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Exposure to a low dose of bisphenol A impairs pituitary-ovarian axis in prepubertal rats Effects on early folliculogenesis

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ABSTRACT

The research work studies the effect of providing a low dose of bisphenol A (BPA), on the reproductive axis of prepubertal female rats. Wistar mated rats were treated with either 0.1% ethanol or BPA in their drinking water until their offspring were weaned on the 21 day of birth. The estimated average dose of exposure to dams was approximately 3 μg/kg/day. The pups were sacrificed at the 30th day of life. Body weight at the moment of the sacrifice was significantly higher in the group exposed to BPA; ovarian weight and its relative weight were not modified. LH and estradiol levels increased significantly, meanwhile FSH ones showed no significant changes. The number of primary, secondary and atretic follicles increased and antral ones was decreased. Our results demonstrated that early exposure to a low dose of BPA disrupts the normal function of the reproductive axis in prepubertal female rats.

1. Introduction

Reproduction is a process regulated by the hypothalamic-pituitary-gonadal (HPG) axis, therefore, disruption of this system may alter its normal function. Within the HPG axis, reproductive maturation and activity are coordinated by the release of gonadotropin releasing hormone (GnRH) (Elkind-Hirsch et al., 1981; Gorski et al., 1975). Gn-RH secretion is pulsatile and its frequency varies during development, plasma LH and FSH levels increase in response to Gn-RH peaks, hence disruption in the normal pulsatile secretion may be an important factor to alter the release of gonadotropins. Moreover, estrogens change the release of the decapeptide being one of

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the factors that regulates its secretion. In this sense, it has been largely demonstrated that Gn-RH release is increased per cycle by estrogens (Kauffman et al., 2007b; Tena-Sempere, 2006) and this mechanism is particularly sensitive to disruption by hormones or hormone-like compounds. In mice prenatally exposed to several endocrine disruptors (ED), an increase in estrogen feedback as well as development of precocious puberty, has been demonstrated (Bateman and Patisaul, 2008; Kauffman et al., 2007a). Bisphenol A (BPA) is one of the most important ED highly produced and used in the industry. It is highly employed in the manufacture of polycarbonate plastic, present in the majority of food recipients, and epoxy resins present in cans, as well as in the synthesis of glues, pipes and dental sealants used in odonthology (Olea et al., 1996; Vandenberg et al., 2009). In vivo studies have suggested that BPA is a weak estrogen which effects are exerted by binding to the nuclear steroid receptors ERa and ERb although some of its effects may be exerted via nongenomic pathways (Adachi et al., 2005; Alonso-Magdalena et al., 2006; Wetherill et al., 2007). Exposure to BPA may have different outcomes depending on the stage of life during which the exposure took place. In female rats, exposure during prenatal or postnatal period, like lactation or prepubertal period, has been found to have an impact on female reproductive physiology, including the timing of pubertal onset, premature vaginal opening, irregularities in the estrous cycle, increment in the uterine weight and disruption of early oogenesis (Crain et al., 2008; Maffini et al., 2006; Rasier et al., 2006; vom Saal et al., 2007). All in all, Suzuki et al. (2002), demonstrated meiotic disturbances like an increase of ovarian tissue occupied by antral follicles and the appearance of multioocyte follicles. These results have demonstrated that BPA is also capable of altering the ovarian function. Considering these previous findings, in this research work we have tried to test whether exposure to a low dose of BPA during pre and early postnatal period adversely affects the pituitary- gonadal axis. We have investigated its effect on LH, FSH, estradiol serum levels and on early folliculogenesis.

2. Materials and methods

2.1. Animals and treatments

Wistar female rats (weighing 250-300 g) from the Department of Physiology, School of Medicine, University of Buenos Aires, were singly housed in metallic cages. The animals were housed in a temperature and light controlled environment (lights on from 07:00 to 19:00 h, T: 22–24 °C), and have had free access to a pellet diet (ACA Animal Nutrition Division, Complete balanced animal aliment, Protein 23%, fibre 6%, minerals 10%, Argentine Industry) and tap water. The diet contains soybean meal, but as all animals were exposed to the same levels of phytoestrogen the feed intake was equivalent for control and BPA treated rats. Moreover, the same lots of diet were provided to animals from both groups at the same time during the course of the study, so as to control across groups for possible variation in the content of diet. After acclimatization to the light/dark cycle for one week, the experiment has been started. Males Wistar (weighing 300-350 g) rats and female rats were co-housed (1:1) until mating was confirmed by observation of a copulatory plug. The day the mating was confirmed was recorded as gestation day (GD) 0. At this moment mated female rats were separated and housed isolated in metabolic cages. They were treated with either 0.1% ethanol (control group, n 10) or BPA (4, 4′ Isopropylidene-Diphenol, MP Biomedicals, LLC, Germany) dissolved in ethanol (BPA group, $n = 10$) in their drinking water until their offspring were weaned at 21st day of age. BPA was dissolved in 100% ethanol (Funabashi et al., 2004; Cardoso et al., 2010) at the concentration of 0.3 mg/ml and further diluted 1:10 to make a BPA concentration of 30 μg/ml. This was further diluted 1:1000 with drinking water to reach a final concentration of 30 μg/l. The estimated average dose of exposure (ADE) to dams was approximately 3 μg/kg/day (a dose that approximates BPA levels in the environment) (Akingbemi et al., 2004). 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 the following day. The assessments assume that all the water lost from the bottle was consumed. They do not account for possible leakage or evaporation of the water or for potential loss of BPA activity during the 24-h period. The actual level of BPA affecting the fetuses during gestation or that was ingested postnatally by the offspring during the period of lactation was not estimated in this study. It is very important to note that the Lowest Observed Adverse Effect Level (LOAEL) established by the U.S. EPA for BPA is 50 mg/kg/day, and the dose considered safe for human being is 50 μg/kg/day (Diamanti-Kandarakis et al., 2009). It is also important to mention that oral route of administration was chosen, intending to mimic best the most common route of human exposure to the ED. On day 21st of life, female pups ($n = 10$) were separated from the mother and housed in metal cages until their sacrifice on post natal day (PND) 30 when the different endpoints were studied. This age group of rats was used for the experiment because the prepubertal period is a time of active reproductive tract development and hormonally active chemicals are known to exhibit greater potency during sexual differentiation in rodents and humans. Animal care was carried out according to the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (Guide for the Human Care and Use of Laboratory Animals, National Research Council, National Institutes of Health, Publication No. 86-23, Washington, DC, 1985).

2.2. Effects of BPA on development

After birth, the number of newborn rats was determined, a thorough physical examination was performed and body weight was appropriately measured on days 1st, 7th, 14th, 21st and 30th of life. On sacrifice day, ovarian and body weight were determined, and relative ovarian weights were calculated ([organ weight/body weight-organ weight] × 100).

2.3. LH, FSH and estradiol determination

These studies were designed to evaluate the effect of BPA on gonadotropins and estradiol secretion. For this purpose, animals were sacrificed by decapitation and trunk blood was collected to determine serum LH, FSH and estradiol

concentrations by radioimmunoassay and electro quimioluminiscence methods. LH and FSH serum concentrations were measured in duplicate using a double antibody radioimmunoassay (RIA). The material for that assay was provided by the NIAMDD Rat Pituitary Program. Specificity of the antiserum, in terms of its reactivity in RIA with anterior pituitary hormones other than LH and FSH, was challenged with high purified preparations of rGH, rFSH, rTSH and rPRL. Intra and inter assay coefficient of variation for LH and FSH were 8% and 10%, respectively. Values were expressed as ng/ml in terms of the reference preparation (rat LH-IRP 1 and rat FSH-IRP 1). Estradiol serum concentrations were measured by a competitive immuno assay provided by VITROS (Immuno diagnostic Products Estradiol Reagent Pack, Ortho Clinical Diagnostics by Johnson & Johnson Company). Intra and inter assay coefficients of variation were 3.1% and 7.0%, respectively. Values were expressed as pg/ml.

2.4. *Histological studies*

After dissection ovaries were fixed during 24 h in Bouin fluid. Samples were then dehydrated using increasing concentrations of ethanol, cleared in xylene and embedded for 8 h in Paraplast (Fisherbrand, Fisher, WA, USA). Transversal sections of 7 μm were mounted in charged slides (Fisherbrand Superfrost/plus, Fisher, WA, USA) and stained with the Masson Trichromic technique. Finally, sections were examined and digitally photographed with a Nikon microphot Fx (Nikon, Coolpix 4500).

2.5. *Quantification*

Growing follicles, consisting in primary, secondary and antral (Graafian) follicles, as well as atretic follicles were quantified in randomly chosen 7 μm sections of each ovary taken from three different areas within the organ, and then obtained an average for each sample expressed in number of follicles per section. We considered as primary follicles to those which were surrounded by one layer of follicular cells (cuboidal in shape), secondary follicles to those which were surrounded by 2–8 follicular cells layers (granulose cells), antral (Graafian) follicles to those with one single large antrum of follicular fluid. Atretic follicles were considered when they showed oocyte and granulose cells in degeneration and the presence of a glassy membrane resulting of thickening of the basement membrane between the granulose and theca layer.

In the pool of preantral follicles (primary and secondary), only those follicles in which the oocyte nucleolus was present were scored. The majority of Graafian follicles did not contain an oocyte in the sections examined. Therefore, they were scored using the antrum as a marker.

2.6. *Statistical analysis*

Results are expressed as the mean ± S.E.M. The differences between the means of the two experimental groups were calculated by Student's "t" test. $p < 0.05$ was considered significant.

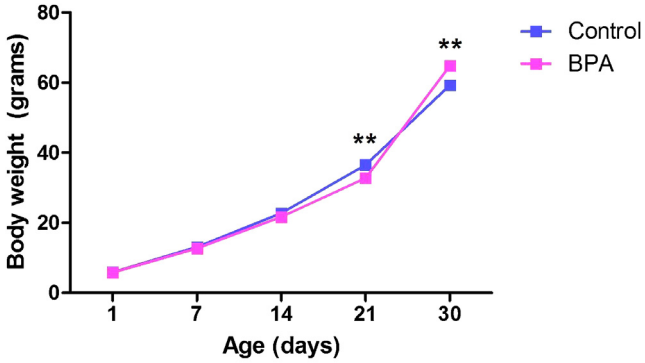

Fig. 1 – Body weight since the day of birth until the day of sacrifice (days 1, 7, 14, 21 and 35). **$p < 0.01$ vs. control.

3. Results

Body weight was regularly determined since the day of birth until the day of sacrifice (days 1, 7, 14, 21 and 35). Animals exposed to BPA exhibited a significant decrease in their body weight at PND 21, and a significant increase at PND 30 (Fig. 1, $p < 0.01$ vs. control). Table 1 describes body, ovarian weight and relative ovarian weight, absolute and relative ovarian weight were not modified by exposure to BPA. Fig. 2 also describes LH serum levels increased significantly in treated animals ($p < 0.05$), meanwhile FSH was not modified. Fig. 2 also depicted estradiol serum levels which were also increased by treatment ($p < 0.01$). In order to compare and evaluate the differences in follicular populations between control ovaries and BPA treated animals the average number of follicles per section was used. Fig. 3 shows a significantly increase in the total number of follicles in development in animals exposed to BPA ($p < 0.01$). The number of primary and secondary follicles was higher in BPA treated animals ($p < 0.05$, $p < 0.01$), meanwhile, the number of antral follicles were lower in this group ($p < 0.01$). In coincidence with the mayor follicular recruitment, atretic follicles were increased significantly in the ovaries of the animals exposed to BPA ($p < 0.05$). Fig. 4 shows histological sections of the ovaries of control and exposed animals to BPA. Fig. 5 shows a microphotograph of an atretic follicle.

4. Discussion

Early exposure to an ED, like fetal life and/or suckling period, has a priming effect on the hypothalamus pituitary axis. It was suggested in some rats studies that exposure to ambiental disruptors produces changes in gonadotropins and steroids

Table 1 – Body and ovarian weight and relative weights.

	Control	BPA
Body weight (g)	59.3 ± 0.8	64.8 ± 0.6***
Ovarian weight (g)	0.0170 ± 0.0009	0.0160 ± 0.0007
Relative ovarian weight	0.0260 ± 0.0007	0.0270 ± 0.0006

*** $p < 0.001$ vs. control.

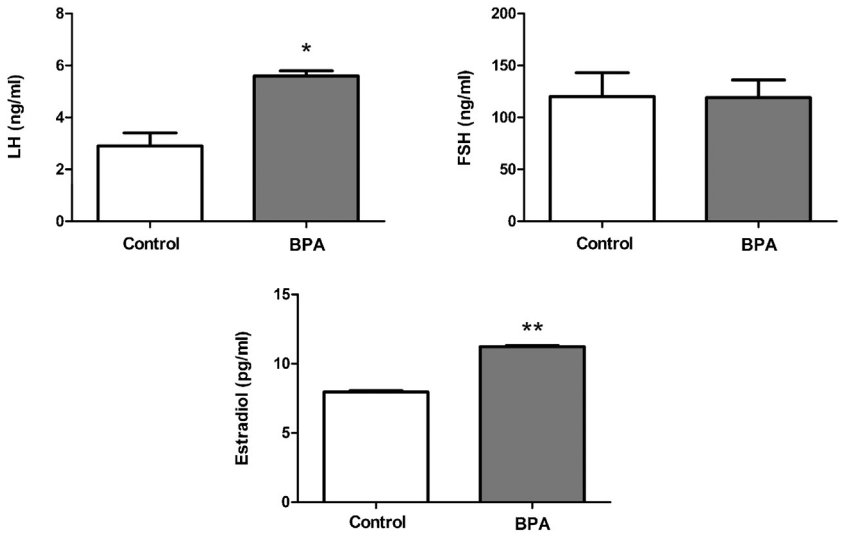

Fig. 2 – LH, FSH and estradiol serum levels. Each column represents the mean ± SEM ($n = 6–11$), $^{*}p < 0.05$, $^{**}p < 0.01$, vs. control.

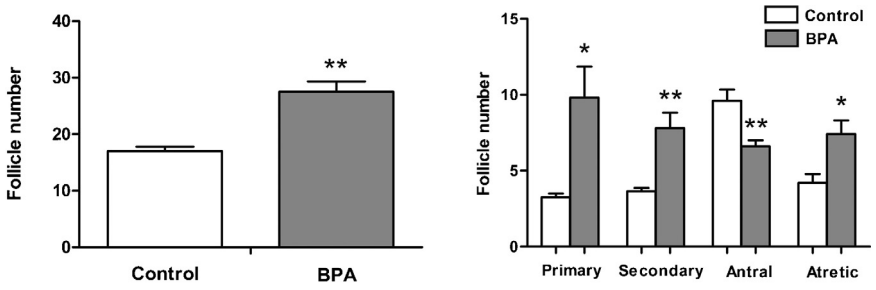

Fig. 3 – Number (mean ± SEM) of growing follicles (primary, secondary, antral) and atretic follicles in ovarian sections of control and BPA treated animals. $^{*}p < 0.05$, $^{**}p < 0.01$.

serum levels. These interferences may be due to their effect on hypothalamic neuronal or pituitary gland cells (Mostafa et al., 2007). As it is mentioned above, reproductive function is coordinated by GnRH pulsatile release which frequency varies during development, leading to an increase of gonadotropins levels. Therefore, it is reasonable to think that gonadotropins secretion may be affected by many factors altering Gn-RH pulsatile release. In the present study, we showed that exposure to a low dose of BPA during gestation and lactation periods affects the normal function of the pituitary-gonadal axis in prepubertal female rats. The parameters of development analyzed, show a significant decrease in body weight of treatment animals at PND 21, together with a significant increase of this parameter at PND 30. Other authors have previously reported variations in body weight, being the outcomes controversial. Kwon et al. (2000) observed no changes in body weight of rats of the strain Sprague-Dawley exposed to high doses of BPA, administered orally, during gestation and lactation period.

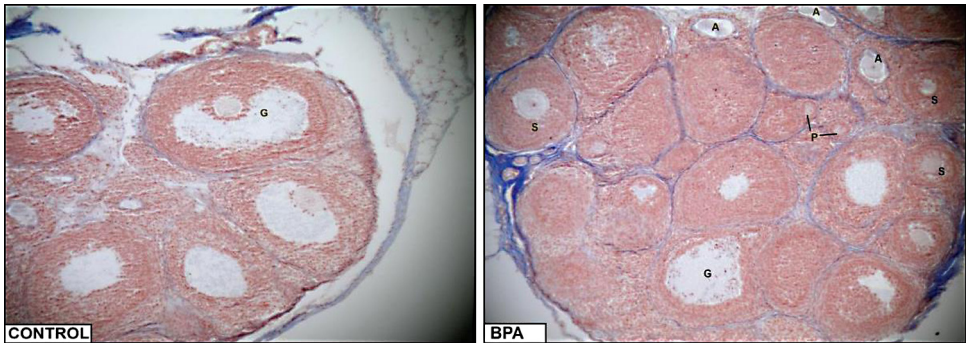

Fig. 4 – Microphotograph of ovarian sections of control and BPA treated animals, showing growing follicles - primary (P); secondary (S); Graafian (G)- and atretic follicles (A). Zoom: 100×.

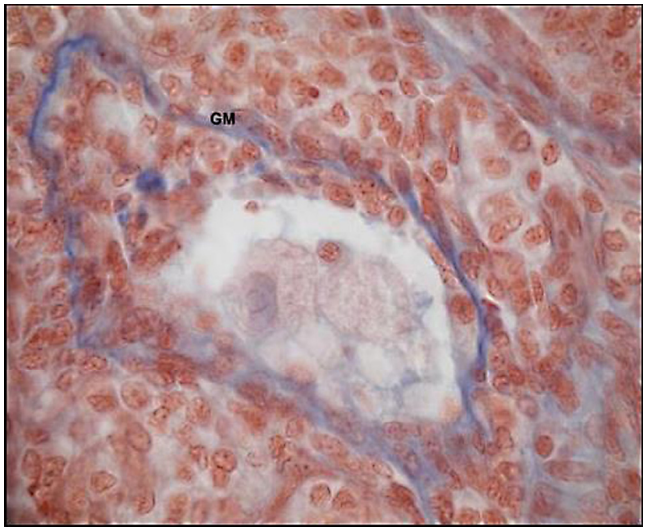

Fig. 5 – Microphotograph of an atretic follicle showing degeneration of oocyte and granulose cells and thickening of the basement membrane between the granulose and theca layer (glassy membrane, GM). Zoom: 600×.

Rubin et al. (2001) reported a rise in body weight of female offspring of mothers exposure to BPA administered in drinking water from day 6 of pregnancy and during lactation period. The different results obtained by the groups may be associated with the time of exposure, dose, via of administration, or to the animal strains used, as it has been previously reported (Watanabe et al., 2003). The fact that exposure to BPA produces an increase in body weight at PND 30 in our animals, could be related to its action on adipocyte. Metabolism of adipose tissue is known to be modulated by steroid hormones, and could therefore be affected by EDs. In vitro studies with human cell lineages have demonstrated that BPA stimulates lipid storage (Wada et al., 2007). Moreover, Masuno et al. (2002) showed that BPA enhance adipocyte differentiation and lipid accumulation in target cells in a dose dependent manner.

Data from Phrakonkham et al. (2008) indicates that BPA increase gene expression of adipogenic transcription factors in 3T3-L1 preadipocytes.

BPA exposed animals showed no changes in ovarian weight and their respective relative weight. Rivera et al. (2011) reported an important decrease in ovarian weight of prepubertal lambs neonataly exposed to BPA, in contrast with Evans et al. (2004) findings who showed no changes in ovarian weight of lambs exposed to BPA from PND 30 until PND 50. Moreover, studies of Rodriguez et al. (2010), showed small ovarian size of rats treated with estradiol valerato in contrast with control group. These discrepancies in the results may also be explained by considering the animal strains used, dose, time of exposure and via of administration. Hormone analysis showed that LH and estradiol serum levels were increased significantly in animals exposed to the ED, meanwhile FSH ones were not modified. These changes in gonadotropins may reflect an anomalous function of Gn-RH generator pulse. In this sense, Fernández et al. (2010) demonstrated that exposure to BPA increased Gn-RH pulse frequency leading to an increase in LH levels. It has also been reported that exposure to BPA during early stages of development increases the sensitivity of the pituitary to Gn-RH action and up regulates ER β at the hypothalamic levels. Altogether these effects may be responsible of increased LH serum levels observed in exposed animals.

Increase in Gn-RH pulse frequency after BPA exposure may also explain the unaltered FSH secretion. It has been found a differential regulation of gonadotropin subunit gene expression by Gn-RH, high frequency pulses of the decapeptide induce a selective increase of the ARNm of LH-β chain, without any effect on ARNm of FSH-β, leading to higher levels of LH than FSH (Weiss et al., 1993; Kaiser et al., 1995). In our experiment, estradiol serum levels were increased significantly in the group of animals exposed to BPA. Several studies have shown different effects on ovarian steroidogenesis. Peretz et al. (2011) show that in mice BPA (44-440 μM) inhibits growth of antral follicles in vitro and reduces production of estradiol and expression of the enzymes of steroidogenesis (Peretz et al., 2011). Conversely, Grasselli et al. (2010), demonstrated that in cultured porcine granulosa cells a low dose of BPA (0.1 μM) increased estradiol levels, meanwhile high doses ranging from 1 to 100 μM decreased its production. The controversy in results may be related to differences in experimental approaches such as, species, doses and length of exposure. It is important to consider that the elevated estradiol levels observed in exposed animals, may be another factor which contributes to an anomalous secretion of Gn-RH, as it is observed in PCOS, and may also explain the inappropriate secretion of gonadotropins which we have observed and registered. Considering these previous findings it may not be discarded, that an increase in Gn-RH/LH pulsatility could be a cause and/or consequence of alterations in sex hormone levels. Another finding in our work has been that animals exposed to the disruptor, have shown more primary, secondary, and atretic follicles, and fewer antral ones compared to the control group.

According to difference sources, in the embryonic rodent ovary the oocytes are arranged in clusters named nests. After birth, selected oocytes undergo atresia and surviving ones become surrounded by squamous pregranulosa cells forming the primordial follicle. This primordial follicle pool is very important for fertility because it represents the total population of oocytes available to a female during her reproductive lifetime (Kesele and Skinner, 2003). This process named follicular assembly, is completed within 4 days of postnatal rodent development and is completely gonadotropin independent (Pepling et al., 2006) Moreover, it has been stated that in rodents, the high levels of estradiol and progesterone during pregnancy and their drops after birth may regulate follicular development in the neonate. The hypothesis of the authors is that high levels of maternal and fetal estradiol and progesterone retard early follicle development and that the decline after birth allows primordial follicle assembly and development to be initiated (Weisz and Ward, 1980; Thau et al., 1976).

Considering previous data and taking into account estrogen BPA effect, it may be speculated that during gestational period it may retard follicular development. Later in the newborn animals, the number of primary and secondary follicles increased under BPA exposure. This event may be explained by an increase in initial recruitment of primordial

follicles and their transition to primary follicles, as has been previously demonstrated. Studies of Rodriguez et al. (2010), suggest that BPA could stimulate proliferation of granulose cells by acting through ERβ and/or ERα in the ovary. Moreover, they demonstrated that BPA stimulates early folliculogenesis by increasing the proliferation rate of ERβ and ERα expression in granulose cells of primary, transitional and preantral follicles in the rat. In our experiment, we have observed that BPA exposure decreased the number of antral follicles by interfering with their development and increased the number of atretic follicles.

The transition of the follicle from the preantral (secondary) to early antral stage is the "penultimate" stage of development in terms of gonadotropin dependence and follicle destiny, growth or atresia. Follicles selected for further development receive gonadotropic and intra-ovarian regulatory signals for survival, being follicular atresia a consequence of inadequate growth (Orisaka et al., 2006). Follicular growth during the preantral and early antral transition is regulated by granulosa-theca cell interactions. In this process, both FSH and LH are necessary for ovarian follicular maturation and the synthesis of ovarian steroid hormones. LH promotes the production of androgens (dehydroepiandrosterone, androstenedione, and testosterone) from cholesterol and pregnenolone, by stimulating 17α-hydroxylase activity in the thecal cells. The androgens then diffuse to the granulosa cells where FSH stimulates the expression of the cytochrome P450 aromatase, which converts the androgens to estrogens (Hillier and van den Boogaard, 1980). These findings, suggest that abnormal gonadotropins levels may alter these phases of follicular development. In our experiment, the unaltered FSH levels observed in animals exposed to BPA accompanied by elevated LH ones may impair the normal follicle development. LH may stimulate androgen synthesis by theca cells which may be converted in estradiol by granulose cell under FSH stimulation. However, under the influence of constant FSH levels follicular growth may be continuously stimulated but not to the point of full maturation as has been previously reported (Fauser, 1994).

In relation with follicular growth and atresia, Rivera et al. (2011), demonstrated that BPA administration on PND 14 to lambs stimulated follicular development and increased follicular atresia at PND 30, suggesting that in the prepubertal stage an accelerated folliculogenesis may lead to an increased follicular atresia Our results, demonstrated that exposure to a low dose of BPA can adversely affect the normal function of the pituitary-gonadal axis in prepubertal female rats. We speculated that the pattern of gonadotropins and estradiol levels accompanied by an altered folliculogenesis observed in exposed animals may lead to reproductive dysfunctions such as PCOS in adult ones. Further studies are necessary to shed light over conclusions about BPA effect on reproductive failures in adulthood.

Conflict of interest

The authors state that they have no financial or personal relationship with people or organizations that could have inappropriately influenced their work.

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