ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Boldenone Undecylenate Injection Consequences on Male Rabbit Behavioral Response, Fertility and Testicular Ultrastructural Changes with Special Respect to the Withdrawal Effects

*** Samah R. Khalil[1], Hesham H. Mohammed[2], Yasmina M. Abd-Elhakim[1], Wafaa A.M. Mohamed[3]**

1. Department of Forensic Medicine and Toxicology, Faculty of Veterinary Medicine, Zagazig University, Egypt.
2. Department of Veterinary Public Health, Faculty of Veterinary Medicine, Zagazig University, Egypt.
3. Department of Clinical Pathology, Faculty of Veterinary Medicine, Zagazig University, Egypt.

Manuscript Info

Manuscript History:
Received: 12 November 2013
Final Accepted: 20 December 2013
Published Online: January 2014

Key words:
Anabolic androgenic steroids; Testesterone; Boldenone undecylenate; Sexual behavior; Aggressive behavior.

Abstract

Boldenone esters (mainly the undecylenate form) are heavily used in the field of animal production in order to increase the productivity and reduce breeding expense. The current study was conducted to evaluate boldenone undecylenate (BOL) effect on behavioral and reproductive responses. Therefore, 18 mature male New Zealand rabbits and 10 females were used in this study. The Animals were assigned to two groups, the control group received 0.25 ml corn oil ⁄ kg bwt and BOL-treated group received 4.5 mg⁄ kg bwt BOL, 3 intramuscular injection with two weeks interval. Six rabbits from BOL-treated group were withdrawn from the treatment which served as a BOL withdrawal group (6 weeks post 3rd injection). Blood, semen and testis specimens were collected for serum hormonal assay, semen evaluation and ultrastructural investigation. Sexual behavior was tested using a receptive female and aggressive behavior was assessed via a male intruder. The BOL-treated animals showed a significant increase in serum testosterone, diminished LH and estradiol levels, elevated sperm count and motility percentage. In contrast, diminished testosterone and elevated estradiol levels were recorded in animals withdrawn from BOL. Besides, low sperm count and motility percentage and severe ultrastructural alterations. Males treated with BOL showed significant increases in both sexual and aggressive behaviors. While, BOL-withdrawal group showed significantly lower levels of sexual behavior compared with control and did not show any heightened aggression. Considering the results of this study, BOL injection affects animal future fertility even after cessation of its use

Introduction

The natural male hormone, testosterone (TES) is the unique hormone in human and animals bodies that have both anabolic (muscle growth) and androgenic (masculinizing) effects. Hence, anabolic androgenic steroid (AAS) was developed as synthetic derivatives of TES with enhanced anabolic activity and reduced androgenic activity. They were used to improve the food producing animal growth rate through promoting protein synthesis and muscle growth (Mottram and George, 2000). Also, they can exert potent effects on the human body that may be beneficial for athletic performance (Hartgens and Kuipers, 2004).

Boldenone, is a synthetic AAS which differs from TES only by one double bond at the 1-position, synthesized by the dehydrogenation of TES, often produced and administered as esterified molecules in a ready-to-use anabolic preparations; boldenone undecylenate (BOL). It is applied as a growth promoter on meat production farms (De Brabander et al., 2004), in order to increase the productivity and to reduce breeding expense, therefore might be abused to achieve more efficient meat production. Also it is used to improve athletic, body builder and racing horse performance in sports (Ho et al., 2004).The abuse of esters of AAS in cattle fattening and sports is hard to control. In

Egypt, BOL has been used heavily in the field of animal production. It is represented under the trade name Equipoise, Ganabol, Equigan and Ultragan.

There is a growing disquiet that these synthetic hormones used in veterinary treatment are making their way into surface waters and even ground water via human and animal wastes, mainly by their incomplete removal during passage through wastewater treatment plants (Soto et al., 2004). It exhibits relatively more stability in aqueous media and more resistance to microbial degradation (Schiffer et al., 2001) leading to accumulation and persistence in the environment and endanger consumers with a permanent exposure. Beside the danger of consuming the meat of animals that treated with them.

The abuse of AAS was proved to cause serious and irreversible organ damage (Maravelias et al., 2005). Among the most common adverse effects of AAS that have been described are reduced fertility (Dohle et al. 2003), cardiovascular disorders (Sader et al., 2001), hepatic neoplasms and carcinoma (Velazquez and Alter, 2004), tendon damage (Battista et al., 2003), psychiatric and behavioral disorders in both sexes (Clark and Henderson, 2003).

According to the International Agency for Research on Cancer (IARC), BOL is classified in class 2A (growth promotors – steroids), as a probable human carcinogen with a high carcinogenic index (IARC Monograph, 1987). In light of this carcinogenic potential of AAS residues and obvious human health risks, the European Community banned the use of steroid hormones as growth-promoting agents in livestock breeding (EC, 1996), but in other countries orderly applied for meat production and human uses (Schiffer et al., 2001). Hence, the presence of boldenone or its metabolites in biological samples is proposed to be a marker for illegal hormone administration in various animal species especially in cattle fattening (De Brabander et al., 2004).

The potential behavioral effects of AAS abuse in human populations have received prominent coverage in a number of reviews (Christiansen, 2001 and Kuhn, 2002). However, less attention has been paid to the behavioral effects using animal models. Beside, the effects of AAS on reproductive capacity have been hardly studied in human as well as in many animal species (Thabet et al., 2010).

Rabbit is a good model to study different aspects of sexual behavior and performance (Villagran et al., 2003). Hence, mature male New Zealand rabbit was used in this work as a preclinical animal model to compare between the immediate effects of BOL exposure and after withdrawal time on various reproductive alterations and behavioral changes induced in meat-producing animal. The examined parameters include body weight gain, weight and histopathological analysis of testis, sperm characteristics as well as determination of serum concentrations of TES, luteinizing hormone (LH) and estradiol. In addition, examination of the behavioral consequences on both aggressive and sexual behavior.

Materials and Methods

Animals

Eighteen apparently healthy mature male New Zealand rabbits, 5– 6 months old (weighing 2800-3500 gm), in addition to ten sexually receptive females (for sexual behaviour assessment) were obtained from the Laboratory Animal's farm of Faculty of Vet. Medicine, Zagazig University and acclimated to the laboratory environment for 2 weeks prior to use. Animals were housed separately in metal cages, fed pelleted commercial feed (Abou Amer Co., Cairo, Egypt) and water were supplied. Rabbits were administered a prophylactic dose of ivermectin as a safe guard against mange and gastrointestinal nematodes. Adult male rabbits were trained to serve an artificial vagina for semen collection. All the experiment was approved by the ethical committee of Cairo University in accordance with the guidelines of the National Institutes of Health (NIH) for the Care and Use of Laboratory Animals.

Chemical and reagent

Equi-gan® vial was obtained from Laboratorios Tornel, Co., (S.A. Mexico). Each vial containing oily solution (50mg BOL/ml vehicle). All other chemicals, reagents and stains were obtained from El- Gomhoria Chemical Company (Egypt) and Sigma (St. Louis, MO, USA).

Experimental design

After 2 weeks of acclimatization, rabbits were assigned to two groups, vehicle control group (n=6) and BOL-treated group (n=12). Control group were received corn oil in a dose of 0.25 ml ⁄ kg bwt as a vehicle, BOL- treated group were received 4.5 mg⁄kg bwt BOL, 3 injections intramuscularly with two weeks interval for 4 weeks (Paget and Barnes, 1964). Six rabbit from BOL-treated group were withdrawn from the experimental treatment which served as a BOL withdrawal group (6 weeks post 3rd injection). Body weight for each animal was obtained weekly in order to calculate the injection volume required to adjust the desired dose. During the experiment, body weight was recorded on the first day before injection (initial) and on the day of sacrifice (final) and the weight gain was calculated.

Behavioral assessment

To evaluate the aggressive behavioral consequences of BOL exposure, resident-intruder model of offensive aggression (Christie and Barfield, 1979) was applied. Animals from BOL-treated and withdrawal groups were presented in their home cage with a stimulus male animal of equal age and weight from the control group. Number of bites, and latency and frequency of fight over a 10-min test period was scored.

For sexual behavior, all males used in this study were given a sexual rest before initiation of the study. Thereafter, female was introduced to the male's cage for a period during which the copulatory sexual behavior stages were recorded, then the female was immediately withdrawn and semen collected with artificial vagina, then a new female was introduced to the male's cage. The procedure continued until the male was not interested in the newly introduced female. When there was no longer response to the newly introduced female, the male was considered sexually exhausted (sexual satiety). The sexual behavior was scored through recording of different behavioral patterns observed during mating until sexual activity ceases such as: mount number and mount latency (time from introduction of the female until the first mount with pelvic thrusting), ejaculation latency (time between mounting and ejaculation), post ejaculatory refractory period, (time from one ejaculation until the next mount), furthermore the number of ejaculations was also recorded (sexual satiety) and its abnormal sexual behavior towards other male (Nelson, 1995; Agmo, 1996).

Semen collection and sperm characteristics

Ejaculates were collected from each male rabbit on the day of sacrifice with artificial vagina. The semen was evaluated immediately after collection for the seminal picture. The percent of motile spermatozoa was microscopically estimated at 400× magnification (mass and individual sperm motility) and sperm cell concentration was estimated according to Bearden and Fuquay (1980), the count was repeated five times for each sample to minimize the error. Sperm abnormalities were recorded according to Evans and Maxwell (1987). In order to assess the incidence of abnormalities in head, neck/midpiece and tail, at least 500 spermatozoa were observed per animal.

Sampling

Rabbits of control and BOL-treated groups were sacrificed after the last injection of vehicle and BOL respectively by one day while 6 weeks later in the BOL-withdrawl group. After decapitation trunk blood was collected then centrifuged at 3000 rpm for 10 min for separation of serum which stored at -20°C for hormonal analysis. After dissection, the testicles were removed, trimmed off the attached tissues, grossly examined and weighed. The relative weight of testis was calculated (testis weight/total body weight×100).

Hormonal assays

Serum hormonal TES, Estradiol and LH levels were determined using an enzyme linked immunosorbent assay (ELISA) with commercial kits, following manufacturer's instruction. Serum TES, Estradiol were evaluated using (Oxis International, Inc, USA. Catalog No. 11150 and 11110, respectively) kit, the sensitivity of assays were 0.05 ng/ml and 10 pg/ml respectively, the level expressed respectively as ng/ml and pg/ml. Serum LH was quantified using (BioCheck, Inc. USA. catalog No. BC-1031) kit, the sensitivity of assay was 1 mIU/ml.

Histopathological and ultrastructural investigation

Specimens from testis were fixed in Bouin's solution and were processed, stained with hematoxylin and eosin for histopathological investigation using light microscope according to Bancroft and Stevens (1996). For ultrastructural investigation, very small specimens were fixed by immersion in 3% glutaraldehyde solution for 24 hrs, washed by cacodylate buffer 0.1M, followed by post-fixation in 1% osmium tetraoxide (O_2O_4) in 0.1 M phosphate buffer (pH 7.3) for 2 hrs at 4°C. Then the tissues were dehydrated in upgraded series of ethanol and finally embedded in araldite 502 resin. Semi-thin sections (1 μm) were stained with uranium acetate and lead citrate and investigated with a transmission electron microscopy (TEM).

Statistical analysis

Data of the current study was statistically analyzed using the computer program SPSS/PC+2001. The statistical method was one way ANOVA test, followed by Duncan's multiple range test (Duncan, 1955). Data are presented as means plus or minus the standard error. The minimum level of significance was set at $P \leq 0.05$

Results

Body weight and relative testis weight

Table 1 shows that BOL injection has no significant effect on the body weight gain of both BOL-treated and withdrawal groups compared with the control group (-43.3 ± 61.19). While there is a significant decrease in BOL-withdrawal group (-73.3 ± 21.86) compared with treated one (83.3 ± 17.64). The relative testis weight was significantly increased in BOL-treated group (0.38 ± 0.02), while in the withdrawal group showed a significant decrease (0.19 ± 0.02)($P \leq 0.05$) compared with the control group (0.28±0.03).

Sperm characteristics

Semen characteristics was affected after BOL injection, rabbit treated with BOL showed significant elevation of sperm count (601.83±17.29) with high percentage of morphological abnormalities (13.80±0.95) than control group. In addition, sperm individual and mass motility were significantly increased (40.00±1.15, 86.67±1.67, respectively). In contrast, the BOL withdrawal group showed a significant decrease of sperm count (283.17±28.37) compared to those of the control group (372.50±22.55) (Fig 1). In addition the incidence of abnormal spermatozoa was significantly high (15.78±0.88) compared to control (8.24±0.28). As illustrated in Fig (2), the primary pathologic spermatozoa were (detached, microcephalic & pyriform head; detached, rudimentary &double tail), while the secondary abnormalities were (coiled & looped tail and protoplasmic droplets). There was no obvious difference in the incidence abnormalities between BOL-treated and withdrawal groups.

Hormonal analysis

The mean serum TES level was elevated significantly in male injected with BOL in comparison with control group (210.67 ± 7.31 and 146.00 ± 11.27, respectively) while in the withdrawal group, the level of serum TES decreased (166.0 ± 2.65), but still non significantly higher when compared with control group. LH serum levels was significantly diminished in both BOL-treated and withdrawal groups (1.07 ± 0.03 and 1.13 ± 0.04, respectively) compared with control one (1.42 ± 0.02). Serum Estradiol showed a significant decrease in BOL treated group (3563.33 ± 58.08), this diminished level was significantly increased in BOL-withdrawal group (5905.00 ±348.77) compared with control group (4283.00 ± 35.95) as illustrated in Fig (3)

Behavioral study

Animals treated with BOL showed significantly heightened measures of offensive aggression when presented with an intruder of equal size and weight. Male rabbits treated with BOL showed a significant increase in bites number (2.83 ± 0.48) ($p \leq 0.05$) over control animals (1.17 ± 0.17). In addition, BOL-treated rabbits displayed a significantly quicker aggressive response towards intruders (fight latency and frequency) (22.50 ± 2.81, 2.00 ± 0.37, respectively) than control animals (10.83 ± 0.83, 1.00 ± 0.26). The BOL-withdrawal animals did not score even a single bite on the intruder during the entire test period with short fight latency compared with control animals as shown in table 2.

Concerning sexual behavior, BOL administration evoked a significant difference on the number and latencies of mounts, intromissions and ejaculations. In general, BOL facilitated sexual behavior in BOL-treated group and reduced in withdrawal group. Data analyses showed a trend toward a significant increase number of mounts (4.67 ± 0.33) in BOL-treated relative to control group (3.17 ± 0.48). In contrast, animal withdrawn from BOL showed significantly fewer mounts (1.83 ± 0.40). The same pattern of results was found for latencies to show sexual behavior, reduced latencies and post ejaculatory interval in BOL-treated and lengthened of them in withdrawal one as shown in table 2. In BOL-treated group, male rabbits reach sexual satiety (i.e., they are unable to continue copulating on the same day) after (5.33 ± 0.33 ejaculations) while in BOL-withdrawal group, after (1.33 ± 0.21 ejaculations).

Histopathological investigation

The testis of control rats showed uniform seminiferous tubules with complete spermatogenesis and interstitial connective tissue (Fig 4A). While, the testis of BOL-treated-group showed moderate testicular degeneration with improvement of spermatogenesis. The latter was represented by presence of numerous elongated spermatids and huge numbers of spermatozoa in the lumina of the seminiferous tubules. Moreover, the testicular degeneration was evident by vacuolation and desquamation of the spermatocytes. Sometimes, the seminiferous tubules were lined by single or 2-3 layers of degenerated germ cells (Fig 4B). The Leydig cells were slightly depleted and degenerated. In comparison with the aforementioned findings of control and treated groups, the testis of withdrawal animals showed severe degenerative changes, atrophy and rarely necrosis in the majority of the seminiferous tubules with irregular, buckled basement membrane and incomplete spermatogenesis (Fig 4C). The seminiferous tubules were almost devoid of spermatids and spermatozoa. The Sertoli were focally proliferated and restored the Leydig cells (Fig 4D). Few leukocytes of mostly neutrophils were infiltrated the tubular structures and the interstitium. Regarding to the interstitium, there were congestion of the interstitial blood vessels.

The Transmission Electron Micrographs of control group showed normal basement membrane, primary spermatocytes, spermatogonia, Sertoli cell (Fig 5A) and normal Leydig cell with normal nucleus, mitochondria and smooth endoplasmic reticulum (Fig 5B). Normal spermatids and spermatozoa were noticed with electron dense, myelin bodies, lipid droplets, mitochondria, flagellum, and other organelles. The BOL-treated group revealed moderate empty vacuolar spaces in the Sertoli cells, spermatogonia and spermatocytes with numerous spermatids (Fig 6A). Numerous spermatozoa in the lumen of seminiferous tubules were detected (Fig 6B). Irregular and thickened basement membrane with normal basal lamina was visualized besides hydropic degeneration and rarefaction of the cytoplasm of germinal cells and fibrinous edema among these cells (Fig 6C). The Leydig cells were rarely detected and the remaining ones were vacuolated with swollen mitochondria (Fig 6D). The BOL-withdrawal testis showed widely separated layers of basal lamina, severe testicular degeneration with large empty

vacuoles in spermatocytes and Sertoli cells. The latter showed phagocytic vacuoles containing myelin bodies (Fig 7A). Wide interstitial space and vacuoles in the germ cells were seen (Fig. 7B). Focal proliferation of the Sertoli cells was seen besides the vacuolated germinal cells (Fig 7C). Few leukocytic infiltrations of mostly neutrophils were seen on the degenerated cells and the interstitial tissue. The Leydig cells were normal (Fig 7D).

Fig. 1. Effect of BOL intramuscular injection on sperm charactaristics of male New Zealand rabbit (Mean ± SE, n=6). Different superscripts depict significant differences among groups ($p \leq 0.05$)

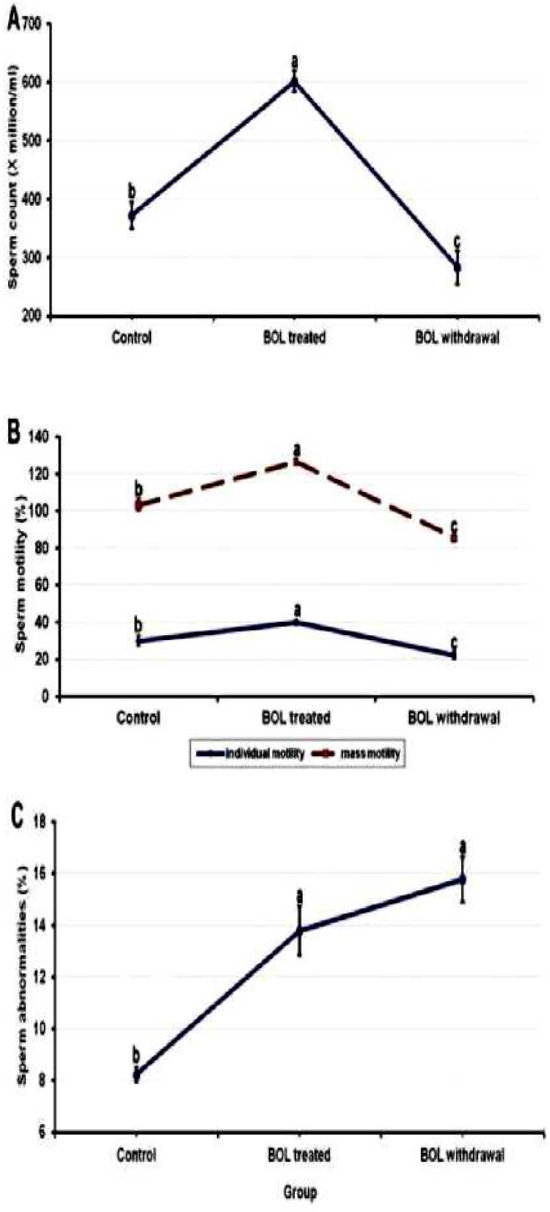

Fig. 2. Effect of BOL intramuscular injection on sperm morphology of male New Zealand rabbit (Eosin - nigrosin, X100).

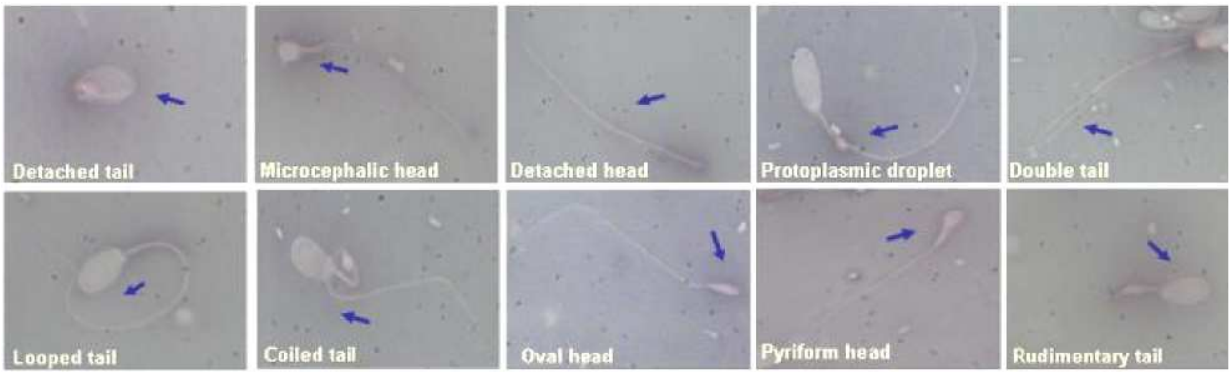

Fig. 3. Effect of BOL intramuscular injection on serum hormonal level (Testosterone, Luteinizing and Estradiol hormone) of male New Zealand rabbit (Mean ± SE, n=6). Different superscripts depict significant differences among groups ($p \leq 0.05$)

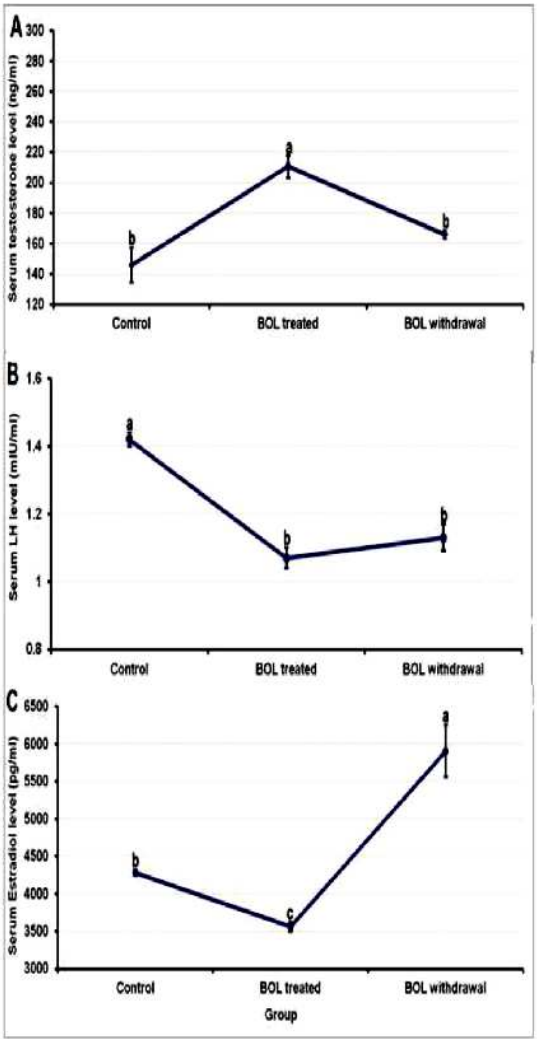

Fig. 4. Light microscopy of rat testis from: A, control with normal seminiferous tubules and spermatogenesis. B, treated group with moderate testicular degeneration (arrow) and improvement of spermatogenesis (arrowheads). C, D, withdrawal group with severe testicular degeneration, incomplete spermatogenesis and proliferated Sertoli cells (arrow) beside few leukocytes infiltration. (HE X. Bar = 100 µm).

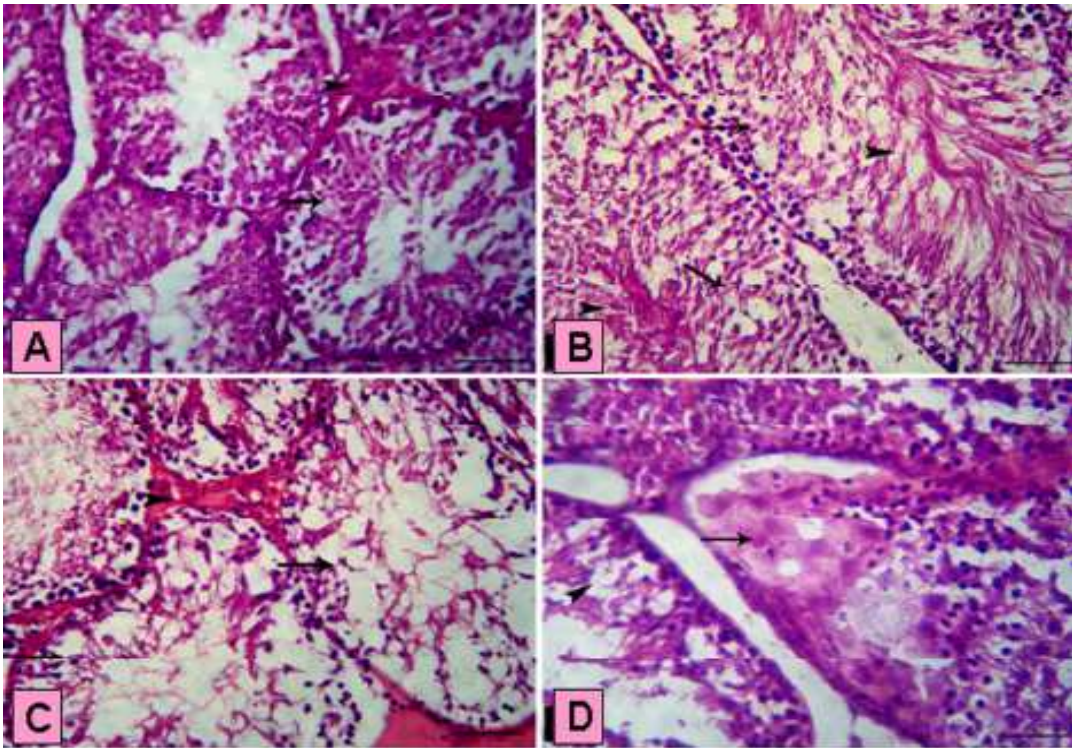

Fig. 5. Electron microscopy (TEM) of rat testis from the control group showing: A, normal basement membrane (BM), primary spermatocytes (PS), spermatogonia (SG), Sertoli cell (S), Sertoli cell junction (SJ) and interstitial spaces (ITS). B, normal Leydig cell (LC) with normal nucleus, mitochondria (M) and vesiculated smooth endoplasmic reticulum (SER). X. Bar = 2 microns.

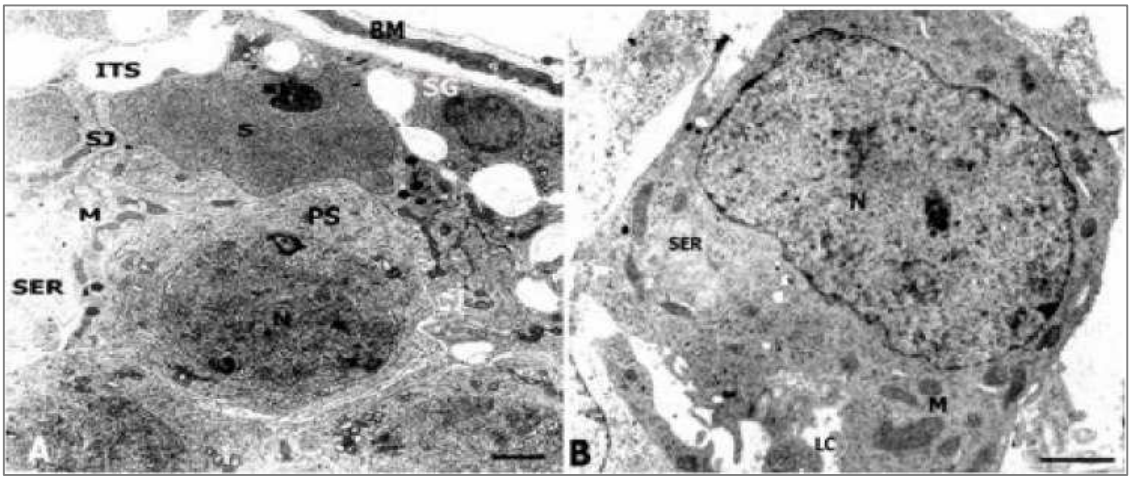

Fig. 6. Electron microscopy (TEM) of rat testis from the treated group showing: A, moderate empty vacuolar spaces (V) in the Sertoli and spermatocytes with numerous spermatids (SP). B, numerous spermatozoa in the lumen of seminiferous tubule (arrows). C, irregular (arrows) and thickened basement membrane (BM) with normal basal lamina (BL), rarefaction of the cytoplasm of primary spermatocytes (PS) and fibrinous edema among these cells (FE). D, vacuolation (V) in the Leydig cell (LC). X. Bar = 2 microns.

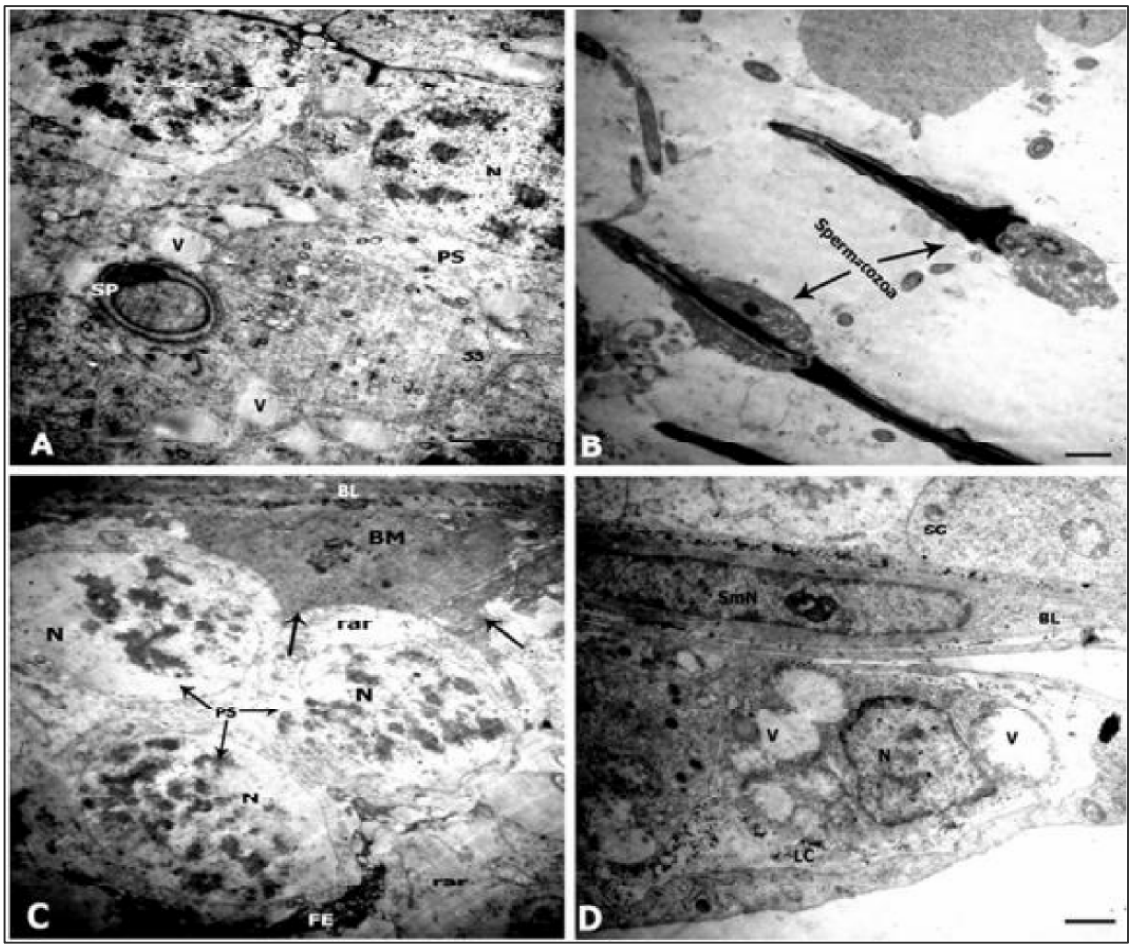

Fig.7. Electron microscopy (TEM) of rat testis from the withdrawal group showing: A, widely separated layers of basal lamina (BL), large empty vacuoles in spermatocytes and Sertoli cell (V) and phagocytic vacuole in Sertoli cell containing myelin, B, Wide interstitial space (ITS) and vacuoles in the germ cells (V). C, vacuolations in the germ cells (V) and proliferated Sertoli cells (SC). D, normal Leydig cell (LC) and leukocyte (Neu). X. Bar = 2 microns.

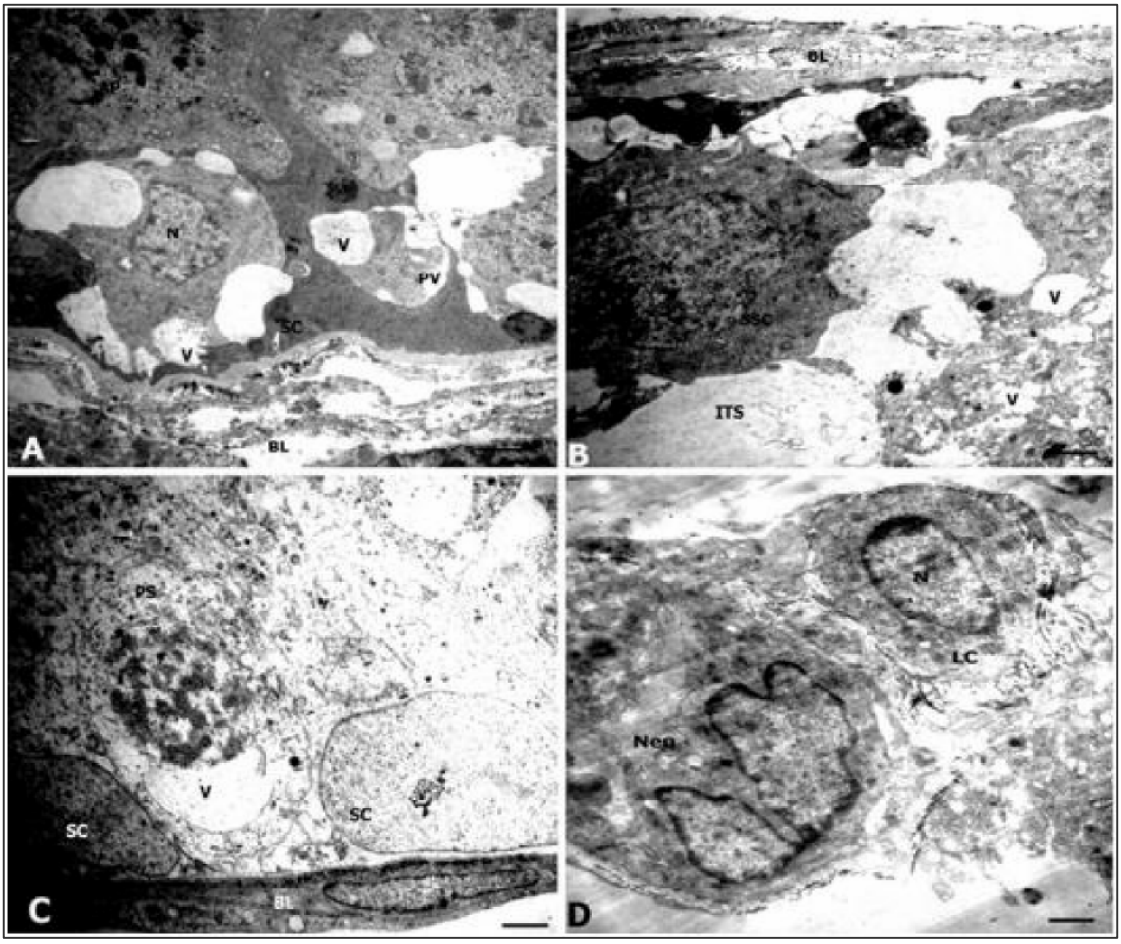

Table 1. Effect of BOL intramuscular injection on body weight gain and relative testis weight (g) of male New Zealand rabbit (Mean ± SE, n=6)

Parameter / Groups	Initial body weight (g)	Final body weight (g)	body weight gain (g)	Relative testis weight (g)
Control	2926.7±61.7 [a]	2883.3 ± 37.1 [a]	-43.3 ±61.19 [ab]	0.28±0.03 [b]
BOL- treated	3226.7±295.8 [a]	3310.0 ±278.4 [a]	83.3 ±17.64 [a]	0.38 ±0.02 [a]
BOL- withdrawal	3410.0±57.7 [a]	3336.7 ±76.2 [a]	-73.3 ±21.86 [b]	0.19±0.02 [c]

Means within the same column carrying different superscripts are significantly different ($p \leq 0.05$)

Table 2. Effect of BOL intramuscular injection on aggressive and copulatory sexual behavior of male New Zealand rabbit (Mean ± SE, n=6)

Parameter / Groups	Aggressive behavior			Copulatory sexual behavior				
	Fight latency (sec)	Fight frequency	Bites No.	Mount latency (sec)	No. of mounts	Ejaculation latency (sec)	Post-ejaculatory interval	No. of ejaculation
Control	10.83 [b] ±0.83	1.00 [b] ±0.26	1.17 [b] ±0.17	88 [b] ±6.69	3.17 [b] ±0.48	70.5 [b] ±5.68	105 [b] ±7.06	3.50 [b] ±0.22
BOL-treated	22.50 [a] ±2.81	2.00 [a] ±0.37	2.83 [a] ±0.48	61 [c] ±4.15	4.67 [a] ±0.33	53.5 [c] ±2.73	72.5 [c] ±5.50	5.33 [a] ±0.33
BOL-withdrawal	0.80 [c] ±0.51	0.17 [c] ±0.17	0.00 [c] ±0.00	108 [a] ±3.19	1.83 [c] ±0.40	101.5 [a] ±6.31	143.5 [a] ±9.81	1.33 [c] ±0.21

Means within the same column carrying different superscripts are significantly different ($p \leq 0.05$).

Discussion

Few studies have assessed the impact of BOL treatment on male reproductive function and behavior. Consequently, this study was performed to evaluate the effects of BOL on body weight gain, testis weight, hormonal level, sperm characteristics and histopathological investigation of mature male New Zealand rabbit testis. Besides, behavioral interactions especially aggressive and reproductive behaviors with special concern to the withdrawal impacts.

A conflicting data was recorded on the assessment of AAS effects on body weight gain in human and laboratory animals. Our study revealed that, BOL injection had no significant effect on the body weight gain of both BOLtreated and withdrawal groups compared with the control group. Similar results have been reported in horses (Maher et al., 1983), in female rats (Howe and Morello, 1985) and in veal calves (Cannizzo et al., 2007). In contrast, Thabet et al., (2010) and Toussan et al., (2012) revealed that injection of BOL evoked a significant increase in body weight of immature rabbit. Also, in veal calves (Toffolatti et al., 2006). Multiple factors, including administration time-course, dose, composition, and target-tissue metabolism have been contributed the AAS effects on body weight gain. Furthermore, androgens are known to cause alterations in many physiological processes, all predisposing the animal to changes in weight gain during the treatment period (Kochakian, 1990).

The elevated serum TES level recorded in the present work after administration of BOL is correlated with regular BOL injection which provides a continuous supply of TES into the blood producing a stable TES level. These findings agreed with other investigators (Muraoka, 2001; Shiono, 2001; Rasul and Aziz, 2012) in rats, (Tousson et al., 2012) in rabbit, Gabr and Shaker (2006) in ram using other TES derivatives. While, Simontacchi et al. (2004) showed that TES administration to bull calves did not induce any variation in plasma TES. In contrast, Oda and El-Ashmawy (2012) and Thabet et al. (2010) revealed that injection of anabolic steroid BOL to male rabbit evoked a significant decrease of serum TES level. This elevation of serum TES level was declined till became non significant with control group after 6 weeks of treatment stoppage, this may be attributed to the cessation of exogenous TES administration.

The serum estradiol level was diminished throughout BOL injection course in this study but elevated after stoppage of administration in withdrawal group. This may be attributed to aromatization of BOL whereas it converted in tissues to estradiol, subsequently estradiol strongly suppresses the spermatogenesis process (Torres-Calleja et al., 2001). Therefore, decreasing the sperm count as observed in BOL-withdrawal group of the present study.

Serum LH level was significantly decreased in both BOL-treated and withdrawal groups. Dohle et al., (2003) and Eklof et al., (2003) revealed that the exogenous treatment with synthetic TES usually followed by suppression of gonadotropin-releasing hormone production, LH production and intratesticular TES production via the negative feedback loop. The resultant intratesticular TES suppression leads to suppression of antioxidant enzyme expression, an increase in peroxidative damage, the disruption of spermatogenesis and an increase in germ cell apoptosis within the seminiferous tubule. Interestingly, the suppression of antioxidant activity largely affects the Leydig cells that contain most of the catalase and GPx activities (Chaki et al. 2006).

As the size and function of sexual organs are highly dependent on the level of TES, so the increased relative weight of testis recorded in BOL-treated was parallel with the significant increase in serum TES level observed in present work. On the other hand, BOL-withdrawal group showed significant decrease in relative weight of testis compared with the control one. The reduced testis weight may be as a result of the decrease of developing germ cell number and absence of different stages of spermatogonia as well as severe degenerative changes detected during

histopathological examination. The later finding is consistent with the previous results of (Thabet et al., 2010) after stoppage of BOL administration by several weeks. In the same line Brower (2002); Pope and Brower (2005) confirmed that the sudden cessation of AAS administration after a long period of use leading to hypogonadism consequently impaired sexual functioning and future infertility (Menon, 2003; de la Torre Abril et al., 2005). Usually, the hypothalamic-pituitary-testicular function recovers within weeks to months, but several reports have described hypogonadism persisting for more than a year after AAS were discontinued (Boyadjiev et al., 2000; Menon, 2003; van Breda et al., 2003), this can explain the persistence of decrease of testis in BOL -withdrawal group To further explore the effects of BOL on the male reproductive system, semen characteristics were investigated. The elevated sperm count and morphological sperm abnormalities observed in BOL-treated group may be attributed to elevated serum TES level as a result of regular use of BOL which consequently stimulate the spermatogenesis as TES essential for the attachment of different generations of germ cells in seminiferous tubules. Depending on histological and ultrastructural study, the present work reported that intramuscular injection of male rabbits with BOL is deleterious to the structure of testis which exhibited different alterations (more marked in BOl-withdrawal group). These changes manifested in BOL-treated group as moderate testicular degeneration with improvement of spermatogenesis which represented by presence of numerous elongated spermatids and huge numbers of spermatozoa in the lumina of the seminiferous tubules. Moreover, the testicular degeneration was evident by vacuolation and desquamation of the spermatocytes. Also, the Leydig cells were slightly depleted and degenerated. The enhanced spermatogenesis explained by continuous BOL administration consequently stimulates the spermatogenesis. Inhibited spermatogenesis was the prominent feature in BOL-withdrawal group. This in parallel with the significant reduction in serum TES level and extensive testicular structural perturbations recorded in this group, as TES level is directly linked to spermatogenesis (Amory et al., 2006). In addition, synthesis TES treatment could deplete intratesticular TES and arrest spermatogenesis (Park and Yi, 2002). Also, the decrease in sperm count may occur due to increase free radical formation initiating germ cell apoptosis and subsequent male infertility (Blanco-Rodriguez and Martinez-Garcia, 1998). Our results were similar to those reported in Thabet et al. (2010) where the sperm count significantly decreased after 30 and 45 day post last administration of BOL in male rabbit. The count and morphology of the sperm may be abnormal for months after drug withdrawal (Bahrke et al., 2000). In present work elevated morphological sperm abnormalities has been recorded, these results were similar to those reported by Wolfe et al. (1991) in bull, Brown (2005) and Ciocca (2005) in athletes and Cannizzo et al. (2007) in veal calves. On other hand, Oda and El-Ashmawy (2012) reported that no significant sperm abnormalities after BOL injection in rabbit.

There was obvious cessation of spermatogenesis in BOL-withdrawal group. The majority of seminiferous tubules showed severe degenerative changes, atrophy and rarely necrosis with irregular, buckled basement membrane. The seminiferous tubules were almost devoid of spermatids and spermatozoa. Also, severe damage of Sertoli cells consequently affects sperm production. The TEM has confirmed these results. These findings may be attributed to the diminished serum and intratesticular TES levels in BOL-withdrawal group. Consequently, leading to detachment of germ cells from seminiferous epithelium and may initiate germ cell apoptosis and subsequent male infertility (Blanco-Rodriguez and Martinez-Garcia, 1998). Cannizzo et al. (2007) suggested that most of the histopathological changes seen in testis can be explained by estradiol, this was confirmed by the increase in estradiol level recorded in the present study. leukocytic infiltrations (neutrophils) on the degenerated cells and the interstitial tissue by chemotaxic agents that produced by damaged inflammatory tissues. Normal Leydig cell seen in testis of withdrawn animals may be as a result of its proliferation to compensate the reduced TES level following exogenous TES stoppage.

The testicular lesions were similar to those described by Groot and Biolatti (2004); Thabet et al. (2010) and Oda and El-Ashmawy (2012). Tousson et al. (2012) showed the same results after BOL injection in rabbit, which increased in dose dependent way. These changes suggested that BOL adversely affects spermatogenesis which may lead to a continuous damage of the testicular function and structure and subsequent future infertility following BOL cessation, explaining the common genital progressive disturbances of athletes and body builder.

The behavioral responses to AAS depend on the chemical structure of the steroid administered, the age of animal, dose and duration of treatment (Clark and Henderson, 2003). This experiment demonstrated that treatment of male rabbit with BOL led to increased levels of aggressive and reproductive behaviors. In contrast, BOL-withdrawal animals showed significantly lower levels of sexual behavior and did not show heightened aggression when compared with control group.

A marked increase in sexual intercourse frequency was detected after BOL administration. A significant difference was observed in the number and latency of both mounts and ejaculations as a result of BOL treatment. While withdrawn animals showed absolute frigidity. In addition, it has been observed that the male animal mounts other males, also, long intromissions latency and decrease ejaculation number are indicative of the onset of

sexual satiety (Arteaga et al., 2000), which is the inhibition of masculine sexual behavior after repeated ejaculations. These results are in agreement with previous work in which the treatment with small doses of naloxone increased libido in bucks (Fuentes et al., 1997). The varaiation of sexual behavior between BOL-treated and withdrawal groups may be linked with variable TES level observed in this experiment, where as TES is one of the hormones that modulates sexual behavior (Crews, 1993).

This finding stands in contrast to previous studies, adult male rats treated with TES and TES cypionate do not display altered reproductive behaviors when compared to controls (Farrell and McGinnis, 2003). However, TES administered to intact adolescent male rats increased sexual behaviors in adulthood (Wesson and McGinnis, 2006). Also, long-term treatment of male rats with oxymethelone, stanozolol, nandrolone, or 17α-methyltestosterone during adolescence and continuing to adulthood results in a decrease in sexual behaviors (Farrell and McGinnis, 2003; Feinberg et al., 1997).

In fact, we found that BOL-withdrawal decreases sexual behavior, similar reductions in sexual behavior in response to exposure to supraphysiological levels of androgens have been reported in rats up to 9 weeks after withdrawal (Farrell and McGinnis, 2004) and hamsters (Meek et al., 1997). The decrement in sexual behavior induced by high levels of exogenous androgen may be secondary to a general suppression of endogenous hypothalamic–pituitary– gonadal axis function.

Conclusion

The results of this work suggested that the use of exogenous androgen, BOL had no effect on body weight gain but induced a deleterious effect on hormonal and behavioral events, which can be reflected by changes in the weights of the testis which confirmed by the histopathological examination of testis of treated male rabbit, hormonal status and sperm characterstics alterations. Besides, aggressive behavior and sexual behavior disruptions. Hence, affect in animal future fertility even after cessation of BOL. Considering the results of the study, further studies needed to clarify more BOL alterations including before the puberty period also longer withdrawal period over 6 weeks.

Acknowledgments

The authors are thankful to Prof. Dr. Mohamed Hamed Mohamed, Professor of Pathology, Faculty of Veterinary Medicine, Zagazig University for his guidance and support in histopathological study.

References

Agmo, A., Raul, G., Paredes, J., Ignaci Ramos, Contrerasso, J.L. (1996). Dopamine and Sexual Behavior in the Male Rabbit. Pharmacology Biochemistry & Behavior. 55 (2), 289-295.

Amory, J.K., Page, S.T., Bremner, W.J. (2006). Oral testosterone in oil: pharmacokinetic effects of 5*α* reduction with finasteride or dutasteride and food intake in men. J. Androl. 27,72–78.

Arteaga, M., Motte-Lara, J., Velazquez-Moctezuma, J. (2000). Temporal pattern of recovery from sexual satiety in male golden hamster (Mesocricetus auratus). Physiol. Behav. 68(4), 591–4.

Bahrke, M.S., Yesalis, C.E., Kopstein, A.N., Stephens, J.A. (2000). Risk factors associated with anabolic-androgenic steroid use among adolescents. Sports Med. 29, 397–405.

Bancroft, J.D., Stevens, A. (1996). Theory and Practice of Histological Technique. 4th Ed., Churchill, Livingston, New York, London, San Francisco, Tokyo.

Battista, V., Combs, J., Warne, W.J. (2003). Asynchronous bilateral Achilles tendon ruptures and androstenediol use. Am. J. Sports Med. 31, 1007–1009.

Bearden, H,J., Fuquay, J. (1980). Applied Animal Reproduction. Reston, VA: Reston Publishing Co. Inc, 158–160.

Blanco-Rodriguez, J., Martinez-Garcia, C. (1998). Apoptosis precedes detachment of germ cells from the seminiferous epithelium after hormonal suppression by short-term estradiol treatment of rats. Int. J. Androl. 21, 109–115.

Boyadjiev, N.P., Georgieva, K.N., Massaldjieva, R.I., Gueorguiev, S.I. (2000). Reversible hypogonadism and azoospermia as a result of anabolic-androgenic steroid use in a bodybuilder with personality disorder. A case report. J. Sports Med. Phys. Fitness. 40, 271–274.

Brower, K.J. (2002). Anabolic steroid abuse and dependence. Current. Psychiatry Rep. 4, 377–387.

Brown, J.T. (2005). Anabolic steroids: what should the emergency physician know? Emergency Med Clinics of North Amer. 23, 815-826.

Cannizzo, T.F., Zancanaro, G., Spada, F., Mulasso, C., Biolatti, B. (2007). Pathology of the Testicle and Sex Accessory Glands Following the Administration of Boldenone and Boldione as Growth Promoters in Veal Calves. J. Vet. Med. Sci. 69(11), 1109–1116.

Chaki, S.P., Misro, M.M., Gautam, D.K., et al. (2006). Estradiol treatment induces testicular oxidative stress and germ cell apoptosis in rats. Apoptosis. 11, 1427-37.

Christiansen, K. (2001). Behavioural effects of androgen in men and women. Jorunal of Endocrinology. 170, 39–48.

Christie, M.H., Barfield, R.J. (1979). Effects of castration and home cage residency on aggressive behavior in rats. Horm. Behav. 13(1), 85–91.

Ciocca, M. (2005). Medication and supplement use by athletes. Clinics in Sports Med. 24, 719-738.

Clark, A.S., Henderson, L.P. (2003). Behavioral and physiological responses to anabolic-androgenic steroids. Neurosci. Biobehav. Rev. 27, 413–436.

Crews, D. (1993). Diversity hormone-behavior relation in reproductive behavior. In: Becker JB, Breedlove SM, Crews DA (Eds.) Behavior Endocrinology. The Bradford Book. MIT Press, Cambridge, MA, USA (Chapter 5).

De Brabander, H.F., Poelmans, S., Schilt, R., Stephany, R.W., Le Bizec, B., Draisci, R., Sterk, S.S., et al. (2004). Presence and metabolism of the anabolic steroid boldenone in various animal species: A review. Food Additives and Contaminants. 21, 515–525.

de la Torre Abril, L., Ramada Benlloch, F., Sanchez Ballester, F., et al. (2005). Management of male sterility in patients taking anabolic steroids. Arch. Esp. Urol. 58, 241–244.

Dohle, G.R., Smit, M., Weber, R.F. (2003). Androgens and male fertility. World J. Urol. 21, 341–345.

Duncan, D.B. (1955). Multiple range and multiple F-test. Biometrics. 11,1-42.

EC. (1996). Council directive 96/22/EC of 29 April 1996 concerning the prohibition on the use in stockfarming of certain substances having a hormonal or thyrostatic action and of beta-agonists, and repealing directives 81/602/EEC, 88/146/EEC and 88/299/EEC. Official Journal of the European Communities. 125, 3–9.

Eklo., A.C., Thurelius, A.M., Garle, M., Rane, A., Sjoqvist, F. (2003). The anti-doping hot-line, a means to capture the abuse of doping agents in the Swedish society and a new service function in clinical pharmacology. Eur. J. Clin. Pharmacol. 59, 571 –577.

Evans, G., Maxwell, W.M. (1987). Handling and examination of semen. In: Salamon's Artificial Insemination of Sheep and Goats, p. 93 (ed W.M.C. Maxwell), Sydney, Australia: Butterworths.

Farrell, S.F., McGinnis, M.Y. (2003). Effects of pubertal anabolic–androgenic steroid (AAS) administration on reproductive and aggressive behaviors in male rats. Behav. Neurosci. 117 (5), 904–911.

Farrell, S.F., McGinnis, M.Y. (2004). Long-term effects of pubertal anabolic–androgenic steroid exposure on reproductive and aggressive behaviors in male rats. Horm. Behav. 46, 193-203.

Feinberg, M.J., Lumia, A.R., McGinnis, M.Y. (1997). The effect of anabolic– androgenic steroids on sexual behavior and reproductive tissues in male rats. Physiol. Behav. 62 (1), 23–30.

Fuentes, V.O., Fuentes, P.!., Garcia, A. (1997). Chronic treatment with nal oxone enhances libido in the male goat during anoestrus. Vet. Rec. 141(2), 52.

Gabr, F.!., Shaker, M.H. (2006). Effect of anabolic steroids (Bol denone Undecylenate) on metabolic, hormones and reproductive performance of mature rams. j. Egypt Vet. Med. Assoc. 66(2), 235-248

Groot, M.J., Biolatti, B. (2004). Histopathological Effects of Boldenone in Cattle. J. Vet. Med. A. 51, 58–63.

Hartgens, F., Kuipers, H. (2004). Effects of androgenic-anabolic steroids in athletes. Sports Med. 34, 513–554.

Ho, E.N., Yiu, K.C., Tang, F.P., Dehennin, L., Plou, P., Bonnaire, Y., et al. (2004). Detection of endogenous boldenone in the entire male horses. J Chromatogr B: Anal. Technol. Biomed. Life Sci. 808(2), 287–94.

Howe, G.R., Morello, C.J. (1985). Effects of an anabolic steroid on reproduction in female rats. Steroids. 45, 497–501.

IARC Monograph, (1987). Suppl. 7, 11: http://monographs.iarc.fr/ ENG/ Monographs/ suppl7/suppl 7.pdf (accessed June 15, 2006).

Kochakian, C.D. (1990). History of anabolic-androgenic steroids. In: Lin, G.C; Eirinoff L, eds. Anabolic steroid abuse. N IDA Research Monographs 102, 29–59.

Kuhn, C.M. (2002). Anabolic steroids. Recent Prog. Horm. Res. 57, 411–34.

Maher, J.M., Squires, E.L., Voss, J.L., Shideler, R.K. (1983). Effect of anabolic steroids on reproductive function of young mares. J. Am. Vet. Med. Assoc. 183, 519–524.

Maravelias, C., Dona, A., Stefanidou, M., Spiliopoulou, C. (2005). Adverse effects of anabolic steroids in athletes A constant threat. Toxicol. Lett. 158, 167–175.

Meek, L.R., Romeo, R.D., Novak, C.M., Sisk, C.L. (1997). Actions of testosterone in prepubertal and postpubertal male hamsters: dissociation of effects on reproductive behavior and brain androgen receptor immunoreactivity. Horm. Behav. 31 (1), 75–88.

Menon, D.K. (2003). Successful treatment of anabolic steroid-induced azoospermia with human chorionic gonadotropin and human menopausal gonadotropin. Fertil. Steril. 79(Suppl 3),1659–1661.

Mottram, D.R., George, A.J. (2000). Anabolic steroids. Baillieres Best Pract. Res. Clin. Endocrinol. Metab. 14, 55–69.

Muraoka, K. (2001). Effects of testosterone replacement on renal function and apoptosis on mesangial and renal tubule cells in rats. Yonago Acta Medica. 41, 37-44.

Nelson, R.J. (1995). An Introduction to Behavioral Endocrinology. Signer Associates Inc. Sunderland, Massachusetts, USA.

Oda, S.S., El-Ashmawy, I.M. (2012). Adverse effects of the anabolic steroid, boldenone undecylenate, on reproductive functions of male rabbits. I nt. J. Exp. Path. 93, 172–178

Paget, G.E., Barnes, J.M. (1964). Evaluation of Drug Activities, Toxicity Tests. Pharmacometrics, vol. I. London and New York: Academic Press, pp. 135.

Park, C.S., Yi, Y.J. (2002). Comparison of semen characteristics, sperm freezability and testosterone concentration between Duroc and Yorkshire boars during seasons. Animal Reprod. Sci. 73, 53–61.

Pope, H., Brower, K. (2005). Anabolic-Androgenic Steroid Abuse. In: Sadock, B, Sadock, V Comprehensive Textbook of Psychiatry. Philadelphia, PA: Lippincott Williams & Wilkins. p. 1318-1328.

Rasul, K.H., Aziz, F.M. (2012). The Effect of Sustanon (Testosterone Derivatives) Taken by Athletes on the Testis of Rat. Jordan Journal of Biological Sciences. 5(2), 113 – 119.

Sader, M.A., Griffiths, K.A., McCredie, R.J., Handelsman, D.J., Celermajer, D.S. (2001). Androgenic anabolic steroids and arterial structure and function in male body builders. J American College of Cardiology. 37, 224-230.

Schiffer, B., Daxenberger, A., Meyer, K., Meyer, H.D. (2001). The fate of trenbolone acetate and melengestrol acetate after application as growth promoters in cattle: environmental studies, Environ. Health Perspect. 109,1145–1151.

Shiono, M. (2001). The effect of aging and exogenous testosterone replacement on nitric oxide concentration and activity of nitric oxide synthase in the rat corpus cavernosum. Yonago Acta Medica. 44, 45-53.

Simontacchi, C., Perez de Altamirano, T., Marinelli, L., Angeletti, R., Gabai, G. (2004). Plasma steroid variations in bull calves repeatedly treated with testosterone, nortestosterone and oestradiol administered alone or in combination. Vet. Res. Commun. 28(6), 467-77.

Soto, A.M., Calabro, J.M., Prechtl, N.V., Yau, A.Y., Orlando, E.F., Daxenberger, A., et al. (2004). Androgenic and estrogenic activity in water bodies receiving cattle feedlot effluent in Eastern Nebraska, USA. Environ. Health Perspect. 112(3), 346-52.

Thabet, N.S., Abelrazek, E.M., Ghazy, E.M., Elballal, S.S. (2010). Effect of the anabolic steroids, boldenone undecylenate on reproductive performance on male rabbits. J. Reprod. I nfertil. 1, 8–17.

Toffolatti, L., Rosa Gastaldo, L., Patarnello, T.C., Romualdi, R., Merlanti, C., Montesissa, L., et al. (2006). Expression analysis of androgen-responsive genes in the prostate of veal calves treated with anabolic hormones. Domestic Animal Endocrinology. 30, 38–55.

Torres-Calleja, J., González-Unzaga, M., DeCelis-Carrillo, R., et al. (2001). Effect of androgenic anabolic steroids on sperm quality and serum hormone levels in adult male bodybuilders. Life Sciences. 68, 1769–1774.

Tousson, E., El-Moghazy, M., Massoud, A., Akel, A. (2012). Histopathological and immunohistochemical changes in the testis of rabbits after injection with the growth promoter boldenone. Reprod. Sci. 19(3), 253-9.

van Breda, E., Keizer, H.A., Kuipers, H., Wolffenbuttel, B.H. (2003). Androgenic anabolic steroid use and severe hypothalamic-pituitary dysfunction: a case study. Int. J. Sports Med. 24, 195–196.

Velazquez, I. and Alter, B.P. (2004). Androgens and liver tumors: Fanconi's anemia and non-Fanconi's anemia. Am. J. Hematol. 77,257–267.

Villagran C, Navarro J, Fuentes VO (2003) Sexual exhaustion in White New Zealand male rabbits of different ages. Animal Reproduction Science. 76, 251 –255.

Wesson, D.W., McGinnis, M.Y., (2006). Stacking anabolic androgenic steroids (AAS) during puberty in rats: a neuroendocrine and behavioral assessment. Pharmacol. Biochem. Behav. 83(3), 410–419.

Wolfe, D.F., Carson, R.L., Mysinger, P.W., Duran, B.S., Rahe, H.S., (1991). Effects of anabolic steroid, boldenone undecylenate, on certain reproductive parameters in bulls. Theriogenol 35, 893-906.