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Bisphenol A Down-Regulates The mRNA Expression of Steroidogenic Genes And Induces Histopathological Changes in Testes Of Rats

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Abstract:

Bisphenol A (BPA) is an endocrine disruptor with a weak estrogenic effect used in industry as a component of food cans. We aimed to study the toxic effects of BPA on mRNA expression of steroidogenic genes and testicular structure in mature male rats. Animals were divided into 3 groups: vehicle control rats as first group, while second group received 10 μg/kg BW and third group received BPA 15 μg/kg BW orally every alternate day for a period of 105 successive days. Serum testosterone level, mRNA expression of genes related to steroid synthesis, histopathological examination, spermatogenesis index and number of Leydig cells were evaluated in this study. Lower serum hormone levels were observed in both BPA-treated groups as compared to the control group. The gene expression patterns of *steroidogenic acute regulatory protein (StAR), cytochrome P450 17a (CYP17a)* and *3β-Hydroxysteroid dehydrogenase* (3β-HSD) were significantly down-regulated in BPA-treated rats compared to control group. Meanwhile, the expression of *aromatase* (CYP19) and lutinizing hormone receptor (LHR) was significantly up-regulated. Histopathological lesions were observed in the testes and epididymis of BPA-treated rats. Spermatogenesis index and the number of Leydig cells were significantly decreased in BPA-treated groups compared with the control group. This study highlights negative effect of BPA on steroidogenic genes and testicular structure in male rats.

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Introduction

Bisphenol A (BPA) is a ubiquitous environmental chemical that is integrated in the manufacturing process of many industrial products such as plasticizers, as well as in the production of materials used for food and potable water, such as epoxy lining of food and beverages cans [1-4]. It leaches from those products causing health hazards for humans and animals [2]. Its presence in biological and nonbiological samples was previously verified [5]. BPA is an endocrine disruptor; it binds to estrogenic receptors mimics the action of estrogen hormone [6], also, it can bind to androgen receptors, blocking endogenous androgen action [7] resulting in impairment of male reproductive function [8, 9]. BPA at a dose of 10 mg/kg bw/day orally for 14 days reduced activity of testicular mitochondrial enzymes in micewith subsequent stimulation of oxidative stress through inhibiting the activities of antioxidant enzymes [10]. Alternatively, extremely low concentrations of BPA inhibited activity of steroidogenic enzymes in human and rat testicular microsomes [11]. Reproductive toxicity of BPA was previously studied using different experimental animals such as mice and rats [12-16], evidenced by alterations in sperm parameters as well as reductions in sex hormones, testicular antioxidant enzymes and weights of reproductive organs. .

Enzymes of steroidogenesis perform a vital role in biosynthesis of different hormones. They consist of several specific cytochrome P450 enzymes (CYPs), hydroxysteroid dehydrogenases (HSDs), and steroid reductases [17]. It was reported that BPA reduced the expression of the *steroidogenic acute regulatory protein (StAR)* and *3β -hydroxysteroid dehydrogenase (3β-HSD)* [18,19]. Also, protein expression of the luteinizing hormone receptor (LHCGR) in male rats was suppressed following BPA exposure, impairing the secretion of androgen hormone by testicular Leydig cells [20].

Here, using environmentally relevant doses of BPA and for long duration of exposure, we investigated the downstream pathway of chronic exposure of BPA via evaluating its effect on the expression of genes encode steroidogenesis including *StAR, 3β-HSD,CYP17a, CYP19 and LHR*. Moreover, its effect on serum testosterone level, the index of spermatogenesis and the number of Leydig cells. Histopathological examination of the testes and epididymis were also investigated.

Materials and Methods

Experimental Animals

Thirty mature Sprague Dawley rats weighted 250-280 g were used. Animals were purchased from the Animal House, Helwan University, Egypt and housed in separate cages in the department of Physiology, Faculty of Veterinary Medicine, Mansoura University. Rats were kept in a controlled environment, maintained under a 12 h light; dark cycle, 24ºC (±3ºC) and 50-70% humidity and were provided with a standard diet and water *ad-libitum*. Animals received human care in compliance with the guidelines of animal care of the National Institutes of Health, and all animals producers were performed in accordance with the Ethics Committee of the National Research Centre, Egypt, registration number (09/189).

Chemicals

Bisphenol-A (4,4 isopropylidenediphenol) (Sigma, Aldrich, Germany) with a molecular weight of 228.29 g/mol was dissolved in corn oil as a vehiclebefore administration. Diethyl ether was used for anesthesia.

Experimental Design

Rats were kept in polycarbonate cages. The cages as well as the water bottles used in this study were washed, rinsed, and dried several times a week in order to decrease the release of BPA from polycarbonate cages and water bottles [21]. Therefore, exposure of experimental animals to phytoestrogens and BPA from these sources was minimal and equal for all groups. Animals were divided into three groups (n=10). A vehicle control group orally gavaged with corn oil, secondgroup received 10 µg/kg BW of BPA in corn oil, while third group received 15 µg/kg BW of BPA in corn oil. Administration was continued every alternate day for 105 days according to [22].

Blood and Tissue Sampling

At the end of the experimental period, all experimental rats were anaesthetized using diethyl ether. Blood samples were collected via cardiac puncture and centrifuged at 3000 rpm for 15 minutes for serum separation and stored at -20°C until analysis. Both testes were removed; one testis was frozen in liquid nitrogen

and stored at -80°C for RNA extraction, while the other testis was washed with normal physiological saline and fixed in 10% formaline for histopathological examination.

Biochemical Analysis

Serum testosterone hormone was evaluated using commercial ELISA kits (Biodiagnostic Co., Catalog number: LKTW1, Egypt) according to [23] and measured using Immulite 2000 device.

RNA Extraction and Reverse Transcription

Total RNA was extracted from 50 mg of testis using Trizol reagent according to the manufacturer's instructions (Direct-zol™ RNA MiniPrp, catalog No. R2050). The quantity and purity were measured by using Nanodrop technique (UV-Vis spectrophotometer Q5000/USA). The cDNA of each sample was synthesized following the manufacturer's protocol (SensiFasttm™ cDNAsynthesis kit, Bioline, catalog No. Bio-65053).

Quantitative Real Time PCR

Relative quantification of mRNA levels of *StAR, CYP17a, aromatase, LHR and 3β-HSD* in testes of rats was performed by real-time PCR using SYBR Green PCR Master Mix (2× SensiFast™ SYBR, Biolline, catalog NO. Bio-98002). Primer sequences and the size of each amplified PCR product are shown in Table 1. The house keeping gene (B-actin) was used as an internal control. The reaction mixture was carried out in a total volume of 20 µl consisting of 10 µl 2× SensiFast SYBR, 3 µl cDNA, 5.4 µl H_2O, 0.8 µl of each primer. The PCR cycling conditions were as follows: 95°C for 2 min followed by 40 cycles of 94°C for 15 sec, annealing temperatures as shown in Table 1 for 30 sec, and 72°C for 20 sec. At the end of the amplification phase, a melting curve analysis was performed to confirm the specificity of the PCR product. The relative expression of the gene in each sample was compared to that of the internal control (*B-actin*) and calculated according to [24].

Histopathological Examination

Twenty cross sections of testes and epididymis were taken from each group, fixed in 10% formaline and then processed routinely until being embeded in a paraffin wax. Paraffin sections of 5 µm thickness were prepared and stained with H&E according to [25]. Histopathological changes were examined by light microscopy. Seminiferous tubules were evaluated for their modified spermatogenesis index in all groups using Johnson's score in which scoring method ranged from 1 (tubular section without any cell) to 10 (tubular section with regular thickness of germinal epithelium with complete spermatogenesis stages) according to [26]. The number of Leydig cells was counted in ten random fields of each testicular section per animal in H&E stained slides using (100X) objective power according to [27].

Statistical Analysis

All the data obtained from the experiment were expressed as means ± SEM. Statistical analysis of data was carried out by software SPSS program package version 17 (SPSS, 2004) [28] using the one-way analysis of variance ANOVA followed by Duncan's Range Test (DMRT) for testing the significant differences between variables. Results were considered significant only at the level of ($P < 0.05$). Scores of spermatogenesis index were tested by using a Chi-Square test among the three groups.

Results

Serum Testosterone Level

We examined the effect of environmentally relevant doses of BPA on serum level of testosterone. A significant reduction ($P < 0.05$) was observed in serum level of testosterone (1.5 ± 0.41; 2.1 ± 0.21 ng/dl) at 10 and 15 µg/kg BPA, respectively compared with the control group (3.7 ± 0.29 ng/dl). However, no significant difference was observed between both BPA-treated groups (Figure 1).

Gene Expression Patterns

Steroidogenic enzymes are key factors for the biosynthesis of various steroid hormones. BPA at either 10 or 15 µg/kg significantly down-regulated the relative mRNA expression of *StAR* (0.67 ± 0.09, 0.53 ± 0.88 , respectively) compared to control group (1.15 ± 0.16). Expression of *CYP17a* gene was reduced ($P < 0.05$) at doses of 10 (0.60 ± 0.12) and 15 µg/kg BPA (0.40 ± 0.15) compared to control group (1.17 ± 0.23). The testicular mRNA expression of *3β-HDS* gene was suppressed after exposure to either 10 or 15 µg/kg BPA (0.75 ± 0.07 and 0.53 ± 0.12, respectively) compared with control group (1.44 ± 0.12)

Table 1. Oligonucleotide primer sequences, annealing temperature and PCR prduct size of the studied genes.

Gene	Oligonucleotide sequence	Annealing temp. (ºC)	Size (bp)
StAR	f5′- GGGCATACTCAACAACCAG-3′ r5′- ACCTCCAGTCGGAACACC-3′	58	111
CYP17a	f5′- CTCTGGGCACTGCATCAC-3′ r5′- CAAGTAACTCTGCGTGGGT-3′	58	114
Aromatase	f5′- GCCTGTCGTGGACTTGGT-3′ r5′- GGTAAATTCATTGGGCTTGG-3′	58	142
LHR	f5′- CATTCAATGGGACGACTCTA-3′ r5′- GCCTGCAATTTGGTGGA-3′	55	130
3β-HSD	f5′- TGTGCCAGCCTTCATCTAC-3′ r5′- CTTCTCGGCCATCCTTTT-3′	56	145
Β-Actin	f5′- TCGTGCGTGACATTAAAGAG-3′ r5′- ATTGCCGATAGTGATGACCT-3′	56	134

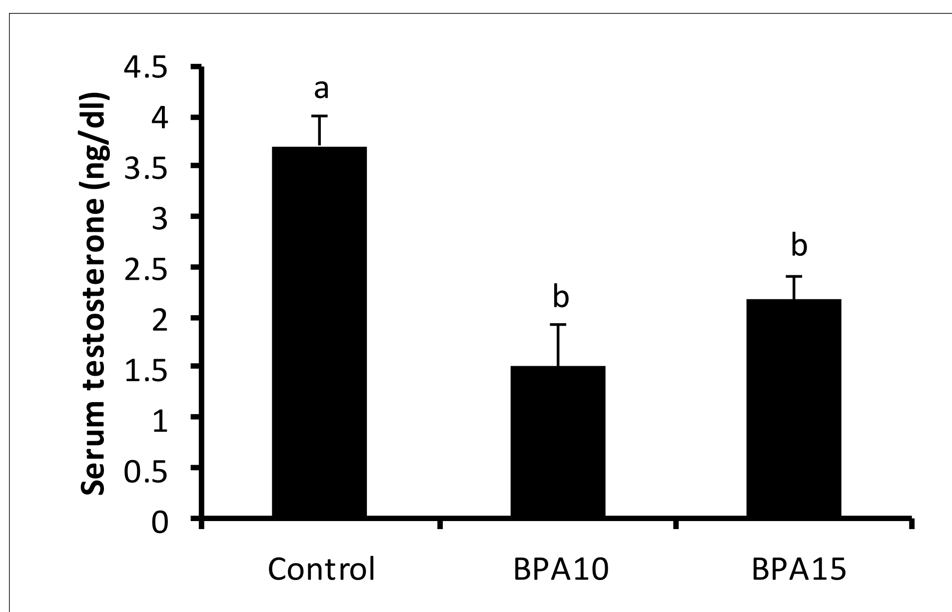

Figure 1. Serum level of testosterone (ng/dl) in control and BPA treated rats.

(P < 0.05).Nevertheless, BPA significantly up-regulated mRNA relative expression of *CYP19* at 10 (16.14 ± 0.25) and 15 μg/kg (10.17 ± 0.29) in comparison with control group (1.68 ± 0.67). Likewise, LHR gene expression was significantly increasedin second (2.09 ± 0.52) and third groups (2.04 ± 0.19) compared to control group (0.67 ± 0.09). There was no significant difference between both BPA treated groups in all selected genes (Figure 2).

Histopathological Results

In the control group, the testes and epididymis displayed normal histological structures (Figure 3a and 3b). The testes in the second group (10 μg/kg BW BPA) showed some seminiferous tubules with few or no late spermatids and a wide, emptied lumen. Vacuolar degeneration was demonstrated in the germinal epithelium (Figure 3c) and partially emptied ducts were encountered in the epididymis (Figure 3d). Meanwhile, testes of the third group (15 μg/kg BW BPA) showed decreased sperm production as indicated by fewer spermatids, no late spermatids and a much wider lumen. Vacuolar degeneration and necrosis were demonstrated in the germinal epithelium (Figure 3e). Partially emptied ducts with marked edema widely separating epididymal ducts were observed (Figure 3f). Concerning index of spermatogenesis, the control group showed highest percentage (80%) of testicular secions scored a 10. However, the highest percentage (79%) and (76%) of testicular sections scored a 7 and 6 (Chi-Square test; value = 447.050a, df = 8, (P < 0.0001) in BPA-treated rats at 10 and 15 μg/kg BW, respectively. Moreover, necrotic interstitial Leydig cells were observed only in several sections of testes from BPA-treated groups (Figures 4a to 4c). The numbers of Leydig cells were significantly lower in BPA-treated groups compared to control (Figure 5).

Discussion

Despite its presence in low levels in the environment, exposure to BPA is common among living beings producing several deleterious effects on various physiological mechanisms particularly reproduction [29]. The reproductive toxicity of BPA was extensively studied both *in vivo* and *in vitro* in different animal species and cell lines [30-33] with wide range of doses and concentrations of BPA particularly in case of *in vitro* studies. Even in case of *in vivo* studies that used very low doses of BPA, pregnant dams or their pups were used as experimental model, meaning that the exposure to the endocrine disruptor was applied in critical periods of life [34]. But concerning to *in vivo* studies in adult animal species, most of them testified high doses of BPA (1 to 240 mg/kg) in adult male rats and mice [14, 15, 33, 35]. Whereas, the current reference dose of BPA according to Environmental Protection Agency (EPA) is 50 μg/kg bw/day [36]. Additionally, it is plausible that BPA is integrated in several daily-used products, thus, exposure of humans to this chemical occurs over prolonged periods of time, as evidenced by the presence of BPA in measurable levels in blood [37]. In vivo studies that examined low relevant doses of BPA in adult males are relatively few. Adult male rats exposed to 2 ng to 200 mg /kg/day for 6 days showed reduction in testicular weight and daily production of sperms starting at dose of 20 μg/kg of BPA, but lower doses showed no obvious effects, it could be due to the short exposure period [38].

Moreover, chronic postnatal exposure of rats to environmentally relevant dose of BPA (2.4 μg/kg/day) for 70 days (from 21 to 90 day) resulted in elevation in serum levels of LH without variation in serum testosterone levels between BPA treated group and control group [39]. While testosterone production in Lyedig cells was reduced in ex vivo experiment. Likewise, BPA at oral doses of 0.2–20 μg BPA/kg/day for 45-60 days decreased epididymal and testicular weights and induced epididymal oxidative stress in adult rats [40, 41]. Also, adult male mice received BPA at very low doses (2, 25 and 100 ng/kg) for 28 days showed reduction in their fertility represented by lower pregnancy rate and increased resorption sites in female coubled with BPA-exposed males with reductions in the absolute weights of the testes [42]. Similarly, BPA at a dose of 2.4 μg/kg/day for 15 day decreased serum levels of LH and testosterone as well as Leydig cell androgen biosynthetic capacity in postnatal BPA-exposed rats. While BPA at a dose of 10 μg/kg/day for 15 day showed no difference in LH or testosterone concentrations [39], suggesting that the effect of BPA influenced by the administered dose and the exposure period.

Taken together, we used in the present study,

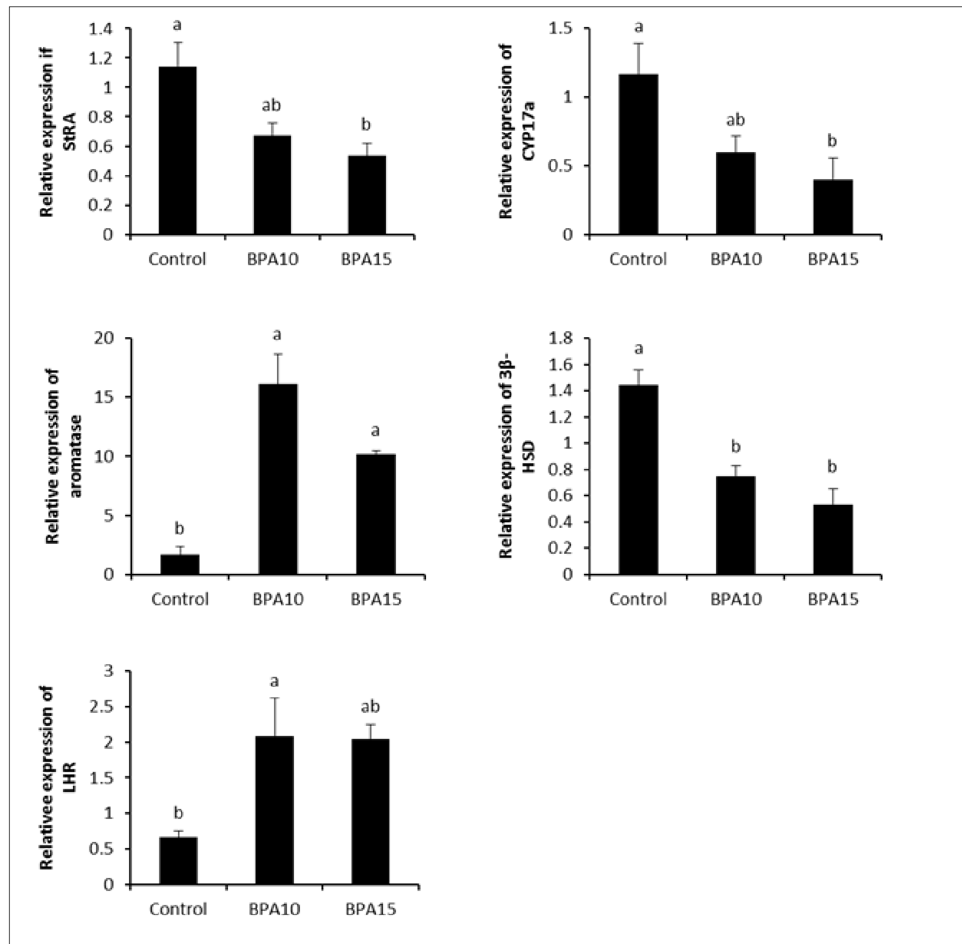

Figure 2. mRNA levels of StAR, CYP17a, aromatase, LHR and 3β-HDS in the control and BPA-treated groups. Small alphabetic letters show significance when ($P < 0.05$).

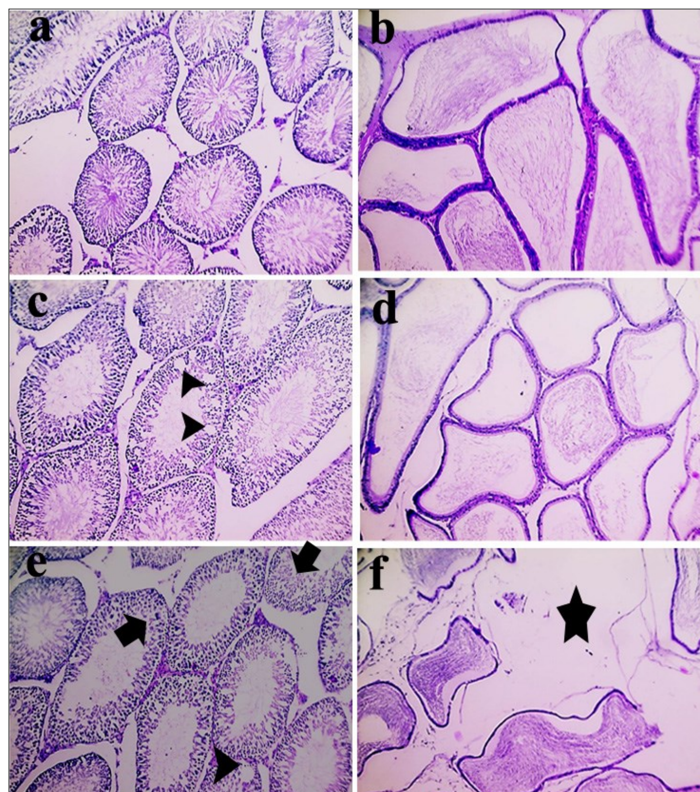

Figure 3. Sections from control group; the testes (a) and epididymis (b) display normal histological structures. In second group (BPA 10 µg/kg); testes (c) with arrowheads pointing to vacuolar degeneration in germinal epithelium lining some seminiferous tubules. While, epididymis (d) shows partial emptying epidydmal ducts. In third group (BPA 15 µg/kg); Testis (e) with arrowhead pointing to vacuolar degeneration in germinal epithelium lining some seminiferous tubules and arrows pointing to necrosis in germinal epithelium. Meanwhile, epididymis (f) with a star marking severe edema and widely separating epididymal ducts (X: 100).

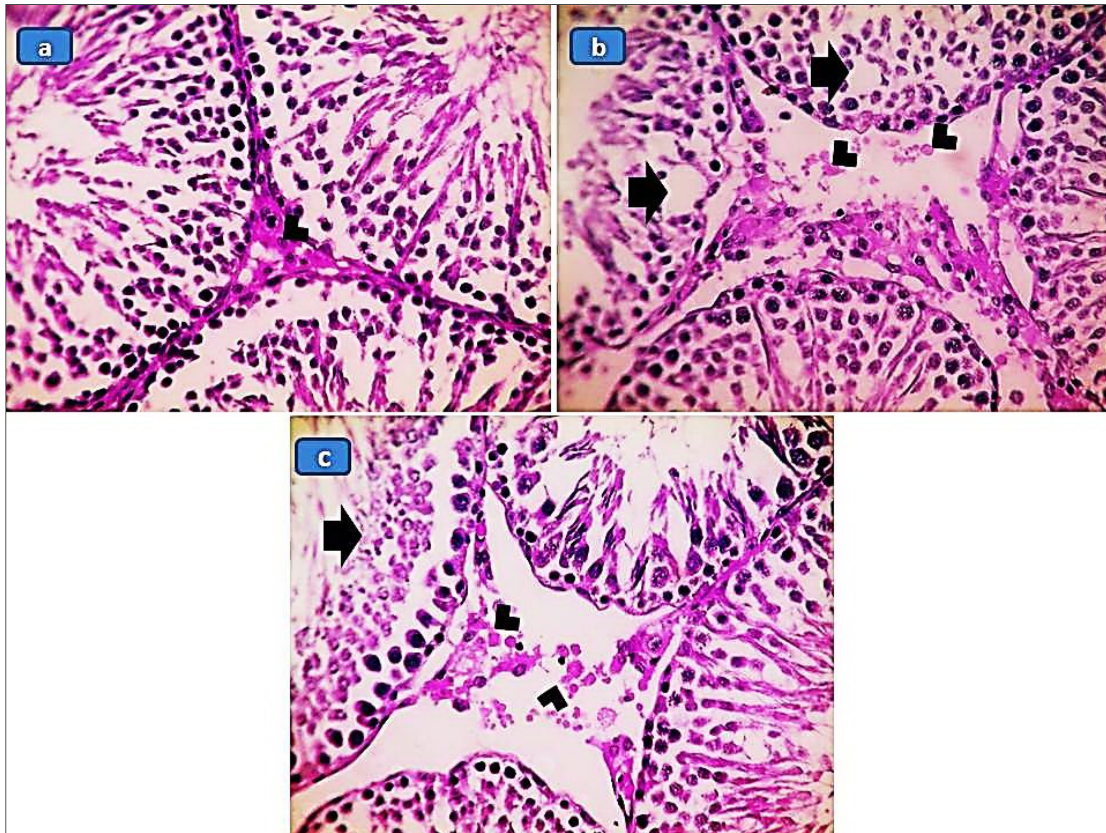

Figure 4. (a) Testis from control group shows normal germinal epithelium and interstitial Leydig cells (arrowhead). **(b)** Testis from second group (BPA 10 μg/kg) shows vacuolated spermatocytes (arrows) and necrotic interstitial Leydig cells (arrowheads). **(c)** Testis from third group (15 μg/kg) shows necrotic spermatocytes (arrow) and necrotic interstitial Leydig cells (arrowheads) (H&E, X:200).

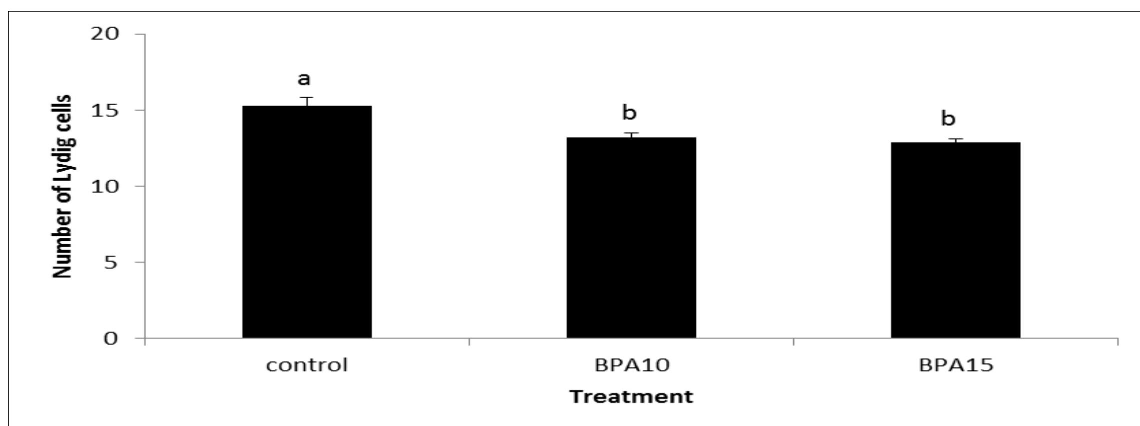

Figure 5. Number of Leydig cells in testis from control and BPA-treated groups (10 or 15 μg/kg BW). Small alphabetic letters show significance when (P < 0.05).

10 and 15 µg/kg bw BPA, hence, the BPA doses, used here, were lower than the reference limit for humans, also we expand the exposure period to 15 weeks.

In consistence with previous in vitro and in vivo studies [14, 39], the results of the present study indicated that the exposure of mature male rats to BPA at either 10 or 15 µg/kg BW for 105 successive days significantly decreased the number of Leydig cells and serum testosterone level compared to the control group. Leydig cells are the major producer of testosterone under the influence of luteinizing hormone (LH), thus, lowering in the number of Leydig cells negatively affect testosterone production [43]. Such reductions may be attributed to the endocrine disrupting properties of BPA [9), which possesses a weak estrogenic action [6] and anti-androgenic effects [44]. Moreover, BPA acts as a mitogen in Leydig cells in rats and interfers with the proliferative activity and development of Leydig cells [20]. Likewise, production of testosterone was reduced in BPA-exposed TM3 murine Leydig cells [45].

It was proposed that *StAR, CYP17a, 3β-HSD* and CYP19 *mRNAs* were predominately expressed in gonads [46]. These enzymes are responsible for the process of steroidogenesis aiming to formation of endogenous male hormones (testosterone and androstenedione) via specific cascade of reactions [43]. Recent studies have been focused on the effects of BPA on steroid biosynthesis in different animal models. In mammals, BPA at various doses modulates sex hormone levels and changes the expression of steroidogenic genes including *StAR, CYP17a, 3β-HSD* and *CYP19* [47-49]. These data could support the present results where BPA significantly down-regulated the expression of mRNA of selected steroidogenic biomarkers with subsequent reduction in the level of testosterone attributing to the direct inhibitory action of BPA on steroidogenesis and potentially disrupts StAR phosphorylation and cholesterol transport to mitochondria [50, 51]. Also, the inhibition of testicular steroidogenesis by BPA was associated with decreased steroidogenic enzyme gene expression in rat Leydig cells [39]. Reduction in the activity of *3β-HSD* and inhibition of *CYP17a* following BPA exposure were also observed in both rats and human testes microsomes [11].

Aromatase enzyme (CYP 19) is a specific form of Cytochrome P450 and is a key enzyme that catalyzes the conversion of androgen to estrogen in the steroid genesis pathway in the gonads [52]. In rat testicular Leydig R2C cells, BPA induced an increase in *CYP19* gene expression and its enzyme and reduced testosterone synthesis [53]. On the other side, the expression of gene encode aromatase enzyme was decreased in vitro using rat Lyedig cells [39]. The current data showed that BPA significantly up-regulated the expression of the *CYP19* gene, it could be via the estrogenic effects of BPA [54]. In the same respect, BPA stimulated *CYP19* mRNA expression and activity mediated by regulation of PKA, AKT and MAP kinase signaling pathways in mouse MA-10 and rat Leydig cells [32, 53]. The induction of *CYP19* expression may be contribute to decreased serum levels of testodterone.

Previous studies that examined the effect of BPA on LH levels varied in their outcomes, Several studies reported inhibitory effect of BPA on seum levels of LH in rats [39, 55], others observed an increase in LH level after exposure of male rats to BPA [35, 56]. But measuring of testicular expression of LHR was scarce. The expression of testicular LHR was redeuced in rats received a dosage of 200 mg/kg/day bisphenol AF (BPAF) [57]. Perinatal BPA at doses of 2.5 and 25 µg/kg bw/day suppressed protein expression of the luteinizing hormone receptor (LHCGR) [20].

In the current study, the expression of LHR gene was investigated to monitor the responsiveness range of gonads to gonadotropins when exposed to BPA. A significant up-regulation of LHR in BPA-treated groups was recorded.

On the other hand, expression of LHR gene was suppressed in mice exposed to BPA [58, 59]. Binding of LH to its receptor (LHR) in Leydig cells triggers a cascade of events that are catalysed by the steroidogenic enzymes to form testosterone [60]. Hence, lower LHR as well as *CYP19* expressions suggested a kind of struggling of steroidogenesis. Alternatively, despite BPA could reduce the production of androgen, an evidence was exist that BPA could interfere with LHR binding [61] uncoupling of LH from its receptor possibly contributes to diminished LH stimulation of steroidogenesis. The Higher levels of serum LH in BPA-treated rats were presumably resulted from a reduction in the negative

feedback regulation by testosterone.on the hypothalamus and pituitary [39].

Histopathological lesions were detected in the testes and epididymis from BPA-treated rats. Seminiferous tubules showed vacuolar degeneration and necrosis inBPA-treated rats similar to those reported by Khafaga and Bayad [62]. Also, seminiferous epithelial damage was observed in testes of rats exposed to BPA for 42 days represented by disruption of intercellular junctions and sloughing of germ cells into the seminiferous tubular lumen [63]. Indeed, the decrease of spermatogenesis index and Leydig cell numbers in BPA-treated groups is in consistent with lower testosterone levels in BPA-treated groups compared to control group. The index of spermatogenesis is one of the most important indicators of the state of spermatogenic layer [64]. The decrease in this indicator always indicates disturbances of spermatogenesis and decreased functional activity of seminal gland as well as a reduction of the functional activity of the testes. [64]. Moreover, the current results are in agreement with previous studies reported the opposing effect of BPA on spermatogenesis and total sperm count [65-67].

Conclusion

BPA supplementation could affect steroidogenesis via lowering serum levels of testosterone , altering the expression patterns of steroidogenic genes, exerting histopathological lesions in the testes and epididymis and lowering index of spermatogenesis as well as the number of Leydig cells in adult male rats.

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Conflict of interest

The authors declare no conflicts of interest.

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