

# In Utero Exposure to Low Doses of Bisphenol A Lead to Long-term Deleterious Effects in the Vagina<sup>1</sup>

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## Abstract

The origins of the “endocrine disrupter hypothesis” may be traced to reports on adolescent daughters born to women who had taken the highly potent synthetic estrogen, diethylstilbestrol, while pregnant, and who developed a rare form of vaginal cancer and adenocarcinoma. Bisphenol A (BPA) is an estrogenic chemical that is highly employed in the manufacture of a wide range of consumer products. Some observational studies have suggested that the amounts of BPA to which we are exposed could alter the reproductive organs of developing rodents. We examined the influence of BPA at low doses to address the questions of (a) whether *in utero* exposure affects the vagina of the offspring and (b) which mechanisms cause the toxic effects. Gravid Sprague-Dawley dams were administered either 0.1 (low dose) or 50 mg/kg per day BPA, the no observed effect level, or 0.2 mg/kg per day 17 $\alpha$ -ethinyl estradiol by gavage. Striking morphological changes were observed in the vagina of postpubertal offspring leading us to examine vaginal estrogen receptor (ER) expression because BPA binds to the ER $\alpha$ , which is important for growth of the vaginal epithelium. We show that the full-length ER $\alpha$  is not expressed during estrus in the vagina of female offspring exposed to either dose of BPA when compared to the control group, whereas ER $\alpha$  expression does not differ from the control group during the diestrus stage. ER $\alpha$  downregulation seems to be responsible for the observed altered vaginal morphology.

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**Keywords:** bisphenol A, low dose, estrogen receptor, rat vagina, endocrine disrupter.

## Introduction

Endocrine-disrupting industrial chemicals (EDCs) are released into the environment and interfere with normal hormonal processes. Some of these chemicals can produce toxic effects at surprisingly low doses. Many researchers hypothesize that exposure to these EDCs during critical periods of development, i.e., early postnatal or *in utero*, could result in adverse effects to wildlife and humans [1,2]. They may influence growth, reproduction, and development.

The origins of the “endocrine disrupter hypothesis” may be traced to reports on adolescent daughters born to women who

had taken the highly potent synthetic estrogen, diethylstilbestrol (DES), while pregnant. These daughters developed a wide range of reproductive tract abnormalities, including a rare form of vaginal cancer, vaginal adenocarcinoma [3].

Bisphenol A (BPA) is a chemical that has been widely discussed as a prime candidate for endocrine disruption. BPA is composed of two unsaturated phenolic rings that resemble DES. It is among those estrogenic industrial compounds that are highly and widely used in the manufacture of epoxy, polyester-styrene, and polycarbonate resins and which are used for the manufacture of dental fillings, baby bottles, and food packaging. The ability of BPA to migrate from polymer to food has been described [4,5]. *In vitro* studies demonstrated that BPA binds to the estrogen receptors (ERs), induces estrogen-dependent gene expression/responses and is weakly estrogenic when compared to 17 $\beta$ -estradiol (E2) or DES [6–8].

Minuscule amounts of EDCs were shown to alter the reproductive organs of developing mice [9,10], sparking alarm within the scientific community and regulatory agencies. Fred vom Saal's group at the University of Missouri, Columbia showed that early developmental exposure to low doses of DES increased prostate weight in offspring, but levels above this had the opposite effect [11]. A second study by vom Saal showed even more disturbing effects. It demonstrated that exposure of female mouse fetuses to a very low dose of BPA alters their postnatal growth rate and results in precocious puberty [12]. They found significantly reduced number of days between vaginal opening and first vaginal oestrus. Alarmed about the implications of these results, some laboratories, mainly industrial ones, tried to reproduce these data but failed [13–15]. Faced with conflicting studies and aware of other new research that might resolve the question, the Environmental Protection Agency (EPA) enlisted the help of an expert panel, which met and conducted an extensive review of raw data from submitted studies [16]. The expert panel finally concluded

Abbreviations: BPA, bisphenol A; DES, diethylstilbestrol; EDCs, endocrine-disrupting industrial chemicals; EPA, Environmental Protection Agency; ER, estrogen receptor; E2, 17 $\beta$ -estradiol

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that some estrogenic chemicals can cause biologic effects at levels below those normally found safe. Discrepancies between the studies may be attributed to variable sensitivity to estrogenic chemicals by laboratory animals. Indeed, a study has demonstrated that rodent strains can vary dramatically in their response to estrogenic compounds [17]. Furthermore, the issues of dose and binding affinities to the ERs seem to be the heart of the controversy regarding xenoestrogens. Pointing to these uncertainties, the expert panel recommended new screening and testing procedures because traditional toxicology tests, particularly standard reproductive and developmental assays, appear to be inadequate for screening programs. The scope of the new testing program should include assays for (1) *in vitro* and *in vivo* screening of ER expression and functions and (2) genetic analysis. Robert Kavlock, director of the reproductive division of the National Health and Environmental Effects Research Laboratory at the U.S. EPA, currently stated that the inconclusive results concerning effects of BPA on reprotoxicology, can only be solved by understanding the mechanisms [2].

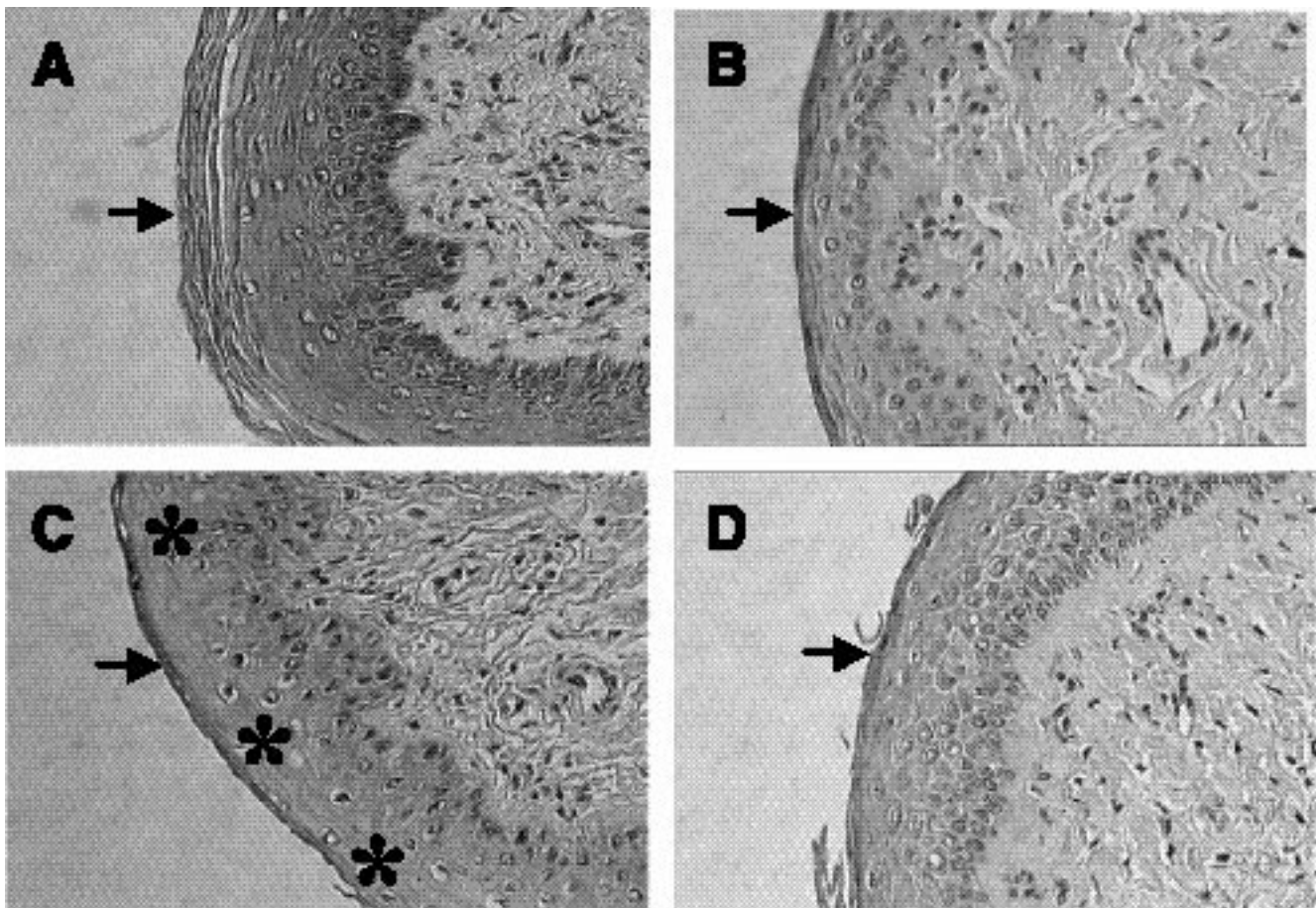
We examined the influence of BPA on several reproductive endpoints during early development and adulthood at

low doses to address the questions of (1) whether *in utero* exposure interferes with the reproductive system of the offspring and (2) what are the mechanisms leading to long-term deleterious effects after exposure of the developing fetus to exogenous estrogens.

### Materials and Methods

Female Sprague-Dawley rats with sperm-positive vaginal smears were treated with either 2% cornstarch (Mondamin) at 10 ml/kg per day, 0.1 mg/kg per day BPA, 50 mg/kg per day BPA or 0.2 mg/kg per day E2. Cornstarch served as the vehicle for BPA and pharmacological grade peanut oil was used as the vehicle for E2. The gravid dams were treated by gavage on gestation days 6 through 21.

Intact female offspring were maintained on a 12/12 hour light:dark cycle, and beginning at approximately 3 months of age, estrous cycle stage was determined by vaginal swabbing for 3 weeks. Each estrus group contained 22 offspring in the cornstarch group, 13 offspring in the 0.1 mg/kg per day, and 12 offspring in the 50 mg/kg per day BPA group, as well as 19 offspring in the 0.2 mg/kg per day E2 group. The diestrus group contained 20 offspring in the



**Figure 1.** Representative histology of the rat offspring vagina at estrus. (A) Control (cornstarch-treated animals) group. The vaginal epithelium is formed by 6–10 layers, of which superficial layers are cornified (↓). ×400. (B) Rat vagina at estrus. 0.1 mg/kg BPA. No keratinization of the superficial layers (↓). Vaginal epithelium is formed by less than four layers. ×400 (C) Rat vagina at estrus. 0.2 mg/kg E2 (positive control). No keratinization at the surface of the epithelium (↓). Slight desquamation of the superficial layers (\*). Vaginal epithelium is formed by ≤6 layers. ×400. (D) Rat vagina at estrus. 50 mg/kg BPA. Process of slight keratinization at the surface of the superficial layers (↓). Mostly no keratinization can be observed. Vaginal epithelium is formed by ≤6 layers. ×400.



weights were determined. Animals were maintained in accordance with the Guide for the Care and Use of Laboratory Animals from the physiological society of Germany. BPA was purchased from Sigma-Aldrich Chemicals (Steinheim, Germany) and E2 from Aldrich Chemical (Milwaukee, WI).

### Histology

Vaginal tissue was fixed in Bouin solution, embedded in paraffin, and sectioned at 3  $\mu\text{m}$ . After processing tissue sections were stained with hematoxylin and eosin for light microscopy. The thickness of vaginal epithelium and the number of vaginal epithelial cell layers were measured using a microscope (Axiophot, Zeiss, Göttingen, Germany) and the public domain Scion image analysis software (Scion Image 1.62c MacOs, Scion, Frederick, MD).

### Western Blot Analyses

Western blot analyses were performed on vaginal tissue of each *in utero* treated offspring. Homogenization of rat vaginal tissue samples was performed for 15 minutes in an ultrasonic bath on ice in homogenization buffer including a cocktail of protease inhibitors (complete; Boehringer Mannheim, Germany). Homogenates were centrifuged at 20,000 $\times g$  for 30 minutes. The resulting supernatants were retained for further experiments. Protein concentrations were determined in triplicate according to Bradford using BSA as a reference standard. Fifteen micrograms protein was separated by SDS-PAGE using 10% gels and then electrotransferred to nitrocellulose membranes using a semidry blot transfer method. The quality as well as equal loading of protein blots was determined by Ponceau S staining of nitrocellulose and using a monoclonal antibody against  $\beta$ -actin (Sigma, Germany) at 1:15,000. Blots were incubated overnight with polyclonal antibodies against ER $\alpha$  (MC-20, sc-542; Santa Cruz, Heidelberg, Germany) at a 1:100 dilution. The  $M_r$  of the immunoreactive bands were determined using molecular weight marker protein stocks SDS-PAGE 7b (Sigma, Germany). After washing, blots were incubated for 90 minutes with anti-rabbit or anti-mouse IgG-conjugated peroxidase antibody at a 1:5000 dilution (Biogenes, Berlin, Germany). Blots were finally rinsed, and immune complexes were visualized using ECL reagent (Amersham, Freiburg, Germany). Specificity of the obtained immunoreactive bands was assessed by using peptide absorbed antiserum against ER $\alpha$ -peptide (MC-20, sc-542P; Santa Cruz).

## Results

### Histology of Vaginal Epithelium

Striking morphologic changes in the differentiation, stratification, and cornification of the vaginas could be observed during estrus from the same *in utero* treated animals when compared to the negative control group (Figure 1A). No keratinization at the surface of the epithelium was observed following exposure to 0.1 mg/kg BPA (Figure 1B) when compared to the control group

(Figure 1A). The thickness of the total epithelium was reduced following exposure to 0.1 mg/kg BPA (Figure 1B) when compared to the control group (Figure 1A). The 50-mg dose caused a similar effect, but was less pronounced (Figure 1D). Exposure to 0.2 mg/kg E2 (positive control; Figure 1B) resulted in a morphologic picture similar to the 0.1 mg/kg BPA group (Figure 1B) with slight desquamation of the superficial layers. In diestrus, no major differences of the vaginal epithelium could be observed between the control and treated animals (data not shown).

We could not observe any further morphological and structural changes of the vagina in offspring.

### Western Blot Analyses

We clearly demonstrate that the full-length ER $\alpha$  variant at 64 kDa is not expressed during estrus at the protein level in the vagina of all female offspring exposed to either dose of BPA compared to the negative control group, whereas ER $\alpha$  expression does not differ from the control group during the diestrus stage (Figure 2A). The anti-ER $\alpha$  antibody specifically reacted with three bands at 64, 56, and 42 kDa from homogenates of rat vagina from control animals (Figure 2A). Offspring exposed to 0.2 mg E2/kg body weight during gestation showed either a decreased or no expression of ER $\alpha$  in their vagina during estrus (Figure 2B). Binding to all immunopositive bands was eliminated when antibody was preincubated with antigen peptide (not shown).

In addition, we could not observe ER $\beta$  expression at the protein level in the rat vaginal tissue from any group (data not shown).

## Discussion

Vaginal epithelium cornifies or mucifies in response to the changing levels of circulating steroids during the estrous cycle [18]. Estrogens stimulate vaginal epithelium proliferation *in vivo* [19] and play a critical role in vaginal growth, epithelial morphogenesis, cytodifferentiation, and secretory activity. EDCs have been suspected to have adverse environmental effects because prenatal exposure to DES, another synthetic estrogen, was reported to be associated with clear cell adenocarcinoma of the vagina in female offspring. We performed Western blot analyses to study the expression of ERs and their variants in the vagina of 4-month-old (postpubertal) offspring exposed to BPA *in utero*, because ER $\alpha$  expression is important for growth, differentiation, and cornification of the vaginal epithelial cells [20]. The expression of ER $\alpha$  was investigated because we recently demonstrated [21] that those *in utero* treated animals exhibited altered times to vaginal opening and striking morphologic changes in the differentiation, stratification, and cornification of the vaginas during estrus. Western blot analyses were used to elucidate the possible mechanisms for the effects we observed in our observational studies. For the first time, we clearly demonstrate that the full-length ER $\alpha$  variant at 64 kDa is not expressed during estrus at the protein level in the vagina of all female offspring exposed to either dose of BPA compared to the negative

control group, whereas ER $\alpha$  expression does not differ from the control group during the diestrus stage (Figure 2A). The anti-ER $\alpha$  antibody specifically reacted with three bands at 64, 56, and 42 kDa from homogenates of rat vagina from control animals (Figure 2A). These shorter bands (56 and 42 kDa) are derived from the alternative usage of initiation ATG or splicing [22,23]. Specificity of immunoreactivity was analyzed by using peptide-preabsorbed antibodies. Furthermore, offspring exposed to 0.2 mg E2/kg body weight during gestation showed either a decreased or no expression of ER $\alpha$  in their vagina during estrus (Figure 2B). Our findings clearly indicate that *in utero* exposure, not neonatal exposure [24], of rats to low doses of BPA, similar to amounts typically found in the environment, elicits long-term changes in ER $\alpha$  expression. BPA significantly downregulates the expression of ER $\alpha$  leading to our findings showing striking morphologic changes in the differentiation and cornification of vaginas. Our data are consistent with previous reports showing a downregulation of ER $\alpha$  expression in the uterus after neonatal exposure to DES [24,25]. The mechanisms underlying the downregulation of ER $\alpha$  expression may be complex, as is the case with the tissue-specific agonist/antagonist action of tamoxifen [26]. BPA may antagonize the action of endogenous estrogens because there are differences in the existence of tissue-specific ER coactivators [27]. Adult organisms have compensatory mechanisms that may minimize disturbances; these mechanisms may be imperfect in the very young or unborn who are still in the process of developing homeostasis and undergoing genetic imprinting.

In summary, this is the first study that shows that the full-length ER $\alpha$  is not expressed during estrus in the vagina of female offspring exposed *in utero* to either dose of BPA when compared to the control group, whereas ER $\alpha$  expression does not differ to the control group during the diestrus stage. ER $\alpha$  downregulation seems to be responsible for the observed altered vaginal morphology.

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