Bisphenol A alters differentiation of Leydig cells in the rabbit fetal testis

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ABSTRACT The endocrine disruptor Bisphenol A (BPA) crosses the placental barrier and reaches the fetal organs, including the gonads. In the testis, fetal Leydig cells (FLC) produce testosterone required for the male phenotype and homeostatic cell-cell signaling in the developing testis. Although it is known that BPA affects cell proliferation and differentiation in FLC, results concerning the mechanism involved are contradictory, mainly due to differences among species. Fast developing fetal gonads of rodents lack cortex and medulla, whereas species with more extended gestation periods form these two tissue compartments. The rabbit provides a good subject for studying the disruptive effect of BPA in fetal Leydig and possible postnatal endocrine consequences in adult Leydig cells. Here, we investigated the impact of BPA administered to pregnant rabbits on the FLC population of the developing testes. Using qRT-PCR, we assessed the levels of S*F1*, *CYP11A1*, *3β-HSD,* and androgen receptor genes, and levels of fetal serum testosterone were measured by ELISA. These levels correlated with both the mitotic activity and the ultrastructural differentiation of the FLC by confocal and electron microscopy, respectively. Results indicate that BPA alters the expression levels of essential genes involved in androgen paracrine signaling, modifies the proliferation and differentiation of the FLCs, and alters the levels of serum testosterone after birth. Thus, BPA may change the postnatal levels of serum testosterone due to the impaired FLC population formed by the proliferating stem and non-proliferating cytodifferentiated FLC.

KEY WORDS: *Bisphenol A, Leydig cell, testis, rabbit, steroidogenesis*

Introduction

Due to prevalent use, BPA (BPA, 4,4'-dihydroxy-2,2-diphenyl propane) has become a ubiquitous environmental contaminant to which all organisms are extremely exposed. High temperatures induce the release of BPA monomers that contaminate human food and beverages and pollute the environment worldwide (Vom Saal *et al.*, 2007). BPA enters the body through the respiratory tract, the skin, and orally, where it accumulates in various tissues (Geens *et al.*, 2012). BPA crosses the placental barrier in both rodents and humans, meaning that maternal exposure to BPA may affect the developing fetus (Balakrishnan *et al.*, 2010). In humans, BPA is present in urine, blood, amniotic fluid, and umbilical cord blood (Minatoya *et al.*, 2017).

Male reproductive disorders, collectively known as the "Testicular Dysgenesis Syndrome" (TDS), result from alterations in testis development during fetal life (rev Skakkebaek *et al.*, 2016). BPA modifies the metabolism of steroid hormones, their receptor proteins, and the gene transduction pathways, disturbing fetal development (N'Tumba-Byn *et al.*, 2012). Thus, BPA is a potential

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candidate for engendering postnatal TDS. Fetal exposure to BPA can affect the establishment of the FLC population by disrupting the steroidogenic process in developing testis. Thus, even short interruption to the steroidogenic process in the fetal testis can lead to disturbing consequences in postnatal life (Shima et al.,2015).

SF1 regulates the expression of the genes that code steroidogenic enzymes such as CYP11A1 (P450 side-chain cleavage), CYP17A1 (cytochrome P450C17), 3β-HSD (3β-hydroxysteroid dehydrogenase), 17β-HSD (17β-hydroxysteroid dehydrogenase) and StAR (steroidogenic acute regulatory protein) (Shima et al., 2015). Mild functional changes in SF1 are associated with severe reproductive phenotypes. SF1 deficient mice show defects in terms of gonad and adrenal gland development, indicating its essential role in the establishment of fetal steroidogenic tissue (Sadovsky et al., 1995).

Studies on the role of the androgen receptor (AR) during fetal testis development indicate that the spatiotemporal cellular specificity of AR expression differs between species (Rouiller-Fabre et al., 2015). In mice, AR is expressed in germ cells and peritubular cells (Merlet et al., 2007). Although in AR knockout (KO) mice, FLC develops normally, maturation of adult Leydig cells (ALC) is impaired (Kaftanovskaya et al., 2015). Organ culture studies reported that BPA reduces the endocrine activity of the fetal testis in humans, rats, and mice (N'Tumba-Byn et al., 2012) and in vitro studies in Leydig TM3 cells indicated that BPA inhibits the action of androgens via the AR (Teng et al., 2013).

Fetal Leydig cells develop during fetal life, their main function is to produce testosterone, and they are responsible for the masculinization of the male urogenital system. The FLC is differentiated from the interstitial tissue (rev Habert et al., 2001). In mice, the first FLC population appears at 12.5 dpc (days post-coitus). It starts producing testosterone (Merchant-Larios and Moreno-Mendoza, 1998), in rats at 15 dpc, and in humans at eight weeks (Voutilainen, 1992). Their number increases considerably in early fetal testis, and subsequently, FLCs gradually enter mitotic arrest, and the population stabilizes, to almost disappear during the peri-

TABLE 1

EFFECTS OF *IN UTERO* EXPOSURE TO BPA ON FETAL LEYDIG CELLS AND STEROIDOGENIC FETAL TESTIS FUNCTION

Species	Doses of BPA and Experimental design	Major effects induced by BPA	References
Rat (IGS strain)	4, 40, and 400 mg/kg administered by gavage at 6 dpp through lactation day 20. Testis analyzed at 9 and 36 weeks old.	Increased testosterone levels in serum.	Watanabe et al., 2003
Rat (Long-Evans strain)	2.4 μg/Kg/day of BPA administered 12 dpc-21 dpp. Testis analyzed at 90 dpp	Decreased testosterone levels in serum.	Akingbemi et al., 2004
Rat (Sprague-Dawley strain)	0.002–400 mg BPA/kg/day administered at 11 -20 dpc. Testis analyzed at 20 dpc.	-BPA induced downregulation of StAR, CYP17A1, and CYP11A1. -The expression of 3β-HSD was not modified.	Naciff et al., 2005
Rat (Sprague-Dawley strain)	0.2, 2, 20, and 200 μg/mL BPA daily in drinking water at 1-22 dpc. Testis analyzed at 22 or 23 dpp (2 hours after birth).	200μg/mL of BPA exposure decreased serum T levels only in newborn rats 2 hours after birth.	Tanaka et al., 2006
Human fetal testis of GW 6-11. Rat Mouse	10^{-12} to 10^{-5} M of BPA In vitro organ culture of fetal testis and culture during 72 h with BPA.	Only in human fetal testis: -A dose of 10^{-8} M reduced testosterone secretion regardless of the age of the fetus.	N'Tumba-Byn et al., 2012
Rat (Long-Evans strain) and Leydig cell culture previously exposed to BPA	2.5 or 25 μg/kg/day BPA administered at 12-21 dpc. Testis analyzed at 21 dpc and 35 and 90 days of age.	-Increased Leydig cell numbers at 90 days of age -Testosterone production of the Leydig cell decreased. However, the levels of serum T were similar to controls. -Decreased levels of LH receptor and 17β-HSD.	Nanjappa et al., 2012
Rat (Sprague-Dawley strain)	0.02, 0.5, and 400 mg/kg/day BPA 11 dpc, subcutaneously. Testis analyzed at 16, 18, and 20 dpc.	400mg/kg/day of BPA decreased the amount of StAR protein in Leydig cells and gene expression at 20 dpc.	Hortsman et al., 2012
Rat (Sprague-Dawley strain) (Wistar strain)	10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M of BPA Organ culture of fetal testis at 14.5 dpc for 72 h.	- 10^{-7}-10^{-5} M for 72h Inhibits Insl3 secretion. -10^{-8} M and 10^{-5}M for 72h, reduced T secretion.	Ben Maamar et al., 2015
Human testis of 8 – 12 GW	Organ culture incubated 72 h with 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} M of BPA.	inhibited testosterone production	Ben Maamar et al., 2015
Mouse (Kunming strain)	50, 500, or 2500 mg/kg/day. BPA from day 1-21 dpc. Testis analyzed at 8 weeks of age.	-Serum T reduced and was negatively correlated with the dosages of BPA. -mRNA levels of StAR decreased -Doses of 50 and 2500 mg/kg/day: the expression of CYP11A1 decreased -500 mg/kg/day: the expression of Cyp11a1 increased.	Ma et al., 2017
Human fetal testis of GW 5 – 12 and GW 14-18	0.01 to 10 μM of BPA in Fetal testes in organ culture 3 days, and 6 weeks xenografts in nude mice treated with 10 μM of BPA in drinking water.	-Testis GW 5 – 12 and GW 14-18 -No significant differences between control and BPA in serum testosterone and expression of StAR, Cyp11A1, CYP17A1, and CYP19 genes.	Eladak et al., 2018
Rat (Sprague-Dawley)	4, 40 or 400 mg/kg/day In utero exposure on 12 to 21 dpc. Testis analyzed at 21 dpc	-Decreased serum testosterone levels -Disrupts number, distribution, and proliferation of fetal Leydig cell. -Down-regulated mRNA expression of Insl3, 17β-HSD, CYP11A1, and CYP17A1. -Down-regulated protein expression of LH receptor, CYP17A1, and 17β-HSD.	Lv et al., 2019
Mouse (Kunming)	2.5, 5, 10, 20, and 40 mg/kg/day. BPA at 0.5–17.5 dpc. Testis analyzed at 21 and 56 dpp.	-Doses of 20mg/kg BPA significantly reduced serum testosterone levels	Wei et al., 2019
Rabbit (New Zealand)	50mg/kg/day. BPA administered orally at 15 – 31 dpc. Testes analyzed at 17, 21, 26, 27, 28, 29, 30, 31 dpc and 1-3 dpp (proliferative and differentiated) fetal Leydig cells)	BPA treated testes decreased levels of CYP11A1, 3β-HSD, and AR at 27 dpc and reduced the number of proliferating Leydig cells at 21-28 dpc. Increased level of serum T at 3 dpp. Leydig cell clusters are formed by both differentiated cells with abundant SER and less differentiated cells with predominant RER in both control and BPA-treated testes.	Current results, 2020

M: molar, GW: gestational weeks, PND: postnatal day, dpc: days post-coitus, dpp: days post-partum, AR: androgen receptor, SER: smooth endoplasmic reticulum, RER: rough endoplasmic reticulum, T: testosterone.
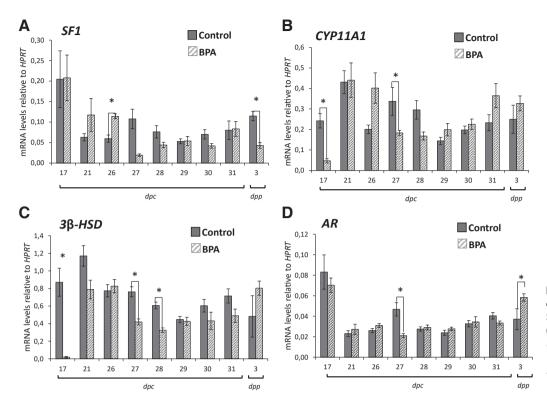
Fig. 1. **Effects of bisphenol A (BPA) on gene expression levels on (A)** SF1, **(B)** CYP11A1, **(C)** 3β-HSD *and* **(D)** AR. *Each bar represents the mean of relative expression of each gene to* HPRT ~ *SEM. Asterisks indicate a significant difference to the control.* *P < 0.05.

natal period (Barsoum *et al.,* 2013). However, there are important variations according to different species, in terms of the effects of *in utero* exposure to BPA on FLC and steroidogenic fetal testis function (Table 1).

The effect of BPA on the proliferative activity of Leydig cells remains controversial. Whereas during the prepubertal period, Leydig cells in rats increased their proliferation *in vivo* and *in vitro* (Chen *et al.*, 2018), other studies reported that BPA inhibits the proliferation of Leydig cells *in vitro* (Nanjappa *et al.*, 2012). Moreover, the effect of BPA on the FLC in mice, rats, and humans are qualitatively similar; however, the lowest no observed adverse
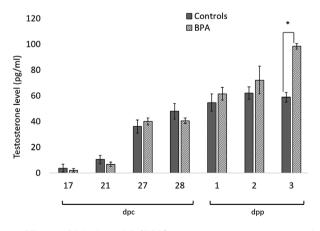
effect level (NOAEL) is more than 100-fold lower in humans than rodents. Furthermore, specific signaling pathways for BPA differ between rodents and humans (N'Tumba-Byn *et al.*, 2012).

Thus, those concerned about the prevalent use of rodents as models to assess the risk of endocrine disruptors on human fetal testis (rev in Habert *et al.*, 2014), have recommended using alternative animal models in which the fetal testis shows a developmental pattern closer to humans (Rouiller-Fabre *et al.*, 2015). Compared to the mouse, the rabbit has advantages for evaluating the effects of toxic agents on early testis development (rev Madeja *et al.*, 2019). For example, the gonadal pattern of development in the rabbit resembles that of humans; both form a bipotential gonad with two tissue compartments: the medulla and the cortex, in which the somatic-germ cell interactions occur according to the compartment (Díaz-Hernández *et al.*, 2019).

Importantly, in contrast to the almost synchronized germ cells in mice (Bullejos and Koopman, 2004), the proliferating germ cell population coexists for a long time with germ cells entering meiosis or mitotic arrest in ovary and testis, respectively (Frydman *et al.*, 2017). Thus, compared to rodents, the effect of BPA can differ in species with a longer gestation period. Like humans, the FLC population contains proliferating stem cells and non-proliferating differentiated cells. Thus, stem and differentiated Leydig cells are simultaneously exposed to BPA, when critical paracrine somatic-germ cell interactions occur (Díaz-Hernandez *et al.*, 2008; Daniel-Carlier *et al.*, 2013).

Here, we investigated the effect of BPA on the fetal steroidogenic process during proliferation and differentiation of the FLC population, using the rabbit as an animal model. For this purpose, we measured the expression profiles of the *SF1*, *CYP11A1*, *3β-HSD* and *AR* genes, and serum testosterone levels. Moreover, the proliferation pattern of the FLC was correlated. Our results indicate that BPA

Fig. 2. **Effects of bisphenol A (BPA) on testosterone concentration, measured in serum.** *The significantly higher level was evident in puppies born from BPA treated mothers at three* dpp. *The asterisk indicates significant differences compared to the respective control. Bars represent the mean ~ SEM, *P < 0.05.*
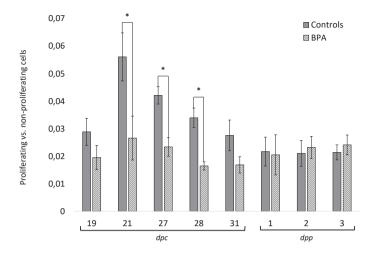
Fig. 3. The ratio of proliferating Leydig cells in the control and bisphenol A (BPA)-treated testis. *There are apparent significant differences in the number of proliferative Leydig cells in BPA- treated testes compared to controls at 21-28* dpc, *(*p<0.05). Bars represent the mean ~ SEM.*

alters the expression patterns of essential genes involved in steroid paracrine signaling, modifies the proliferation and differentiation of the FLC, and alters the levels of testosterone after birth.

Results

Levels of SF1, CYP11A1, and 3β-HSD were lower in BPA treated testis at 27 and 28 dpc

In order to discern how the BPA may perturb the regulatory pathways of steroidogenesis during the establishment of the fetal Leydig population, the expression levels of three factors were analyzed: a nuclear receptor *SF1,* and the steroidogenic enzymes *CYP11A1,* and *3β-HSD*. High levels of *SF1* expression were detected at 17 *dpc* in both control and BPA groups (Fig. 1A). Subsequently, although the levels decreased in both groups, the BPA treated group showed significantly higher levels than controls at 26 dpc. Then, the expression profile of the BPA group diminished and remained similar to, or lower than the control group, up to 31 *dpc*. However, at three *dpp*, the relative levels of *SF1* in the BPA group decreased significantly below the controls (Fig 1A).

Likewise, even though *CYP11A1* was detected in the testes of both groups at 17 *dpc*, levels in the BPA group appeared significantly lower (Fig. 1B). In contrast, four days later, at 21 *dpc,* the levels of *CYP11A1* in the BPA group increased to match those of the control group. The levels of the BPA group then decreased again, down to 27 *dpc,* when levels registered as significantly lower than the controls. The difference between groups of *CYP11A1* levels was not significant, up to 3 *dpp* (Fig. 1B). Like *CYP11A1*, the transcript profile of *3β-HSD* was significantly lower in testes treated with BPA than among controls. Overall, the BPA expression profile was below the profile of the controls, with significant differences at 17, 27, and 28 *dpc*. However, levels of *3β-HSD* increased at three *dpp* (Fig. 1C). Interestingly at 17 *dpc*, A*R* levels were similar in both BPA treated and control testes. After that, the levels decreased significantly at 27 *dpc* in the BPA group, remaining similar in both groups up to day 31. In contrast, after birth, AR expression increased significantly in puppy testes of BPA treated mothers, compared to their controls (Fig 1D).

Fetal testosterone level is higher in BPA-treated puppies at 3 dpp

To define whether the steroidogenic disrupting effect of BPA affects the testosterone secreted by the developing testis, the levels of serum testosterone in fetuses and newborns were determined. Fig. 2 presents testosterone levels measured in serum from samples of the fetus at 17, 21, 27, and 28 *dpc* and newborns at 1-3 *dpp*. Even though during the fetal period, testosterone levels were similar in the testes of both BPA and control groups, a significantly higher level was evident in puppies born from BPA treated mothers, at three *dpp* (Fig. 2).

The number of proliferating fetal Leydig cells is lower in BPA-treated testes

To study the effect of BPA on steroidogenic tissue in developing rabbit testis, we correlated the expression of CYP11A1 and KI67. As the antibody against CYP11A1 marks the first enzyme in the steroidogenic process, this probably identifies younger proliferating and non-proliferating mature Leydig cells, respectively. As differentiated Leydig cells cease proliferating and gradually enter quiescence, an analysis of proliferating and non-proliferating Leydig cells reveals the effect of BPA on the normal time course during the establishment of the Leydig cell population *in situ.* Two populations
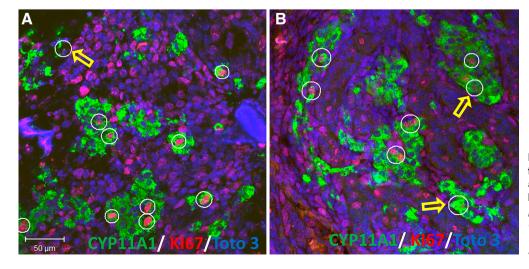

Fig. 4. Representative immunostained frozen sections of testis of control (A) and bisphenol A (BPA) treated (B) embryo at 21 *dpc*. *Proliferating Leydig cells, positive for KI67, are enclosed in white circles. Yellow arrows point to proliferating Leydig cells in which the red nucleus is in another optical layer of the confocal stack.*
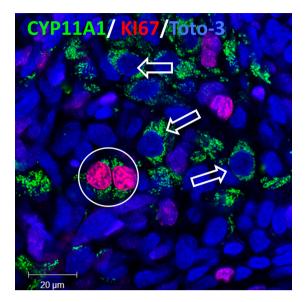
Fig. 5. High-resolution immunofluorescent colocalization of CYP11A1 (green) and KI67 (red) *to identify proliferating (circle) and no-proliferating (arrows) fetal Leydig cells of a control testis at 28 dpc. Leydig cells express CYP11A1 in the mitochondria and KI67 in the nucleus.*

of Leydig cells were counted: (1) proliferating Leydig cells, positive to KI67 (CYP11A1 + / KI67 +) and (2) non-proliferating Leydig cells, negative to KI67 (CYP11A1 + / KI67-). The ratio of proliferating Leydig cells in the control population appeared similar in BPA treated and control testis at 19 *dpc*. Subsequently, however, the number of

proliferative Leydig cells became significantly lower in BPA treated testes than in controls at 21-28. Once again, differences were not significant at day 31 and among newborn puppies (Fig.3).

As the Leydig cell count presented in Fig. 3 was made using 30 μm thick frozen sections (Suppl. Fig. S1), stacks of 2.0 μm thick confocal images were made to increase the resolution of proliferating and non-proliferating Leydig cells. Fig. 4 shows representative frozen sections of control and BPA treated embryo at 21 *dpc*.

Fig. 5 shows a representative immunofluorescent colocalization in fetal Leydig cells of a control testis at *28 dpc*. Leydig cells identified with CYP11A1 (green) were expressed in mitochondria. KI67 (red) was localized in the nucleus of all types of proliferating cells in the testis. The confocal merge image ensured precise identification of proliferating and non-proliferating Leydig cells.

Fetal Leydig cell clusters contain differentiated cells with abundant smooth endoplasmic reticulum and less differentiated cells with the predominant rough endoplasmic reticulum

We undertook an ultrastructural study in order to assess cytodifferentiation in proliferating and non-proliferating Leydig cells, during BPA treatment. At 21 *dpc*, rabbit testis shows the seminiferous cords surrounded by the stromal tissue, among which clusters of Leydig cells are undergoing differentiation (Fig. 6 A,B). Besides being larger than other stromal cells, the differentiated Leydig cells develop two conspicuous cell organelles: the mitochondria and the smooth endoplasmic reticulum. In contrast to the mitochondria of adult Leydig cells with tubular cristae, the fetal and newborn Leydig cells of the rabbit mostly maintain lamellar cristae in their mitochondria (Figs. 7B, 8C). However, the smooth endoplasmic

Fig. 6. Representative light and electron microscope images of control rabbit fetal testes at 21 *dpc*. (A) *Semi-thin section stained with toluidine blue shows the seminiferous cords (SC) connected to the rete testis (RT), surrounded by clusters of Leydig cells (LyC) among the stromal tissue.* **(B)** *This electron micrograph shows a Leydig cell beginning to form smooth endoplasmic cisterns (yellow arrows). The inset in (A) shows a high magnification of the white square area in B. mt, roundish mitochondria; Nu, nucleus; Ncl, nucleolus.*

Fig. 7. Light and electron micro-graphs of a bisphenol A (BPA)-treated testis at 28 *dpc*. (A) *Semi-thin section showing the seminiferous cords (SC) surrounded by clusters of Leydig located among stromal tissue (LyC).* **(B)** *Ultrastructure of part of the cytoplasm of two attached Leydig cells in a cluster. The red points indicate the intercellular space. The upper cell shows predominant smooth endoplasmic reticulum (SER). In contrast, the cell at the bottom has predominant rough endoplasmic reticulum (RER), which probably corresponds to non-proliferating and proliferating cells, respectively.*

reticulum gradually displaces other organelles and occupies most of the cytoplasmic space (Figs. 7B, 8C). As fetal testes grow considerably with age, mainly due to the increased space occupied by the stromal tissue (blood vessels, fibroblast, and extracellular matrix), the clusters of Leydig cells at 28 *dpc* become more dispersed (Fig. 7A). Interestingly, the clusters included Leydig cells with different degrees of the smooth and rough endoplasmic reticulum. Cells with predominantly rough endoplasmic reticulum probably correspond to Leydig cells that are still proliferating (Fig. 7B).

Discussion

Studies of the toxicity of BPA on development and reproduction among rodents led the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) to propose that 50 mg/kg per day represent the lowest-observed-adverse-effect level (LOAEL) in mammals. Accordingly, we treated pregnant rabbit females daily with 50 mg/kg BPA. The treatment started at 15 *dpc*, corresponding to the establishment of embryonic genital ridges, followed by the onset of specification of Sertoli and Leydig cells in the rabbit (Díaz-Hernández *et al.*, 2008). To assess the effect of BPA on the differentiation of the FLC population, we analyzed the expression profiles of three gene encoding factors involved in testosterone production: *SF1, CYP11A1, 3β-HSD* and the *AR*, during the fetal period and at day three post-partum (*3 dpp*).

Reports employing rodent models found that *SF1* plays a central role in the specification of Leydig cells, regulating the genes that encode steroidogenic enzymes (Hatano *et al.*, 1994; Leers-Sucheta *et al.*, 1997). Changes in *SF1* levels alter the differentiation and steroidogenic capacity of Leydig cells during the fetal stage, which in turn compromises the population of adult Leydig cells (Barsoum *et al.*, 2013). The expression of *SF1* in Leydig cells lasts throughout fetal development in rodents, whereas in humans, *SF1* continues to be expressed at constant levels during the first trimester of pregnancy (Mamsen *et al.*, 2017).

Current results from the rabbit fetal testis control showed the highest levels of *SF1* at 17 *dpc*; then, levels decreased but showed considerable variations throughout the period analyzed. As SF1 during testis determination dimerized with SRY and AMH in preSertoli and Sertoli cells, respectively, the level of *SF1* profiles detected in the current study, correspond to two kinds of cell populations. The testis is determined by preSertoli cells and specifies the stem FLCs also. Thus, the significantly higher levels of *SF1* seen at 27 *dpc* and the lower levels detected at three *dpp* in BPA-treated rabbits, possibly result from alterations to the two kinds of testicular cells, induced by the endocrine disruptor.

Besides the fact that the transcript levels of *SF1* correspond to Sertoli and Leydig cells in the rabbit fetal testis, the lack of a direct correlation between the levels of *SF1*, *CYP11A1*, *3β-HSD*, and *AR* may be due to the multiplicity of pathways involved in the regulation of the steroidogenic process that can be altered by BPA. At the testicular level, BPA acts as an androgen receptor antagonist (Wolstenholme *et al.*, 2011)*,* and can inhibit *CYP11A1* and *3β-HSD expression* (Peretz and Flaws 2013; Ye *et al.*, 2011). Furthermore, BPA disrupts the steroid hormone balance in the testis, altering the levels of *CYP11A1* and *CYP19* throughout the JNK/c-jun signaling pathway, independently of SF1 (Lan *et al.*, 2017).

Most mechanistic studies of the effect of BPA in Leydig cells correspond to studies *in vitro* or involving postnatal rodents. In the present work, the experimental design to observe the effect of BPA on fetal Leydig cells intended to mimic the real condition in which BPA affects the fetal testis from a holistic point of view. As the BPA was orally supplied to pregnant rabbits, the alteration of both proliferation and steroidogenesis of the fetal Leydig cell population found here results from multiple factors. BPA may disrupt the gene expression of S*F1* and the steroidogenic enzymes *CYP11A1* and *3β-HSD*, through alternative signaling pathways, including JNK/c-jun, steroid feedback control, and hormone metabolism. Thus, the lack of parallel correlation between the gene levels of the nuclear receptor SF1 and the steroidogenic enzymes may be explained by the multifactorial effects of BPA on the developing fetal Leydig cell population.

Although BPA has adverse effects on steroidogenic enzymes of Leydig cells, results vary depending on the animal model and the experimental design. Treatment *in utero* of pregnant rats with 400 mg/Kg of BPA, downregulated their levels of *CYP11A1* mRNA and protein (Lv *et al.*, 2019). Among male mouse pups exposed to 50 mg/Kg/day of BPA during the prenatal period, there was an
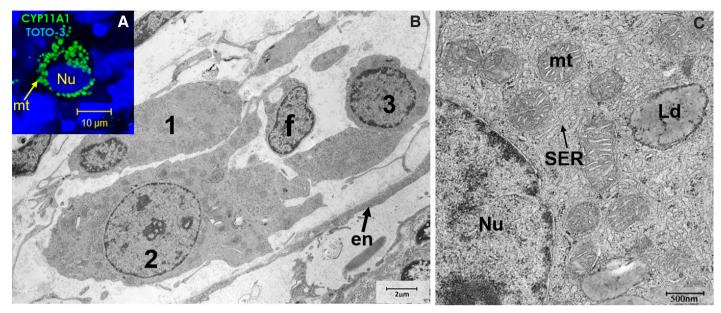
Fig. 8. Immunofluorescence and electron microscopy of a control testis section at 28 *dpc*. (A) *Immunostained CYP11A1 (green), localized in the mitochondria of the Leydig cell, the nuclei are stained with toto-3 iodide (blue).* **(B)** *Electron micrograph displaying a cluster of stromal cells: three Leydig cells (1, 2, and 3), a cross-sectioned fibroblast (f), and an endothelial cell (en).* **(C)** *Ultrastructure of a Leydig cell, the cytoplasm, shows abundant prominent mitochondria with lamellar cristae, smooth endoplasmic reticulum (SER), and lipid droplets (Ld). Nu, nucleus.*

evident decrease in levels of *CYP11A1* in the testis (Xi *et al.*, 2011).

In mice, the testicular amplification of both *CYP11A1* and *3β-HSD* was detected from 13 to 20 *dpc* (Greco and Payne, 1994). Recent work reported that in human testis, the expression of *CYP11A1* and *3β-HSD* can be detected after the 8th to 9th gestational weeks (GW). However, although C*YP11A1* showed significant activation at 11-12 GW, levels of *3β-HSD* were constant until 12 GW (Savchuk *et al.*, 2019).

In pregnant does, C21 steroids of placental origin are potential precursors for testosterone biosynthesis before the start of this function in the fetal testis, where the initiation of testosterone synthesis by 3β-HSD occurs between 17 and 19 *dpc* (Wilson and Siiteri, 1973). Using qRT-PCR in control rabbit FLCs, we confirmed that the levels of *CYP11A1* and *3β-HSD* start at 17 *dpc* and peak at 21 *dpc*. Subsequently, the transcript expression levels of the two genes oscillate by up to 3 *dpp*.

In contrast, to control testes at 17 *dpc*, two days after initiation of treatment, levels of *CYP11A1 and 3β-HSD* were significantly lower in BPA-treated testes. Although the levels increase at 21 and 26 *dpc*, on day 27, *CYP11A1* becomes significantly lower than in controls, whereas *3β-HSD* is lower at 27 *dpc* and 28 *dpc*. Finally, although during the perinatal period (20 *dpc* to 3 *dpp*), the different transcript levels of the two genes appeared among control and BPA treated testes, these are not significant.

A proposed mechanism for the anti-androgenic effect of BPA is that it competes with androgens for AR, preventing its translocation to the cell nucleus. Thus, the BPA inhibits the formation of functional complexes required for transcription (Teng *et al.*, 2013). Moreover, BPA antagonizes the interaction of AR with co-repressors such as SMRT and NCoR in Fetal Sertoli cells (Wang *et al.*, 2017).

Besides Sertoli cells, peritubular cells and gonocytes also express the AR in fetal testis. Thus, the disturbing effects caused by BPA in the differentiation, proliferation, and steroidogenic activity of the FLC population also impair the signaling timing among cells, which may differ among species (Hazra *et al.*, 2013). Leydig cells of human fetal and testes of newborns express the AR (Boukari *et al.*, 2009). In contrast, FLCs in rodents are independent of androgens (Shima *et al.*, 2015). In male fetal rat gonad, the AR was expressed at 17 *dpc* and subsequently increased. Our current results show that compared to the controls, the AR levels of BPA treated animals are significantly lower at 27 *dpc*. However, after birth, the testes of puppies at three *dpp* manifest significantly higher levels.

Like human patients with androgen insensitivity syndrome (Brinkman, 2001), AR knockout mice (ArKO) show male to female sex reversal (Yeh *et al.*, 2002). Although ArKO mice develop smaller fetal testis with degenerated Sertoli and germ cells, the Leydig cells become hypertrophic. This result suggests a paracrine interaction between the three kinds of cells for healthy testis development. Although we found that the levels of the AR in BPA-treated rabbit fetal testis appeared considerably disrupted, the postnatal consequences remain unknown.

The enzyme CYP11A1 is the first and rate-limiting step that regulates the conversion of cholesterol to pregnenolone in the mitochondria. Then the 3β-HSD, together with other enzymes located in the smooth endoplasmic reticulum, complete the synthesis of testosterone. At the ultrastructural level, differentiated FLCs in the rabbit have roundish mitochondria, most of them with lamellar cristae and abundant cisterns of the smooth endoplasmic reticulum. However, in developing rabbit testis, FLCs represent a heterogeneous population in terms of differentiation. At 21 *dpc*, most cells of the FLC population contain less smooth cisterns of the smooth endoplasmic reticulum; however, they formed more massive interstitial clusters than FLC population cells in the later fetal testis. Interestingly, at 21 *dpc*, the levels of *CYP11A1* and *3β-HSD* are higher, as are the number of proliferating FLCs. Thus, although the proliferating Leydig cells are less differentiated, their more significant number correlates with the significantly higher levels of the two steroidogenic genes.

Using a Leydig cell regeneration model in rats, Chen *et al.,* (2018) found that BPA accelerates the differentiation of postnatal Leydig cells without affecting their proliferation. In contrast, current results in the rabbit show altered differentiation and a significantly decreased proliferation of fetal Leydig cells in BPA treated mothers. Besides species differences, the two experimental designs differ considerably. Studies focused on the BPA effect on the rat analyzed the regeneration of postnatal Leydig cells in the two-month-old testis. In contrast, here, we analyzed the earlier fetal Leydig cells in developing testis of BPA treated mothers. Thus, the different developmental responses to BPA are hardly surprising, considering the spatio-temporal testis contexts of the two populations of Leydig cells: inside and outside the uterus.

In the rabbit, the Müllerian and Wolffian ducts develop similarly in both sexes between 15 and 20 *dpc*. Present results show that Leydig cells start producing testosterone as early as 17 *dpc*. In classical experiments, rabbit male fetuses were castrated at 19–21 *dpc* and developed a female phenotype (Jost, 1947). Our present results show no significant differences in fetal testosterone serum levels among BPA-treated and controls. Thus, the male somatic phenotype was undisturbed.

Levels of serum testosterone measured in BPA treated rat fetuses at 21 *dpc* appeared lower than controls (Lv *et al.,* 2019). Similarly, our results show that the serum levels of testosterone were more moderate in both control and BPA-treated fetus at 17 and 21 *dpc*. Subsequently, levels increased in both BPA-treated and untreated testes without significant differences up to 28 *dpc*. However, significant differences occurred three days after birth, being higher in the testis of BPA-treated rabbits. The substantial increase in serum testosterone in postnatal pups, correlates positively with the increased levels of *AR* in BPA treated FLCs.

Bjerregaard *et al.,* (1974) state in their ultrastructural study that the development of Leydig cells in the rabbit fetal testis, can be divided into three phases: Phase 1 (16-17 *dpc*) immature Leydig cells appear, Phase 2 (18-21 *dpc*), the fetal Leydig cells become differentiated with growing presence of smooth endoplasmic reticulum and decreasing rough endoplasmic reticulum and Phase 3 (22 *dpc* onwards). The authors stated that during phases 1 and 2, differentiation in the Leydig cells was nonsynchronous, subsequently evolving into synchronous differentiation at phase 3.

However, our current results show that the differentiating rabbit fetal Leydig cells persist as a nonsynchronous population, during phase 3. We found that besides the variable presence of the smooth endoplasmic reticulum among the Leydig cells, the proliferative capacity of the less differentiated Leydig cells suggests the presence of an asynchronous Leydig cell population. Therefore, the disrupting effect of BPA acting on an asynchronous fetal Leydig cell population probably increases the multifactorial disturbing effect of BPA on the normal steroidogenic pathway.

Overall, the current results demonstrate the complexity of the BPA disrupting effects inflicted on developing fetal Leydig cells *in situ*, from a holistic perspective. We found that in the rabbit, the fetal Leydig cells constitute a nonsynchronous population that may compensate for the harmful effects caused to the normal steroidogenic pathway during the fetal period. However, although most cytodifferentiated fetal Leydig cells disappear at around 10 *dpp*, the levels of AR and serum testosterone were higher in BPA treated puppies at 3 *dpp*. Thus, the long-lasting effects of BPA on adult Leydig cells remain to be studied.

TABLE 2

PRIMER SEQUENCE

Gene	Primers 5'→ 3.'	Length	Melting temperature	Sequence reference NCBI
Steroidogenic factor 1				
SF1	F: CGCAGGTGCATGGTCTTCAA	112 bp	60°C	XM_008273334.2
	R: CGTACTGGATCTGGCGGTAG		60°C	
Cytochrome P450 family 11 subfamilies A member 1				
CYP11A1	F: CCGTGGTATCCTCTACAGCC	136 bp	60°C	XM_008253734.2
	R: CCCCATCTCATACAGGTGCC		60°C	
3 beta-Hydroxy-steroid dehydrogenase				
3β-HSD	F: CAGGTGTCATTCACCGAGACA	131 bp	60°C	XM_002715686.3
	R: GTTGGGTCCAGCTACTTCCA		60°C	
Androgen receptor				
AR	F: GTGCCCTATCCCAATCCCAG	107 bp	60°C	NM_001195724.1
	R: ACGTGGTCCCTAGCAGTCTC		60°C	
Hypoxanthine guanine phosphoribosyltransferase				
HPRT	F: GCAGACCTTGCTTTCCTT	103 bp	60°C	EF219063.1
	R: GTCTGGCCTGTATCCAACACT		60°C	
Sex-determining region Y				
SRY	F: GTGGTCCCAACATCAGA	202 bp	56°C	Díaz-Hernández *et al.*, 2008
	R: ACCTCGTCGGAAGGTTAAAATC		56°C	

F= forward primer, R= reverse primer.

Materials and Methods

Animals and treatment

Individually caged New Zealand rabbits received food and water at libitum. As rabbits have mount-induced reflex ovulation, copulation day was considered day "0" of pregnancy. Pregnant females received daily oral doses of 50 mg/kg/day of BPA (Sigma-Aldrich, USA), dissolved in ethanol, from 15 to 31 days *post-coitus* (*dpc*). Pregnant females in control received only ethanol. Treatments began at 15 *dpc,* corresponding to the onset of genital ridge establishment (Díaz-Hernández *et al.*, 2008). Pregnant females and newborns were euthanized with an overdose of sodium pentobarbital (PiSA, Mexico). Fetal testis was sampled at 17, 21, 26, 27, 28, 29, 30, and 31 *dpc* and postnatal puppies at 1, 2, and 3 *dpp*. From pairs of gonads removed from at least five dissected animals, one was processed for immunofluorescence and the other for RNA extraction. Sex of undifferentiated gonads at 17 and 21 *dpc,* was identified using an *SRY* amplification, as described by Díaz-Hernández *et al.,* 2008. Subsequently, testes and ovaries were visually identified. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the Instituto de Investigaciones Biomédicas, UNAM.

RNA Extraction and cDNA synthesis and qPCR

Dissected testes were quickly frozen and stored at -70°C. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Germany) following the manufacturer's instructions. RNA was treated with RNase-Free DNase (Qiagen) at room temperature for 30 min. The concentration and quality of the total RNA were assessed by determining the optical density at A260: A280 ratio (2.0 ~ 0.15) in a NanoDrop 2000 spectrophotometer (Thermo Scientific) and by gel electrophoresis (1.5% agarose gel with ethidium bromide). For cDNA synthesis, total RNA (2 μg) was placed in 20 μl of 10 UI of transcriptor reverse transcriptase (Roche, Life Science). Negative controls were prepared without the template.

Gene expression determined with real-time PCR system (Applied Biosystems, California, USA) at 17, 21 26-31 *dpc,* and 3 *dpp*. The primers were created from rabbit specific sequences *SF1, CYP11A1*, 3β-*HSD,* and *AR.* Four housekeeping genes were tested (Table 2): oligonucleotides of *H2AX* and *YWHAZ,* designed by

Daniel-Carlier *et al.,* (2013). As under our conditions, *HPRT* turned out to be the most stable option; it was used as an internal control to validate the levels of gene expression. Primers and melting temperatures are presented in Table 2. Optimal alignment of the primers was determined on a

temperature gradient, as well as the amplification efficiency of each gene obtained, to standardize the qPCR conditions for each gene. Fragment authenticity was verified by sequencing.

The qPCR reactions were implemented using SYBR Select Mix (Applied Biosystems, California, USA) 1X, oligonucleotides (10 mM), 1 μl of cDNA diluted in 9 μl of RNase-free water, and ultrapure water in a total volume of 10 μl. In all tests, negative controls were conducted without a template, and reactions were performed in triplicate. Conditions for cycles were set at 50°C for 2 min, 95°C for 2 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min. Melting curves were generated to ensure the amplification of a single product. Gene expression was quantified by Ct relative comparison. Ct is defined as the fractional cycle number at which reporter fluorescence reaches a certain level. The $2^{-\Delta\Delta Ct}$ method was applied to calculate relative changes in expression.

Immunofluorescence and measurement of Leydig cell proliferation

For immunofluorescence, samples were fixed in 4% paraformaldehyde in PBS, dehydrated in saccharose gradient, and embedded in Tissue-Tek®. Frozen samples were stored at –70°C. Colocalization of anti-KI67 (M740, clone MIB-1, Dako, Denmark) as a nuclear marker and anti-CYP11A1 (sc-18043, Santa Cruz, Biotechnology) as a cytoplasmic marker were used to analyze Leydig cell proliferation. Testes at 19, 21, 27, 28, and 31 *dpc* and 1, 2, and 3 *dpp* were processed. 30 μm thick serial sections of longitudinally oriented testis were alternately placed on one of four slides, to include adjacent sections of each testis on each slide (Suppl. Fig. S1). The slides were washed with ethanol to remove lipid droplets. Heat-induced antigen retrieval was performed by placing slides for 45 min at 90°C in Tris-buffer pH 10. These were then transferred into PBS and 0.5% Triton X-100 in PBS in a rack sequence for immunofluorescence. 10% of horse serum in PBT (0.5% Triton 100X/PBS) for two h at room temperature to block unspecific staining. Subsequently, sections were washed and incubated overnight at 4°C with primary goat anti-CYP11A1 antibody, diluted at 1:70. These were then incubated with the Alexa Fluor 488 donkey anti-goat secondary antibody (A11055, Molecular Probes, Invitrogen) for 20 min at room temperature and left overnight at 4°C, with the second primary mouse monoclonal anti-KI67, diluted at 1:150. The Alexa Fluor 555 donkey anti-mouse (A31570, Molecular Probes, Invitrogen) was used as a secondary antibody. Cell nuclei were counterstained with TOTO-3 iodide (blue) (T3604, Molecular Probes, Invitrogen) and mounted with fluorescent medium (S3023, Dako, Agilent Technologies, Denmark). Images were collected and processed with a confocal Zeiss Pascal LSM5 microscope. In negative controls, the primary antibodies were omitted and incubated exclusively with secondary antibodies. Leydig cells were stained green, and KI67 were stained red. The number of cells labeled with colocalized CYP11A1 and KI67 was measured with Zeiss LSM5 Image Software on four cryosections for each testis (n= 4 testis per age category). Labeled cells were identified in 3D confocal stacks of 3.0 μm optical sections, and their number per unit area (325.67 μm²) was counted.

Serum collection and testosterone assay

The blood collected in Eppendorf tubes without anticoagulant was allowed to stand for 1hr at room temperature to promote coagulation and then kept at 4°C for 24 h, centrifuged 15 min at 3000 rpm at 4°C, and stored at -20°C until use. Samples from at least five animals in each age category were analyzed. Serum testosterone was quantified by competitive ELISA immunoassay (ENZO, testosterone ELISA kit, Farmingdale, NY) according to the manufacturer's instructions.

Semi-thin sections and transmission electron microscopy

Testes from each group were fixed in Karnovsky solution and subsequently postfixed with 1% OsO4 in Zetterqvist's buffer. After, the testes were dehydrated with an ascending series of ethanol concentrations and embedded in Epon 812. Semithin sections (1 μm thick) were cut with an ultramicrotome (Power Tome Microtomy XL, RMC) and stained with toluidine blue. Thin sections (60nm) were stained with uranyl acetate and lead citrate. Images were collected and processed either with a light or transmission electron microscope (Jeol EM1010).

Statistical analysis

Data are presented as mean and standard error (SE). Statistical differences between groups were evaluated and compared using a one-way ANOVA test, followed by Welch's correction. Statistical significance was set as $p < 0.05$.

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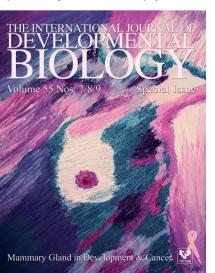Int. J. Dev. Biol. (2013) 57: 225-239
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