouse uterotrophic assay as an end point for determining the estrogenicity of suspected environmental estrogens. *Key words*: bisphenol A, CD-1 mouse, endocrine disruptors, lactoferrin, morphometrics, nonmonotonic dose–response curves, uterotrophic assay. *Environ Health Perspect* 109:55–60 (2001). [Online 12 December 2000] *http://ehpnet1.niehs.nih.gov/docs/2001/109p55-60markey/abstract.html*

Estrogens exert a powerful influence on the development, regulation, and endocrine control of the female genital tract and mammary glands. Their capacity to induce cell proliferation in estrogen-target tissues underlies the critical role that these steroid hormones also play in carcinogenesis. Therefore, the disturbing revelation that synthetic chemicals have the capacity to mimic the effects of estrogens presents a very real concern for human health, particularly when exposure occurs at stages of tissue organization and development such as in prenatal or neonatal individuals. The fact that these chemicals do not necessarily share a similar structure to estrogen is further confounding. One such chemical is bisphenol A (BPA). This monomer is used in the manufacture of polycarbonates and epoxy resins from which a plethora of products are generated, including food and beverage containers, dental sealants, and babies' bottles. The propensity of BPA to leach from such products under normal conditions (*1*–*5*) highlights the need to investigate its potential for inducing developmental and reproductive abnormalities in humans.

Numerous studies conducted *in vitro* attest to the estrogenic character of BPA. This chemical has been shown to induce cell proliferation in MCF-7 cells (*2*,*6*,*7*), stimulate the release of prolactin from pituitary GH3 cells (*8*), and induce transcriptional activation of the estrogen receptor (ER) in both yeast-based assays (*9*) and human embryonal kidney cells via the estrogen response element (*10*). BPA has also been shown to up-regulate the expression of vitellogenin mRNA in primary hepatocytes derived from male *Xenopus laevis* (*11*). However, few studies have addressed the effects of *in utero* exposure to environmentally relevant doses of BPA *in vivo*. One study in male CF-1 mice described a significant increase in adult prostate weight after *in utero* exposure to BPA concentrations as low as 2 and 20 µg (*12*). Although this research establishes effective doses of BPA in males using prostate weight as an end point, it is limited when extrapolating to studies in the female reproductive tract. Because this is the focus of our work, it is imperative to establish a dose–response range in female mice using an appropriate end point in a classical estrogen-target organ such as the uterus.

One established method for determining the estrogenicity of a chemical is the rodent uterotrophic assay, which measures an increase in wet weight of the uterus (*13*). Yet, a review of this method reveals a confounding range of results that points to species-specific and even strain-specific differences in the magnitude of the uterotrophic response to BPA. Fischer 344 rats (ovariectomized), the strain that is most sensitive to BPA exposure, exhibit an approximately 2-fold increase in uterine wet weight after 3 days exposure to 0.3 mg/kg BPA delivered via a subcutaneous implant (*14*). Sprague Dawley rats (ovariectomized) exhibit less sensitivity because the same dose elicits no effect (*14*); 10 mg/kg BPA administered orally for 4 days induces a 1.4-fold increase in wet weight (*15*). Immature Alpk:AP rats exhibit a uterotrophic response to 400 mg/kg BPA as evidenced by 1.3-fold and 1.5-fold increases in wet weight after oral gavage and subcutaneous injection, respectively (*16*). The few studies conducted in mice reflect a greater uterotrophic resistance to BPA. Immature CFLP mice do not respond to 3 days subcutaneous injection of 0.5 mg BPA/mouse (approximately 16.7 mg/kg), yet 5 mg (approximately 167 mg/kg) causes toxicity to the animals (*17*). CD-1 mice show particular resistance to the effects of estradiol on the basis of testicular measurements (*18*), and they may be equally unresponsive to estrogen mimics such as BPA.

These data clearly demonstrate the absence of a systematic dose–response curve to BPA in any species or strain of rodent. In this paper we address the topic of susceptibility to BPA using the immature CD-1 mouse as a model. Because CD-1 mice are outbred for large litter size, they are useful for studies on the developmental and reproductive consequences of environmental hormones. Therefore, there is a need to establish the suitability of this strain for toxicology studies in female development and reproduction. In addition, we investigated the validity of the classical mouse uterotrophic assay because much of the confounding data regarding the effect of BPA *in vivo* is based on this method. We accomplished this by comparing the changes in uterine wet weight with three other end points that reflect estrogenic activity within the uterus, namely

Address correspondence to A. Soto, Department of Anatomy and Cellular Biology, Tufts University School of Medicine, Boston, Massachusetts 02111-1800 USA. Telephone: (617) 636-6954. Fax: (617) 636-6536. E-mail: asoto@infonet.tufts.edu

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epithelial cell morphology, induction of the estrogen-inducible protein lactoferrin, and expression of proliferating cell nuclear antigen (PCNA).

Materials and Methods

Animals. Immature female CD-1 mice (Charles River Laboratories, Wilmington, MA) were maintained in a temperature-controlled room on a 14 hr light:10 hr dark cycle in the Tufts University-New England Medical Center animal facility. Mice were fed RMH 3000 rodent diet (Agway Inc., Syracuse, NY) that tested negligible for estrogenicity, and water was supplied from glass bottles only. Cages and bedding also tested negative for estrogenicity using the E-SCREEN assay (*19*). All experimental procedures were approved by the Tufts University-New England Medical Center Animal Research Committee.

Vaginal opening. Before 23-day-old female mice were exposed to dimethyl sulfoxide (DMSO), BPA, or estradiol (E_2), they were checked to see if their vaginas had opened. This typically occurs in mice around 35 days of age under the influence of estrogens and represents the initial stages of attaining sexual maturity. Before sacrifice at 26 days of age, mice were checked for vaginal opening again. We established two populations: one in which the vagina showed the beginning of a perforation but was not completely canalized (termed partial opening), and another showing complete canalization and patency (termed complete opening).

Uterotrophic assay. At 23 days of age, mice were weighed and divided randomly into 10 experimental groups (n = 4–22). We performed morphometric analyses on a subset of these animals (n = 4–12) that were randomly chosen. Nine of the groups were implanted with Alzet osmotic pumps (Alza Corp., Palo Alto, CA) containing either DMSO (vehicle), BPA, or E_2 (Sigma, St. Louis, MO); another group acted as an untreated control (no pumps were implanted). BPA and E_2 were dissolved in DMSO, and the pumps were prepared in accordance with the manufacturer's instructions to deliver BPA to mice in concentrations of 0.1, 0.5, 1, 5, 50, 75, and 100 mg/kg body weight/day, and E_2 at a concentration of 5.0 µg/kg body weight/day. This dose of E_2 has been shown to induce a 2-fold increase in uterine wet weight (*20*). Pumps were implanted subcutaneously in mice under aseptic conditions. After 3 days mice were weighed and sacrificed by cervical dislocation, and their uteri were dissected out. Each uterus was blotted and the wet weight recorded. Data were expressed as a percentage of the body weight.

Histology

The uteri were immediately fixed in 4% formaldehyde in 0.1 M phosphate buffered saline (PBS; pH 7.4) for 10 hr. One horn of each uterus was dissected transversely into three segments. Tissue was processed through a series of alcohols and xylene, infiltrated with Paraplast paraffin (Fisher, Pittsburgh, PA) under vacuum, and embedded in paraffin. Five-micron sections were cut on a Sorvall JB-4 microtome (DuPont, Wilmington, DE), mounted on Superfrost positive charged slides (Fisher) and stained to determine *a*) changes in the luminal and glandular epithelium by morphometric analysis; *b*) immunolocalization of lactoferrin, an estrogen-inducible protein; and *c*) immunolocalization of PCNA to assess cell proliferation.

Morphometric analyses. Sections of uterus were stained with hematoxylin and eosin and prepared for light microscopy. The relative area of the uterine endometrium occupied by the mucosa (luminal epithelium, glandular epithelium, and lamina propria) and myometrium, and the height of the luminal epithelium were determined using the program Bioscan Optimus (Version 1.13; Media Cybernetics, Silver Springs, MD). We measured relative areas of uterine tissue compartments using the 10× objective and epithelial cell height using the 20× objective. For epithelial cell height, we measured only areas of epithelium in which the nucleus and basement membrane of single cuboidal/columnar epithelial cells could be seen. Four areas in each of three transverse sections of uterus were analyzed per animal. For the parameter of epithelial cell height, four measurements were made within four areas of the three transverse sections of each uterus per animal.

Immunohistochemistry

Lactoferrin. Lactoferrin was localized within the uterine epithelium by immunofluorescence (*21*). Sections were hydrated and microwaved in 10 mM citrate buffer (pH 6) for antigen retrieval (*22*); nonspecific binding was blocked with 5% normal goat serum and 5% normal rabbit serum in 0.01 M PBS. Sections were incubated overnight at 4°C in a humid chamber with a rabbit antibody raised to mouse lactoferrin (monoclonal IgG, 1:100; supplied by Christina Teng, National Institute of Environmental Health Sciences, Research Triangle Park, NC). Biotinylated goat anti-rabbit IgG (1:400; Roche Diagnostics Corp., Indianapolis, IN) was applied to sections, and lactoferrin was visualized by streptavidin Alexa Fluor conjugate (Molecular Probes, Eugene, OR). Sections were counterstained with Hoechst 33258 (1:1000; Sigma) and mounted in glycerol/0.01 M PBS (1:1). Fluorescent images were captured using a SPOT-Real Time digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI) attached to a Zeiss Axioskop (Carl Zeiss, Inc., Thornwood, NY) and analyzed in the SPOT-Real Time program.

We semiquantitatively determined (using the 20× objective) the expression of lactoferrin within the luminal and glandular epithelium of the uterus by assessing the intensity of staining within both the apical and basal regions of the cells. The intensity of staining ranged from 0 to 3, with 3 representing the most intense staining, similar to that observed in the estradiol group. The four scores (luminal apical, luminal basal, glandular apical, and glandular basal) were summed; thus maximal staining was represented by a score of 12. Four areas in each of the three transverse sections of uterus were analyzed per animal.

PCNA. Immunohistochemical staining of PCNA was performed using the avidin-biotin-immunoperoxidase method. Sections were hydrated and microwaved in 10 mM citrate buffer (pH 6) for antigen retrieval; both endogenous peroxidase and nonspecific binding were then blocked with 0.3% H_2O_2 in methanol and 1.5% normal goat serum in 0.01 M PBS, respectively. Sections were incubated overnight in a humid chamber at 4°C with anti-PCNA mouse antibody (monoclonal IgM; Beckman Coulter, Miami, FL) at a dilution of 1:500. Mouse IgM preimmune serum was used at the same concentration as the primary antibody to provide an isotypic control. We applied the biotinylated secondary antibody (IgM) and avidin-peroxidase (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA) to sections according to the manufacturer's instructions and visualized peroxidase activity by diaminobenzidine (Sigma). Sections were lightly counterstained with hematoxylin and prepared for light microscopy.

The expression of PCNA within the luminal epithelium of the uterus was determined by densitometric image analysis using Scion Image (Scion Corporation, Frederick, MD). Video images of the sections were captured using the 40× objective and calibrated so that the cytoplasm read 0 (0%) average gray value (AGV) and the darkest nucleus read 255 (100%) AGV. Those nuclei with an AGV near 217 (representing 15% of the highest intensity range) were counted as positive for PCNA. Data are presented as the number of PCNA-positive stained nuclei per 1,000 µm basement membrane. We assessed a total length of 5000–10,000 µm basement membrane from three cross-sectional areas of the uterus per animal.

Statistical Analysis

There was no significant difference between the control group and vehicle group for all

variables, so data were pooled; analysis was then performed against these combined data. We analyzed uterine wet weight (as a percentage of body weight) and the relative area of luminal and glandular epithelium by one-way analysis of variance using a Tukey's post hoc comparison. Body weight, uterine wet weight (absolute), the relative area of lamina propria and myometrium, epithelial cell height, and PCNA labeling were analyzed by a Kruskal-Wallis test because data were not normally distributed. Lactoferrin induction was analyzed by a Kruskal-Wallis test, as this data was nonparametric in nature. We determined differences between the control group relative to each treatment group using Bonferroni-adjusted Mann-Whitney tests, and evaluated the percentage of mice showing vaginal opening on day 26 (sacrifice) using a two-sample *Z* test. The analysis compared the control group to each treatment group. Results were considered significant at $p < 0.05$. We performed a Pearson's correlation test on the body weight and uterine wet weight data for each treatment group; results were considered significant at $p < 0.01$.

Results

There was an overall significant difference between the control group and the experimental treatments (BPA groups and E_2) for body weight ($p < 0.05$), uterine wet weight ($p < 0.0001$), uterine wet weight per body weight ($p < 0.0001$), relative area of luminal epithelium ($p < 0.0001$), relative area of lamina propria ($p < 0.0001$), height of the luminal epithelial cells ($p < 0.0001$), and lactoferrin expression ($p < 0.0001$). The Pearson's correlation test revealed a significant correlation between body weight and uterine wet weight after exposure to 75 mg/kg BPA and estradiol only ($p < 0.01$). There was no significant difference between the control group and experimental treatment groups for the relative area of glandular epithelium and myometrium and PCNA labeling. All data are presented in Tables 1, 2, and 3.

Vaginal Opening

The proportion of mice showing vaginal opening (completely open and partially open combined) on day 26 after 3 days exposure to DMSO, BPA, or E_2 was significantly different between groups (Table 1). Relative to the control group, a greater proportion of mice exposed to 0.1 mg/kg BPA ($p < 0.0001$), 100 mg/kg BPA ($p < 0.0001$), and E_2 ($p < 0.0001$) showed vaginal opening. Although not statistically significant ($p = 0.0512$), mice exposed to 75 mg/kg BPA also showed an increased incidence of vaginal opening (completely open and partially opened combined) relative to the control group.

Body Weight

BPA induced a significant increase in body weight at a concentration of 0.5 mg/kg ($p < 0.05$) and a significant decrease in body weight at 100 mg/kg ($p < 0.05$; Table 1). These represented changes in body weight of 12% and 10%, respectively.

Uterotrophic Assay

The administration of BPA at concentrations of 0.1–75 mg/kg body weight had no effect on the wet weight of the uterus relative to the control group. There was a 53% increase in uterine wet weight in response to 100 mg/kg body weight BPA ($p < 0.05$) and a 308% increase in uterine wet weight in response to E_2 (positive control group; $p < 0.0001$) relative to the control group. Wet weight of the uterus, calculated as a percentage of the body weight, showed a similar pattern of change (Table 1, Figure 1).

Uterine Morphology

Luminal epithelium. The relative area of luminal epithelium within the uterus was not affected by BPA at any concentration. E_2 treatment induced a significant increase in this parameter by 115% relative to the control group ($p < 0.0001$) (Table 2, Figure 1).

The uterus exhibited an increase in epithelial cell height in response to BPA at concentrations of 5 ($p < 0.05$), 75, and 100 mg/kg ($p < 0.0001$), which represents increases of 14, 42, and 63%, respectively. Although not statistically significant ($p = 0.056$), epithelial cell height also increased in response to 50 mg/kg BPA. Treatment with E_2 induced an 83% increase ($p < 0.0001$) in

Table 1. Body weight, uterine wet weight (expressed as absolute values and as a percentage of body weight), and the percentage of mice showing vaginal opening at sacrifice in immature CD-1 mice implanted for 3 days with subcutaneous pumps containing DMSO vehicle, BPA, or E_2.

Treatment	Body weight (mg)	Uterine wet weight (mg)	Uterine wet weight (per body weight)	Vaginal opening at day 26 (%)	
				Complete	Complete and partial
Control	15. 89 ± 0.38	19.01 ± 1.16	0.1195 ± 0.0064^{ab}	27 (13/48)	40 (19/48)
E_2 (5 µg/kg)	16.90 ± 1.06	77.62 ± 4.97*	0.4612 ± 0.0159^{c}	91 (20/22)*	95 (21/22)*
0.1 mg/kg BPA	17.50 ± 0.72	24.30 ± 4.11	0.1374 ± 0.0020^{abd}	100 (5/5)*	100 (5/5)*
0.5 mg/kg BPA	17.80 ± 0.32*	20.35 ± 1.40	0.1143 ± 0.0078^{ab}	55 (6/11)	64 (7/11)
1 mg/kg BPA	16.22 ± 0.35	16.08 ± 1.84	0.0984 ± 0.0096^{ab}	45 (5/11)	45 (5/11)
5 mg/kg BPA	16.32 ± 0.63	15.54 ± 1.89	0.0938 ± 0.0086^{a}	41 (7/17)	47 (8/17)
50 mg/kg BPA	15.82 ± 0.64	19.13 ± 1.40	0.1236 ± 0.0105^{ab}	24 (4/17)	59 (10/17)
75 mg/kg BPA	14.92 ± 0.50	23.74 ± 1.94	0.1580 ± 0.0078^{bd}	67 (4/6)	67 (4/6)
100 mg/kg BPA	14.39 ± 0.44*	29.08 ± 2.87*	0.2013 ± 0.0169^{d}	100 (6/6)*	100 (6/6)*

Values for each group are expressed as mean ± SEM. The control group represents pooled data from the control and vehicle groups. For uterine wet weight (per body weight), mean values with no superscripts in common are significantly different ($p < 0.05$).
*Significantly different from the control group ($p < 0.05$).

Table 2. Morphometric analyses of uterine tissue from immature CD-1 mice implanted for 3 days with subcutaneous pumps containing DMSO vehicle, BPA, or E_2.

Treatment	Relative area (%)				Height of epithelium
	Luminal epithelium	Glandular epithelium	Lamina propria	Myometrium	
Control	12.11 ± 0.78^{a}	1.69 ± 0.17^{a}	48.52 ± 1.38	36.93 ± 1.83	14.39 ± 0.54
E_2 (5 µg/kg)	25.98 ± 1.09^{b}	1.34 ± 0.19^{a}	40.16 ± 1.28*	33.78 ± 1.26	26.31 ± 1.12*
0.1 mg/kg BPA	11.76 ± 1.12^{a}	2.07 ± 0.55^{a}	51.83 ± 3.07	35.50 ± 3.74	14.56 ± 1.11
0.5 mg/kg BPA	15.07 ± 1.84^{a}	1.33 ± 0.30^{a}	50.50 ± 1.10	33.75 ± 1.78	16.46 ± 0.92
1 mg/kg BPA	11.77 ± 1.92^{a}	1.77 ± 0.37^{a}	49.69 ± 0.81	36.78 ± 2.23	15.83 ± 0.76
5 mg/kg BPA	12.36 ± 0.88^{a}	2.10 ± 0.33^{a}	42.27 ± 1.46*	43.05 ± 2.37	16.46 ± 0.60*
50 mg/kg BPA	12.93 ± 0.71^{a}	1.30 ± 0.21^{a}	48.01 ± 1.56	37.75 ± 1.65	16.13 ± 0.47
75 mg/kg BPA	12.61 ± 0.97^{a}	1.95 ± 0.39^{a}	53.00 ± 0.56	32.44 ± 0.80	20.43 ± 0.97*
100 mg/kg BPA	15.45 ± 1.27^{a}	1.80 ± 0.40^{a}	49.17 ± 4.24	34.56 ± 4.81	23.46 ± 0.74*

Values for each group are expressed as mean ± SEM. For luminal epithelium and glandular epithelium, mean values within each column with no superscripts in common are significantly different ($p < 0.05$).
*Significantly different from the control group ($p < 0.05$).

epithelial cell height relative to the control group (Table 2, Figures 1 and 2).

Glandular epithelium. The relative area of glandular epithelium within the uterus was not affected by treatment with BPA or E_2 at any concentration compared to the control group (Table 2).

Lamina propria and myometrium. Compared to the control group, the relative area of lamina propria within the uterus was not affected by any concentration of BPA except 5 mg/kg ($p < 0.01$); E_2 also induced a significant decrease in the relative area of lamina propria ($p < 0.01$). The relative area of myometrium within the uterus was not affected by treatment with BPA or E_2 at any concentration (Table 2).

Lactoferrin

Within the luminal and glandular epithelium of the uterus, BPA induced a significant increase in the expression of lactoferrin by 373% at a concentration of 75 mg/kg ($p < 0.01$) and by 438% at a concentration of 100 mg/kg ($p < 0.01$) relative to the control group. In some animals, lactoferrin expression was induced at a concentration of 50 mg/kg BPA, although this was not statistically significant (Figure 3). At all lower BPA concentrations, lactoferrin expression was not induced. The expression of lactoferrin was increased by 405% in the E_2 group ($p < 0.01$), whereas the control group showed no response (Table 3, Figure 3).

PCNA

There was no significant difference in the expression of PCNA in the luminal epithelium between treatment groups (Table 3).

Discussion

BPA has the capacity to induce proliferative and stimulatory changes in estrogen target tissues that are analogous to those induced by estrogens. These effects have been identified in various strains of rat after exposure to concentrations from 0.3 to 800 mg BPA/kg body weight. The ensuing morphologic changes include proliferation of mammary gland epithelium (*23*), cell proliferation and cornification of the vagina (*14,24*), and an increase in the wet weight, epithelial cell height, and mucous secretion of the uterus (*14,16*). In the present study we have demonstrated that CD-1 mice are also responsive to BPA and that concentrations from 5 to 100 mg/kg body weight can induce a statistically significant increase in the height of luminal epithelial cells; we have also demonstrated that 0.1 mg/kg BPA can induce vaginal opening. Only 100 mg/kg BPA can induce a uterotrophic response. These changes were not accompanied by a change in the relative area of uterus occupied by luminal epithelium, glandular epithelium, lamina propria (except at 5 mg/kg BPA), and muscle, which demonstrates that the relationship between different tissue compartments within the uterus remain the same with BPA treatment. In contrast, the E_2 group did show a significant increase in both epithelial cell height and relative area of epithelium within the uterus. This demonstrates that the single E_2 dose used in the study (known to induce a maximal response) has a more profound effect than the BPA doses that were assayed.

Although this study demonstrates that BPA induces changes in the mouse reproductive tract at doses as low as 0.1 mg/kg, it highlights the phenomenon that each end point measured reflects a different dose–response profile. The data for uterine wet weight suggest a U-shaped profile because weight is increased at 0.1 (although not statistically significant) and 100 mg/kg BPA, yet drops between 1 and 5 mg/kg BPA. Similarly, incidence of vaginal opening is significantly increased at 0.1 mg/kg BPA and again at 100 mg/kg BPA. Lactoferrin expression shows a profile in which the middle concentrations of BPA (e.g., 1 mg/kg) increase this variable, whereas doses on either side have absolutely no effect until they reach 75 and 100 mg/kg. These data suggest that exposure to BPA induces a nonmonotonic response in the reproductive parameters measured, a finding that is consistent with other studies in which E_2 and the potent estrogen diethylstilbestrol (DES) induce an inverted U-shaped dose–response curve for prostate weight (*25*).

The glycoprotein lactoferrin has been detected in significant amounts in the lactating mammary gland (*21,26*), uterus, uterine luminal fluid, cervix, vagina, ovary, and

Table 3. Labeling of PCNA and lactoferrin within the uterus of the immature CD-1 mice implanted for 3 days with subcutaneous pumps containing DMSO vehicle, BPA, or E_2.

Treatment	PCNA labeled luminal epithelium[a]	Luminal and glandular epithelium[b]	Lactoferrin expression[c]
Control	20.75 ± 3.79	1.62 ± 0.25	0
E_2 (5 µg/kg)	21.79 ± 5.34	8.18 ± 0.99*	100
0.1 mg/kg BPA	NA	NA	NA
0.5 mg/kg BPA	22.29 ± 10.85	1.28 ± 0.26	0
1 mg/kg BPA	30.64 ± 7.93	1.79 ± 1.15	17
5 mg/kg BPA	30.64 ± 12.57	1.18 ± 0.47	0
50 mg/kg BPA	20.37 ± 8.16	2.36 ± 0.71	33
75 mg/kg BPA	NA	7.67 ± 1.26*	100
100 mg/kg BPA	NA	8.71 ± 0.60*	100

NA, not available. Values for each group are expressed as mean ± SEM.
[a]Number of labeled cells/1,000 µm basement membrane. [b]Arbitrary units to a maximum of 12. [c]Score > 4 out of a possible 12 (%). *Significantly different from the control group ($p < 0.05$).

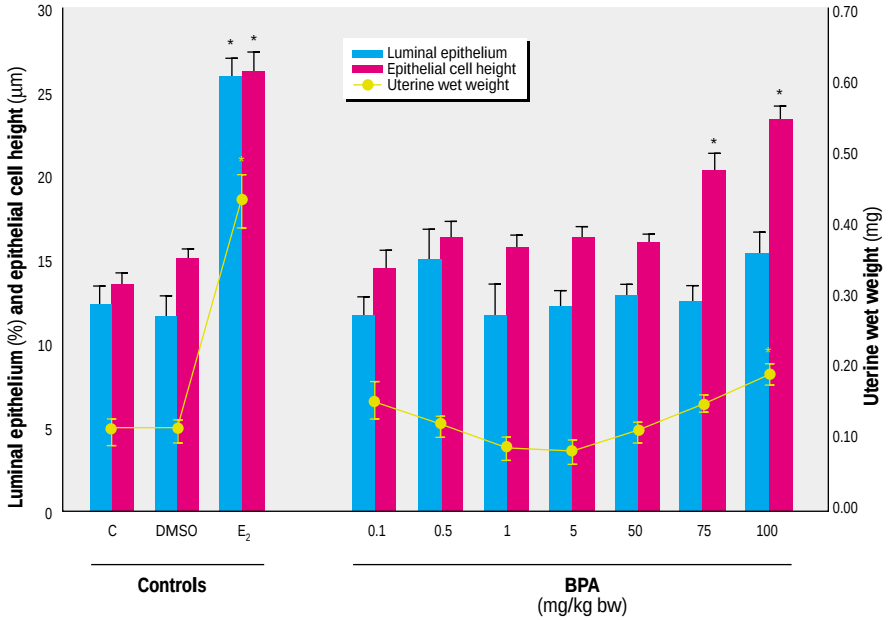

Figure 1. Differences in relative area of luminal epithelium (the percentage of uterus occupied by luminal epithelium), height of luminal epithelium, and uterine wet weight of immature mice exposed to BPA concentrations ranging from 0.1 to 100 mg/kg/day. C, untreated control. Bars represent mean values ± SEM.
*Significantly different ($p < 0.05$) relative to C and DMSO control groups combined.

oviduct of the mouse (*21*,*27*,*28*). A member of the transferrin family, lactoferrin is thought to provide bacteriocidal protection for gametes (possibly both male and female) in the uterus due to its chelating properties (*29*,*30*) and may be implicated in fetal growth and development (*31*). The expression of lactoferrin in the mouse is under estrogenic control and shows fluctuations in concentration during the estrous cycle (*32*), and in response to DES in a time- and dose-dependent manner (*21*,*33*). As such, lactoferrin is often used as a marker of estrogen action. In the present study we demonstrated that 75 and 100 mg/kg BPA induced a > 300% increase in the immunolocalization of lactoferrin in both the luminal and glandular epithelium of the uterus. This increase is of the same magnitude as that induced by E_2 in the current study and as described in previous work (*27*), demonstrating the estrogenic nature of BPA *in vivo*. The difference in the sensitivity of individual mice to BPA, which was particularly evident in the measurement of lactoferrin expression in the 50 mg/kg BPA group, may be a consequence of intrauterine position during development. The intrauterine position has been shown to determine the level of endogenous estrogens to which a developing fetus is exposed on the basis of position relative to either a male or a female (*34*,*35*).

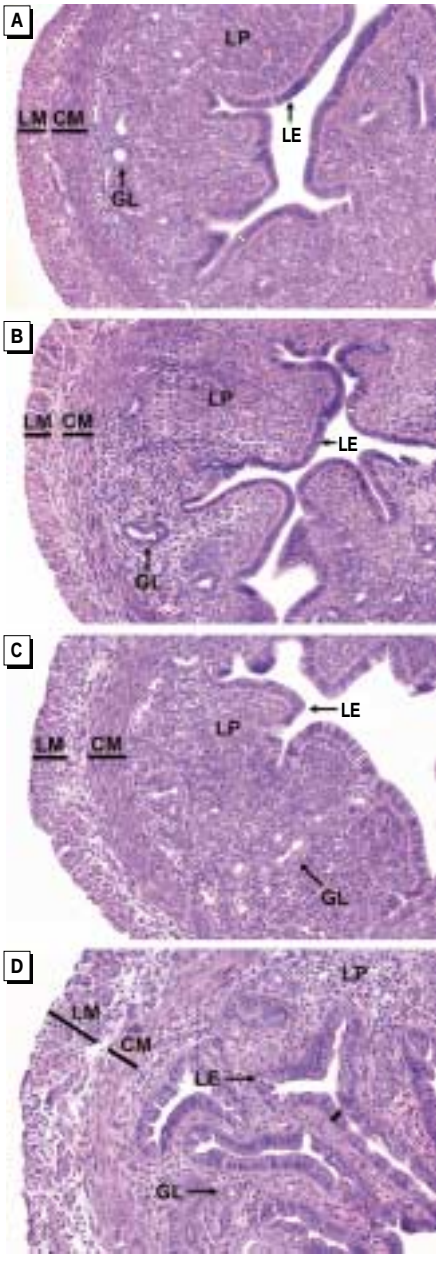

Figure 2. Photomicrographs showing the luminal epithelium (LE), glandular epithelium (GL), lamina propria (LP), longitudinal smooth muscle (LM), and circular smooth muscle (CM) of the uterus from immature mice exposed for 3 days to (*A*) DMSO, (*B*) 50 mg/kg BPA, (*C*) 75 mg/kg BPA, and (*D*) E_2. Note the increase in the height of the luminal epithelium in uteri exposed to 75 mg/kg BPA and E_2. H&E; magnification 470×.

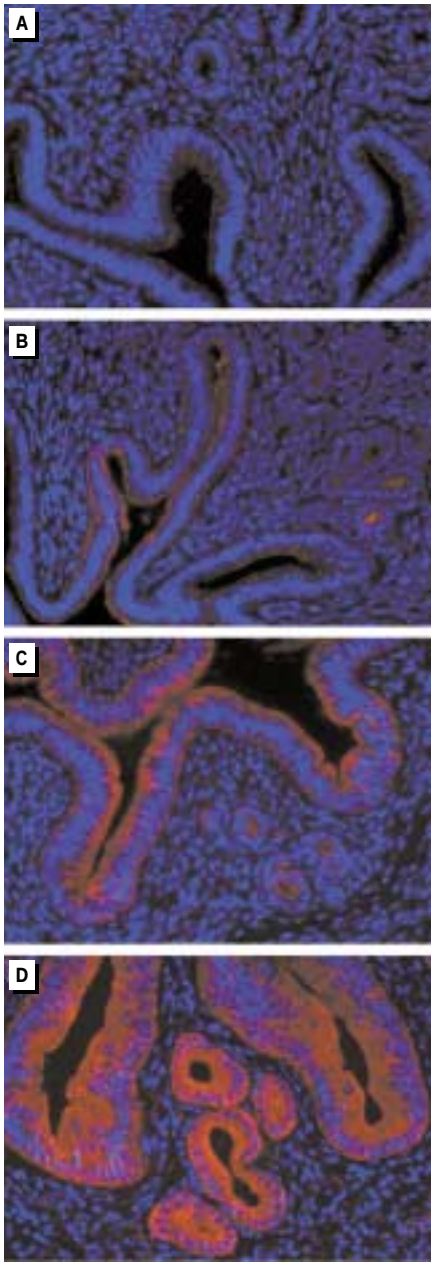

Figure 3. Immunolocalization of lactoferrin in the luminal epithelium and glandular epithelium of the uterus from immature mice exposed for 3 days to (*A*) DMSO, (*B*) 50 mg/kg BPA, (*C*) 75 mg/kg BPA, and (*D*) E_2. Note the increase in lactoferrin expression (red) in uteri exposed to 75 mg/kg BPA and E_2. The uterus exposed to 50 mg/kg BPA (*B*) represents one animal that showed a maximal lactoferrin response to BPA at this dose. Magnification 950×.

The null effect of BPA on the expression of PCNA within the mouse uterus was most likely due to high variation within treatment groups. In retrospect, labeling of bromodeoxy uridine (BrdU) may have provided a more accurate account of cell proliferation because it specifically indicates DNA synthesis, whereas PCNA is also expressed in cells involved in RNA transcription and cell repair (*36*). PCNA has been shown to increase expression within the mouse uterine epithelium during development (*37*) and pregnancy (*38*). In one study Karlsson et al. (*39*) describe a decrease in PCNA expression in luminal epithelial cells of the rat uterus in response to tamoxifen and toremifene that is concomitant with an increase in the expression of BrdU.

One of the aims of this study was to provide insight into effective doses of BPA *in vivo* such that subsequent studies on the effects of *in utero* exposure on development and reproduction could be undertaken. The current study revealed reproductive changes within the immature CD-1 mouse (vaginal opening) following 3 days exposure to BPA at 0.1 mg/kg body weight. Yet recent work in CF-1 mice, a strain also outbred for large litter size, has revealed that *in utero* exposure to 2.4 µg BPA/kg body weight significantly advances the onset of puberty in females (*35*). Despite the lack of data on the effects of BPA exposure on female reproductive tract morphology in that study, the comparison with our study demonstrates that caution must be exercised in extrapolating the effective dose for *in utero* exposure from studies carried out in immature or adult animals.

Earlier estimations of the estrogenic potency of BPA suggested that this chemical was a "weak" estrogen mimic, exhibiting a relative binding affinity to both the ER-α and β approximately 1:2,000 that of 17β-estradiol (*6*,*40*). Because these are *in vitro* studies, they do not take into account factors such as uptake, transportation, and metabolism of BPA specific to the live animal, which modify the concentration of chemical available to bind the ER or other serum proteins such as albumin, sex hormone-binding globulin, and corticosteroid-binding globulin (*12*,*40*–*42*). BPA binds both human sex steroid-binding protein (0.01%) and trout sex

steroid-binding protein (0.1%) with low affinity relative to [^{3}H]dihydrotestosterone, and to rat α-fetoprotein with negligible affinity (*43*). The presence of α-fetoprotein is particularly important during fetal and neonatal development because it is believed to prevent early exposure of the organism to endogenous estrogens, thus preventing inappropriate sexual differentiation of the brain (*44*). The pharmacokinetics of BPA most likely acts to increase its effective concentration in circulation, making it more readily available to the ER and thus enhancing its estrogenic activity relative to the protein-bound estradiol.

The uterotrophic assay has been traditionally used to establish the estrogenic activity of sex steroids and suspected environmental estrogens (*13*,*45*). Our present study reveals that BPA is able to induce a uterotrophic effect, that is, an increase in wet weight of the uterus, at only the highest concentration used (100 mg/kg body weight). This assay suggests that BPA is not a very potent estrogen mimic in the CD-1 mouse, although at 5 mg/kg body weight, BPA induced a significant increase in the height of luminal epithelial cells. These data demonstrate that the uterotrophic assay is of limited value in determining the estrogenicity of a suspected environmental estrogen because changes at the cellular level were observed at significantly lower doses than those at which a change in wet weight occurred. Moreover, there was a significant effect on vaginal opening at even lower BPA doses (0.1 mg/kg body weight).

In conclusion, this work contributes to the body of evidence showing that BPA acts as an estrogen *in vivo* by inducing cellular and biochemical changes in the mouse uterus that are consistent with estrogenic activity. Also, BPA was capable of inducing changes in vaginal opening, uterine wet weight, epithelial cell height, and lactoferrin expression in the CD-1 mouse, which establishes that this strain is suitable for investigating the effects of *in utero* exposure to BPA on development and reproduction. Finally, this work argues against using the mouse uterotrophic assay as an end point for determining estrogenicity of synthetic chemicals, and demonstrates that it is essential to develop alternative, more sensitive *in vivo* assays.

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