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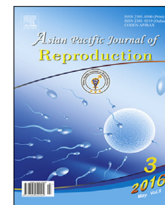

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Prenatal progesterone exposure of male rats induces morphometric and histological changes in testes

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ABSTRACT

Objective: To investigate the effects of prenatal progesterone exposure of male rats on testicular morphometry and histology.

Methods: Twenty male Wizard rats born to dams treated with hydroxyprogesterone hexanoate (HPH) during the first 2 weeks of gestation were employed to evaluate the effects of HPH on testicular morphometry, histology and cells quantities. Twenty male rats born to untreated dams were used as control. The rats were kept until they reached 90 days old, sacrificed, testes dissected, weighed and their dimensions recorded. Histological sections were prepared and stained with H & E. The diameter of the seminiferous tubule (ST), height of germinal epithelium, and thickness of the interstitial space (IS) were measured microscopically. Furthermore testicular cells were quantified.

Results: The results showed that experimental rats had significantly ($P < 0.001$) different testicular morphometry, histology and cells quantities compared to the control. The histological sections taken from testes of experiment group showed ST with unusual configuration, detached and/or folded basal lamina, thin germinal layer, wide IS with few LC, Sertoli cells (SC) experienced varying degrees of apoptosis and the lumen of ST contained cells debris and very rare few sperms.

Conclusions: Prenatal HPH exposure of male rats adversely affects their testicular morphometry and histological structure and presumably has crucial negative effects on their future fecundity.

1. Introduction

Recent data suggest deterioration of male reproductive health and this deterioration by no means is attributed to xenobiotic chemicals [1–3]. Xenobiotic hormones are widely used in human medicine. The xenobiotic hormones given to women during pregnancy are considered among the endocrine disrupting chemicals capable of modulating and disrupting the endocrine system of the male off-springs leading to deleterious effects on their reproductive health [4]. Prenatal exposure to steroid chemicals is known to induce testicular developmental disorders and consequently reduction in the fertility of adult males [5,6]. Irreversible testicular growth alteration, reduced sperm production and suppressed steroidogenesis have been reported after exposure to steroids [7,8].

Synthetic progesterone (P_4) is widely used in fertility clinics for many therapeutic purposes. It is traditionally used as contraceptive [9], ovulation inducer in primary infertility cases [10], and pregnancy support for threatened miscarriages during the first trimester, abnormal uterine bleeding inhibitor [2] and as luteal supporter for transplanted IVF embryos [11]. This situation entails that many women around the world are exposed to higher dose of xenobiotic P_4. These hormones are accused of eliciting adverse effects on reproductive health and fertility of future generations [9].

Prenatal exposure to P_4 reduced the body weight, testicular weight, total sperm count and damaged the ST and devoid them of sperm [12]. Histopathological study following in utero P_4 administration revealed severe testicular damage, decrease in

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seminiferous tubules diameters, reduction in number of spermatogenic cells, widening of tubular lumen and interstitial spaces with increment of necrozoospermia [12,13]. Xenobiotic agents interfere with synthesis, secretion, transport, metabolism, binding, elimination of natural blood hormones causing disorder of homeostasis and consequently hinder reproduction and development [14].

Exposure of embryos to the xenobiotic HPC reduced the circulating testosterone levels, sperm cell count and increased necrozoospermia; indicating a probable inhibition of androgen synthesis [2,15]. In the light of the above mentioned studies; the current study investigated the effects of prenatal HPH exposure of male rats on testicular morphometry, histology and cells quantities.

2. Materials and methods

2.1. Animals' management

Eight to ten weeks old females (n = 14) and males (n = 4) of albino Wistar rats were obtained from the animal house of the Faculty of Pharmacy – Jazan University – Kingdom of Saudi Arabia. They were transferred to the animal house of the Department of Anatomy, Faculty of Medicine, Najran University and grouped into two groups. Each group consisted of seven females and two males. They were kept at 12:12 h light/dark cycle (28 ± 7) °C temperature, fed on commercial pellet and offered water *ad libitum*. The female rats were examined 3 times a day for the presence of cervical plugs and those proved mated were kept separated from males. The pregnant females were grouped into two groups and kept separate away from any stress in (90 cm × 45 cm × 15 cm) in sterilized polypropylene cages lined with woody husk. Group I served as control and groups II as the experimental group. The females in the experiment group were subcutaneously injected with 10 mg/kg BW of proluton depot (Hydroxyprogesterone hexanoate; Schering AG; Germany) on the 1st, 7th and 14th of gestation. The dams' body weights were taken on each day of injection to adjust the dose. The prescribed dose of P_4 is within the range of the regular clinical dose given to pregnant women [12]. The females in the control group were injected with a placebo.

2.2. Tissue collection and preparation

Animals were anesthetized with chloroform and sacrificed with cervical dislocation. The testes were dissected and left testes and epididymides were weighed then fixed immediately in aqueous Bouin's solution for 18 h, dehydrated into 70%, 90% and 100% ascending grades of alcohol, cleared with xylene and embedded in paraffin wax. Sections were cut at 5 µm thickness by using an American Optical microtome (A0-821. USA). The sections were mounted onto glass slides, deparaffinized and stained with H & E [16]. The testes were separated from their adjacent epididymides and their diameters and lengths were measured. Relative testes and epididymides weights were then calculated per final body weight [16].

2.3. Histometry

Morphometric measurers of testicles were done according to Thienpot *et al*. [17] and Batra *et al*. [18]. Longitudinal sections of each rat testis were stained with H & E. Then 10 round or nearly round ST were chosen randomly to measure their diameters, the height of germinal epithelium (GE), the thickness of interstitial space (IS) and the number of GE cells using Olympus BX-40 light microscope supported with an image Pro Plus program (100×). Two tubular diameters for each tubule were mapped and their mean recorded. The thickness of IS was measured by measuring three dimensions of spaces assumed to connect the center of each space to the basement membrane of the surrounding ST. The mean of the three dimensions was calculated and multiplied by 2 to obtain the whole thickness of the IS. The GE height was obtained for the same tubules used to determine tubular diameter. GE epithelium was assumed from the basement membrane to the latest stage of GE (spermatids).

2.4. Cells quantitation

The testes of each rat were prepared for light microscopy. After animal scarification, testes were fixed by perfusion with Bouin's fixative for 30 min. The testis was then cut into three vertical longitudinal slices, the middle slice including the mediastinum. After immersion and fixation in Bouin's for another 1.5 h, the slices were dehydrated in ethanol, cleared with xylene and embedded in paraffin wax. From each testis slice five sections of 5 µm were cut, thus 15 sections were obtained and mounted individually onto slides. LC of 15 IS of each rat testis were counted under Olympus BX-40 microscope supported with an Image Pro Plus program. The mean counts of LC were calculated for each group. Also mean counts of the SC and GE cells (spermatogonia type A & B and primary spermatocytes cells) were also recorded [19,20].

2.5. Experimental design

This experiment is a one factorial design to investigate the effects of prenatal exposure to HPH on pubertal rats' testicular morphometry, histology and cells quantities. Forty puppies born to the experiment group (n = 20 puppies) and control group (n = 20 puppies) were allowed to grow for 90 d where they reached maturity. The testes were collected and prepared as above. Morphometry, histometry and cell quantitation were carried as described above.

2.6. Statistical analysis

Statistical analyses for all obtained parameters were performed by using SPSS-16.020 (Chicago, USA). Data were subjected to one way ANOVA and were expressed as mean ± SD. The level of significance was set at $P < 0.05$.

3. Results

3.1. Morphometric aspects

3.1.1. Testicular and epididymal weights

The experimental rats group showed a significant ($P < 0.001$) reduction in testicular and epididymal weights (Figure 1) compared to control group. The mean testicular weights of the control and experimental groups were (2.11 ± 0.33) and (1.60 ± 0.14) g and the mean epididymal weights were (0.67 ± 0.10) and (0.43 ± 0.06) g in the same respective. The relative testicular weight for the control and experimental rats

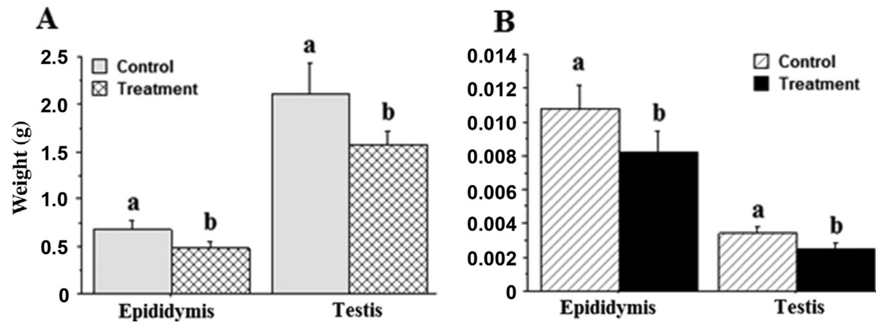

Figure 1. Mean testicular and epididymal weights (A) and relative weights (B).
Bars represent the mean ± SD of 20 replicates.[a,b] For the same organ differ at $P < 0.01$.

were (0.01 ± 0.01) and (0.01 ± 0.01) g and the mean relative epididymal weight were (4.0 ± 0.0) and (2.0 ± 0.0) mg, respectively.

3.1.2. Testicular dimensions

Figure 2 shows that the testicular dimensions of the control and the experiment rats are quite different ($P < 0.001$). The mean length of the right testis of the control (17.85 ± 0.04) mm and that of the experiment rats was (15.57 ± 0.04) while the width was (10.87 ± 0.04) and (8.49 ± 0.02) in the same respective.

3.1.3. Measurements of ST, GE and IS

The diameter of the ST, GE height and the thickness of the IS of the control and experimental rats varied significantly ($P < 0.001$). The mean diameters of ST of the control and experimental rats were (281.5 ± 17.2) and (255.5 ± 14.4) μm, respectively. The height of the GE was (93.7 ± 9.6) and (72.6 ± 7.7) μm, in the same respective as above. The thickness of the IS of the control was (110.5 ± 11.3) μm and that of the experiment rats was (132.1 ± 6.1) μm (Figure 3).

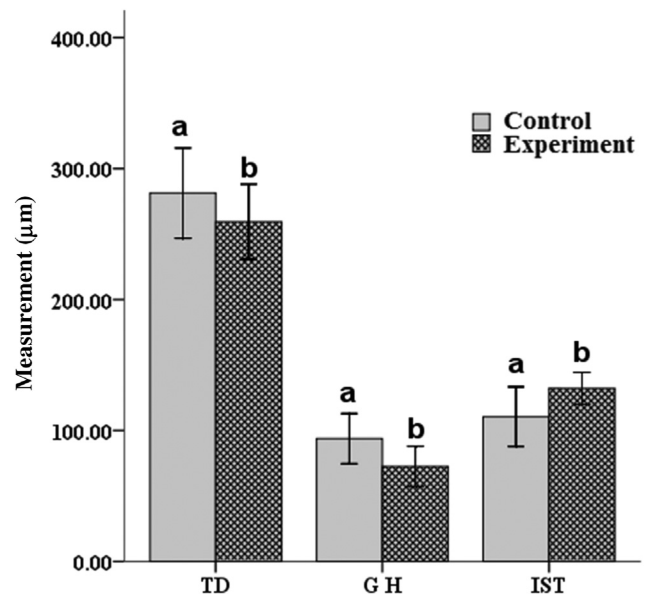

Figure 3. Compares the measurements of seminiferous tubule diameter (TD), germinal epithelium height (GH) and interstitial space thickness (IST) of control and experimental rats.
Bars represent the mean ± SD of 20 replicates.[a,b] $P < 0.01$.

3.2. Histological aspects

Figure 4 shows the rats' testicular parenchyma formed of ST and IS. Figure 4(A) shows that the ST of control rats are separated from the IS with an intact basement membrane. The cross sections of the ST appeared rounded or oval in shape containing spermatogenic and SC and resting on well-formed basement membrane formed of thin elongated simple epithelial cell layer and fibrous tissue. The different types of the spermatogenic cells are easily identified and so is the mitotic activity as revealed by the different stages of the spermatogenesis. The individual cells of the ST and interstitial tissue are clear and with normal features and the lumen contains obvious mass of sperms. Figure 4(B) illustrates many prominent abnormal features of the testis of experimental rats born to dams treated with HPC during pregnancy. The sections taken from testes of experiment group showed thin germinal layer compared to that of the control. The ST loses their usual configuration with detached and/or folded basal lamina. The IS are wide with large blood vessels due to hypoplasia, shrinkage and destruction of ST. Figure 4(C) shows control rat ST at high magnification (notice the normal germination and sperm masses). Figure 4(D) shows experimental rat ST with reduced layers of GE, sparse or no sperm masses and a wider ST lumen with obvious necrotic cellular debris.

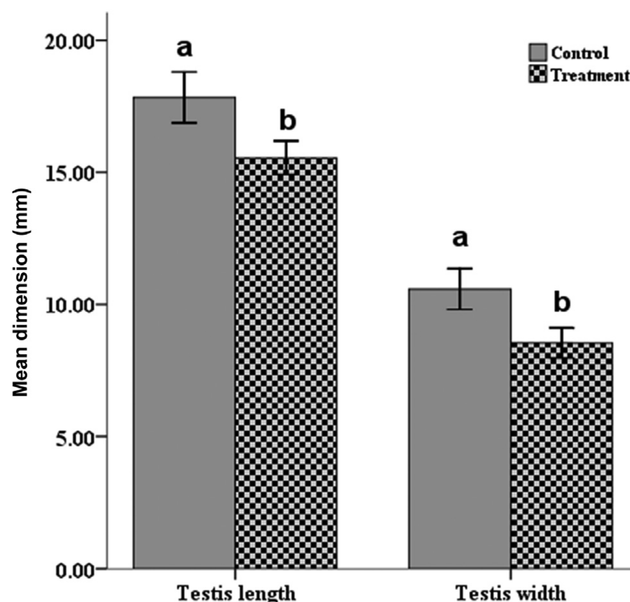

Figure 2. Compares dimensions of testes of control and experimental rats.
Bars represent the mean ± SD of 20 replicates.[a,b] $P < 0.01$.

As in Figure 5(A) the IS (A) of experimental rats testes appeared wider, contained large blood vessels and semi-empty cells except of few LC, connective tissue cells

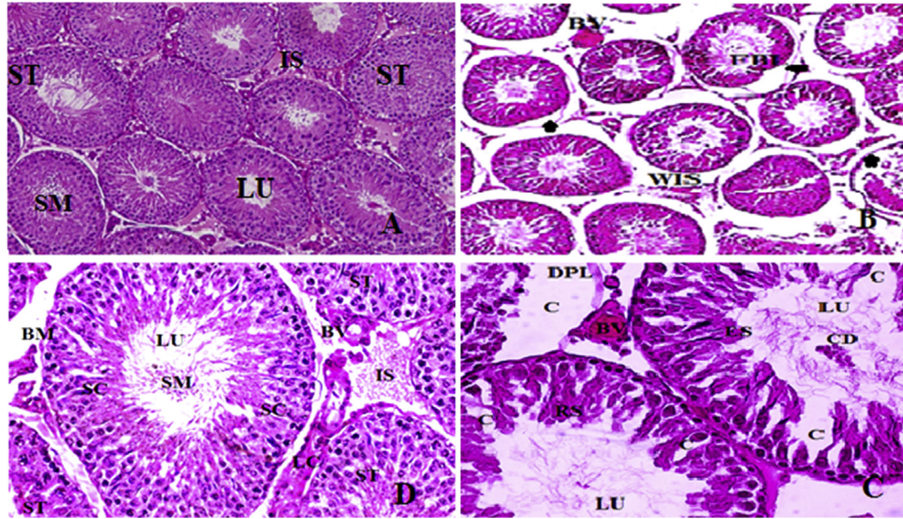

Figure 4. Light photomicrographs of rats testes parenchyma.
Control rat testis (A) and experimental rat testis (B) (×100). Control rat testis (D) and experimental rat testis (C) (×400). Seminiferous tubules (ST), interstitial spaces (IS), intact basement membrane (BM), Lumen (LU), sperm mass (SM), Leydig cell (LC), Sertoli cell (SC), wide interstitial space (WIS), detached basement membranes (star), detached basal laminae (arrow heads); folded basal laminae (FBL), elongated spermatids (ES), round spermatids (RS), detached basal laminae (DPL), debris in the lumen (CD) and blood vessels (BV).

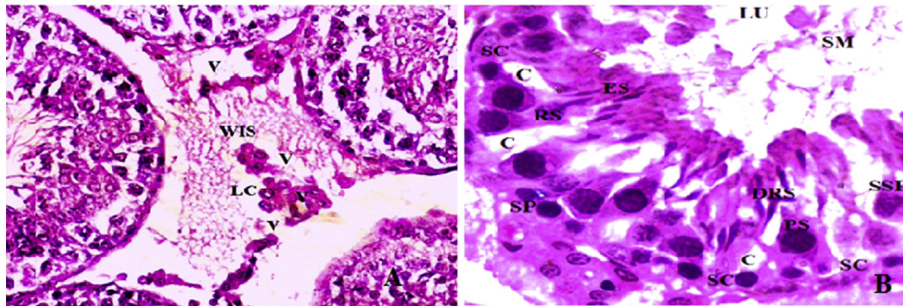

Figure 5. Micrograph A the interstitial space of experimental rat testis and micrograph B Sertoli cells with germinal epithelium of experimental rat.
WIS: with wider interstitial spaces; LC: Leydig cells; and V: vacuole (×400). SC: Sertoli cell; C: cytoplasmic vacuole; SP: spermatogonia; PS: primary spermatocyte, SSP: small size secondary spermatocyte with faintly stained chromatin; DRS: distorted elongated spermatids; ES: little number of elongated spermatids; RS: rounded spermatids; LU: tubular lumen (LU) and SM: sperm mass (×1 000).

and interstitial vacuoles. Figure 5(B) shows degenerative spermatogenic cells with apoptotic changes in the form of inter- and intra-cellular cavitations. The SC are few in most sections with great vesicular cytoplasm cavities resulting in abnormal spacing (vacuoles) between the germ cells of the seminiferous epithelium.

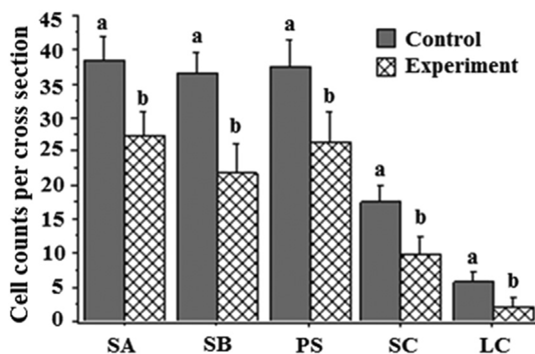

Figure 6. Comparison of seminiferous tubule cell counts.
SA: (spermatogonia type A); SB (spermatogonia type B); PS: primary spermatocytes; Sertoli cells and LC: Leydig cells. Bars represent the mean ± SD of 20 replicates.[a,b] For the same cell type differ at $P < 0.01$.

3.3. Testicular cells quantitation

Figure 6 shows that the counts of the different testicular cells per cross section varied significantly ($P < 0.01$) with treatment. The mean counts of spermatogonia type A of the control rats and the experimental rats were (38.6 ± 3.5) & (27.4 ± 3.6) and type B were (36.5 ± 3.1) & (21.8 ± 4.5), respectively. The primary spermatocytes were (37.5 ± 4.0) & (26.6 ± 4.5), SC were (17.7 ± 2.3) & (9.8 ± 2.8) and LC per a single interstitial space were (5.8 ± 1.5) & (2.1 ± 1.3) in the same order as above.

4. Discussion

Morphometric, histologic examination and cell quantitation revealed that the testes of rats borne to dams treat with HPH had undergone different gross and histological abnormal changes. The testis weight and dimensions are lesser than those of the controls. The ST appeared necrotic with folded basal laminae and they lost their usual configuration. The GE and SC suffered degenerative changes and lost their order. The diameter of the ST, thickness of the GE, luminal spermatozoa and the LC count were also affected.

These adverse effects are probably due to the effects of HPH and/or its metabolites. This findings support that of O'Donnell

et al. [21] and Abney [22]. Also this finding agrees with that of Harini *et al*. [12] and Pushpalatha *et al*. [13]. The rats born to females treated with HPH in this study suffered degeneration of SC and reduction in the number of LC. Proper male gonads development during prenatal life depends mainly on differentiation and development of LC and SC, which are necessary for the development of reproductive tract [23–25]. The fetal LC produce androgens during prenatal life [26] to develop the masculine characteristics of males [3] and SC; the first to be identified in fetal testis; are the nurses of spermatogenesis and play an important role in development of the ST and LC [26,27]. Since in this study SC are seriously affected and LC number is reduced the differentiation and maturation of the GE is affected [28]. The main mission of SC is to orchestrate the epithelium germination through sequential phases of mitosis, meiosis, and differentiation. This mission is accomplished by providing hormonal, nutritional, and physical support.

The SC of the experimental rats had undergone apoptotic changes such as cytoplasmic vacuoles and numerous lipid droplets. A similar phenomenon that affected spermatogenesis and sperm parameters was reported as a result of exogenous stimulant [29]. Furthermore empty vacuolar spaces were observed between SC (Places where spermatogonia and spermatocytes should germinate). The apoptotic changes observed in the GE agree with the findings of Blanco *et al*. [30] who described apoptosis in hamster testis following treatment with anabolic androgenic steroids. Apoptosis of germ cells that occurs in the testicular epithelium serves as a mechanism to reduce the germ cell population to the level that the SC can support. Some drugs and toxic agents injure or disrupt the function of SC and reduce their supportive role, resulting in increment of the apoptotic elimination process [31].

The detachment of germ cells and miss-location of spermatids as well as spermatozoa to positions that are closely related to the basement membrane are probably due to disruption of SC-germ cell interactions a phenomenon reported by Richburg [31].

Toxic agents enhance production of reactive oxygen species (ROS) in cells & tissues these ROS exert oxidative stress (OS), which in turn increases the rates of cellular damage [32]. It seems that HP and/or its metabolites; which can reach the fetal circuit [33]; have the capability to reach the fetal testes and induce similar oxidative stress. These oxidative stresses affect the testicular antioxidant system and lipid peroxidation leading to these cellular changes [34].

The decrement of spermatocytes and increment of the lumen of the ST in the present study are consistent with the study of Goyal *et al*. [8].

The decrements in testicular weights and dimensions recorded in this study could be attributed to germinal and somatic cell loss, ST shrinkage and/or hypotrophy due to prenatal exposure to HP. The weight of the testis is largely dependent on the mass of differentiated spermatogenic cells and it has been used as a measure of spermatogenesis in rats [35]. A positive correlation was observed between weight of testis and number of germ cells [36]. Since the epididymis is the storehouse of sperms the decrement in its weight is presumably due to low sperm production. Similar findings were reported by Pushpalatha *et al*. [13] and Harini *et al*. [12].

In conclusion, prenatal hydroxyprogesterone hexanoate exposure of male rats seriously affects their testicular morphometry, histology and cells quantities and presumably affects their future fecundity.

Conflict of interest statement

We declare that we have no conflict of interest.

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