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Exposure to bisphenol A in young adult mice does not alter ovulation but does alter the fertilization ability of oocytes

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ABSTRACT

Follicle growth culminates in ovulation, which allows for the expulsion of fertilizable oocytes and the formation of corpora lutea. Bisphenol A (BPA) is present in many consumer products, and it has been suggested that BPA impairs ovulation; however, the underlying mechanisms are unknown. Therefore, this study first evaluated whether BPA alters ovulation by affecting folliculogenesis, the number of corpora lutea or eggs shed to the oviduct, ovarian gonadotropin responsiveness, hormone levels, and estrous cyclicity. Because it has been suggested (but not directly confirmed) that BPA exerts toxic effects on the fertilization ability of oocytes, a second aim was to evaluate whether BPA impacts the oocyte fertilization rate using an in vitro fertilization assay and mating. The possible effects on early zygote development were also examined. Young adult female C57BL/6J mice (39 days old) were orally dosed with corn oil (vehicle) or 50 μg/kg bw/day BPA for a period encompassing the first three reproductive cycles (12–15 days). BPA exposure did not alter any parameters related to ovulation. Moreover, BPA exposure reduced the percentage of fertilized oocytes after either in vitro fertilization or mating, but it did not alter the zygotic stages. The data indicate that exposure to the reference dose of BPA does not impact ovulation but that it does influence the oocyte quality in terms of its fertilization ability.

1. Introduction

Bisphenol A (BPA) or 2,2-bis-4-hydroxyphenyl propane is a chemical monomer used in the production of polycarbonate and epoxy resins. Food and beverage containers subjected to extensive aging, heating or cleaning may represent inadvertent sources of BPA. Studies of human urine samples have revealed that more than 90% of individuals have measurable levels of BPA, reflecting the widespread human exposure to this compound (Calafat et al., 2008).

BPA toxicity has received much attention because it is suspected to be associated with adverse health outcomes, including reproductive dysfunction (reviewed in Caserta et al., 2014; Peretz et al., 2014; Rochester, 2013). Epidemiological studies of women have associated BPA exposure with polycystic ovary syndrome (Kandaraki et al., 2011), recurrent miscarriage (Sugiura-Ogasawara et al., 2005), premature delivery (Cantonwine et al., 2010), reduced ovarian response (Bloom et al., 2011; Ehrlich et al., 2012b; Mok-Lin et al., 2010), abnormal oocyte meiotic maturation (Machtinger et al., 2013), abnormal oocyte fertilization (Ehrlich et al., 2012b; Fujimoto et al., 2011), and implantation failure (Ehrlich et al., 2012a). In animal studies, neonatal or perinatal exposure to BPA has been reported to cause early puberty onset (Honma et al., 2002), irregular estrous cyclicity in adult life (Rubin et al., 2001; Wang et al., 2014), implantation failure (Varayoud et al., 2011; Xiao et al., 2011), and impaired meiotic maturation of the oocyte (Eichenlaub-Ritter et al., 2008; Hunt et al., 2003; Lawson et al., 2011; Susiarjo et al., 2007).

In humans, infertility is present in approximately 10–15% of couples, and ovulatory defects and failures in the fertilization ability of oocytes are among the important determinants (Practice Committee of the American Society for Reproductive Medicine, 2006). Ovulation is a process that allows for the release of a fertilizable oocyte from a mature

Abbreviations: BPA, bisphenol A; bw, body weight; CEO, cumulus-enclosed oocyte; CFO, cumulus-free oocyte; DES, diethylstilbestrol; eCG, equine chorionic gonadotropin; FSH, follicle stimulating hormone; GD, gestational day; hCG, human chorionic gonadotropin; LH, luteinizing hormone; PND, post-natal day.

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follicle; this process begins at puberty and continues its cyclic occurrence until oocyte depletion. Specifically, at the start of each estrous cycle in mice, a cohort of primordial follicles grows into later follicular stages, such as primary, preantral, and antral follicles, which are stimulated by both follicle stimulating hormone (FSH) and luteinizing hormone (LH) to become a preovulatory follicle. By mid-cycle, LH triggers a cascade of events that culminate in the expulsion of a mature oocyte capable of being fertilized and the formation of a corpus luteum (McGee and Hsueh, 2000; Richards et al., 2002). It has been suggested that at high doses (10–50 mg/kg bw/day), BPA may impair ovulation in animal models, as indicated by a reduced number of corpora lutea (Adewale et al., 2009; Kato et al., 2003; Suzuki et al., 2002), but the mechanisms of toxicity have not yet been elucidated. In this context, the first aim of this study was to evaluate whether exposure to the referenced safe dose of BPA (50 μg/kg bw/day; EPA, 1988) during the young adult stage impacts ovulation by altering folliculogenesis, the number of corpora lutea or eggs shed to the oviduct, circulating levels of estradiol, LH and FSH, ovarian gonadotropin responsiveness and/or estrous cyclicity.

Decreased fertility has been demonstrated in animal models exposed to BPA. For instance, Sprague–Dawley female rats subcutaneously injected with 50 μg/50 μL BPA during the neonatal period showed a reduced number of pups delivered in a mating protocol (Fernandez et al., 2010). In addition, CD-1 female mice perinatally exposed to 0.025–25 μg/kg body weight (bw)/day BPA showed decreased fertility and fecundity over time in a forced breeding protocol (Cabaton et al., 2011), and FVB female mice perinatally exposed to 50 μg/kg bw/day BPA had a reduced litter size at six months of age (Wang et al., 2014). However, whether the referenced safe dose of BPA elicits toxic effects on the fertilization ability of the oocyte is unknown. Therefore, a second aim of this study was to evaluate the potential toxic effect of BPA exposure on the oocyte fertilization rate using an *in vitro* fertilization assay and natural mating. Moreover, possible effects on early zygote development were also investigated.

2. Material and methods

2.1. Chemicals

BPA (99% purity), diethylstilbestrol (DES; 99% purity), equine chorionic gonadotropin hormone (eCG), human chorionic gonadotropin hormone (hCG), and all reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise specified.

For the 50 μg/kg bw/day BPA dose or the 10 μg/kg bw/day DES dose, stock solutions were prepared by dissolving 500 μg BPA or 100 μg DES in 3.75 μl absolute ethanol, and then mixed with tocopherol-stripped corn oil (MP Biomedicals, Illkirch, Alsace, France) up to 10 ml (final concentration of 50 μg/ml BPA or 10 μg/ml DES). Thus, the final ethanol concentration into tocopherol-stripped corn oil was 0.0375%. The compounds were delivered in volumes of 15–20 μl corn oil based on the mouse bw.

2.2. Animals and dosing

Male and female C57BL/6 J mice were obtained from a breeding colony at the Cinvestav animal facility. All mice were housed in filtered polysulfonate cages and maintained in a facility with a 12 L:12D photoperiod, a temperature of 21 ± 1 °C and a relative humidity of 50%. Food (Formulab Diet for rodents 5008; LabDiet, Brentwood, MO, USA) and high purity water were provided ad libitum. All animal use procedures for this study were approved by the Institutional Laboratory Animal Use and Care Committee at Cinvestav and were performed in accordance with the International Guidelines for the Use and Care of Experimental Animals.

Female mice on post-natal day (PND) 28–32 were checked for vaginal opening daily and were randomly assigned to control, BPA or DES groups. Once vaginal opening occurred, the females were considered to be in the young adult stage and were monitored daily for estrous cycles by examination with vaginal swabs according to Caligioni (Caligioni, 2009). Females manifested the first estrus approximately seven days after vaginal opening. From the day the first estrus was observed until the completion of 3 estrous cycles, females were orally dosed with tocopherol-stripped corn oil in 0.0375% ethanol (vehicle control), BPA (50 μg/kg bw/day), or DES (10 μg/kg bw/day; positive control) every 24 h by placing a pipette tip containing the dosing solution into the mouth. DES was used as a positive control to ensure that the mice were responsive to estrogenic chemicals (Halling and Forsberg, 1990; Rodriguez et al., 2010; Wang et al., 2014). The dose of 10 μg/kg bw/day DES was selected because it has been shown to cause reproductive toxicity in various windows of exposure (Odum et al., 2002). BPA was administered at a dose of 50 μg/kg bw/day because this is the safe exposure limit referenced by the United States Environmental Protection Agency (U.S. EPA) (EPA, 1988). The chosen window of exposure corresponds to three waves of cyclic follicle recruitment, which may represent a critical window of exposure for reproduction (McGee and Hsueh, 2000).

2.3. Tissue collection and exogenous gonadotropin treatments

At the end of the 3-estrous-cycle dosing period, females at estrus were either euthanized by cervical dislocation to collect ovaries containing follicle populations (Hernandez-Ochoa et al., 2010) and corpora lutea (Barnett et al., 2007), or primed with 5 IU of eCG to promote antral follicle growth to the preovulatory stage. Forty-eight hours later, these females were either euthanized to collect ovaries containing preovulatory follicles (Kim et al., 2008) or primed with 5 IU of hCG to mimic the LH surge. Fourteen to sixteen hours after hCG was administered, the oviducts were removed using Watchmaker's forceps under a dissecting microscope, and the cumulus-enclosed oocytes (CEO) were allowed to burst free from the ampulla portion of each oviduct and were gently teased apart to count the number of oocytes (Fig. 1).

2.4. Hormone assays

Blood samples were collected from the control and dosed females between 5:30 and 6:00 PM. Briefly, the mice at the third proestrus were anesthetized by *i.p.* injection of ketamine/xylazine (150/16 mg/kg bw), and blood samples were collected from the retro-orbital sinus using sterile capillary tubes. Serum samples were obtained through centrifugation and were then shipped to the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core to measure the circulating levels of LH, FSH and estradiol.

2.5. Ovarian histology

Ovaries (one per mouse) free of oviduct, fat, and bursa were fixed in Dietrich's solution for at least 72 h and were then processed for histology using standard protocols. Briefly, the ovaries were washed for at least 3 h with running water, dehydrated in ethanol series (70% and 96% twice and 100% twice, at intervals of 1 h each), cleared in xylol/ethanol (1:1 *v*/*v*), infiltrated with paraffin wax, and serially sectioned (thickness, 8 μm) throughout the entire ovary. Sections were mounted and stained on slides in hematoxylin and eosin-phloxine B using standard procedures. Follicle populations were counted at every 10th section of the entire ovary and were scored as primordial follicles, primary follicles, preantral follicles or antral follicles, based on their morphological appearance, as detailed in (Hernandez-Ochoa et al., 2010). Follicles were scored as preovulatory follicles if they contained CEO attached to mural granulosa cells, an antral space and a delimiting layer of theca cells. All ovaries were analyzed without treatment knowledge to avoid bias. Only follicles containing an oocyte with a visible nucleus were counted to prevent double counting of large follicles, such as preantral, antral and preovulatory follicles. To estimate the total follicle number, the follicles counted per ovary were multiplied by 10.

To morphologically assess the size of preovulatory follicles, the diameter in the marked sections was measured in two perpendicular axes (vertical and horizontal) using the Image-Pro Premier 9.0 software (Media Cybernetics, Inc., Rockville, MD, USA). Corpora lutea were classified based on their morphological appearances because, in the ovary, those units contain luteinized cells and are delimited by a layer of regressed theca cells. Each corpus luteum was followed through consecutive sections of the entire ovary to ensure that it was counted only once (Barnett et al., 2007). The total number of corpora lutea were those counted in the entire ovary.

2.6. In vivo fertilization assay

Following the exogenous eCG/hCG treatment, as described above, an in vivo fertilization assay was performed according to Zudova and colleagues (Zudova et al., 2004), with minor modifications. Briefly, after the hCG injection, females were mated overnight with unexposed proven breeder 15-week-old males (2:1) and then checked for vaginal plugs the following morning to ensure mating. At 48 h after hCG injection, the mated females were *i.p.* injected with 2 μM colchicine in 0.2 ml of distilled water to arrest zygotic development primarily at the 8-cell zygote stage. At 5 h after the colchicine injection, the eggs were flushed from the isthmus portion of each oviduct into Hanks' balanced salt solution and then incubated in Hanks' balanced salt solution containing 10 μg/ml hyaluronidase for 12 min at room temperature to remove the cumulus cells. Cumulus cell-free eggs were counted, incubated in 0.03% sodium citrate for 30 min, fixed with methanol-acetic acid (3:1 *v*/*v*, 5 min), stained with 20 μM Hoechst 33342 for 15 min, washed two times with PBS, and mounted on slides for assessment under fluorescence microscopy. The eggs were scored as 2-cell, 4-cell, and 8-cell zygote stages if they contained 2, 4, or 8, respectively, small nucleated cells (known as blastomeres) inside the eggs. The eggs were considered to be unfertilized if they showed oocyte DNA and if blastomeres were not present. The *in vivo* fertilization assay was performed in three independent dosing experiments using at least three mice per treatment, and the means are reported.

2.7. In vitro fertilization assay

Following the exogenous eCG/hCG treatment, as described above, the oviducts were removed 14–16 h post-hCG and placed in 1 ml of M-16 medium. The ampulla portion of each oviduct was punctured, and the CEO were directly subjected to insemination or subjected to the removal of cumulus cells, as described above, and then subjected to insemination. Untreated spermatozoa were incubated separately in 4 mg/ml BSA-supplemented M-16 medium (1 h/37 °C/95% air–5% CO_2) for capacitation. Fifteen to twenty CEO or cumulus-free oocytes (CFO) were inseminated for 6 h with capacitated spermatozoa (1×10^4 cells) in 20-μl drops of 4 mg/ml BSA-supplemented M-16 medium covered with oil. The incubation conditions were 36.5 °C in 95% air and 5% CO_2. Samples were fixed in PBS containing 3% formaldehyde and then stained with 20 μM Hoechst 33342 for 15 min. The samples were washed three times with 1 ml of PBS, mounted on slides using VECTASHIELD mounting medium (Vector Laboratories Inc., Burlingame, CA) and examined under fluorescence microscopy to assess the fertilization rate. Oocytes were considered to be fertilized when development to the 2-cell stage was observed. *In vitro* fertilization assays were performed in three independent dosing experiments using at least six mice per treatment, and the means are reported.

2.8. Statistical Analysis

The data obtained from the independent experiments were analyzed using SPSS statistical software (SPSS Inc., Chicago, IL, USA). Comparisons of BPA and DES treatment groups from the control group were conducted using one-way analysis of variance (ANOVA), followed by the Dunnett's *post hoc* test. Data were expressed as the means ± standard error of the mean (SEM). Statistical significance was assigned at $p \leq 0.05$.

3. Results

3.1. Effects of BPA on ovarian follicle populations

The growth of follicle populations, which eventually leads to the emergence of preovulatory follicles, plays a role in determining ovulation (McGee and Hsueh, 2000). Thus, as a first approach to determine the effect of BPA exposure on ovulation, follicle growth was determined in ovarian sections by counting the relative numbers of follicles at each stage of development (Table 1). Similar numbers of primordial, primary, preantral, and antral follicles were observed in the control, BPA and DES treatment groups (Table 1).

3.2. Effects of BPA on gonadotropin responsiveness

Dosing females with exogenous gonadotropins that have FSH- and LH-like properties allows for an evaluation of ovarian gonadotropin responsiveness (Barnett et al., 2007). Thus, as a second approach to determine whether BPA alters ovulation, ovarian responsiveness to eCG was examined using two separate protocols. In the first protocol, the ability of ovarian follicles to grow to the preovulatory stage in response to eCG treatment was examined by counting the number of preovulatory follicles in ovaries from the control and BPA- and DES-treated mice (Table 1). Morphological assessment of follicular size provides information about the actual size of follicles in vivo (Benedict et al., 2003); thus, the diameters of preovulatory follicles were also measured. After treatment with 5 IU of eCG, no significant differences were observed between any treatment group in the number of preovulatory follicles or in their diameters (Table 1).

An alternative method for measuring ovarian responsiveness to eCG was utilized by counting the number of oocytes shed into the ampulla portion of the oviduct following a superovulation protocol with exogenous gonadotropin treatments (Fig. 2A). No significant differences existed between any treatment groups in terms of the number of shed oocytes (Fig. 2A). To extend these findings to the number of shed oocytes, females subjected to a superovulation protocol were immediately mated with untreated males and then checked for the number of zygotes in the isthmus portion of each oviduct (Fig. 2A). Again, no significant differences were observed in the shed zygotes in the control or BPA- and DES-treated groups (Fig. 2A).

Ovarian follicles that ovulate become corpora lutea (Richards et al., 2002). As a third approach to determine whether BPA alters ovulation, the ability of the controls and the BPA- and DES-treated female mice to ovulate under the stimulus of endogenous gonadotropins at the physiological level was determined based on the number of corpora lutea in the ovaries (Fig. 2B). Compared to the controls, there was a significant decrease in the average number of corpora lutea in the DES-treated females but not in the BPA-treated females (Fig. 2B).

3.3. Effects of BPA on estrous cyclicity

Vaginal smears were taken daily in the morning, from the first day of dosing until the day of euthanasia, to evaluate estrous cyclicity, and the duration for which the females remained in each stage of the estrous cycle was averaged (Fig. 3). For each stage of the cycle, the females in the BPA treatment group had durations similar to those of the females in the control group (Fig. 3). Similar to previously reported studies (Kang et al., 2002), the females in the DES treatment group spent significantly more time in estrus but significantly less time in metestrus, diestrus and proestrus compared to females in the control and BPA groups (Fig. 3).

Table 1
Histological evaluation of follicle types preceding the process of ovulation in ovaries from the control and BPA- and DES-treated mice.

Parameter	Control (corn oil)	BPA (50 μg/kg bw/day)	DES (10 μg/kg bw/day)
Number of primordial follicles	527 ± 76 (64.00 ± 3.27)	577 ± 12 (65.87 ± 2.80)	678 ± 95 (74.62 ± 1.01)
Number of primary follicles	185 ± 46 (20.26 ± 2.17)	147 ± 29 (17.52 ± 1.87)	132 ± 24 (12.97 ± 1.74)
Number of preantral follicles	103 ± 23 (11.45 ± 1.25)	90 ± 16 (10.85 ± 1.25)	83 ± 8 (8.65 ± 1.33)
Number of antral follicles	40 ± 10 (4.29 ± 0.71)	40 ± 6 (5.76 ± 1.71)	43 ± 13 (3.76 ± 0.94)
Number of preovulatory follicles	58 ± 9	47 ± 14	65 ± 6
Diameter of preovulatory follicles (μm)	435 ± 13	402 ± 15	409 ± 7

Primordial, primary, preantral and antral follicles were counted in ovaries from unprimed mice, whereas preovulatory follicles were counted and measured in ovaries from mice primed with 5 IU of eCG. The percent of follicle types in unprimed ovaries are shown in parentheses. Data are presented as the mean ± SEM from n = 6 ovaries per group. No statistically significant differences were observed among the groups, according to ANOVA.

3.4. Effects of BPA on circulating LH, FSH and estradiol levels

Elevated circulating levels of estradiol trigger releases of FSH and LH, which promote the oocyte release from preovulatory follicles (Caligioni, 2009). Hence, serum levels of estradiol, FSH and LH at the proestrus stage were compared in BPA-treated mice versus the controls (Fig. 4). The data show that the serum levels of estradiol and FSH were not significantly different in the BPA- or DES-treated females compared to the control females (Figs. 4A and 4B). In addition, the serum levels of LH were not significantly different in the BPA-treated females, but they were significantly reduced in the DES-treated females compared to the control females (Fig. 4C).

3.5. Effects of BPA on the fertilizing ability of the oocyte.

To evaluate whether BPA exposure impairs the fertilizing ability of the oocyte, mating as well as *in vitro* fertilization assays were performed with both CEO and CFO (Fig. 5). A detrimental effect of BPA on fertilization was expected to increase the frequency of unfertilized eggs. As shown in Fig. 5, BPA exposure significantly reduced the frequencies of fertilized oocytes in both fertilization models.

To evaluate the effects of BPA on early zygote development stages, the cell division rate at 48 h after natural mating was determined. An effect of BPA on early zygote development was expected to reduce the frequency of each zygotic stage as compared to control females. Although there was a significant decrease in the percent of 4-cell and 8-cell zygotes compared to the 2-cell zygotes in the BPA-treated females, the rates for zygote development to the 2-cell, 4-cell and 8-cell stages were not significantly different in the BPA- or DES-treated females compared to the control females (Table 2).

4. Discussion

The current study provides evidence that oral dosing at the referenced safe exposure limit of BPA (50 μg/kg bw/day) during the young adult stage alters the fertilizing ability of oocytes without affecting early zygote development to the 8-cell stage. Because BPA may target the ovaries, the exposure windows in our study spanned the first reproductive cycles to be consistent with human exposure when ovarian function begins. In an attempt to characterize the mechanisms underlying the effect of BPA toxicity on ovulation, our study examined several endpoints, including folliculogenesis, the number of corpora lutea and eggs shed to the oviduct, ovarian gonadotropin responsiveness, circulating hormone levels and estrous cycles. We found that BPA exposure did not significantly alter any parameters related to ovulation, suggesting that BPA (50 μg/kg bw/day) exposure during the young adult stage is safe for immediate ovulatory cycles. We cannot exclude the possibility that ovulatory defects could take somewhat longer to manifest because previous studies reporting detrimental effects of BPA on the number of corpora lutea have indicated that the window of vulnerability to BPA extends beyond the exposure period. Specifically, mice exposed prenatally

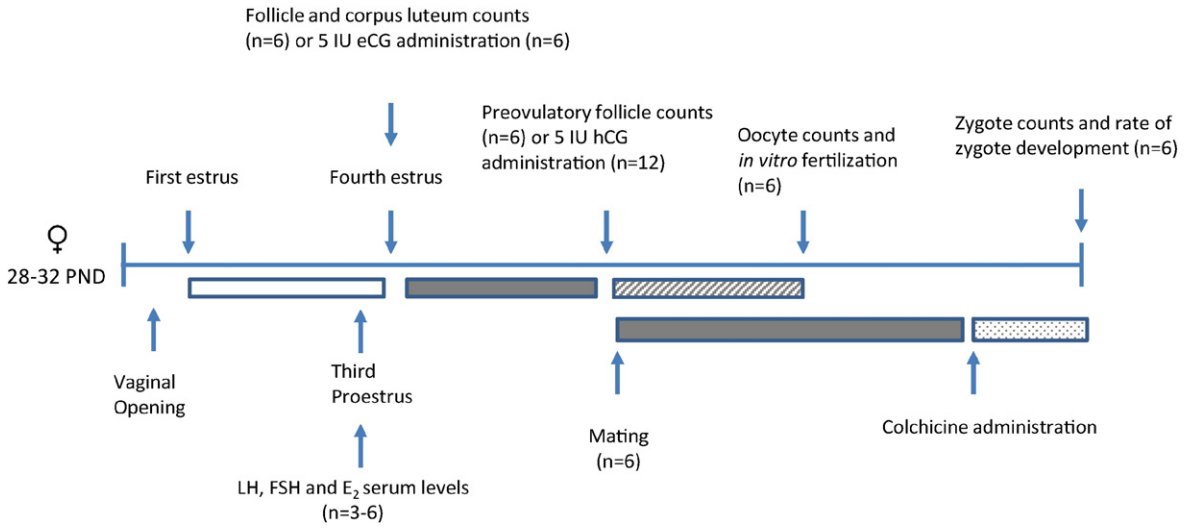

Fig. 1. Experimental design. Open box indicates daily dosing (vehicle, BPA or DES) and estrous cyclicity for three estrous cycles; filled boxes indicate a 48 h period; dashed box indicates a 14-16 h period; dotted box indicates a 5 h period. Note: twelve females per dose group were administered with hCG to be assigned into subgroups for *in vitro* fertilization (n = 6 mice per dose group) and mating (n = 6 mice per dose group).

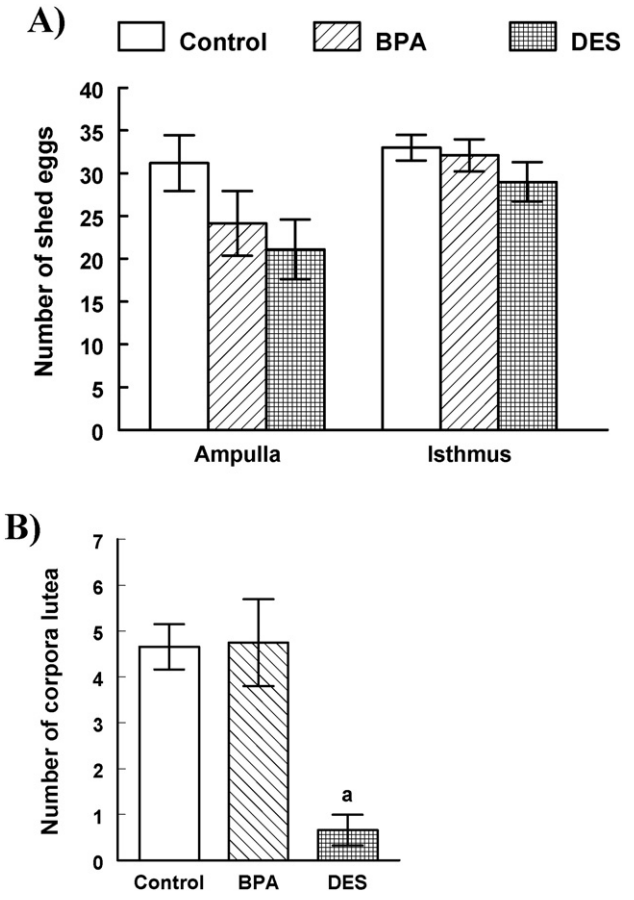

Fig. 2. Effects of 50 μg/kg bw/day BPA on the numbers of shed eggs and corpora lutea. Panel A: At 24 h after exposure, the mice at estrus were *i.p.* injected with 5 IU of eCG, and 48 h later, the mice were *i.p.* injected with 5 IU of hCG. Eggs were collected from the ampulla and isthmus portions and then counted. Panel B: At 24 h after exposure, ovaries from unprimed mice at estrus stage were collected and then subjected to histological evaluation. Corpora lutea were counted in one ovary per mouse. Each bar in the graphs represents the mean ± SEM from 6 mice per group. The letter "a" above the bars indicates a significant difference ($p \leq 0.05$) from the control based on one-way ANOVA followed by the Dunnett's *post hoc* test.

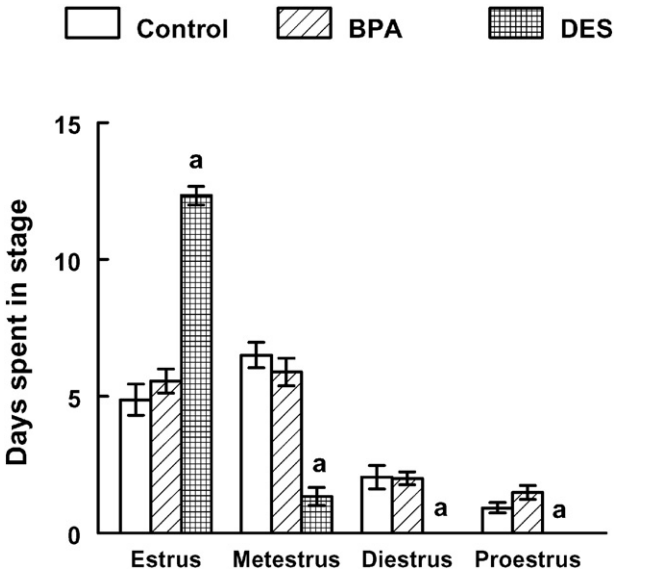

Fig. 3. Impact of 50 μg/kg bw/day BPA on estrous cyclicity. Fresh vaginal swabs were obtained daily to determine the estrous cycle stage, and the duration for which the mice remained in each phase was averaged per dose group and reported. Each bar in the graph represents the mean ± SE from 12 mice per group. The letter "a" above the bars indicates a significant difference ($p \leq 0.05$) from the controls, based on one-way ANOVA followed by the Dunnett's *post hoc* test.

[10 mg/kg bw/day BPA; subcutaneous injection (sc)] on gestational days (GD) 10–18 (Suzuki et al., 2002) and rats exposed postnatally [50 mg/kg bw/day or 4 mg/pup BPA (sc)] at the first days of life (PND 0–9) have been shown to develop ovulatory defects, as evidenced by a decreased number of corpora lutea when the animals reach puberty (Adewale et al., 2009; Kato et al., 2003). Thus, future studies should examine whether BPA exposure at a dose of 50 μg/kg bw/day, administered during the early reproductive period, affects the parameters related to ovulation later in the reproductive period.

In the present study, BPA exposure did not alter the estrous cycle. Although some experimental models have shown mixed effects of BPA on ovarian cyclicity (reviewed in Caserta et al., 2014; Peretz et al., 2014), data similar to those found in our study were observed in a study of *in utero* exposure to 50 μg/kg bw/day BPA (Wang et al., 2014). In mice, the estrus and metestrus stages correspond to the ovarian phase in which the growing pool of recruited preantral and antral follicles is stimulated by both FSH and LH to synthesize estradiol, which promotes their own preovulatory follicle growth. The diestrus stage corresponds to the ovarian phase in which estradiol levels start to increase, peaking during the proestrus stage. Consequently, oocyte expulsion from preovulatory follicles occurs due to the slow release of LH from the pituitary gland (Caligioni, 2009). Our estrous cyclicity data indicate no changes in hormone levels and no changes in the number of preantral, antral or preovulatory follicles in BPA-treated females compared to control females. However, other studies have shown that BPA exposure (4.4–440 μM or 1–100 μg/ml) reduces antral follicle growth and estradiol levels in in vitro cultures (Peretz et al., 2012; Peretz et al., 2011; Ziv-Gal et al., 2013) and reduces the number of ovarian antral follicles in perinatally exposed mice (Gámez et al., 2015). In addition, our data did not indicate a reduced primordial follicle pool, which has been previously reported in mice, rats and lambs perinatally exposed to low doses of BPA (Peretz et al., 2014; Rivera et al., 2011; Rodriguez et al., 2010; Wang et al., 2014). From the fetal stage to the prepubertal stage, the size of the primordial follicle pool drops drastically through programmed cell death (i.e., atresia) (McGee and Hsueh, 2000), and BPA has been shown to reduce follicle growth by accelerating follicular atresia (Peretz et al., 2012; Rivera et al., 2011; Wang et al., 2014). Thus, our contrasting findings on late follicle growth, the primordial follicle pool and estradiol levels may be explained by the origin of the samples, including the age, experimental model and window of exposure.

Based on the reduced fertilization rates shown in the present study, we suggest that animal exposure to BPA during the first reproductive cycles may strongly influence oocyte quality in terms of the fertilization ability. Because the LH surge triggers cumulus cell expansion around the oocyte to provide a microenvironment for oocyte fertilization (Tanghe et al., 2002) and because BPA negatively impacts cumulus cell expansion (Mlynarcíková et al., 2009), we focused on determining whether the detrimental effects on oocyte fertilization occurred through the impact of BPA on cumulus cells. However, we observed reduced oocyte fertilization in BPA-treated mice, regardless of the presence or absence of cumulus cells, which suggests that BPA targets the oocyte. Due to the LH surge, oocytes resume meiosis from prophase I to metaphase II, a stage at which the oocytes remain until fertilization (Richards et al., 2002). It has been speculated that metaphase II oocytes with meiotic chromosome abnormalities cannot progress through the second division triggered by fertilization (Pacchierotti et al., 2008). Thus, the reduced fertilization competence of BPA-exposed oocytes may result from alterations in the meiotic process, as reported elsewhere (Can et al., 2005; Eichenlaub-Ritter et al., 2008; Hunt et al., 2003; Lenie et al., 2008; Machtinger et al., 2013). We cannot exclude the possibility that other intra-follicular mechanisms altered by the 3-estrous-cycle dosing BPA period may contribute to the detrimental effect on the oocyte. Although aging renders oocytes less receptive to fertilization (Hunt and Hassold, 2008), the reduced fertilization competence of

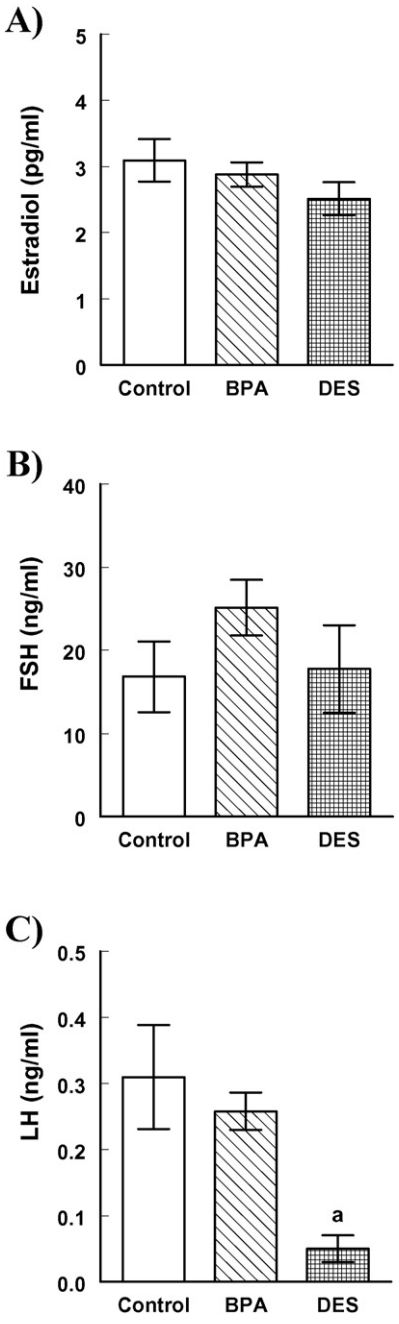

Fig. 4. Impact of 50 μg/kg bw/day BPA on circulating levels of estradiol and gonadotropin hormones. Serum samples were collected at the third proestrus to measure levels of estradiol (panel A), FSH (panel B), and LH (panel C). Each bar in the graph represents the mean ± SEM from 3 to 6 mice per group. The letter "a" above the bars indicates a significant difference ($p \leq 0.05$) from the control, based on one-way ANOVA followed by the Dunnett's *post hoc* test.

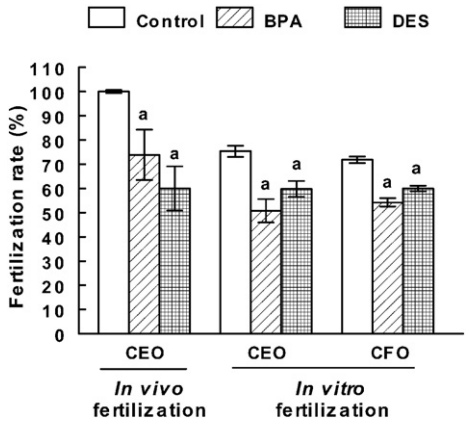

Fig. 5. Impact of 50 μg/kg bw/day BPA on in vivo and in vitro fertilization of the oocyte. Cumulus-enclosed oocytes (CEO) were collected at 14–16 h post-hCG stimulus and were then incubated with hyaluronidase to obtain cumulus-free oocytes (CFO). Both CEO and CFO were separately subjected to in vitro fertilization assays. Each bar in the graph represents the mean ± SEM from 6 mice per group. The letter "a" above the bars indicates a significant difference ($p \leq 0.05$) from the control, based on one-way ANOVA followed by the Dunnett's *post hoc* test.

oocytes observed in the present study cannot be explained by a natural process of oocyte deterioration because the fertilization experiments were performed when the BPA-treated females were still considered to be young (50–55 days of age).

We found a greatly reduced percent of 4-cell and 8-cell zygotes compared to the 2-cell zygotes in the BPA-treated females, but this finding was not statistically significant when compared to the control females. Thus, we cannot suggest that BPA alters the ability of fertilized oocytes to divide and reach the 8-cell zygote stage. Our suggestion is in contrast to previously reported studies that have shown that low BPA doses increase development to the 8-cell zygote stage, whereas high BPA doses decrease development to the blastocyst stage. Specifically, 2-cell mouse zygotes incubated in media containing 1 nM to 3 nM (0.23 μg/L to 0.68 μg/L) BPA for 2 days demonstrated increased development to the 8-cell stage, whereas 2-cell mouse zygotes incubated in media containing 100 nM (23 mg/L) BPA for 2 days demonstrated reduced development to the blastocyst stage (Takai et al., 2000). Furthermore, pregnant C57BL6 females sc exposed to 100 mg/kg bw/day BPA during the first three days of gestation, a period that is critical for embryo transport, showed delayed development to the blastocyst stage (Xiao et al., 2011). Thus, more in-depth studies of early zygote development using our study design are warranted.

The 3-estrous-cycle dosing period in our study resulted in a decreased number of corpora lutea with a concomitant decrease in LH serum levels in the unprimed DES-treated females. However, no alterations were observed in the number of shed eggs in the oviducts from primed (eCG plus hCG) females. Our data on the number of shed eggs are in agreement with other studies that have reported that exogenous gonadotropins are effective in inducing ovulation in DES-treated females (Halling and Forsberg, 1990). Future studies should examine whether DES induces spontaneous rupture of immature follicles that might result in an increased number of shed eggs to the oviduct.

In conclusion, the present study targeting the mouse ovary during the first reproductive cycles indicates that the reference dose of 50 μg/kg bw/day BPA set by the U.S. EPA compromises oocyte fertilization, without adversely impacting ovulation or early zygote development. Our findings on oocyte fertilization support the findings of human studies reporting positive associations between urinary/serum BPA concentrations and decreased fertilization rates in women

Table 2
Effects of BPA or DES on early mouse zygote development.

Zygote stage	Control (corn oil)	BPA (50 μg/kg bw/day)	DES (10 μg/kg bw/day)
2-cell zygote (%)	47.5 ± 24.6	60.3 ± 6.4	46.3 ± 21.3
4-cell zygote (%)	26.5 ± 7.8	15.2 ± 9.1[a]	27.8 ± 9.6
8-cell zygote (%)	26.0 ± 23.9	24.5 ± 13.5[a]	25.9 ± 20.6

Zygotes were flushed from the isthmus portion of each oviduct after the in vivo fertilization assay in the control and BPA- and DES-treated mice; they were then assessed for the number of blastomeres under fluorescence microscopy. Zygotes at each stage of development were calculated as the percentage of total recovered cleaved eggs. Data are presented as the mean ± SEM from three independent experiments; n = 6 mice per group. The letter "a" indicates a significant difference ($p \leq 0.05$) from the 2-cell zygotes for the BPA-treated females, according to one-way ANOVA followed by Dunnett's *post hoc* test.

undergoing *in vitro* fertilization (Ehrlich et al., 2012a; Fujimoto et al., 2011). Whether BPA exposure during the stages used in this study alters ovulation and zygote development later in life remains to be determined.

Conflicts of interest statement

The authors declare that there are no conflicts of interest. The content is solely the responsibility of the authors and does not necessarily represent the official views of the Mexican Council for Science and Technology.

Transparency document

The Transparency document associated with this article can be found, in online version.

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