Low dose of bisphenol A impairs the reproductive axis of prepuberal male rats

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Abstract The objective of the present work was to study the effect of a low dose of bisphenol A (BPA), on the reproductive axis of prepuberal male rats exposed to the endocrine disruptor (ED) during gestation and lactation period. Wistar-mated rats were treated with either 0.1 % ethanol or BPA in their drinking water until their offspring were weaned at the age of 21 days. The estimated average dose of exposure to dams was approximately 3 μg/kg/day of BPA. The pups were sacrificed on the 35th day of life. Body weight was measured during the development and at the moment of the sacrifice; testicular and seminal vesicles weight and their respective relative weights were also measured. LH, FSH and testosterone were determined and histological studies of testicular tissue were also performed. Body weight at the moment of the sacrifice was significantly higher in the group exposed to BPA; testicular weight decreased significantly; seminal vesicles weight and relative weights of testes and seminal vesicles were not modified by treatment. LH and FSH serum levels increased significantly after treatment, meanwhile testosterone showed no significant changes. Histological studies showed the lumen of seminal tubes reduced by the presence of immature cells of the spermatic lineage. Our results suggest that pre- and early postnatal exposure to a low dose of BPA disrupts the normal function of the reproductive axis in prepuberal male rats. The effects of the ED may be exerted at different levels of the axis and may be dependent on the dose, manner of administration, and the moment of exposure to the disruptor.

Keywords Bisphenol A · Reproductive axis · Prepuberal male rats

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Introduction

Endocrine disruptors (EDs) have been defined by the US Environmental Protection Agency as:

> exogenous agents that interferes with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body responsible for the maintenance of homeostasis, reproduction and development and/or behaviour [15].

Available data suggests that EDs have the ability to impact on different levels of the reproductive axis (hypothalamus, pituitary, and gonads). It is currently

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considered that the mechanisms of development and setting of the axis during fetal period may comprise the window of vulnerability to permanently affect the activity of this endocrine axis. Prenatal and early postnatal period seem to be the most vulnerable to this exposure, since it is during these stages when organs and neural systems suffer most rapid changes. The organization of neuroendocrine control of the reproductive axis is not completed at birth, thus during the neonatal period it remains sensitive to the influence of steroids or EDs. Puberty and peri-menopausal period are vulnerable to disruption as well, due to the alterations in serum hormone levels that feature these stages [20].

Bisphenol A (BPA) is an ED used 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 odontology [22, 30]. Previous studies have shown that BPA interferes with the activity of endogenous estrogens by disrupting the proper activity of their nuclear hormone receptors in a diverse set of target tissues. Some of its effects are exerted by binding to the nuclear steroid receptors ERα and ERβ, leading to estrogen receptor (ER)–nuclear signaling pathways that modify estrogen-responsive gene expression [1, 3, 12, 13]. However, it has been shown that BPA can act via nongenomic receptors to activate cell-signaling pathways at very low concentrations [33]. BPA has also been attributed to have antiandrogenic properties, for its binding to androgen receptor (AR), inhibits its interaction with steroid hormones, nuclear translocation of the receptor itself and interaction of the AR with its coactivator [17]. Ramos et al. [25] have also demonstrated that exposure to BPA during pregnancy leads to lower expression of the androgen receptor.

Several studies show a remarkable increase in reproductive diseases, both in animals and human beings, which could be associated with the increasing number of EDs present in our environment. The main effects on male fertility by exposure to these ED are: reduction in the quality of the sperm, high incidence of cryptorchidism and hypospadias, and major incidence of testicular and prostate cancer, among others [5, 7].

Previous reports showed that exposure of male rats to a dose of BPA of 2.4 μg/kg/day between postnatal days 21 and 35 reduces serum levels of luteinizing hormone (LH) and testosterone. This was also associated with an increase in testicular size, reduction in the epididymal weight, and in the daily sperm production [2]. Moreover, it has been recently reported that low dose of BPA impairs spermatogenesis by suppressing reproductive hormone production and promoting germ cell apoptosis in adult male rats [14].

Considering these findings into account, the aim of this work was to study the effects of a low dose of BPA (3 μg/kg/day) on the reproductive axis of prepuberal male rats exposed to the ED during pregnancy and lactation period.

Material and methods

Animals and treatment

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. Animals were housed in a light and temperature controlled environment (lights on from 0700 to 1900 h, *T*: 22–24 °C), and had free access to a pellet diet (ACA Animal Nutrition Division, Complete balanced animal aliment, protein 23 %, fiber 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 1 week, the experiment was started. Male Wistar rats (weighing 300–350 g) and female rats were co-housed (1:1) until mating was confirmed by observation of a copulatory plug. The day that mating was confirmed was recorded as gestation day (GD0). At this moment, mated female rats were separated and singly housed in metallic cages.

BPA was dissolved in 100 % ethanol [11] 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 to 1:1,000 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) [13]. 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 USEPA for BPA is 50 mg/kg/day, and the dose considered safe for human being is 50 μg/kg/day [10]. 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 the 21st day of life, the male pups (n=10) were separated from the mother and housed in metal cages until their sacrifice on post natal day (PND35) when the different endpoints were studied. This age group of rats was used for the experiment because the prepuberal 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 [8].

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).

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. At the day of the sacrifice, testis, seminal vesicle (SV) weight and body weight were determined, and relative weights of each organ were calculated ([organ weight/body weight −organ weight]×100).

Effects of BPA on pituitary gland and reproductive organs

In order to assess the effects of BPA on gonadotropins and on testosterone secretion, trunk blood samples were taken during the light phase 0900–1000 after decapitation to determined LH, FSH, and testosterone serum levels. Moreover, histological studies of testis were performed.

Measurement of LH, FSH and sexual steroid hormones

LH serum concentration was measured in duplicate using a double antibody radio immunoassay (RIA). The material for the assay was provided by the NIAMDD Rat Pituitary Program. Intra- and inter-assay coefficients of variation were 8 and 10 %, respectively. Values were expressed as nanograms per milliliter in terms of the reference preparation (rat LH-IRP 1).

FSH serum levels were measured in duplicate using a double antibody RIA. The material for the assay was provided by the NIAMDD Rat Pituitary Program. Intra- and inter-assay coefficient of variation were 8 and 10 %, respectively. Values were expressed as nanograms per milliliter in terms of the reference preparation (rat FSH-IRP 1).

Testosterone serum levels were measured by a competitive immunoassay provided by VITROS (Immuno diagnostic Products Testosterone 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 nanomol per liter.

Histological studies

After dissection, testes were fixed by immersion in Bouin's fluid for 24 h. Afterwards, samples were sequentially dehydrated, using increasing concentrations of ethanol, cleared in xylene, and finally embedded in Paraplast (Fisherbrand, Fisher, WA, USA). Transversal sections (7 μm) were mounted on charged slides (Fisherbrand Superfrost/Plus, Fisher WA) and stained with the Masson trichromic staining technique. Sections were examined with a NIKON microphot FX microscope and digitally photographed (Nikon, Coolpix 4500).

Statistical analysis

The differences between means of the two experimental groups were calculated by Student's *t* test. A value of $p<0.05$ was considered statistically significant.

Results

Effects of Bisphenol A on development

1. Body weight measurements

   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 1, 7, 14, and 21, and a significant increase at PND 35 (Fig. 1, $p<0.01$; $p<0.001$; $p<0.0001$ vs. control).
2. Testicular weight, seminal vesicle weight, and relative weights

   Testes and SV were weighted after the sacrifice, and relative weight (RW) was calculated ([organ weight/body weight−organ weight]×100), therefore relating the weight of the tissue to the animal body weight. A statistically significant reduction in testicular weight was observed; meanwhile, seminal vesicles' weight was not modify by treatment. No variation was detected among the correspondent relative weights. Body weight at the moment of the sacrifice (PND 35), as it was previously mentioned, showed a significant increase in animals treated with BPA as compared to those from the control group (Table 1) ($p<0.01$; $p<0.00$ vs. control).

Table 1 Body, testicular and seminal vesicle weight, and relative weights

	Control	BPA
Body weight (g)	83.3±1.0	105.5±2.6***
Testicular weight (g)	0.358±0.007	0.304±0.007 **
Seminal vesicle weight (g)	0.050±0.005	0.040±0.006
Relative testicular weight	0.363±0.008	0.339±0.004
Relative seminal vesicle weight	0.058±0.007	0.049±0.004

**$p<0.01$ ***$p<0.001$vs control

Effects of Bisphenol A on the pituitary gland and reproductive organs

Effects of Bisphenol A at the pituitary and testicular levels

1. LH, FSH, and testosterone serum levels

   LH and FSH serum levels increased significantly among animals exposed to BPA, meanwhile testosterone one were not modify (Fig. 2), $p<0.05$ vs. control.
2. Histological studies

   Histological studies showed that spermatogenesis was not altered since control and treated males showed the same stages of spermatogenesis (spermatogonia, spermatocytes I and II). Close inspection of several seminiferous tubules showed the lumen reduced by the presence of immature cells of the spermatic lineage (Fig. 3). The obtained results were clearly consistent between all analyzed specimens

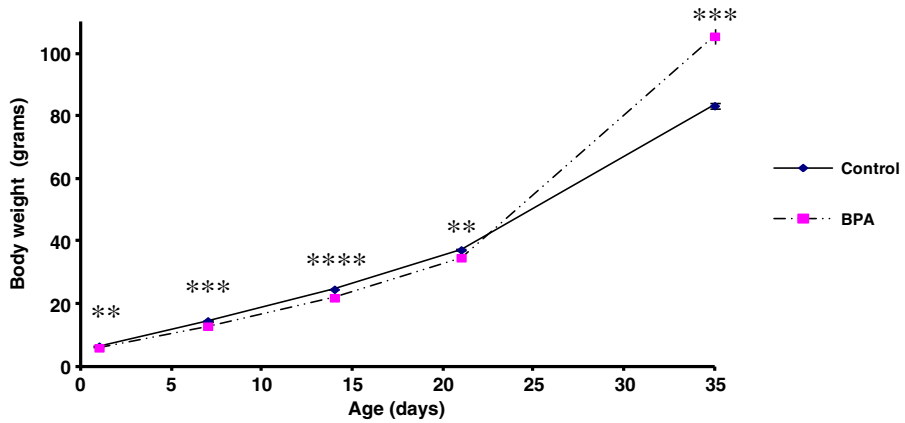

Fig. 1 Body weight since the day of birth until the day of sacrifice (days 1, 7, 14, 21, and 35). **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ vs control

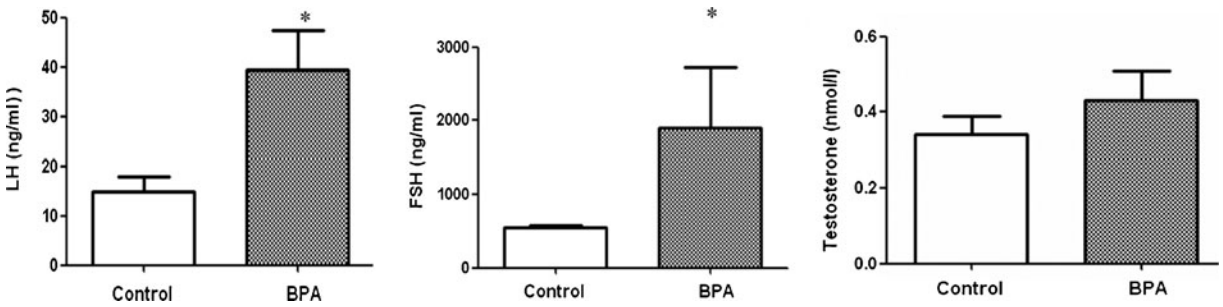

Fig. 2 LH, FSH, and testosterone serum levels. Each column represents the mean±SEM, (n=6–11), *p<0.05 vs control

Discussion

The processes and regulation of male reproductive development are under the control of several mechanisms which begins to operate during fetal life. These mechanisms are sensitive to endogenous and exogenous factors, among which are the EDs, therefore, exposure to them during pre- and early postnatal life may disrupt the normal development of the hypothalamic–pituitary–gonadal axis (HPG).

The data of the present study demonstrated that exposure to low dose of BPA during the mentioned periods, modifies the activity of the HPG axis in prepuberal male rats. The parameters of development analyzed, show a significant decrease in body weight of treatment animals at birth and at PND 7, 14, and 21,

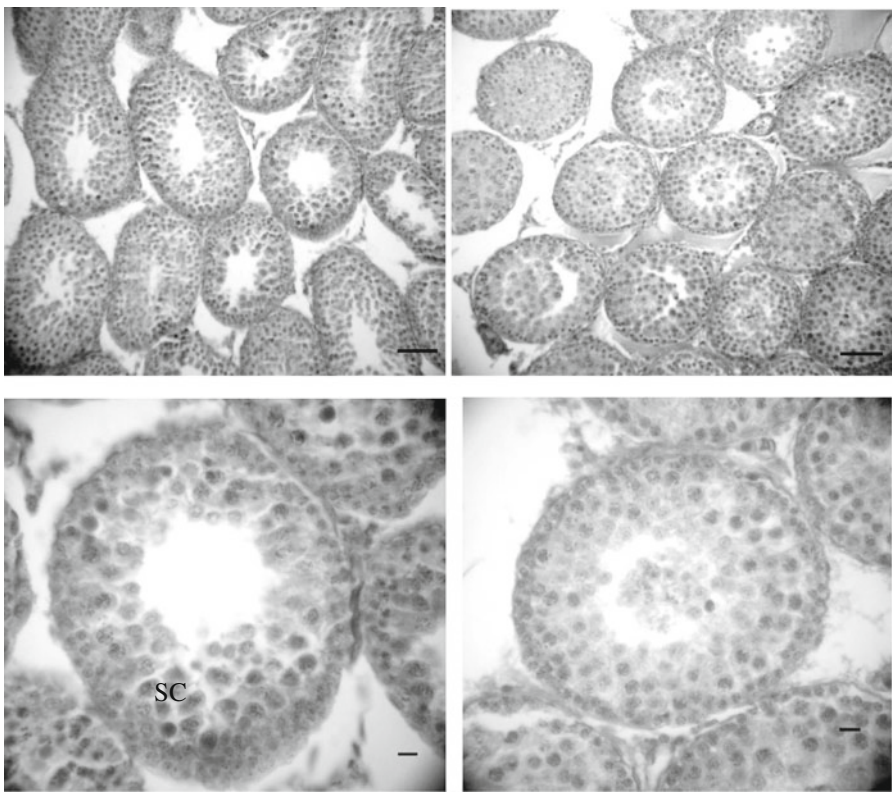

Fig. 3 Transversal section of seminiferous tubule from prepuberal control and exposed to BPA rats. The last shows the lumen of seminal tubes reduced by the presence of immature cells of the spermatic lineage. *SC* Sertoli. *Cells bars*: **a**, **b**=100 μm; **c**, **d**=10 μm

together with a significant increase of this parameter at PND 35. Other authors have previously reported variations in body weight, with the outcomes being controversial. Kwon et al. [16] observed no changes in body weight of rats of the strain Sprague–Dawley exposed to high doses of BPA, administered orally, during the gestation and lactation periods. Rubin et al. [26] 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, an experimental design similar to the one chosen by us. The different results obtained by the different 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 [32]. The fact that exposure to BPA produces an increase in body weight at PND 35 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 this sense, in vitro performed studies with human cell lineages have demonstrated that BPA stimulates lipid storage [31]. It has also been reported that exposure to BPA during peri- and postnatal periods increments the mass of adipose tissue and cholesterol serum levels in mice [19]. Masuno et al. showed that BPA enhance adipocyte differentiation and lipid accumulation in target cells in a dose-dependent manner [18]. Recently, data from Phrakonkham et al. indicates that BPA increase gene expression of adipogenic transcription factors in 3T3-L1 preadipocytes [24].

Absolute seminal vesicles weight and the respective relative weight were not modified by exposure to BPA, whereas absolute testicular weight decreased significantly without changes in relative ones.

The maintenance of seminal vesicle function is known to be androgen-dependent [2], therefore it would be expected that changes in androgens levels may be traduced in modifications in the accessory sex gland weight. In this sense, testosterone serum levels were not modified in animals exposed to BPA and no changes in seminal vesicles weight were observed.

Testis weight depends mainly on the number of Sertoli cells. These cells proliferate in a precise period which begins in fetal life and goes on in neonatal period [27]. Our data showed that absolute testicular weight decreased in animals exposure to BPA. These effects would be a consequence of BPA binding to ERβ present in Sertoli cells. These receptors are already present at early stages of development, more precisely in fetal and ulterior periods [34]. BPA could alter the morphology of these cells by binding to the receptors, and could also decrease the number of cells, consequently modifying testicular weight and tubular organization [6]. Histological analysis demonstrated that the lumen of the tubes was reduced and filled with immature cells belonging to the spermatic lineage and macrophages, suggesting an indirect effect of BPA on the germinal epithelium, as it has been previously described [4]. Translocation of germ cells from the base to the lumen of the seminiferous tubules occurs by conformational changes in the lateral margins of the Sertoli cells, so the presence of immature spermatogenic cells in the tubular lumen may be explained by hypoplasia of these cells after exposure to BPA. However, future studies are necessary and they will be based on detailed analysis of BPA effects on Sertoli and Leydig cell morphology.

LH increase observed in animals exposed to BPA may be explained by a possible reduction in the negative feedback mechanism of testosterone, probably induced by its antiandrogenic action. It has been demonstrated that BPA affects multiple steps of the activation and function of AR, thereby inhibiting the binding of native androgens to AR, AR nuclear localization, AR interaction with its coregulator, and its subsequent transactivation [17–25]. Moreover, the fact that exposure to BPA increases FSH serum levels, may be due to its ability to interfere with inhibin secretion. Tohei et al. reported that testicular contents of inhibin were significantly decreased in adult male rats treated with BPA, although its plasma concentrations were not changed [29]. Therefore, it would be of great importance to determine inhibin levels in future works, in order to verify effects of BPA on Sertoli cells and on the secretion of this hormone. Altogether these findings suggest that BPA may affect the mechanisms of feedback which control the HPG axis. Nevertheless, a direct effect of BPA at the hypothalamic level may not be discarded. Kisspeptin binding to its G-protein coupled receptor KISS 1 R, which is expressed by Gn-RH neurons, stimulates Gn-RH release and activation of the mammalian reproductive axis, being critical for puberty and the regulation of reproduction [9]. It has been recently suggested that the hypothalamic Kiss 1 neurons may be a potential target of BPA action. Studies in rodents demonstrated that early exposure to androgens and estrogens are extremely important for proper sexual differentiation of hypothalamic Kiss1 system. Therefore, it would be expected that inappropriate exposure to synthetic estrogen may disrupt Kiss system impairing reproductive maturation [23]. Results of

Tena Sempere demonstrated that exposure to synthetic estrogens compounds during early critical periods in rodents decreased hypothalamic Kiss1 mRNA levels and kisspeptin fiber density in the hypothalamus, altering gonadotropin secretion and/or Gn-RH neurons activity [28]. Moreover, Xi et al. have recently reported that exposure to BPA during gestation and lactation induced transcript levels of Kiss1 and Gn-RH in the hypothalamus and FSH in the pituitary glands of male mice offspring in a dose-dependent manner. Therefore, the authors conclude that upregulation of expression of hypothalamic *KiSS-1* by BPA may stimulate synthesis and release of Gn-RH and gonadotropins in the hypothalamus and pituitary gland, respectively [35]. On this basis, it may be possible that activation of Kiss 1 system and an increase in Gn-RH release may be responsible of gonadotropins increase in our animals exposed to a low dose of BPA.

As regards the effects of BPA on testosterone, in our experiment, no significantly changes in its levels were observed. It may be speculated that BPA may interfere with LH binding to Leydig cells as has been recently reported by Nanjappa et al. This group demonstrated that administration of low concentrations of BPA from gestational day 12 to day 21 postpartum diminished protein expression of the luteinizing hormone receptor (LHCGR) and the 17beta-hydroxysteroid dehydrogenase enzyme (HSD17B3), which contributes to decrease LH stimulation of the steroideogenesis and androgen secretion by Leydig cells of male rats [21].

Based on our findings, we therefore conclude that pre- and early postnatal exposure to a low dose of BPA disrupts the normal function of the HPG. The effects of the EDs may be exerted at the different levels of the axis and may be dependent on the dose, manner of administration, and the moment of exposure to the ED as has been previously reported by other authors.

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