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Effect of the Anabolic Steroid, Boldenone Undecylenate on Reproductive Performance of Male Rabbits

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Abstract: The present study was conducted to clarify the effect of the anabolic steroid boldenone undecylenate (BOL) on the reproductive performance of male rabbits. The study was carried out on 60 apparently healthy White New Zealand male rabbits. Rabbits were divided into three groups each of 20 rabbits. The first group was injected with an adjuvant substance (sesame oil) and was considered as the control group (C). The second group was administered BOL at the recommended dose for rabbits (4.5mg/kg b.w) and was indicated as group R. The third group was administered BOL at a dose double the recommended dose (9 mg/kg b.w) and was considered as group D. All treatments were given three times at three weeks interval. Blood, semen and tissues specimens were collected three times after the last drug injection with two weeks interval. Evaluated parameters included growth performance parameters [total weight gain (TWG), feed conversion rate (FCR) and feed efficiency (FE)], serum hormonal assays [testosterone (TES) and estradiol (E2)], semen evaluating parameters [ejaculate volume (EV), sperm concentration (SC), total sperm output (TSO), packed semen volume (PSV), mass motility (MM), total motile sperms (TMS), individual progressive motility (IPM), sperm motility index (SMI) and percentage of sperms with morphological abnormalities (Abs)] as well as histopathological examination of testes. Testes weights also have been determined. Results showed that BOL injection in male rabbits resulted in a significant increase ($p \leq 0.05$) in TWG and FE and a significant decrease ($p \leq 0.05$) in FCR. There was a significant decrease ($p \leq 0.05$) in serum concentrations of TES while serum E2 was significantly ($p \leq 0.05$) increased. Evaluation of semen parameters showed a significant ($p \leq 0.05$) decrease in SC, TSO, PSV, MM, TMS, IPM and SMI and a significant ($p \leq 0.05$) increase in the number of morphologically abnormal sperms. BOL administration resulted in also a significant ($p \leq 0.05$) decrease in testes weight in all treated groups. Histopathological examination revealed the presence of severe lesions in testes including stromal proliferation, fibrosis, degeneration of gonocytes, vacuolization and desquamation of germinal epithelium, hemorrhage and absence of different stages of spermatogonia. The present study demonstrates that administration of BOL in male rabbits results in obvious improvement in growth rate, but also many reproductive side effects are produced including clear decrease in serum TES levels and quite increase in serum E2 concentrations. In addition BOL injection produces highly detrimental effects on semen quality, spermatozoal output, production and testis size. Further studies are needed to confirm this conclusion in rabbits. Nevertheless, the results support previous suggestions that the severity of these reproductive side effects is dose-dependent.

Key words: Anabolic steroid · Boldenone undecylenate · Rabbit · Infertility · Growth promoters

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INTRODUCTION

Anabolic androgenic steroids (AAS) are synthetic substances that mimic the male sex hormones; androgens [1]. They are derived from the modification of the testosterone molecule in order to augment or limit certain characteristics of testosterone [2]. Testosterone is the principle hormone in human and animals that produces male secondary sex characteristics (androgenic) and is an important hormone in maintaining adequate nitrogen balance, thus aiding in tissue healing and the maintenance of muscle mass (anabolic) [3,4]. In general, testosterone has been altered to develop agents that are more anabolic and less androgenic than testosterone, thus multiplying the compound's desirable, anabolic, nitrogen-sparing effects and minimizing its generally undesirable androgenic effects [5]. As AAS are either derived from or are closely related to testosterone which has strong genitotropic effects, it will not be surprising that side effects of these agents include the reproductive system which may be influenced to a great extent [2, 6, 7]. Well over a thousand different compounds have been synthesized and studied in the hope of producing compounds that have a more anabolic and less androgenic effect superior to that of testosterone [8]. Boldenone undecylenate (BOL) is a synthetic androgenic steroid hormone with anabolic properties intensively used for growth promoting purposes in animals destined for meat production and as a performance enhancer in athletics [9, 10]. As for other anabolic steroids, its illegal use as a growth promoter to increase body mass and enhance physical conditioning is becoming particularly widespread [8]. Rabbit has since been identified as an economy livestock that could bridge the wide gap in dietary protein intake all over the world [11, 12]. Rabbit when compared with other livestock animals is characterized by a very fast notorious reproductive rate indicated by early sexual maturity, high prolificacy, relatively short gestation length and short generation interval as well as high productive potential, rapid growth and good quality protein content [13-15]. This may be a very important characteristic feature as a part of the strategic promotion of rabbit production to be relevant in solving the problem of meat shortage in developing countries including Egypt and particularly on the level of the small-scale farmers. The effects of AAS on reproductive capacity have been hardly studied in human as well as in many animal species. But, inadequate evidence exists on the AAS-induced impaired fertility in rabbits. For this purpose, the objective of the present article was to evaluate the effect of BOL on reproductive performance in male rabbits with special respect to the effect of dosage.

MATERIALS AND METHODS

Rabbits: Sixty apparently healthy 60 days old male White New Zealand rabbits weighing, 1250-1500g were used in the present study. Rabbits were kept in battery cages and were administered a prophylactic dose of ivermectin (avemic, avico company) as a safe guard against mange and gastrointestinal nematodes. In addition, a prophylactic dose of inactivated vaccine (Hipra, S.A laboratories) against viral hemorrhagic disease was given at a dose of 0.5 ml/rabbit subcutaneously into the fore back.

Experimental Protocol: After two weeks of adaptation and acclimatization, rabbits were divided uniformly by weight into three equal groups each of 20 rabbits. The first group was administered adjuvant substance (sesame oil) and was considered as the control (group C). The second group (group R) was treated with BOL (Tornel laboratories, Mexico) at the manufactured recommended dose for rabbit (4.5mg/kg b.w) according to Paget and Barnes [16]. The third group was treated with BOL at a dose double the recommended dose (9 mg/kg b.w) and was indicated as group D. All treatments were administered intramuscularly three times at three weeks interval. The rabbits were offered a commercial ration pellets (Atmida Co.). Each buck was fed an amount of pellet ration (60gm/kg b.w/day) according to AOAC that provides normal growth and maintains adult body weight. Fresh tap water was supplied *ad libitum*.

Sampling

Blood Samples: Blood samples were collected in plain centrifuge tubes and serum samples were separated and stored at -20°C until used for hormonal assays.

Semen Collection: Adult male rabbits were trained to serve an artificial vagina (IMV, France) and a teaser doe two weeks prior to semen collection. This preliminary period was chosen in order to assure that males were reproductively normal according to their libido and semen characteristics, also to avoid the collection of old spermatozoa accumulated within the epididymis.

Histopathology: For histopathology, tissues from testes were taken, fixed in 10% formalin to be prepared for histopathological examination. Tests weights were determined before fixation.

All blood, tissues and semen specimens were collected three times following the last injection dose of the drug at two weeks interval, i.e. at 15, 30 and 45 days post injection.

Evaluated Parameters

Growth Performance Parameters: Animals were weighed weekly early in the morning before feeding. Feed residues, average feed intake (g) and average body gain (g) were recorded daily. Total weight gain (TWG) (g) was determined as the difference between the weights of rabbits at the beginning and end of experiment. Feed conversion rate (FCR) was determined as feed intake (g) divided by weight gain (g) [17]. Feed efficiency (FE) was determined as daily body gain (g) divided by daily feed intake (g) [17].

Hormonal Assays: Serum concentrations of testosterone (TES) and estradiol (E2) were determined using the electrochemiluminescence immunoassay (ECLIA) intended for use on Elecsys and Cobas immunoassay analysers (Roche, Germany) according to methods described previously [18, 19].

Semen Evaluating Parameters: Semen evaluating parameters included quantitative parameters as ejaculate volume (EV, ml), spermatozoa concentration (SC, $x10^6$/ml), total sperm output (TSO, $x10^6$/ejaculate) and packed semen volume (PSV, %).

Qualitative semen evaluating parameters included mass motility (MM), total motile sperms (TMS, $x10^6$/ejaculate), individual progressive motility (IPM, %), sperm motility index (SMI) and percentage of live sperms with morphological abnormalities (Abs, %).

Histopathology: Tissues from testes prepared for histopathological evaluation were examined microscopically for any abnormal lesions.

Statistical Analysis: Data were subjected to statistical analysis using one way analysis of variance (ANOVA). All data were presented as mean± standard error (SE). Means were compared by the Duncan test at 0.05 level of probability.

RESULTS

Growth Performance: Table 1 shows that injection of BOL in male rabbits resulted in significant ($p \leq 0.05$) increase in TWG and FE, while FCR was significantly ($p \leq 0.05$) decreased. These changes were more prominent when the double dose of the drug was injected compared to rabbits given the recommended dose.

Hormonal Assays: The mean values of serum testosterone showed a significant ($p \leq 0.05$) decrease at 30 and 45 days post injection (Table 2). Significant differences between both doses of BOL was not recorded. Table 2 also indicates that the mean values of serum estradiol levels were significantly ($p \leq 0.05$) higher in BOL treated rabbits as compared to their respective control values. The increase was more significant ($p \leq 0.05$) in rabbits treated with the double dose of BOL compared to animals given the recommended dose.

Semen Evaluating Parameters

Quantitative Semen Evaluating Parameters: The effect of BOL on quantitative semen parameters as shown in (Table 3) indicated that SC showed a significant ($p \leq 0.05$) decrease in both treated groups with the most lower values were recorded in group D compared to the rabbits of group R, particularly at 15 and 45 days post injection. TSO decreased in both treated groups. The decrease was more significant ($p \leq 0.05$) in rabbits treated with the double dose of BOL during all collection periods. PSV decreased after injection of BOL. Significant ($p \leq 0.05$) differences from control values were seen in group R at 30 days post injection and in group D at 15 and 30 days after injection. Significant adverse effects of BOL on EV of male rabbits were not reported in both treated groups at any time of the collection periods (Table 3).

Table 1: Effect of BOL on growth performance parameters in male rabbits (Values are mean ± SE)

	Group		
Parameter	C	R	D
TWG (g)	775±0.02^b	868±0.04^b	1097±1.25^a
FCR	10.55±0.02^a	9.43±0.01^b	7.31± 0..07^c
FE	0.094±0.01^c	0.105±0.00^b	0.136±0.01^a

Means in the same column followed by different letter superscripts are significantly different at ($P \leq 0.05$)

Table 2: Effect of BOL on secretory pattern of some reproductive hormones in male rabbits. (Values are means ±SE)

		Days post injection		
Parameter	Group (N=5)	15	30	45
TES (ng/ml)	C	2.09±0.21[a]	2.82±0.73[a]	2.72±0.73[a]
	R	2.02±0.24[a]	1.65±0.34[b]	1.55±0.34[b]
	D	1.48±0.37[b]	1.4±0.37[b]	1.38±0.37[b]
E2 (ng/ml)	C	5.16±0.01[b]	6.22±0.22[c]	8.52±2.52[b]
	R	6.00±0.02[a]	9.62±8.62[b]	10.00±0.20[b]
	D	6.24±0.01[a]	11.72±6.47[a]	12.02±4.02[a]

Means in the same column followed by different letter superscripts are significantly different at ($P \leq 0.05$)

Table 3: Effect of BOL on quantitative semen parameters in male rabbits. (Values are means ±SE)

		Days post injection		
Parameter	Group (N=5)	15	30	45
EV (ml)	C	0.87±0.04[a]	0.77±0.05[a]	0.70±0.07[a]
	R	0.85±0.05[a]	0.75±0.10[a]	0.66±0.05[a]
	D	0.80±0.02[a]	0.77±0.08[a]	0.76±0.12[a]
SC (x10^6/ml)	C	173.75±3.75[a]	585.00±121.1	408.75±36.98[a]
	R	170.00±3.53[a]	431.25±57.16[b]	350.00±35.35[b]
	D	151.25±9.65[b]	368.00±67.63[b]	281.25±57.16[c]
	C	164.00±0.40[a]	450.45±52.66[a]	286.12±48.29[a]
TSO (x10^6/E)	R	159.25±3.14[a]	323.43±72.09[b]	231.00±7.59[ab]
	D	140.75±7.21[b]	283.36±70.25[b]	213.75±30.83[b]
	C	14.50±0.64[a]	14.25±0.25[a]	13.75±0.75[a]
PSV (%)	R	12.25±0.62[a]	11.75±0.47[b]	13.25±0.75[a]
	D	11.75±0.85[b]	11.60±0.24[b]	13.00±0.57[a]

Means in the same column followed by different letter superscripts are significantly different at ($P \leq 0.05$)

Table 4: Effect of BOL on qualitative semen parameters in rabbits. (Values are means ±SE)

		Days post injection		
Parameter	Group (N=5)	15	30	45
MM	C	7.50±0.28[a]	7.50±0.18[a]	7.75±0.25[a]
	R	6.50±0.28[b]	6.50±0.27[b]	7.25±0.47[a]
	D	5.75±0.25[b]	5.75±0.25[b]	7.50±0.28[a]
TMS (x10^6/ml	C	46.23±0.65[a]	309.68±82.14[a]	199.56±23.96[a]
	R	39.58±2.44[a]	203.76±38.20[b]	159.39±5.55[ab]
	D	25.07±4.62[b]	177.38±39.62[b]	144.28±21.53[b]
IPM (%)	C	72.25±1.10[a]	68.57±1.43[a]	69.74±0.62[a]
	R	66.75±1.18[b]	63.00±1.22[b]	69.00±0.40[a]
	D	61.25±1.31[c]	62.60±0.92[b]	67.50±1.04[a]
SMI	C	516.00±22.25[a]	566.50±30.49[a]	540.21±14.90[a]
	R	433.50±17.78[b]	393.00±28.34[b]	500.75±35.41[a]
	D	352.25±17.60[c]	375.80±29.15[b]	506.25±20.81[a]
Abs (%)	C	15.25±0.47[b]	14.75±0.47[a]	14.71±0.43[b]
	R	16.00±0.40[ab]	16.50±0.64[a]	17.50±0.28[a]
	D	17.25±0.47[a]	16.40±0.50[a]	17.50±0.64[a]

Means in the same column followed by different letter superscripts are significantly different at ($P \leq 0.05$)

Table 5: Effect of BOL on testes weight (g) in male rabbits. (values are mean ± SE)

	Days post injection		
Group (N=5)	15	30	45
C	7.90±0.67[a]	8.26±0.70[a]	9.88±0.30[a]
R	5.80±0.37[b]	5.40±0.61[b]	7.11±0.66[b]
D	6.10±0.67[b]	6.20±0.83[b]	7.50±0.73[b]

Means in the same column followed by different letter superscripts are significantly different at ($P \leq 0.05$)

Qualitative Semen Evaluating Parameters: Table 4 demonstrates that the mean values of MM were significantly ($p \leq 0.05$) decreased in both treated groups particularly at 15 and 30 days without any significant differences were reported between the two doses of BOL. Compared to the control group, the mean values of TMS were decreased in both groups, but this decrease was significant ($p \leq 0.05$) in group D. A significant ($p \leq 0.05$) decrease in IPM percentage was noticed in both BOL

treated groups at 15 and 30 days following last drug administration. Significant ($p \leq 0.05$) differences between both doses were observed only at 15 days after injection. Comparison of the mean values for SMI between the control and treated groups showed a significant ($p \leq 0.05$) decrease in SMI in both treated groups at 15 and 30 after injection. Notable significant ($p \leq 0.05$) differences between both doses of BOL were recorded only at 15 days following drug administration. There was also a significant ($p \leq 0.05$) increase in the percentage of live sperms with morphological abnormalities in both treated group specially at 45 days after injection (Table 4). Significant differences between both doses of BOL were not reported.

Effect of BOL on Testes Weight: Data shown in (Table 5) declared that injection of BOL to male rabbits results in a significant ($p \leq 0.05$) decrease in the testes weight in both treated groups during all collection times. Significant differences between both doses of BOL were not observed.

Histopathology: The results of histopathology as shown in plates 1, 2 and 3 implicated that BOL treated rabbits exhibited severe histopathological lesions in testes which showed reduced development according to what could be expected for the age of the animals. Stromal proliferation and fibrosis were evident. Degeneration of gonocytes, widely separated seminiferous tubules with proliferated leydig cells and desquamation of germinal epithelium with formation of giant cells and debris in the lumina were noticed in some animals. There was also absence of different stages of spermatogonia, some degree of hemorrhage and reduced developing germinal epithelium. In some testes amorphous eosinophilic material and vacuolization of the germinal epithelium was present in the lumen of seminiferous tubules. Details of such histopathological changes are shown in plates 1, 2 and 3.

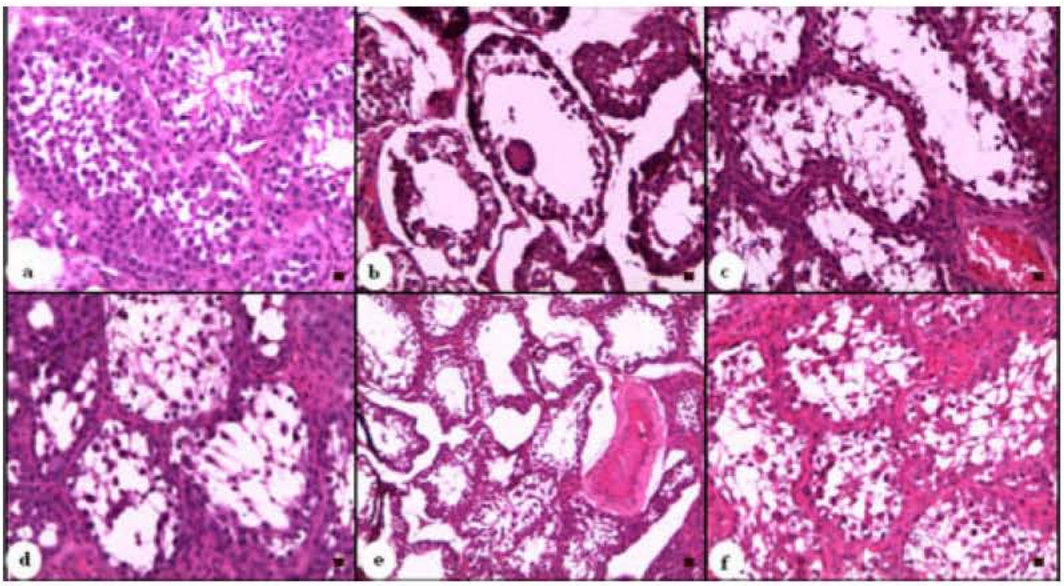

Plate 1: Histopathological changes in testes of rabbits 15 days following BOL injection

a. Group C showing normal testes of immature rabbit with different stages of spermatogonia and some primary spermatocytes (H&E Stain X 20).
b. Group R showing reduced developing germinal epithelium and giant cell formation (H&E Stain X10).
c. Group R showing congestion of blood vessels and absence of different stages of spermatogonia (H&E Stain X 10).
d. Group D showing destructed seminiferous tubules and increase interstitial connective tissue (H&E Stain X 4).
e. Group D showing hemorrhage reduced developing germinal epithelium (H&E Stain X 20).
f. Group D showing fenestrated appearance, severe vaculation and reduced developing germinal epithelium (H&E Stain X 20).

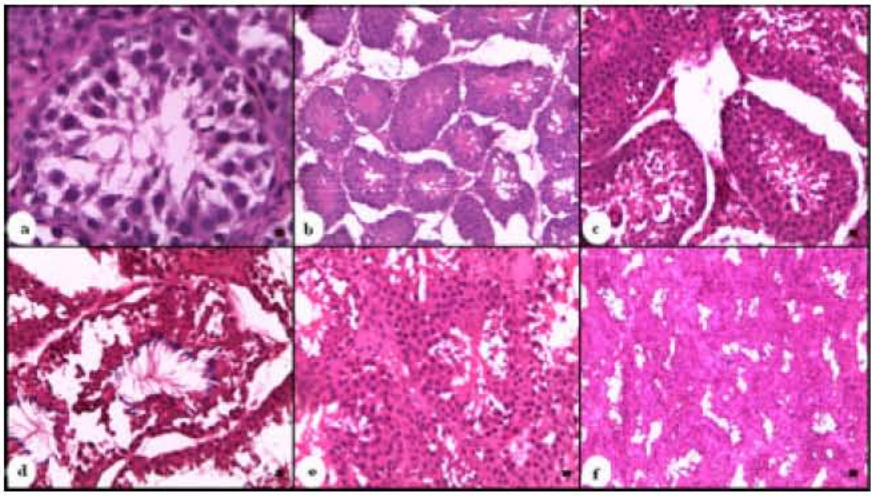

Plate 2: Histopathological changes in testes of rabbits 30 days following BOL injection.

a. Group C showing lumen formation different stages of spermatogonia (H&E Stain X 40).
b. Group R showing amorphous eosinophilic material in the lumen of seminiferous tubules (H&E Stain X 10).
c. Group R showing giant cells and desquamated cells material in the lumen of seminiferous tubules (H&E Stain X 20).
d. Group D showing desquamation and sloughing of lining germinal epithelium (H&E Stain X 20).
e. Group D showing hyperplasic interstitial leydig cells, (H&E Stain X 20).
f. Group D showing increased interstitial connective tissue with atrophy in the seminiferous tubules (H&E Stain X 20).

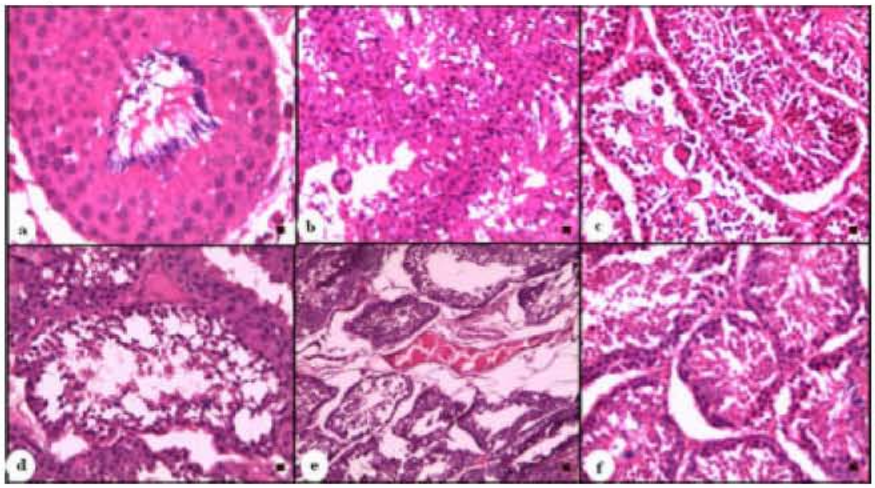

Plate 3: Histopathological changes in testes of rabbits 45 days following BOL injection .

a. Group C showing normal testes (H&E Stain X 40).
b. Group R showing giant cell in the luminal content of seminiferous tubules (H&E Stain X 20).
c. Group R showing normal seminiferous tubules and excess luminal contents of spermatocytic giant cells (H&E Stain X 20).
d. Group D showing degenerated germinal epithelium of seminiferous tubules with proliferated leydig cells, severe vaculation in the center of seminiferous tubules (H&E Stain X 20).
e. Group D showing congestion of blood vessels with widely separated and degenerated seminiferous tubules with proliferated leydig cells (H&E Stain X 10).
f. Group D showing degenerated and desquamated germinal epithelium of seminiferous tubules (H&E Stain X 20).

DISCUSSION

AAS are a class of synthetic steroids usually derived from testosterone and are recognized for their effects on building up muscle, so they are used as performance-enhancing drugs [18, 19]. But, there is no enough available information on their use in rabbits to make firm recommendation for their use in these animals. The present results revealed that injection of the anabolic steroid BOL to male rabbits evoked a significant increase in growth rate as indicated by a significant increase in TWG and FE as well as the improved FCR particularly in rabbits treated with the double dose of the drug (Table 1). This effect could be attributed to promoting the body tissue building processes by the anabolic steroid BOL as a result of increased nitrogen retention, protein synthesis and animal's appetite [1, 2, 20]. Others reported that sex hormones can increase the cellular protein biosynthesis indirectly via stimulation of growth hormone and insulin like growth factor secretion [21]. Furthermore, it was also assumed that BOL reduces glucocorticoid receptor levels and thus lowers sensitivity to endogenous glucocorticoids therefore, the strong growth-promoting potency of BOL is based not only on its anabolic activity as an androgen, but also on its anticatabolic activity as an antiglucocorticoid [19, 22, 23].

Because AAS are either derived from or are closely related to testosterone, these agents have varying degrees of androgenic side effects even with the most relatively mild drugs [8].

In the present work administration of BOL into male rabbits induced a significant decrease in serum testosterone concentrations. AAS have been shown to induce state of hypogonadotropic hypogonadism associated with decreased serum testosterone concentrations [6]. Application of anabolic steroids leads to supra-physiological concentrations of testosterone or testosterone derivatives [5]. Testosterone or testosterone derivatives all act at the hypothalamus to exert negative feedback inhibition upon gonadotropin-releasing hormone (GnRH) [24, 25]. Since GnRH stimulates LH and FSH release in the pituitary, inhibition of GnRH will decrease the release of LH and FSH from pituitary which via the negative feedback loop can inhibit subsequent testosterone production [26-29].

It was also found that this effect could be possibly results from local suppressive effects of excess androgens on the testes leading to the suppression of testosterone production by the Leydig cells [6]. The increase in serum E2 concentration could be attributed as reported in previous studies to aromatization of BOL, as testosterone is the primary substrate for the synthesis of estradiol in male [29-31].

No reports of the direct effects of AAS on semen production and quality in rabbits are available. In regard to this issue, we studied the effect of BOL on both quantitative and qualitative semen parameters and the results have shown that administration of BOL to male rabbits was associated with significant adverse effect on most semen evaluating parameters. These adverse effects included significant decrease in SC, TSO, PSV, MM, TMS, IPM and SMI in addition a significant increase in the number of morphologically abnormal sperms. Changes in the semen quality and impaired sperm production referred to anabolic steroid administration have been recorded in many studies in human and other animal species. Oligo, azoospermia and an increased number of abnormal sperm cells have been reported in athletes using AAS, resulting in a decreased fertility [7, 24, 29, 32]. In stallions, administration of anabolic steroids proved highly detrimental effects to seminal quality, spermatozoal output and production and testis size [33-35]. Similar observations were recorded in dogs, whereas, it showed decreased mean daily sperm output, mean testicular length, LH, FSH and testosterone level in serum following oral administration of methyltestosterone for several months [36]. Bulls treated with BOL was found to have a higher percentage of spermatozoa with primary morphological abnormalities than non treated bulls [10].

The mechanisms by which these reproductive functional abnormalities are produced are due to declining or suppressed testosterone production by leydig cells which induces a state of hypogonadotrophic hypogonadism and will result in a deficient spermatogenesis, despite the high circulating levels of administered hormone [6, 24, 34].

BOL-treated rabbits demonstrated lighter and atrophied testes than controls as indicated by the significant decrease in testes size. Testicular atrophy is a common clinical feature of long-term abuse of anabolic steroids which could result from suppressed TES production and decreased testicular concentrations of TES. Testicular concentrations of testosterone are necessary to maintain normal length of the seminiferous tubules and the reduction in tubule length may be possible reason for the reduction in testes weight seen in BOL treated rabbits [24]. Decrease in testes size, concomitant to serum testosterone levels have been

reported in athletes using AAS as well as in different animal species [29, 32, 35]. These results are further confirmed by the reported histopathological findings of testes shown in plates 1, 2 and 3 which demonstrated reduced development of testes according to what could be expected for the age of the animals including reduction of the number of developing germ cells as well as the degenerative changes in the spermatogonia. Other histopathological findings included marked stromal connective tissue proliferation, fenestrated or vacuolated center of seminiferous tubules, absence of different stages of spermatogonia, desquamation of germinal epithelium and giant cell formation in the lumen of the seminiferous tubules. Similar histopathological findings were reported in cattle [31], calf [30, 37, 38], lamb [38] and horse [34]. The mechanisms by which these pathological lesions are produced could be attributed to suppression of endogenous testosterone production which may lead to reduced testicular development [35]. Interestingly, many authors suggest that most of the histopathological changes seen in testes and accessory sex organs after anabolic steroids administration can be explained by Estradiol [30, 31]. This suggestion is confirmed by the high Estradiol concentration recorded in the present study.

In this work, most of the reproductive abnormalities observed following BOL injection were more significant in rabbits administered the double dose of the drug compared to rabbits given the lower recommended dose, suggesting that the severity of reproductive changes induced by BOL in rabbits is dose dependent. This supports the previous strong indications in many studies that dosage, duration and chemical structure of the anabolic steroids are important for the serum concentrations of gonadotropins and thus for the severity of the reproductive changes seen with anabolic steroids administration [24].

In conclusion, although administration of boldenone undecylenate to male rabbits induces a significant improvement in the growth rate, the drug produces marked adverse reproductive changes including decreased serum levels of TES and increased serum concentrations of E2. In addition BOL results in a highly determinal effects on semen quality, spermatozoal output, production and testis size. These results may not recommend BOL for use as a growth promoter in rabbits. However, further studies are needed to confirm this conclusion. Nevertheless, the results support previous suggestions that the severity of these reproductive side effects is dose-dependent.

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