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Effect of perinatal and postnatal bisphenol A exposure to the regulatory circuits at the hypothalamus–pituitary–gonadal axis of CD-1 mice

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A B S T R A C T

Bisphenol A (BPA) is used in the manufacture of many products and is ubiquitous in the environment. Adverse effects of BPA on animal reproductive health have been reported, however most of the studies relied on the approaches in the assessment of conventional histology and anatomical features. The mechanistic actions of BPA are not clear. In the present study, a murine model was used to study potential effects of BPA exposure during perinatal and postnatal periods on endocrine functions of hypothalamic–pituitary–gonadal (HPG)-axis. At the hypothalamic-pituitary level, BPA exposure resulted in the up-regulation of the expression levels of KiSS-1, GnRH and FSH mRNA in both male and female pups. At the gonadal levels, BPA caused inhibition in the expressions of testicular steroidogenic enzymes and the synthesis of testosterone in the male pups. Conversely exposure to BPA resulted in a greater aromatase expression level and the synthesis of estrogen in the female pups. BPA is a weak estrogen agonist and its effects reported on animal studies are difficult to reconcile with mechanistic action of estrogen. In this study we hypothesized that the effects of BPA on reproductive dysfunction may be due to its actions on gonadal steroidogenesis and so the anomalous releases of endogenous steroid hormones. This non-ER-mediated effect is more potent in affecting the feedback regulatory circuits in the HPG-axis.

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1. Introduction

In the past century the production of synthetic industrial and biomedical chemicals, as well as unexpected by-products, have imposed adverse health consequences on wildlife and humans. Some chemical contaminants are classified as endocrine disrupting chemicals (EDCs) since they can interfere with the synthesis, metabolism and action of endogenous hormones [1,2]. EDCs can affect the hormonal system via (but not limited to) estrogenic, androgenic, anti-androgenic and anti-thyroid mechanisms [1,2], leading to the long-term effects on animal development and health [3–5]. Among different synthetic chemicals, bisphenol A (BPA) is produced in one of the largest-volumes and is used in many products. Currently over 2.7 million metric tons of BPA have been produced for the manufacture of epoxy resins and polycarbonate plastics as constituents of a wide variety of consumer products, including water/milk carboys/bottles, food wrap, food cans and dental fillings [6–12]. Miserably over 100 t are released into the atmosphere annually [11,12].

BPA can be accumulated along food chains and is detectable in tissues of both wildlife and humans [12–15]. More importantly trans-placental transport of BPA was observed and has been demonstrated in both rodents and humans [16–19]. Hence fetus may act as a sink of BPA and would be mostly affected during gestational development [20–22]. Adverse effects of BPA on developmental and reproductive processes in rodents and primates were reported [23–30], including increased prostate weight, decreased epididymis weight, reduced sperm production and decreased concentrations of LH and testosterone in blood serum [31–34]. Although the adverse effects of BPA on reproduction have been reported, most of the studies assessed the impacts only at the levels of conventional histology and the gross comparison of anatomical features/tissue mass. The mechanistic actions of BPA on animal reproductive health have not yet been elucidated.

To fill this knowledge gap, in this study the effects of BPA on expressions of reproduction-related genes along hypothalamus–pituitary–gonadal (HPG)-axis were studied. Both

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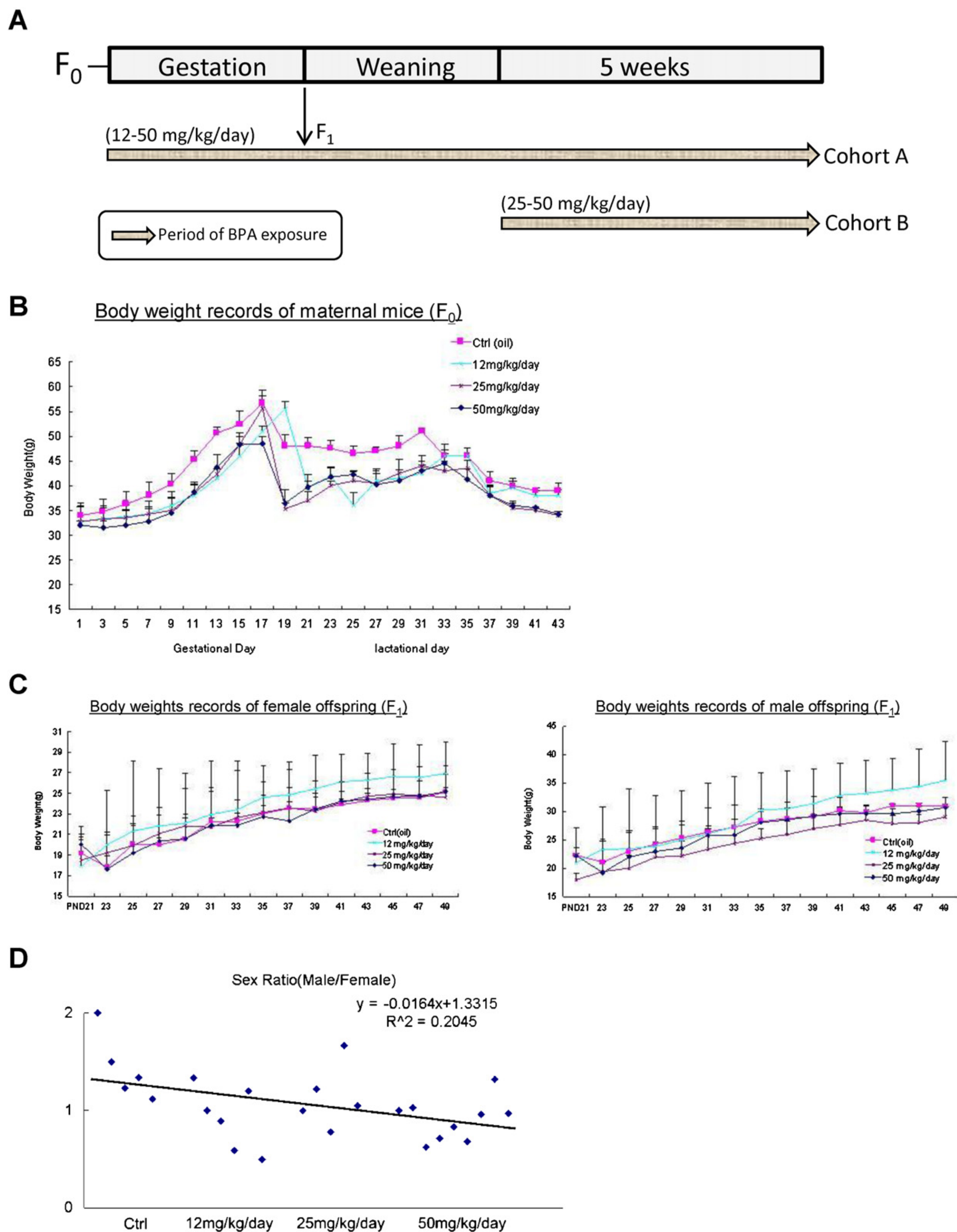

Fig. 1. The study design (A) and the measured gross physiological parameters (i.e. body mass and sex-ratio) of the maternal (F_0) and pups (F_1) from cohort-A (B–D). (B) Body mass records of dams during gestation and lactational periods. (C) Body masses of female (left panel) and male pups (right panel) from PND 21 to 49. (D) Sex ratio of the pups from cohort-A.

perinatal and/or postnatal exposures were investigated to reveal the significance of maternal transfer of BPA to fetus/neonates during gestational and lactation periods. In a previous *in vivo* study, the minimum concentration of BPA that was found to cause statistically significant effects on reproductive performance was 50 mg BPA/kg/day [35]. Accordingly the acceptable human BPA intake was calculated to be 50 μg/kg/day. However recent studies have revealed that human exposure to BPA could be considerably greater than this acceptable level, and daily intake of BPA is not restricted to the diet [36–38]. Concentrations of BPA in human tissues were in the ng per ml or per gram range (i.e. blood (0.2–20 ng/ml), amniotic fluids (1.1–8.3 ng/ml), placenta (11.2 ng/g), breast milk

Table 1
The DNA sequences of primers used in the present study.

	Forward	Reverse
Kisspeptin (*KiSS-1*)	GAATGATCTCAATGGCTTCTTGG	TTTCCCAGGCATTAACGAGTT
Kisspeptin receptor (*GPR54*)	GCTCACT GCATGTCCTACAG	GCCTGTCTGAAGTGTGAACC
Gonadotrophin-releasing hormone (*GnRH*)	GGGAAAGAGAAACACTGAACAC	GGACAGTACAT TCGAAGTGCT
Gonadotrophin-releasing hormone receptor (*GnRH-R*)	CTCTATGTATGCCCCAGCTTTCA	GCAAAGACAATGCTGAGAATCCA
Luteinizing hormone (*LH*)	CCTAGCATGGTCCGAGTACT	GCTACAGGAAAGGAGACTATGG
Follicle-stimulating hormone (*FSH*)	GCTGCTCAACTCCTCTGAAG	GGCAATACCTTGGGAAATTCTG
Growth hormone (*GH*)	AGCAGAGAACCGACATGGAA	GTTGGTGAAAATCCTGCTGAG
Thyroid-stimulating hormone (*TSH*)	TCGGGTTGTTCAAAGCATGA	GGCACACTCTCTCCTATCCA
Prolactin (*PRL*)	CTGCTGTTCTGCCAAAATGTT	CAGGGTATGGATGTAGTGAGAAA
LH receptor (*LH-R*)	GCACTCTCCAGAGTTGTCAG	AGGGAGA TAGGTGAGAGATAGTC
FSH receptor (*FSH-R*)	TCTGCATGGCCCCAATTTTA	GGTAGAACAGAACTAGGAGGATC
Estrogen receptor-α (*ERα*)	AATTCTGACAATCGACGCCAG	GTGCTTCAACATTCTCCCTCCTC
Estrogen receptor-β (*ERβ*)	TTCCCGGCAGCACCAGTAACC	TCCCTCTTTGCGTTTGGACTA
Steroidogenic acute regulatory protein (*StAR*)	GGAACCCAAATGTCAAGGAGATCA	GCACGCTCACGAAGTCTCGA
Cytochrome P450scc (*CYPscc*)	AGCTGGGCAACATGGAGTCA	CCTCTGGTAATACTGGTGATAGGC
Cytochrome P450 17 (*CYP17*)	GATCTAAGAAGCTCAGGCA	GGGCACTGCATCACGATAAA
Cytochrome P450 19a (*CYP19a*)	CTGTCGTGGACTTGGTCATG	GGGGCCCAAAGCCAAATGGC
Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*)	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTG CTGTA

(0.28–0.97 ng/ml), follicular fluids (2 ng/ml), semen (5.1 ng/ml) and urine (1.37 ng/ml) [37,38]. Using concentrations of unconjugated BPA detected in human blood, Vandenberg et al. calculated that to achieve such internal doses, exposures to BPA would need to be approximately 0.5 mg BPA/kg, bw/day [37]. This exposure is approximately 10-fold greater than the dose of 50 μg BPA/kg, bw/day recommended by the USEPA. Using a physiologically based pharmacokinetic model, a comparable exposure (1.42 mg BPA/kg, bw/day) to achieve a steady state human blood level of BPA (0.9–1.6 ng/ml) [39] was estimated. In the present study, doses of 12–50 mg BPA/kg, bw/day were selected. These doses fall into the similar order of magnitude of no observed adverse effects level (NOAEL), 5 mg/kg/day and low observed adverse effects level (LOAEL), 50 mg/kg/day used in rodents for risk assessment purposes and is also used as a base to calculate the tolerable daily intake (TDI) for humans [40].

2. Materials and methods

2.1. Animals and administration procedures

All experimental animals were housed and handled in accordance with Guidelines and Regulations in Hong Kong Baptist University. Six-week-old male and female CD-1 mice were used in this study. The entire study was conducted in replicate with mice that were received in two separate batches. Adult mice were quarantined for 1 week during which time they were observed for any abnormalities. The mice were housed in polypropylene cages with sterilized bedding and were maintained under controlled temperature (23 ± 1 °C) and humidity (55 ± 5%) with a 12 h light–dark cycle (06:00–18:00). The mice were given ad libitum access to standard rodent food Rodentdiet 5002 (Labdiet, IN, USA) and water (in glass bottles). Mice were bred and female mice were checked for vaginal plugs the following morning. Each copulated mouse (F_0) was housed individually, and was randomly assigned to two cohorts (A and B). Each cohort was further divided into 3–4 groups with approximately 5 pregnant mice per group. The 4 groups of cohort A were gavaged in the morning with corn oil (group I-A), or bisphenol A (BPA purity >99.5%, Sigma) in corn oil. The doses were: 12 mg/kg/day (group II-A), 25 mg/kg/day (group III-A), 50 mg/kg/day (group IV-A). The dams were exposed beginning on gestational day 1 until weaning (postnatal day, PND 20) (Fig. 1A). Individual dams were checked for birth at least twice a day and the day when pups were first observed was designated as PND 0. From PND 21 to PND 49, the pups (F_1) produced from cohort-A were dosed by gavage in the morning with the corresponding concentrations of corn oil or BPA.

Pregnant mice from cohort B were divided into 3 groups but not dosed. Starting from PND 20 to PND 49, pups from the respective groups were dosed in the morning by gavage with corn oil (group 1-B), or 25 mg BPA/kg/day (group II-B), 50 mg BPA/kg/day (group III-B).

2.2. Measurement of physical parameters and sampling procedures

Changes in gross anatomy, histology and molecular function were examined. Body masses of dams and neonates were measured by use of an electronic balance (Shimadzu, Kyoto, Japan). The number of pups per dam and the sex-ratio were recorded on PND 1 and PND 15 respectively. Stillbirths and loss of pups during the weaning period were monitored. Male pups produced from cohorts A and B were sacrificed by cervical dislocation in the morning at PND 50. For female pups, vaginal smears were examined at PND 50 ± 2 and the pups were sacrificed on proestrus phase. Blood samples were collected by cardiocenthesis and serum was prepared by centrifugation at 3000 × *g*. Hypothalami, pituitaries and gonads were collected in liquid N_2 and were stored at −80 °C immediately. Real-time PCR assays were conducted to measure expression levels of reproductive-related hormones and receptors for both male and female pups.

2.3. ELISA for gonadotrophins (Gn) and steroid hormones

Serum hormones were quantified in triplicates by use of commercial kits. Follicle stimulating hormone (FSH), luteinizing hormone (LH), prolactin (PRL), progesterone (P) and estradiol (E_2) were quantified by use of kits for the Beckman Coulter ACCESS 2 immunoassay system (Beckman Coulter, Fullerton, CA USA). Concentrations of testosterone (T) in serum were assayed using ELISA kits (MP Biomedicals, Ohio, USA) according to the manufacturer's instruction.

2.4. Real-time PCR

The expression of genes was measured by real-time (quantitative) polymerase chain reaction (Q-PCR). Primers were synthesized (Table 1) and PCR products were cloned into pCRII-TOPO (Invitrogen, Carlsbad, CA) and were subjected to dideoxy sequencing for verification. Cloned PCR fragments with known diluted concentrations (copy number) were then prepared and used for quantification of mRNA by Q-PCR. Tissue or cellular total RNA was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's instruction. Purified RNA with a A260/A280 ratio of 1.8–2.0 was used. Briefly, 0.5 μg of total cellular RNA was reversed transcribed (iScript, BioRad). PCR reactions were conducted using an iCycler iQ real-time PCR detection system using iQ™ SYBR® Green Supermix (Bio-Rad, Pacific Ltd.). The copy number of transcripts was calculated in reference to the parallel amplifications of known concentrations of the respective cloned PCR fragments. Standard curves were constructed and amplification efficiencies were between 0.9 and 0.95. The data were then normalized to expression of GAPDH mRNA. Based on melting curve analyses there were no primer–dimers or secondary products formed. There was only one PCR product amplified for each set of primers. Control amplifications were done either without RT or without RNA.

2.5. Western blot

For Western blotting, samples were homogenized in sodium dodecyl sulfate (SDS) lysis buffer (2% SDS and 25% glycerol in 125 mM Tris/HCl (pH6.8)) and subjected to electrophoresis in 10% polyacrylamide gels. Gels were blotted onto PVDF membranes (PerkinElmer Life Sciences). Western blotting was conducted using rabbit polyclonal antibodies for StAR (1:500, Santa Cruz, USA), CYPscc (1:1000, Chemicon USA) and CYP19a1 (1:300, Abcam, UK), followed by an incubation with horseradish peroxidase-conjugated anti-rabbit antibody (Bio-Rad). Specific bands were visualized with chemiluminescent reagents (Western-lightening Plus, PerkinElmer Life Sciences). Blots were then washed in PBS–0.5% Tween20 and re-probed with rabbit polyclonal antibodies for β-actin (Sigma, USA).

2.6. Estrus cycle monitoring

Estrus cycle in pups was monitored by characterization of vaginal cytology at approximately the same time each day. Fresh vaginal smear samples were collected with fine-tipped eyedroppers by inserting the tip into the vaginal orifice approxi-

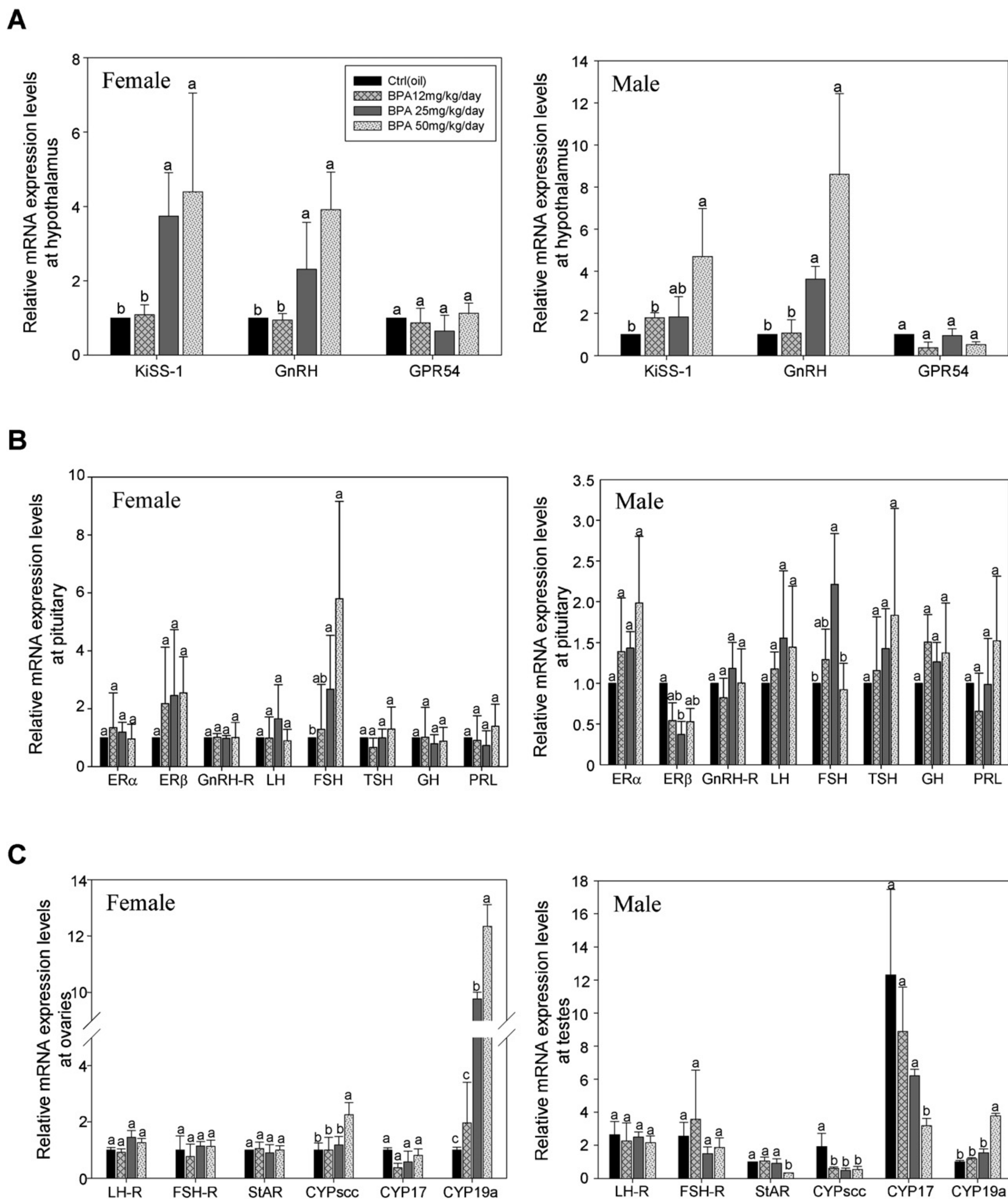

Fig. 2. Effects of BPA exposure on the expression profiles of hormones, receptors and gonadal steroidogenic enzymes at the HPG-axis of female and male pups from cohort-A. The gene expression levels of (A) hypothalamic hormones/receptor, (B) pituitary hormones/receptors and (C) gonadal hormone receptors and steroidogenic enzymes of the female (left panels) and male (right panels) pups. Bars with the same letter are not significantly different according to the results of one-way ANOVA followed by Duncan's multiple range test ($p < 0.05$).

mately 1 cm deep. The dropper contained a small volume (0.2–0.25 ml) of normal saline for flushing. One drop of the solution was placed on a slide. Vaginal smear was evaluated immediately using a light microscope.

2.7. Histological assessment of testis and ovaries

Testis and ovary were fixed in 4% paraformaldehyde, dehydrated in graded ethanol and embedded in paraffin. Serial microscopic sections (5–6 μm) were prepared and 5 slides from each testis were stained with hematoxylin and eosin (H&E, Sigma) for histological assessment. The diameter of seminiferous tubules and diameter of lumen were measured by an ocular grid using light microscopy. An estimate of this parameter was performed by examining 10 fields in 5 histological sections from each testis.

2.8. Statistical analysis

Statistical evaluations were conducted by use of SPSS16. All data were tested to be normally distributed and independent by using the Normal Plots in SPSS and

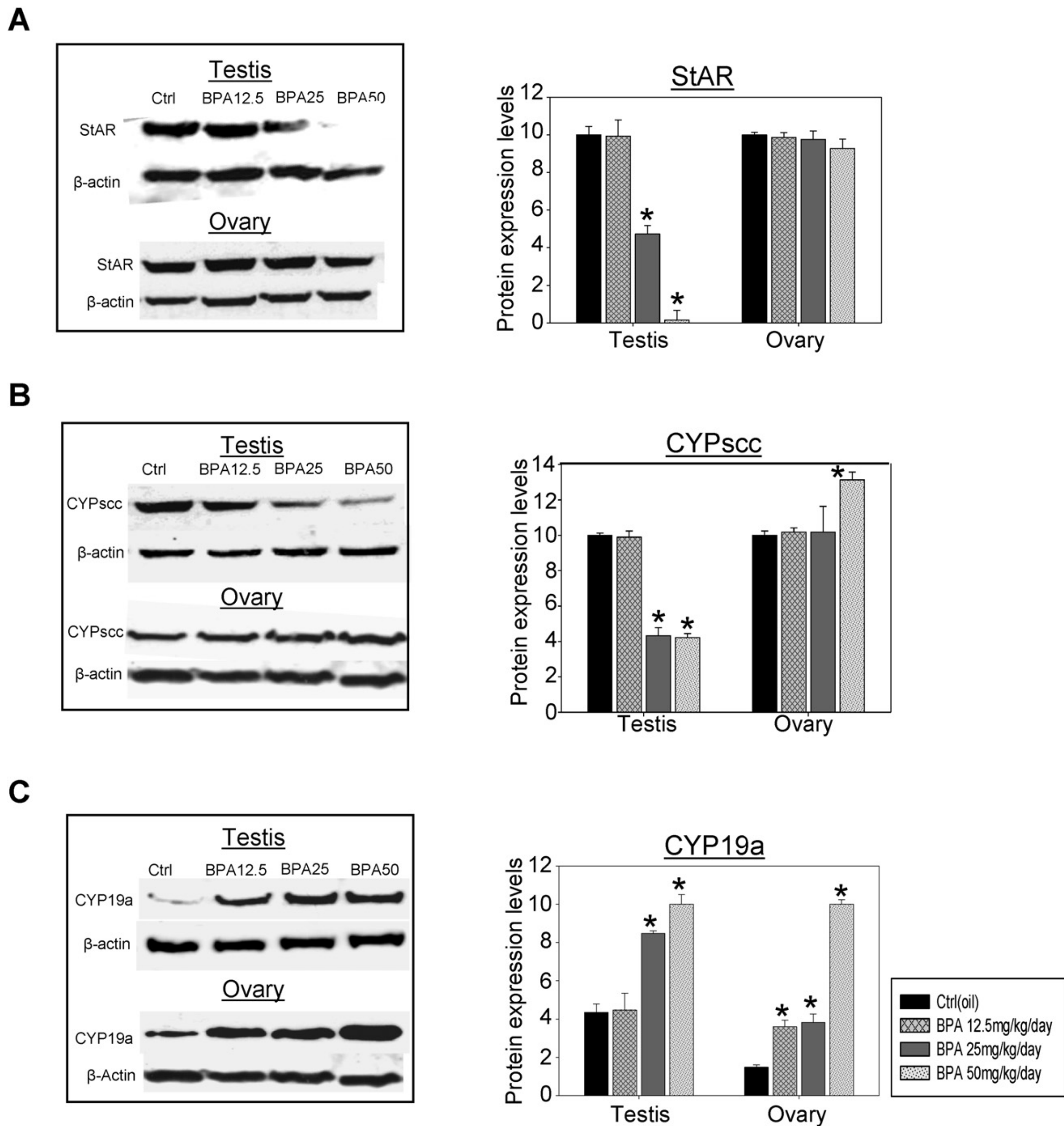

Fig. 3. Effects of BPA on the protein expression levels of gonadal steroidogenic enzymes in female and male pups from cohort-A. Western blot analysis of (A) StAR, (B) CYPscc and (C) CYP19a expression levels in the testes and ovaries. $^*p < 0.05$ as compared to the control.

Shapiro–Wilk significance were 0.05. Differences between treatment groups and corresponding control groups were tested for statistical significance by analysis of variance (ANOVA) followed by Duncan's Mutiple Range test (significance at $p < 0.05$) SPSS16. Associations between expression of genes and concentrations of hormones were investigated by use of Pearson, pair-wise correlations. Results are presented as the mean ± SEM. Groups were considered significantly different if $p < 0.05$.

3. Results

3.1. Survival, growth and reproduction

There were no significant differences in the body masses of maternal animals (during the gestational and weaning periods) (Fig. 1B) and F_1 pups (from PND 21 to PND49) (Fig. 1C) among group 1–4 from cohort-A. No statistically significant differences in perinatal mortality, number of pups per dam (data not shown) or sex ratio of pups (Fig. 1D) between any of the BPA treatments and controls were observed. No differences in the weights and sizes of various organs, such as testis, ovary, seminal vesicles, liver, spleen and thymus of the pups at PND 50 were noted. Histological examination of the testis sections of the male pups showed no noticeable changes in the histology and diameter of the seminiferous tubules (Supplementary Figure 1A). No observable differences in the number of growing follicles in ovary sections (Supplementary Figure 1B) and no noticeable shift in the pattern of estrus cycle were found in the female pups (Supplementary Figure 1C). Similarly for group 1–3 of cohort-B animals, no significant differences in the growth of the pups among the control and the treatment groups were observed (data not shown).

3.2. Hormones and receptors of the HPG-axis

Dose-dependent increases in the expression levels of KiSS-1 and GnRH were observed in hypothalami of BPA-exposed female and male pups of cohort A (Fig. 2A). There were no statistically significant differences in the expression levels of GPR54 mRNA among treatments. No changes in ERα, ERβ and GnRH-R were observed in pituitaries of male or female pups (Fig. 2B), except 25 mg BPA/kg/day which caused a statistically significant down-regulation of ERβ expression in the male pups (Fig. 2B, right panel). Up-regulations of FSH mRNA expressions were observed in female pups exposed to 25 and 50 mg/kg/day BPA and in male pups exposed to 25 mg/kg/day BPA (Fig. 2B). There were no significant differences in the transcript levels of pituitary hormones LH, TSH, GH or PRL of pups exposed to BPA and corn-oil or between the BPA doses. For BPA-exposed pups of cohort B, no noticeable changes in the gene expression levels were detected in hypothalami and pituitaries (data not shown).

The expression levels of steroidogenic enzymes in the gonads of BPA-exposed male and female pups of cohort A were altered (Fig. 2C). In the male pups, both CYPscc and CYP17 expressions were down-regulated (Fig. 2C, right panel). However in the female pups exposed to high dose of BPA, the expression levels of CYPscc were upregulated. In both female and male pups, CYP19a expressions were up-regulated (Fig. 2C). The changes in the transcript levels of the steroidogenic enzymes were consistently demonstrated in the Western blot data (Fig. 3A–C).

Changes in the expression levels of steroidogenic enzymes were associated with changes in the concentrations of E_2 and testosterone respectively in serum of female and male pups. Exposure to BPA resulted in a greater concentration of E_2 in serum of the female (Fig. 4A) while lesser concentrations of testosterone in serum of the male (Fig. 4B). In male pups of cohort-B, the expression levels of the steroidogenic enzymes CYPscc and CYP17 and concentrations of serum testosterone were significantly less in the BPA treatment groups than the controls (Supplementary Figure 2). No noticeable effect on the BPA-exposed female pups was observed.

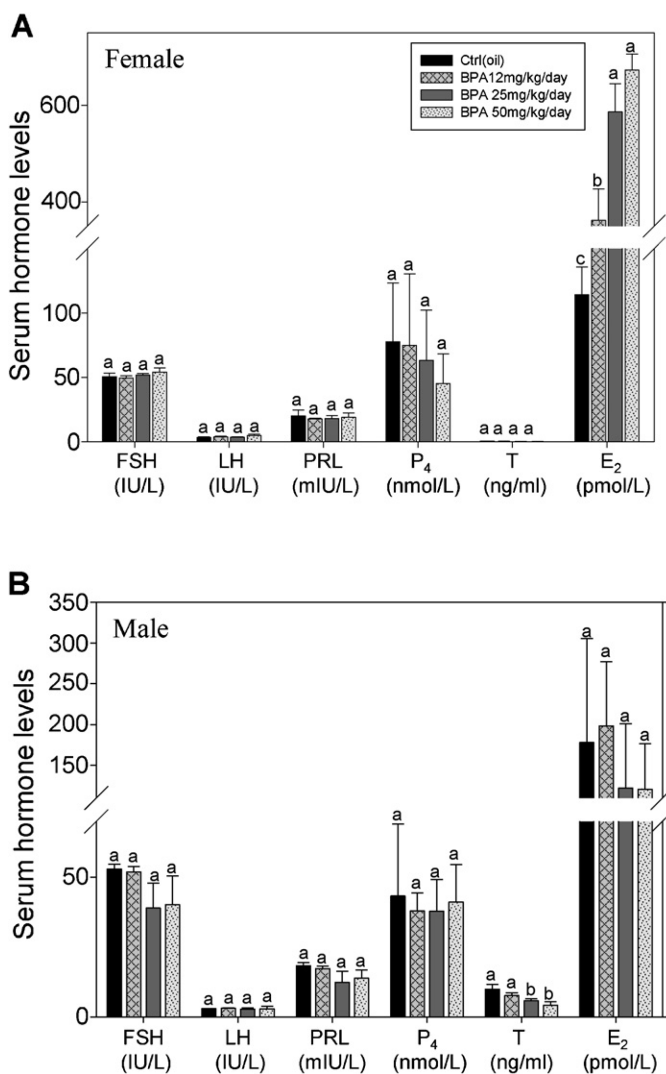

Fig. 4. Effects of BPA on concentrations of pituitary and gonadal hormones in serum of (A) female and (B) male pups of cohort-A. Bars with the same letter are not significantly different according to the results of one-way ANOVA followed by Duncan's multiple range test ($p < 0.05$).

4. Discussion

In the present study, effects of BPA exposure on the reproductive health of offspring were highlighted. Responses of conventional physical and anatomical parameters, such as number of pups per litter, survival and growth of pups were monitored in this study. No statistically significant effects on these parameters were found. The data are generally consistent with those reported by other researchers, using exposure doses from 0.003 to 600 mg BPA/kg, bw/day [41]. However in this study when more sensitive endpoints were recorded (i.e. the expression levels of selected reproductive-related hormone and receptor genes along the HPG-axis), significant effects on the regulatory circuits at the HPG axis were observed. Gestational and lactational BPA exposure induced transcript levels of KiSS-1/GnRH in the hypothalami and FSH in the pituitaries of the male and female offspring. Altered in the transcript levels of steroidogenic enzymes in the gonads and the serum levels of the sex hormones in the offspring were demonstrated.

KiSS-1 functions as a gatekeeper for initiation of puberty and for the regulation of gene expression along the HPG-axis [42,43]. Up-regulation of expression of hypothalamic *KiSS-1* is hypothesized to stimulate synthesis and release of GnRH and Gn in the hypothalamus and pituitary, respectively. Because postnatal exposure to BPA caused no statistically significant effects on the expressions of the genes in either the hypothalami or pituitaries of the pups in cohort-B, the perinatal period seems to be a critical "exposure window" for BPA to affect reproductive neural circuits in hypothalami of both male and female mice. In the studies of rats, BPA exposure were found to affect hypothalamic kisspeptin fiber density, KiSS-1 mRNA expression at the prepubertal stages [44,45], hypothalamic ERα expression [46] and pituitary GnRH-signaling [47]. The present study revealed that the BPA-stimulated hypothalamic KiSS-1 mRNA expressions induced transcript levels of GnRH and FSH in the male and female pups of cohort-A (Table 2). This observation is consistent with the physiological role of the HPG-axis in regulation of puberty and reproduction in animals [48,49] and highlighted the possible mechanistic effects of BPA on the local regulatory circuits of hypothalamus and pituitary [50].

The fact that there are feed-back regulatory mechanisms in place to maintain hormonal homeostasis along the HPG-axis [51,52]. The altered expression levels of hormones at the hypothalamus and pituitary levels may be the cause and/or the consequence of the changes in gonadal steroidogenesis and sex hormone production. Our data illustrated that BPA-elicited differential effects on the expression levels of gonadal steroidogenic enzymes and the concentrations of sex hormones in the serum of BPA-exposed male and female pups. In BPA-exposed female pups from cohort A, the increases of KiSS-1, GnRH and FSH expressions positively correlated with the increased expression level of CYP19a (Table 2) as well as

Table 2
Pearson correlation coefficients (r) between mRNA expressions of the genes along the HPG-axis in pups of cohort-A, after perinatal and postnatal BPA exposure.

Female	Hypothalamus		Pituitary			Ovary		
	KiSS-1	*GnRH*	*FSH*	*LH*	*StAR*	*CYPscc*	*CYP17*	*CYP19a*
Hypothalamus								
KiSS-1	1.000							
GnRH	**0.610***	1.000						
Pituitary								
FSH	**0.850****	**0.707***	1.000					
LH	0.160	0.227	−0.325	1.000				
Ovary								
StAR	0.578	−0.278	−0.108	0.280	1.000			
CYPscc	0.464	0.221	0.111	−0.484	0.066	1.000		
CYP17	−0.418	−**0.517***	0.126	−0.152	−0.283	0.225	1.000	
CYP19a	**0.677***	**0.834****	0.376	0.169	−0.573	−0.430	0.219	1.000

Male	Hypothalamus		Pituitary			Testis		
	KiSS-1	*GnRH*	*FSH*	*LH*	*StAR*	*CYPscc*	*CYP17*	*CYP19a*
Hypothalamus								
KiSS-1	1.000							
GnRH	**0.567***	1.000						
Pituitary								
FSH	0.114	−0.108	1.000					
LH	0.222	−0.157	−0.182	1.000				
Testis								
StAR	−0.326	−0.512	0.111	−0.352	1.000			
CYPscc	−**0.598***	−**0.583***	0.319	−0.092	0.066	1.000		
CYP17	−**0.772***	−**0.674***	0.008	−0.152	0.414	**0.838***	1.000	
CYP19a	**0.819***	**0.947***	0.154	0.169	0.162	−**0.573***	−**0.780***	1.000

Only parameters which have significant differences are shown here.
Significant correlation is indicated by asterisk(s) (*$p < 0.05$; **$p < 0.01$).

Table 3
Correlation coefficients (r) between the concentrations of serum steroid hormones and the mRNA expressions of steroidogenic enzymes in the gonads of pups from cohort-A, after perinatal and postnatal exposure to BPA.

Dose (mg BPA/kg, bw/day)	Female				Male		
	12	25	50		12	25	50
Cohort A							
P_4 vs. *CYP17*	0.152	0.243	0.334	T vs. *CYP17*	0.568*	0.863**	0.746**
P_4 vs. *CYPscc*	0.327	−0.156	−0.243	T vs. *CYPscc*	0.732*	0.804**	0.902**
P_4 vs. *CYP19a*	−0.158	−0.709*	−0.533*	T vs. *CYP19a*	−0.236	−0.558*	−0.659*
P_4 vs. *StAR*	0.332	0.330	0.279	T vs. *StAR*	0.179	0.659*	0.501*
E_2 vs. *CYP17*	0.119	−0.363	−0.298	E_2 vs. *CYP17*	0.133	0.110	0.143
E_2 vs. *CYPscc*	0.047	0.265	0.374	E_2 vs. *CYPscc*	−0.175	−0.123	−0.079
E_2 vs. *CYP19a*	0.65*	0.554*	0.717*	E_2 vs. *CYP19a*	0.246	0.421	0.449
E_2 vs. *StAR*	0.156	0.058	0.079	E_2 vs. *StAR*	0.314	−0.191	0.420

Significant correlation is indicated by asterisk(s) (*$p < 0.05$; **$p < 0.01$).

serum E_2 (Table 3). For the BPA-exposed male pups, although there were stimulations on the expressions of KiSS-1, GnRH and FSH, a negative correlation was observed with the expression levels of testicular steroidogenic enzymes (Table 2). The down-regulation of CYPscc and CYP17 resulted in lesser serum concentrations of testosterone (Table 3). Similar to the male pups of cohort-A, the reduced testosterone concentrations in the serum of male pups from cohort B were directly proportional to the decreased expressions of the testicular enzymes (Table 4). The up-regulations of CYP19a expression in the testes of male pups from cohort A could further reduce

Table 4
Correlation coefficients (r) between the concentrations of serum steroid hormones and the mRNA expressions of steroidogenic enzymes in the gonads of pups from cohort-B, after postnatal BPA exposure.

Female			Male		
BPA (mg/kg/day)	25	50	BPA (mg/kg/day)	25	50
Cohort B					
P_4 vs. *CYP17a*	0.125	−0.026	T vs. *CYP17a*	0.674*	0.826**
P_4 vs. *CYPscc*	0.258	0.152	T vs. *CYPscc*	−0.335	0.524*
P_4 vs. *CYP19a*	0.168	−0.257	T vs. *CYP19a*	0.102	0.458
P_4 vs. *StAR*	0.109	0.046	T vs. *StAR*	−0.275	0.169
E_2 vs. *CYP17a*	0.056	−0.112	E_2 vs. *CYP17a*	0.049	−0.338
E_2 vs. *CYPscc*	0.425	0.130.	E_2 vs. *CYPscc*	0.265	−0.316
E_2 vs. *CYP19a*	0.022	−0.166	E_2 vs. *CYP19a*	0.095	0.098
E_2 vs. *StAR*	0.198	0.279	E_2 vs. *StAR*	−0.044	0.123

Significant correlation is indicated by asterisk(s) (*$p < 0.05$; **$p < 0.01$).

serum testosterone levels. The data of the present study are in agreement with another study where doses of 100–200 mg BPA/kg, bw/day suppressed expressions of steroidogenic enzymes in testes of rats [53]. Furthermore, an *in vitro* stimulatory effect of BPA on CYP19a gene expression was reported in rat Leydig cells [53]. Although it was suggested by another study that the reduced testicular steroidogenesis could be due to lesser concentrations of LH in blood serum [31], no significant changes in the transcript levels of LH in the pituitary or LH-R in the testes of BPA-exposed pups were observed in our study.

BPA is a weak estrogen agonist and so its effects observed in animal studies are difficult to reconcile with the actions as estrogen agonists [6]. According to the "spare receptor" hypothesis, the hormonal system is sensitive to changes in a small proportion of receptor binding (10%), leading to a great change in cellular responses [34,54]. Any further increase in receptor occupancy would only produce a small increase of cellular responses. Therefore it seems unlikely that the observed BPA effects are due to the additional estrogen potency of BPA relative to the existing endogenous estrogen equivalents. Also, BPA has low binding affinity to sex-hormone-binding globulin (SHBG) and further minimizes the potential of BPA to activate membrane SHBG receptor [55–57]. Therefore the contribution of BPA to the total estrogen equivalents in the blood of female pups would be considerably small relative to the estrogen equivalents from endogenous estrogens. Comparatively male pups should be more sensitive to the effects of BPA. The data reported here indicated that perinatal and postnatal exposure to BPA was associated with functional changes in HPG-axis of the animals. These functional changes were unlikely due to the effects of BPA as an ER agonist. Moreover it has been reported that steroidogenesis is a major target for EDCs including BPA [58]. Retrospectively BPA may interfere with steroid hormone synthesis pathways and the release of the more potent endogenous steroid hormones (i.e. E_2 and testosterone) into circulations [6,58–60]. The change in serum sex hormone levels may cause subsequent reproductive dysfunction by interfering with the feedback regulatory mechanisms of the HPG-axis. Our data supported this notion as the altered serum levels of E_2 and/or testosterone were detected in the BPA-exposed pups. Although significant effects of BPA on HPG-regulatory circuits were identified in this study, the doses might not be necessarily reflective of general human exposure to BPA. These data are more relevant for the highly exposed or occupationally exposed individuals [61].

5. Conflict of interest

The author declares that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.reprotox.2010.12.002.

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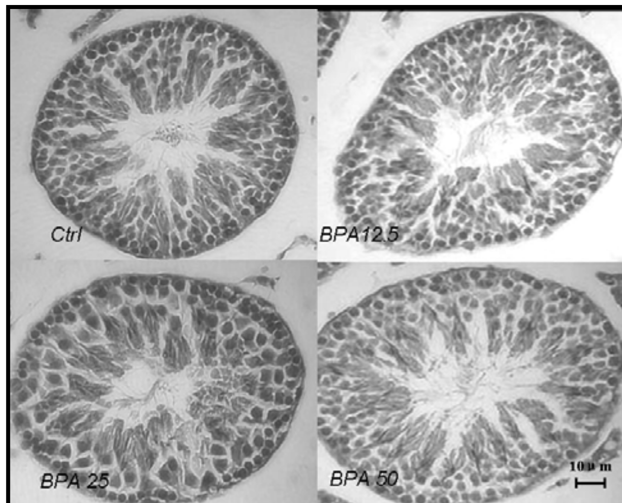
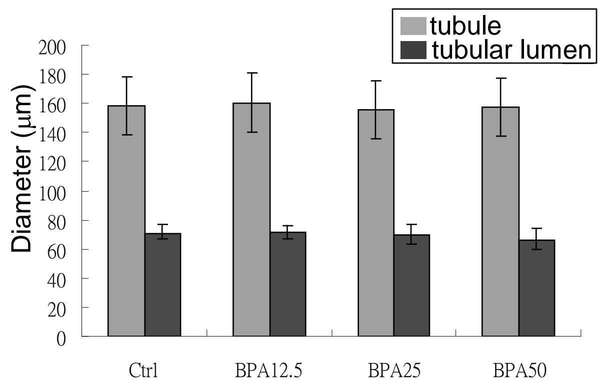
(A)





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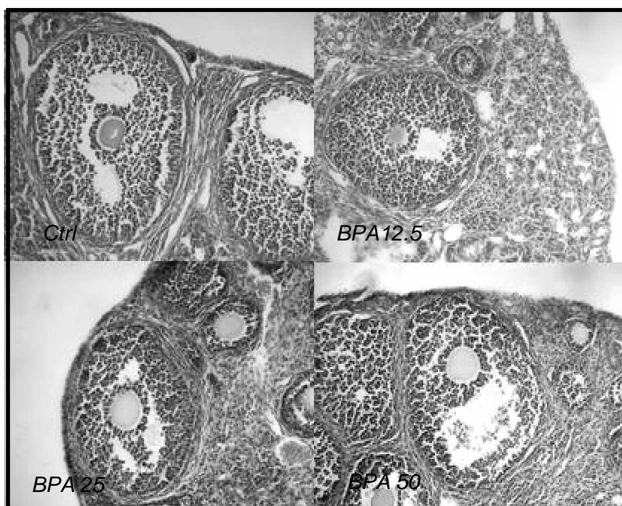

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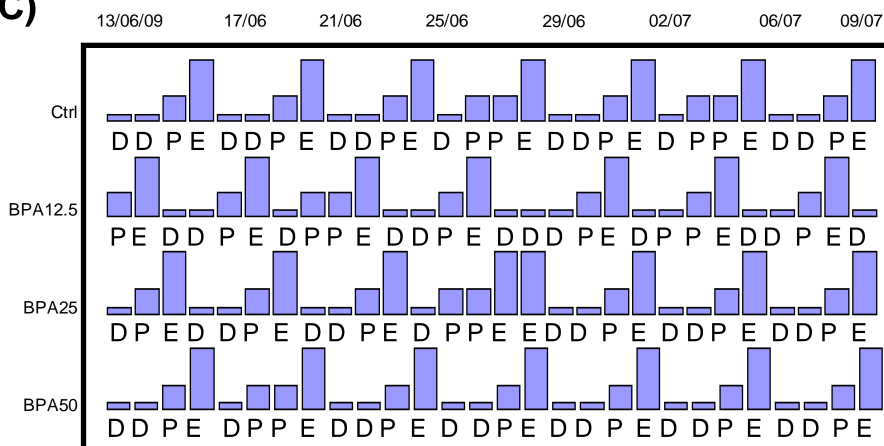
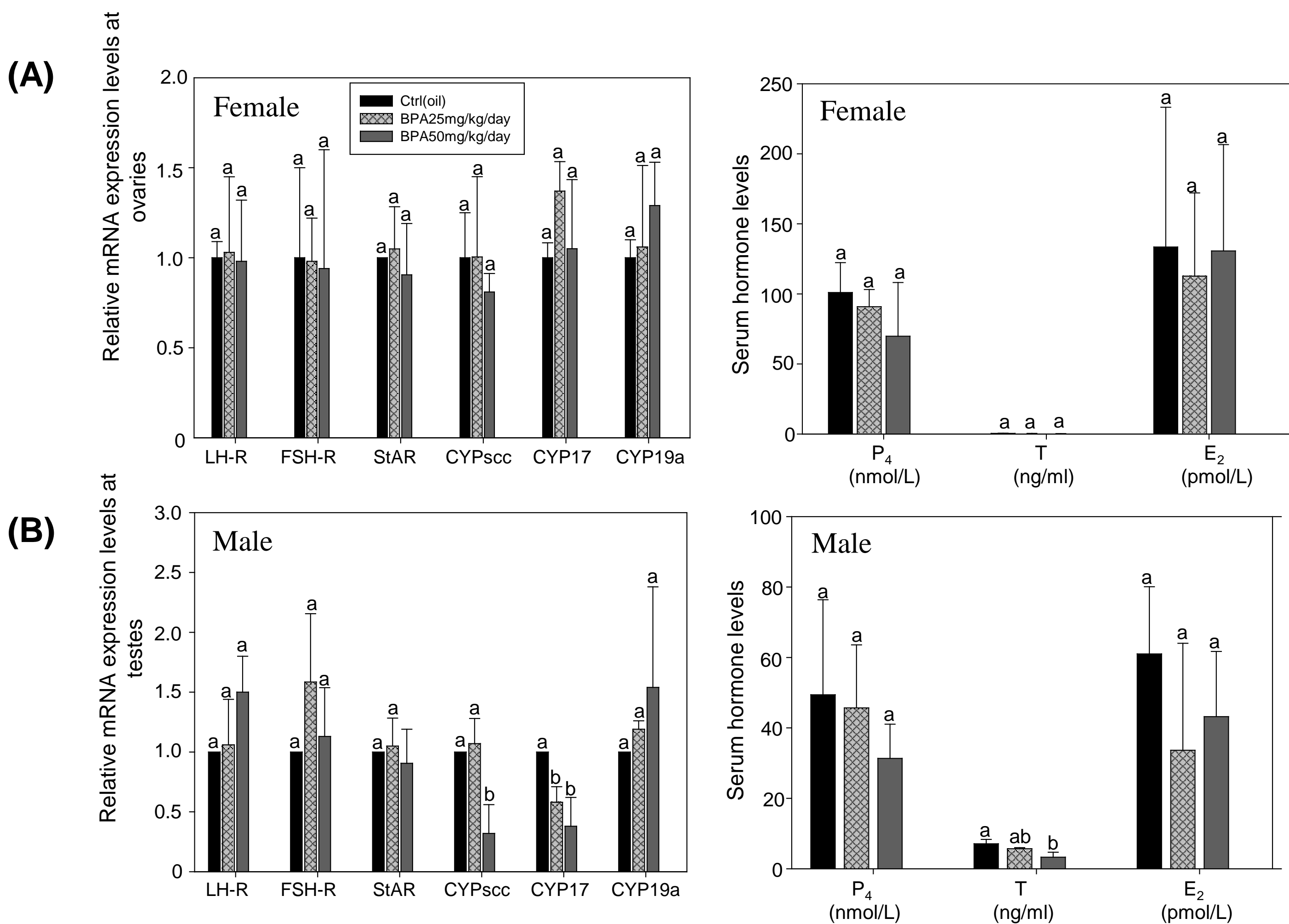


Supplementary FIG.1

Supplementary Figure 1. Effect of BPA exposure on the histological structure of (A) testes, (B)
and (C) estrous cycle of pups from cohort-A. An assessment of the smear was entered. The
symbols D, E and P are entered to indicate diestrus, estrus and proestrus respectively.

Supplementary Figure 2. Effects of postnatal BPA exposure on the expressions of gonadal
steroidogenic enzymes and the concentrations of serum sex hormones of (A) female and (B)
male pups from cohort-B. Bars with the same letter are not significantly different according to
the results of one-way ANOVA followed by Duncan's multiple range test (p<0.05).



Supplementary FIG.2