Protective effect of nanoencapsulated curcumin against boldenone-induced testicular toxicity and oxidative stress in male albino rats

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Background
Boldenone (BOL) (Equigan) is a synthetic anabolic steroid used mainly by veterinarians to treat and promote horses' growth. Recently, body builders have started to use it to enhance their physical performance and muscle growth. Equigan is known to induce testicular injury and reduce fertility in males. Current treatments for reduced fertility are very costly. As alternatives, people are investigating naturally occurring bioactive compounds in plants such as curcumin.
Objective
This study was conducted to evaluate the prophylactic antioxidant effects of nanoencapsulated curcumin (NEC) on BOL-induced testicular toxicity and oxidative stress in male albino rats.
Materials and methods
NEC was prepared using a novel freeze-drying method. For their characterization, ultraviolet–visible spectrophotometry, transmission electron microscopy, and dynamic light scattering were used. Four groups of male rats were used: the first group served as control, the second group received NEC (100 mg/kg orally, once daily), the third group received BOL (5 mg/kg intramascular, once weekly) for 60 days, and BOL and NEC were concurrently administered in the fourth group. Blood was withdrawn from the rats' retro-orbital veins 24 h after treatment. Animals were euthanized immediately; the epididymal sperm reserve was separated. Then, one testis from each rat was kept at –80°C for determination of oxidative stress indices, and the other was fixed in 10% formalin solution for histopathological investigation.
Results and conclusion
Treatment with BOL resulted in significant reproductive damage caused by increased levels of malondialdehyde and nitric oxide and decreased levels of superoxide dismutase and reduced glutathione. Downregulation of the levels of serum testosterone and reduction in semen quantity, sperm count, and motility were also detected in the BOL group. Histopathological examinations showed severe degenerative changes in the testes. Immunohistochemical examination indicated severe reduction in the proliferating cell nuclear antigen-positive spermatogonia in the BOL-treated group as compared with the control. Coadministration of NEC with BOL effectively reduces BOL-induced testicular damage and oxidative stress in male albino rats.

Keywords:
boldenone, nanoencapsulated curcumin, oxidative stress, testicular toxicity

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Introduction

Boldenone (BOL; Equigan) is a synthetic anabolic androgenic steroid used mainly by veterinarians to treat horses and promote animal growth. Recently, body builders have started to use BOL in the off season to enhance their physical performance and muscle growth [1]. The use of BOL is forbidden for humans and food-producing animals and is classified as a schedule III drug [2]. BOL is also classified by the International Agency for Research on Cancer as a possible carcinogen for humans, with a higher carcinogenicity index than that of other anabolics [3,4]. El-Masry *et al*. [5] evaluated Equigan-induced testicular injury, reduced fertility, and oxidative stress in male rats. Nowadays, the current treatments for conditions such as reduced fertility and oxidative stress in humans are very costly. As alternatives, people are investigating naturally occurring bioactive compounds in plants such as curcumin [6].

Curcumin, extracted from the rhizomes of turmeric (*Curcuma longa*), is a natural polyphenolic flavonoid that is often used in Indian cuisine and traditional medicine [7]. Recently, many studies have focused on the protective effect of medicinal plant products that have antioxidant properties, such as curcumin, for curing free radical-induced tissue damage [8,9]. Curcumin has therapeutic potential for the treatment of diseases. Many studies have demonstrated that curcumin possesses anti? inflammatory [10], antioxidant [11], and neuroprotective activities [12]. However, clinical administration of curcumin is limited by its poor oral bioavailability and water solubility, low in-vivo stability, and rapid metabolism and clearance [13].

Nanotechnological research has led to the advancement in nanoparticle production. Nanoparticles have minute size and have large surface areas exhibiting high potential reactivity and enhanced performance [14]. To improve the bioavailability of curcumin, several studies have investigated different carriers such as albumin [15,16], β-lactoglobulin [17], phospholipids [18], chitosan [19], polyvinyl alcohol/polyvinyl alcohol hydrogel [20], cyclodextrins [21], bovine whole-casein micelles [22], and whey protein concentrate [23]. Different methods are available to counteract the difficulties associated with hydrophobic compounds [24,25].

Caseins, which are rich sources of calcium and essential amino acids, are used in the preparation of ready delivery systems for bioactive materials [26]. Casein micelle dissociation to temperatures greater than 60°C in aqueous ethanol 40% was reported [27]. Caseinate-curcumin nanoparticles in aqueous ethanol can be powdered by freeze-drying for ease of application, transportation, and storage.? Dende *et al.* [28] showed that nanoencapsulated curcumin (NEC) has a better therapeutic index and oral bioavailability than native curcumin. NEC could prevent the oxidative stress that was induced during status epilepticus [29].

The aim of the current study was to evaluate the protective effects of NEC against BOL-induced testicular toxicity and oxidative stress in male rats.

Materials and methods

Materials

BOL undecylenate (Equigan injection; 50 mg/ml) was purchased from Tornel S.A. Laboratories, Northwest of Mexico City, Mexico. Curcumin (CCM) was purchased from Merck, Schuchardt (8011 Hohenbrunnbei Munchen, Germany), with a purity of 94% w/w according to the vendor. Sodium caseinate (NaCas) was purchased from Acros (New Jersey, USA). Other chemicals were obtained from either Sigma-Aldrich or Thermo Fisher Scientific (Pittsburgh, Pennsylvania, USA). All chemicals used were of analar grade.

Sodium caseinate nanoencapsulation of curcumin

Overall, 4 g of NaCas was hydrated in 200 ml of 40% v/v aqueous ethanol. After being heated at 60°C using a water bath for 5 min, an excess amount (0.25, 0.50, 1 g) of curcumin was mixed with the NaCas solution by blending at 10 000 rpm for 4 min using a Cyclone I.Q. microprocessor homogenizer (VirTis, Gardine, New York, USA). Then, the mixtures were ultrasonicated at 160 W power and 20 kHz frequency and with 50% pulse (Sonic Vibra Cell, 53 Church Hill Road, Newtown, CT, USA). Centrifugation at 290*g* (model 4540 R, Eppendorf, Hamburg, Germany) for 5 min was carried out to remove the excess curcumin, and the supernatant was obtained. Finally, the nanocapsules were dried by freeze-drying (LABCONCO, USA) to produce solid or powdered nanocapsules. A NaCas sample was processed under the same conditions without curcumin, hereafter referred to as ultrasonicated NaCas.

Estimation of curcumin loading in freeze-dried powder

Overall, 5 μg of freeze-dried powder was suspended in 10 ml of chloroform and stirred overnight at room temperature (21°C). After centrifugation at 6000 g for 10 min (Minispin plus; Eppendorf), the supernatant was transferred and filtered through a PTFE syringe filter with pore size of 0.45 μm (Thermo Fisher Scientific). The permeate was diluted 20 times in chloroform, and the absorbance was measured using a ultraviolet–visible spectrophotometer (Evolution 201; Thermo Scientific, Waltham, Massachusetts, USA) at 419 nm to determine curcumin concentration based on a calibration curve previously established using standard solutions with different amounts of free curcumin dissolved in chloroform [30].

Particle size analysis

In addition to the transparent dispersion with curcumin, two other dispersions were measured by dynamic light scattering: native NaCas and processed NaCas hydrated in deionized water for 6 h. The pH of the dispersion was 6.8. Samples were carried out at 25±0.1°C using a Nano ZS/ZEN3600 Zetasizer (Malvern Instruments Ltd, UK), with a He/Ne laser (λ=633 nm), scattering angle of 90°, scattering optics, and refractive index of 1.54. Samples were diluted and filtered through a

0.45-μm membrane (Millipore, USA) to obtain a count rate in the appropriate range of 100–450 nm to avoid multiple scattering phenomena owing to interparticle interactions. Immediately, the diluted samples were transferred into a polystyrene cuvette for size determination, and the polydispersity index was recorded by dynamic light scattering as described by Soliman and Hassan [31].

Transmission electron microscopy

Transmission electron microscopy (TEM) was done by fixation with glutaraldehyde and phosphotungstic acid solution (2% at pH 7.2). The samples were examined by TEM using a JEOL JEM-1400 Plus (11 Dearborn Rd, Peabody, Boston, MA, USA) TEM instrument with an accelerating voltage of 100 kV at a magnification of ×200 000 [31].

Antioxidant activity

The 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) method [32] was used to determine the antioxidant activity of the WGO. The DPPH radical scavenging activity was calculated as percent inhibition of the reaction.

The DPPH scavenging ability of the test compounds was determined using equation 1 and converted to Trolox equivalent antioxidant capacity (TEAC) using the standard curve for Trolox solutions.

DPPH+scavenging (%)=(1–As/Ac)×100 (1).

Whereas As and Ac are the absorbance values of the test compound and control, respectively.

Animals

Adult male rats were obtained from the animal house colony of the National Research Centre (Cairo, Egypt). Standard laboratory food and water were provided *ad libitum*. Animal procedures were performed in accordance with the Ethics Committee of the National Research Centre and followed the recommendations of the National Institutes of Health's Guide for Care and Use of Laboratory Animals (Publication no. 19-60, revised 1985).

Experimental design

A total of 32 male rats were equally divided into four groups: the first group was kept as control, the second group received NEC (100 mg/kg orally, once daily), the third group received BOL (5 mg/kg intramuscular, once weekly) for 60 days as described by Zahran *et al.* [2] and El-Masry *et al.* [5], and the fourth group was concurrently administered with BOL and NEC as the combination group.

Blood samples

At 24 h after the last injection, the rats were anesthetized, and blood was withdrawn from the retro-orbital vein into a clean test tube for serum separation. After centrifugation, serum was stored at −20°C until being used for estimation of oxidative stress biomarkers and reproductive hormones.

Tissue samples

Animals were euthanized immediately using cervical dislocation; the epididymal sperm reserve was separated, and the testes were rinsed in normal saline and weighed. Then one testis from each animal was kept at −80°C for determination of oxidative stress indices, and the other was fixed in 10% formalin solution for histopathological investigation.

Biochemical assessment

Determination of lipid peroxidation in testicular homogenate and serum

Lipid peroxidation was assayed by measuring the level of malondialdehyde (MDA). MDA forms a 1 : 2 adduct with thiobarbituric acid, which can be measured by spectrophotometry. MDA levels were determined by measuring thiobarbituric acid-reactive species using the method of Ruiz-Larrea *et al.* [33], producing a red colored complex having peak of absorbance at 532 nm.

Determination of reduced glutathione levels in testicular homogenate

Reduced glutathione (GSH) levels were determined by Ellman's method [34]. The procedure is based on the reduction of Ellman's reagent by –SH groups of GSH to form 2-nitro-s-mercaptobenzoic acid. The nitromercaptobenzoic acid anion has an intense yellow color that can be measured spectrophotometrically.

Determination of nitric oxide levels in testicular homogenate and serum

Nitric oxide (NO) was measured in terms of nitrite levels, which were determined using Griess reagent, according to the method of Moshage *et al.* [35], where nitrite, the stable end product of NO radical, is mostly used as an indicator for the production of NO.

Determination of testosterone levels (ng/ml)

Testosterone levels (ng/ml) were assayed using the Testosterone ELISA Enzyme Immunoassay Test Kit [36] using a commercially available enzyme immunoassay kit supplied by Medical Biological Service S.r.l. (Milano, Italy).The sensitivity of the assay was 0.05 ng/ml.

Determination of sperm count

Immediately after the rats were killed, the contents of epididymis were obtained by cutting the caudal epididymis, and sperm samples were collected using the method described by Yokoi *et al.* [37] for sperm count determination. The sperm suspension was carefully mixed with an equal volume of eosin–nigrosin stain for evaluation of sperm viability, where dead sperm were stained pink and live sperm remained unstained [38].

Histopathological examination

Test specimens were fixed in formol buffered saline (10%), dehydrated in graded series of alcohol, cleared in xylene, and embedded in molten paraffin. Paraffin blocks were sectioned at 4–5 μm thickness using a rotary microtome and stained with hematoxylin and eosin [39]. The sections were examined using a light microscope (Olympus CX 41, Japan).

Immunohistochemical assessment of proliferating cell nuclear antigen

Testicular proliferating cell nuclear antigen (PCNA) receptor subunits were detected using the avidin–biotin–peroxidase (ABC) immunohistochemical method [40] (Elite-ABC; Vector Laboratories, Burlingame, California, USA) with a primary monoclonal antibody (Dako, Cambridge, UK). The cellular site of the reaction appeared brown in the cell nuclei.

Statistical analysis

Data were subjected to statistical analysis using Statistical Package for Social Science [41]. Data are represented as the mean±SEM. Simple one-way analysis of variance was performed to identify the effect of treatment on the studied parameters. Duncan's multiple-range test was performed to distinguish between different and significant means at P value less than 0.05.

Table 1 Particle size (nm) of casein micelles, polydispersity index, and encapsulation efficiency of nanoencapsulated curcumin

Samples	Size (nm)	Calculated PDI	Encapsulation efficiency
NaCas, native	249.00 ±8.0[a]	0.155 ±0.011[d]	–
NaCas, ultrasonicated	71.36 ±5.3[e]	0.213 ±0.009[c]	–
NEC, 5% CUR	94.20 ±2.5[d]	0.423 ±0.005[b]	94.10±2.25[a]
NEC, 10% CUR	110.10 ±5.4[c]	0.430 ±0.008[b]	90.75±3.50[b]
NEC, 20% CUR	174.70 ±6.5[b]	0.480 ±0.011[a]	84.95±2.10[c]

The results are presented as the mean±SD ($P<0.05$) using one-way analysis of variance followed by Duncan's multiple-range comparison test; and different superscript letters represent significant differences. NaCas, sodium caseinate; NEC, nanoencapsulated curcumin; PDI, polydispersity index.

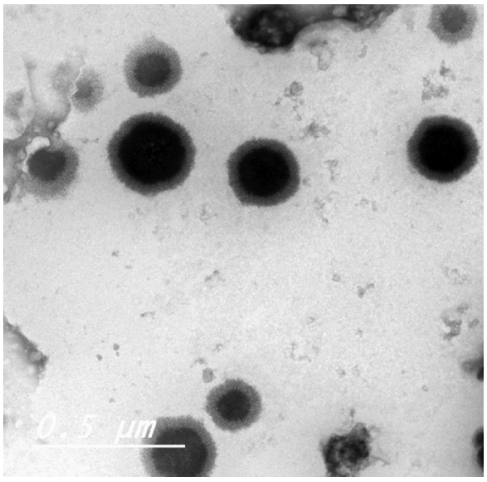
Results and discussions

Particle size, polydispersity index, and encapsulation efficiency of nanoencapsulated curcumin

Table 1 and Fig. 1 show the particle sizes of dispersions prepared from native NaCas, ultrasonicated NaCas, and NEC containing 0.25, 0.50, and 1.00 g curcumin. The dispersion of the ultrasonicated NaCas had a smaller size (71.36 nm) than that of the normal NaCas (249 nm). The dispersions sizes of the NEC loaded with curcumin portions (94.20, 110.10, 174.70 nm) were significantly larger than that of the ultrasonicated NaCas but smaller than that of the native NaCas. The freeze-dried powder contained 4.70, 9.08, and 16.99% curcumin. Compared with the intended weight of curcumin loaded before spray-drying (0.25, 0.50, and 1.00 g of curcumin and 4 g of NaCas for each treatment), the encapsulation efficiency was ~94.10, 90.75, and 84.95%, respectively.

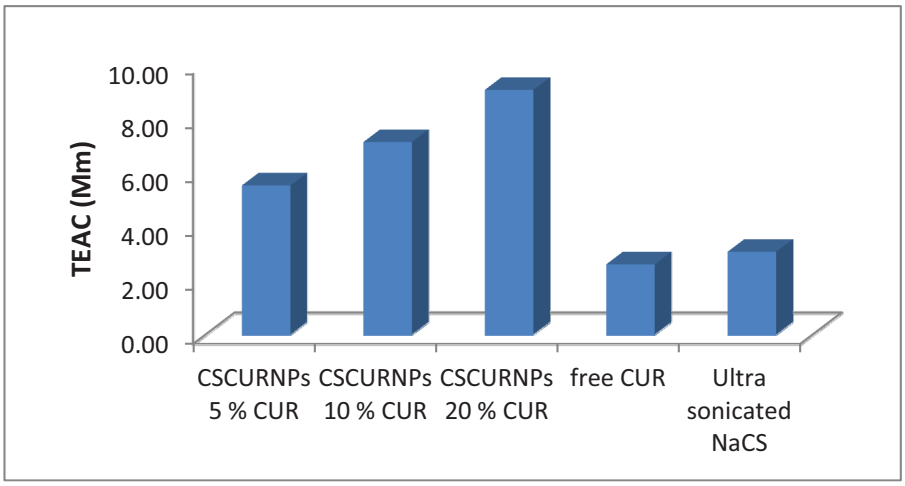
Caseins in warm aqueous ethanol had exhibited structural changes owing to changes in polarity, which affects solvent quality, dissociation, and reassociation of caseins [40]. This was in agreement with ours. After freeze-drying and due to the irreversibility of disrupted physical forces, the dissociated caseins in NEC ? were smaller than those of native NaCas (Table 1). Additionally, caseins have open structures that are evenly distributed and are so tendentious as binders of bioactive compounds [42].

Figure 1



Transmission electron micrograph of NEC 20% curcumin. NEC, nanoencapsulated curcumin.

Figure 2



Antioxidant capacity (Trolox equivalent) of the ultrasonicated sodium caseinate, free curcumin (CUR), and curcumin-loaded (0.25, 0.50, and 1 g loaded to caseinate) NEC at 30°C. NEC, nanoencapsulated curcumin.

Table 2 Body weight, sperm count, viability, and testosterone levels

Groups	Control	NEC	BOL	BOL+NEC	*P* value
Final body weight (g)	199.2±3.53^{b}	211.7±2.60^{ab}	223.3±3.28^{a}	222±1.32^{a}	0.0001
Testicular weight/100 g body weight	1.06±0.03^{b}	1.06±0.02^{b}	0.42±0.009^{a}	0.82±0.05^{ab}	0.0001
Sperm count (million/ml)	89±2.23^{b}	91.67±1.77^{b}	18.86±3.59^{a}	82.83±1.44^{b}	0.0001
Viability %	77.50±1.44^{b}	78.33±1.55^{b}	45±1.89^{a}	73.33±1.15^{b}	0.0001
Testosterone (ng/ml)	3.56±0.187^{b}	3.78±0.41^{b}	1.93±2.519^{a}	2.34±1.312^{ab}	0.0001

Values are expressed as the mean±SE (n=8) using one-way analysis of variance followed by Duncan's multiple-range comparison test; a significantly different from control, b significantly different from BOL ($P \leq 0.05$). BOL, boldenone; NEC, nanoencapsulated curcumin.

Figure 2 showed the antioxidant capacity of curcumin before and after encapsulation.? The antioxidant capacity of the ultrasonicated NaCas was also determined, ?which attributed to amino acid was 307 µM TEAC [43,44]. Free curcumin dissolved in ethanol followed by dilution in distilled water for ? analysis has a ?low antioxidant capacity (260 µM TEAC) owing to the limitation of curcumin solubility. Curcumin has a high value of radical scavenging activity as its ?phenolic –OH bonds give a main role in the oxidation reactions [43]. The NEC with different portions from CUR (5, 10, and 10%) exhibited a higher antioxidant capacity upon dispersion in ?casein nanoparticles (553, 713, and 907 µM Trolox equivalents) ?than free curcumin and ultrasonicated NaCas (P<0.05) [42,45]. Therefore, after dispersion in caseinate nanoparticles, curcumin exhibited improved antioxidant capacity.

Relative body and testicular weights

The final body weight was significantly increased (P<0.0001) in all the treatment groups compared with the control group (Table 2). This effect may be owing to elevated food intake and fluid retention by rats as mentioned by Saleh and Waded [46], Mohammed *et al.* [47], and Elmasry *et al.* [48] or may be a result of the increased muscle size owing to the promotion of positive nitrogen balance by stimulating protein production and decreasing its destruction [1]. However, the relative testicular weight was significantly decreased (P<0.0001) in BOL-treated group as compared with the control, NEC, and the combination groups (Table 2), which was consistent with the results reported by Oda and El-Ashmawy [49] and Khalil *et al.* [50].

Markers of oxidative damage

Our results showed a marked increase in NO and MDA levels and a significant decrease in superoxide dismutase (SOD) and GSH activities in BOL-treated rats compared with the control. Control rats and those treated with CURNPs had the lowest testicular and serum NO levels (P<0.0001) (Table 3). This may be attributed to the toxic and degenerative effects of BOL on testicular tissue and cell membranes, and increase in the levels of free radicals was owing to the increased lipid peroxidation in the fatty content of testicular cells compartments, hence increasing the level of MDA and NO and depletion of the level of SOD and GSH. These results were supported by the findings of Sadowska-Krepa *et al.* [51], El-Moghazy *et al.* [1], and Mayada *et al.* [52] who concluded that treatment

Table 3 Testicular glutathione, nitric oxide, and malondialdehyde levels and serum nitric oxide, malondialdehyde and superoxide dismutase levels

Groups	Control	NEC	BOL	BOL+NEC	*P* value
Testicular GSH (μmol/100 g tissue)	18.41±0.21[b]	18.5±0.17[b]	7.95±0.55[a]	18.32±0.83[b]	0.0001
Testicular NO (nmol/g)	45.5±3.63[b]	43.8±8.71[b]	62.4±8.81[a]	47.0±6.39[b]	0.0001
Testicular MDA (nmol/g tissue)	24.05±0.8[b]	22.7±0.58[b]	31.0±2.73[a]	25.7±1.38[ab]	0.0001
Serum NO (nmol/g)	25.18±1.03[b]	27.73±0.96[b]	46.05±0.86[a]	32.36±1.59[ab]	0.033
Serum MDA (nmol/g)	21.98±0.75[b]	23.26±0.82[b]	37.75±0.37[a]	18.58±2.07[b]	0.0001
Serum SOD (U/ml)	936±66.5[b]	791±17.8[ab]	530±35.2[a]	499±25.6[a]	0.0001

Values are expressed as the mean±SE (n=8) using one-way analysis of variance followed by Duncan's multiple-range comparison test. 'a' Significantly different from control, 'b' significantly different from BOL ($P \leq 0.05$). BOL, boldenone; GSH, glutathione; MDA, malondialdehyde; NEC, nanoencapsulated curcumin; NO, nitric oxide; SOD, superoxide dismutase.

with BOL-induced hepatotoxicity in rabbit through induction of some mediators of the apoptosis such as reactive oxygen species, p53, and TNF-α. Bueno *et al.* [53] found that BOL and stanozolol (1.25 mg/kg for 12 weeks) increased MDA levels in testes. Moreover, treatment with BOL (1.25, 2.5, 5 mg/kg for 12, 8, and 4 weeks, respectively) led to increase in testicular NO levels.

Sperm characteristics

Semen characteristics were severely affected by BOL injection, where four animals out of eight showed complete azoospermia. The sperm count and viability were significantly decreased ($P<0.0001$) in the BOL-treated group compared with the control group. No abnormalities were detected in the sperm morphology (Table 2). This significant reduction may be related to the degenerative effects of BOL on testicular cells, mainly Sertoli cells. These results were similar to those reported by Oda and El-Ashmawy [49], Elmasry *et al.* [48], and Ibrahim and Said [54]. Moreover, this significant decrease may be related to the decreased level of intratesticular testosterone, because testosterone levels are directly linked to spermatogenesis as concluded by Elmasry *et al.* [48] This result is in consistent with those of Thabet *et al.* [55] and Tousson *et al.* [56], who reported that the decrease in sperm count with the increase in dead and abnormal sperm of rats may happen because of the increased free radical formation, inhibiting nucleic acid synthesis and initiating germ cell apoptosis and subsequent male infertility. Reactive oxygen species can indirectly damage the sperm membrane, or directly damage the sperm DNA as mentioned by Meseguer *et al.* [57] and Elmasry *et al.* [48] There was a significant increase ($P<0.0001$) in sperm count and viability in NEC and the combination groups compared with the BOL-treated group. This increment is owing to NEC high value of radical scavenging activity as curcumin ? phenolic –OH bonds give a main role in the oxidation reactions [43]. The NEC exhibited a higher antioxidant capacity upon dispersion in ? casein nanoparticles [45].

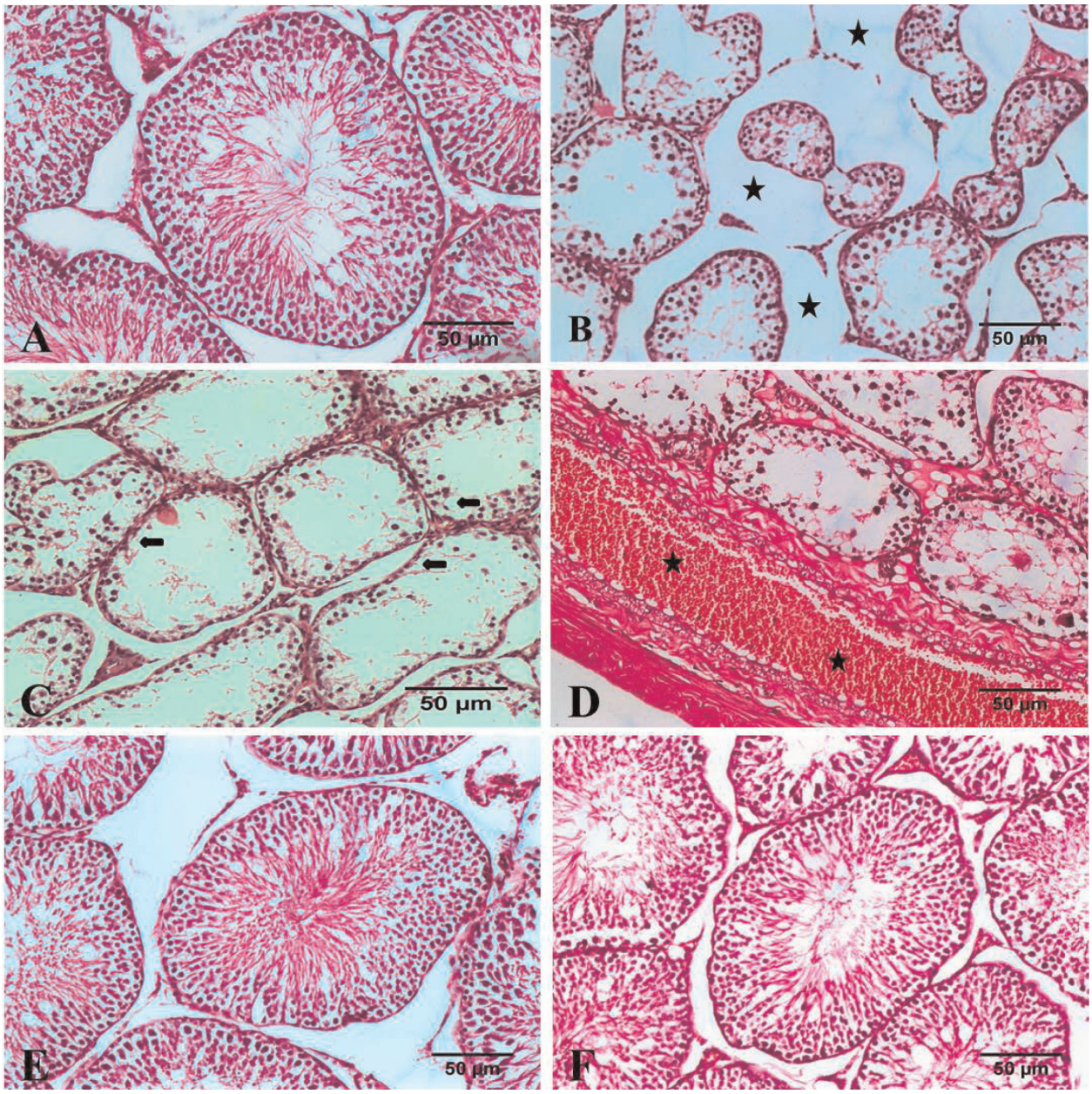
Effect of boldenone on testosterone level

Testosterone levels in serum were significantly decreased in BOL-treated group compared with the control and combination groups and significantly increased in NEC group (Table 2). The decrement in testosterone concentrations may be attributed to the decreased testicular weight and degenerative changes in Leydig cells. This was in consistent with the results of Thabet *et al.* [55], Oda and El-Ashmawy [49], and Mohammed *et al.* [47]. Moreover, this effect might be due to the induction of hypogonadotropic hypogonadism, which associated with decreased testosterone levels after BOL treatment. The results of the present study were consistent with Hall and Hall [58], who concluded that anabolic steroid application leads to decrease in rabbit serum testosterone levels. Dohle *et al.* [59] stated that BOL treatment suppresses gonadotropin-releasing hormone production by the hypothalamus, resulting in the suppression of testicular testosterone production.

Histopathological findings

Histopathological examination of the control testes showed normal histological structure with normal spermatogonial cells, and complete process and stages of spermatogenesis with sperm cell production (Fig. 3a). Microscopically, rat testes treated with BOL showed severe histopathological alterations, where all the examined sections showed atrophy and distortion of seminiferous tubules associated with severe intertubular edema (Fig. 3b), as well as a significant reduction in the number of spermatogonial cells in the seminiferous tubules, which was associated with severe vacuolar degeneration and necrosis of the spermatogonial cell lining epithelium, with absence of spermatids and sperm cells (Fig. 3c,d). Testicular blood vessels showed severe congestion as well as a subcapsular area of the tunica albuginea (Fig. 3d). This finding is

Figure 3



Testes of rat (hematoxylin and eosin; scale bar, 50 µm). (a) Control group, showing the normal histological structure of seminiferous tubules with normal spermatogonial cells and complete process of spermatogenesis. (b) BOL-treated group, showing severe atrophy and distortion of seminiferous tubules with interstitial edema (stars), associated with a significant reduction in the number of spermatogonial cells, severe degeneration and necrosis of spermatogonial cells (arrows) (c). (d) Showing severe vacuolar degeneration and necrosis of spermatogonial cells (arrows) with severe congestion of subcapsular area of the tunica albuginea (star). (e and f) The groups that received NEC and BOL with NEC showing normal seminiferous tubule diameters, normal spermatogonial cells and complete spermatogenesis with sperm production, respectively. BOL, boldenone; NEC, nanoencapsulated curcumin.

consistent with previous studies, which reported that the observed degeneration and loss of spermatogenic series in the seminiferous tubules elucidate the decreased testosterone levels [54], and disrupted the spermatogenic cycle [60]. The use of BOL as an anabolic steroid could also lead to cellular deformity and disorder in the process of spermatogenesis [61]. BOL supplementation increased the levels of 5-alpha reductase and aromatase in the testicular tissue, which may be attributed to alterations in the steroid molecule and their affinity for androgen receptors [62]. Our results are consistent with those of Groot and Biolatti [63], who revealed that administration of the anabolic androgenic steroid BOL induces comparable lesions in the testes of bulls. Bueno *et al.* [64] additionally suggested that treatment with BOL has a significantly harmful effect in the testes of male rat as a result of the increase of oxidative stress and inflammatory markers levels in addition to failure of the antioxidant system, as triggered by the use of BOL.

Groups that received NEC and BOL with NEC respectively showed no histopathological alterations, exhibiting normal seminiferous tubule diameters, normal spermatogonial cells, and complete process of spermatogenesis (Fig. 3e, f).

Immunohistochemical results

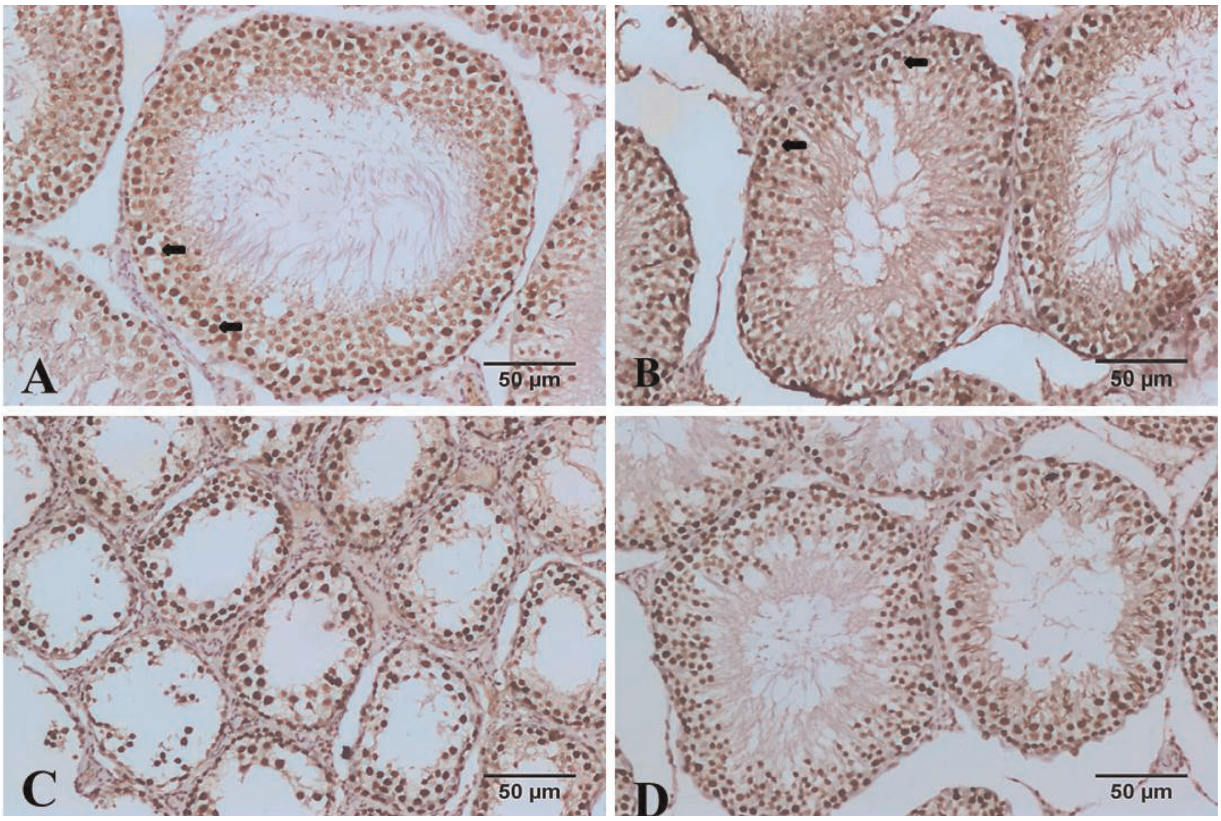
Examined testicular specimens of control and NEC-treated groups showed a strong positive reaction in spermatogonial cells, whereas the other spermatogenic cell types showed a negative reaction (Fig. 4a and b) compared with the BOL-treated group, which showed a weak positive reaction against the monoclonal PCNA antibody, which was associated with a reduction in the number of the PCNA-positive spermatogonia (Fig. 4c). On the contrary, treatment with NEC significantly increased the number of PCNA-positive germinal cells in the testes (Fig. 4d).

The results were in consistent with the results of Tousson *et al.* [56], who concluded that severe reduction in this number in BOL-treated rabbits as counted by PCNA indicates a germinal arrest in the BOL-treated status (defective spermatogenesis). This drastic reduction was recorded and indicates a reduced sperm count [65,66].

Oxidative stress and oxidative cellular damage with its dual of free radical generation and lipid peroxidation are hallmarks of BOL toxicity [67]. Our results indicate that BOL causes severe oxidative tissue damage through increasing the lipid peroxidation in the testicular tissues and decreasing the levels of the antioxidant enzymes GSH and SOD, which is in accordance with Tousson *et al.* [68] Treatment with NEC and BOL showed no histopathological alterations, exhibiting normal seminiferous tubule diameters, normal spermatogonial cells, and complete process of spermatogenesis.

Treatment of male rats with BOL resulted in significant reproductive damage caused by increased lipid peroxidation and NO and decreased levels of SOD and reduced GSH. Downregulation of the levels of serum testosterone and reduction in semen quantity, sperm count, and motility were also detected. Many histopathological alterations in the rats' testes (atrophy, distortion of seminiferous tubules, and

Figure 4



Photomicrographs of proliferating cell nuclear antigen (PCNA) immunoreactivity in rat testes. (a and b) Seminiferous tubules of control and NEC-treated groups showing a strong PCNA-positive reaction of PCNA in numerous spermatogonia (arrows). Photomicrograph (c) of rats received BOL showing a reduction in numbers of PCNA-positive spermatogonia. Photomicrograph (d) of rat testes received BOL and NEC showing a strong positive PCNA-reaction (scale bar 50 µm). BOL, boldenone; NEC, nanoencapsulated curcumin.

reduction in the number of spermatogonial cells in the seminiferous tubules) were detected. Coadministration of a strong antioxidant such as NEC with BOL effectively attenuates and ameliorates the adverse effects caused by BOL in the testes of male rats.

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Nil.

Conflicts of interest
There are no conflicts of interest.

References

1 El-Moghazy M, Tousson E, Sakeran M. Changes in the hepatic and renal structure and function after a growth boldenone injection in rabbits. Animal Biol 2012; 62:171–180.

2 Zahran F, Tousson E, Shalapy MA. Ameliorating effect of ginkgo biloba and moringaoleifera against equigan induced testicular toxicity and oxidative stress in male rat. Am J Biol Chem 2015; 3:39–44.

3 IARC Monograph 1987; Suppl. 7, 11. Available at: http://monographs.iarc.fr/ENG/Monographs/suppl7/suppl7.pdf [Accessed June 15, 2006].

4 De Brabander HF, Poelmans S, Schilt R, Stephany RW, Le BB, Draisci R, *et al.* Presence and metabolism of the anabolic steroid boldenone in various animal species. A review. Food Addit Contam 2004; 21:515–525.

5 El-Masry T, Al-Shaalan NH, Tousson E, El-Morshedy K, Al-Ghadeer A. P53 expression in response to equigan induced testicular injury and oxidative stress in male rat and the possible prophylactic effect of star anise extracts. Annu Res Rev Biol 2017; 14:1–8.

6 Tapal A, Tiku PK. Complexation of curcumin with soy protein isolate and its implications on solubility and stability of curcumin. Food Chem 2012; 130:960–965.

7 Prasad S, Aggarwal B, Gupta S, Tyagi A. Curcumin, a component of golden spice: from bedside to bench and back. Biotech Adv 2014; 32:1053–1064.

8 Agarwal R, Goel SK, Behari JR. Detoxification and antioxidant effects of curcumin in rats experimentally exposed to mercury. J Appl Toxicol 2010; 30:457–468.

9 Elsayed ASI. The curcumin as antioxidant natural herb, with emphasize on its effects against some disease. Inter J Appl Biol Pharm Tech 2016; 7:26–40.

10 Shehzad A, Rehman G, Lee Y-S. Curcumin in inflammatory diseases. Biofactors 2013; 39:69–77.

11 Gao S, *et al.* Curcumin attenuates arsenic-induced hepatic injuries and oxidative stress in experimental mice through activation of Nrf2 pathway, promotion of arsenic methylation and urinary excretion. Food Chem Toxicol 2013; 59:739–747.

12 Zhang L, *et al.* A study on neuroprotective effects of curcumin on the diabetic rat brain. J Nutr Health Aging 2016; 20:835–840.

13 Flora G, *et al.* Nanocurcumin: a promising therapeutic advancement over native curcumin. Crit Rev Ther Drug Carrier Syst 2013; 30:331–368.

14 Boverhof DR, *et al.* Comparative assessment of nanomaterial definitions and safety evaluation considerations. Regul Toxicol Pharmacol 2015; 73:137–150.

15 Bourassa P, *et al.* Resveratrol, genistein, and curcumin bind bovine serum albumin. J Phys Chem B 2010; 114:3348–3354.

16 Mandeville JS, *et al.* Study of curcumin and genistein interactions with human serum albumin. J Pharma Biomed 2009; 49:468–474.

17 Mohammadi F, *et al.* Interaction of curcumin and diacetylcurcumin with the lipocalin member beta-lactoglobulin. Protein J 2009; 28: 117–123.

18 Liu A, Lou H, Zhao L, Fan P. Validated LC/MS/MS assay for curcumin and tetrahydrocurcumin in rat plasma and application to pharmacokinetic study of phospholipid complex of curcumin. J Pharm Biomed 2006; 40:720–727.

19 Shelma R, Sharma CP. Acyl modified chitosan derivatives for oral delivery of insulin and curcumin. J Mater Sci Mater Med 2010; 21:2133–2140.

20 Shah CP, Mishra B, Kumar M, Priyadarsini KI, Bajaj PN. Bindingstudies of curcumin to polyvinyl alcohol/polyvinyl alcohol hydrogel and its delivery to liposomes. Curr Sci 2008; 95:1426e–1432e.

21 Baglole K, *et al.* Fluorescence enhancement of curcuminupon inclusion into parent and modified cyclodextrins. J Photochem Photobiol A Chem 2005; 173:230–237.

22 Sahu A, *et al.* Fluorescence study of the curcumin-casein micelle complexation and its application as a drug nanocarrier to cancer cells. Biomacromolecules 2008; 9:2905e–2912e.

23 Sari TB, *et al.* Preparation and characterization of nanoemulsion encapsulating curcumin. Food Hydrocoll 2015; 43:540–546.

24 Kotta S, *et al.* Exploring oral nanoemulsions for bioavailability enhancement of poorly water-soluble drugs. Expert Opin Drug Deliv 2012; 9:585.

25 Parveen R, Baboota S, Ali J, Ahuja A, Vasudev SS, Ahmad S. Oil based nanocarrier for improved oral delivery of silymarin: in vitro and in vivo studies. Int J Pharm 2011; 413: 245.

26 Kralova I, Sjoblom J. Surfactants used in food industry: a review. J Disper Sci Technol 2009; 30:1363–1383.

27 Dalev PG, Simenova LS. Emulsifying properties of proteinpectin complexes and their use in oil-containing foodstuffs. J Sci Food Agric 1995; 68:203–206.

28 Dende C, Meena J, Nagarajan P, Nagaraj VA, Panda AK, Padmanaban G. Nanocurcumin is superior to native curcumin in preventing degenerative changes in experimental cerebral malaria. Sci Rep 2017; 7:10062.

29 Khadrawy YA, Hussein G, Hosney E. Neuroprotective effect of curcumin nanoparticles against rat model of status epilepticus induced by pilocarpine. J Compl Integr Med 2018; 23:15.

30 Yu H, Huang Q. Enhanced in vitro anti-cancer activity of curcumin encapsulated in hydrophobically modified starch. Food Chem 2010; 119:669–674.

31 Soliman TN, Hassan MA. Preparation and characterization of sustained released zinc citrate encapsulated in whey protein nanoparticles. Pak J Biol Sci 2018; 21:448–453.

32 Rai S, Wahile A, Mukherjee K, Saha BP, Mukherjee PK. Antioxidant activity of nelumbonucifera (sacred lotus) seeds. J Ethnopharmacol 2006 104:322–327.

33 Ruiz-Larrea MB, Leal AM, Liza M, Lacort M, de Groot H. Antioxidant effects of estradiol and 2-hydroxyestradiol on iron-induced lipid peroxidation of rat liver microsomes. Steroids 1994; 59:383–388.

34 Ellman GL. Tissue sulfhydryl groups. Arch Biochem 1959; 82:70–77.

35 Moshage H, Kok B, Huizenga JR, Jansen PL. Nitrite and nitrate determination in plasma: a critical evaluation. Clin Chem 1995; 41:892–896.

36 Heinonen PK. Androgen production by epithelial ovarian tumors in post-menopausal women. Maturitas 1991; 13:117–122.

37 Yokoi K, Uthus EO, Nielsen FH. Nickel deficiency diminishes sperm quantity and movement in rats. Biol Trace Elem Res 2003; 93:141–153.

38 Wyrobek AJ, Gordon LA, Burkhart JG, Francis MW, Kapp RW Jr., Letz G, *et al.* An evaluation of the mouse sperm morphology test and other sperm tests in nonhuman mammals. A report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutat Res 1983; 115:1–72.

39 Bancroft JD, Gamble M. Theory and practice of histological techniques. 6th ed. China: Churchill Livingstone, Elsevier; 2008.

40 Ramos-Vara JA. Principles and methods of immunohisto-chemistry. Methods Mol Biol 2011; 691:83–96.

41 SPSS 2016. Statistical package for social sciences. SPSS Inc., Chicago, IL, USA. Copyright© for Windows version 20.0 SPSS.

42 Esmaili M, Ghaffari SM. Beta casein-micelle as a nano vehicle for solubility enhancement of curcumin; food industry application. LWT-Food Sci Technol 2011; 44:2166–2172.

43 Khopde M, Priyadarsini KI, Venkatesan P, Rao MN. Free radical scavenging ability and antioxidant efficiency of curcumin and its substituted analogue. Biophys Chem 1999; 80:85–91.

44 Kitts D. Antioxidant properties of casein-phosphopeptides. Trends Food Sci Technol 2005; 16:549–554.

45 Takahashi M, Uechi S, Takara K, Asikin Y, Wada K. Evaluation of an oral carriersystem in rats: bioavailability and antioxidant properties of liposome-encapsulated curcumin. J Agric Food Chem 2009; 57:9141–9146.

46 Saleh N, Waded E. Immune response following the administration of the anabolic steroid BoldenoneUndecylenate in rabbits. Stem Cell 2014; 5:80–87.

47 Mohammed HH, Badawi ME, El-Tarabany MS, Rania M. Effects of boldenoneundecylenate on growth performance, maintenance behaviour, reproductive hormones and carcass traits of growing rabbits. Pol J Vet Sci 2016; 19:245–251.

48 Elmasry TA, Al-shaalan NH, Tousson E, El-morshedy K. Star anise extracts modulation of reproductive parameters, fertility potential and DNA fragmentation induced by growth promoter Equigan in rat testes. Braz J Pharma Sci 2018; 54:e17261.

49 Oda SS, El-Ashmawy IM. Adverse effects of the anabolic steroid, boldenone undecylenate, on reproductive functions of male rabbits. Int J Exp Pathol 2012; 93:172–178.

50 Khalil SR, Mohammed HH, Abd-Elhakim YM, Mohamed WAM. Boldenone undecylenate injection consequences on male rabbit behavioral response, fertility and testicular ultrastructural changes with special respect to the withdrawal effects. Int J Adv Res 2014; 2:166–181.

51 Sadowska-Krepa E, Barbara Kłapcińska, Jagsz S, Sobczak A, Chrapusta S, Chalimoniuk M, *et al.* High-dose testosterone propionate treatment reverses the effects of endurance training on myocardial antioxidant defenses in adolescent male rats. Cardiovasc Toxicol 2011; 11:118–127.

52 Mayada RF, Taghred MS, Haytham AA. Boldenone-induced apoptotic, structural, and functional alterations in the liver of rabbits. World Rabbit Sci Assoc 2015; 23:39–46.

53 Bueno A, *et al.* A comparative study of the effect of the dose and exposure duration of anabolic androgenic steroids on behavior, cholinergic regulation, and oxidative stress in rats. PLoS ONE 2017; 12:e0177623.

54 Ibrahim SS, Said AM. Effect of lycopene in amelioration of testicular and renal toxicity induced by boldenone undecylenate in male albino rats. Int J Med Biomed Sci 2019; 3:58–66.

55 Thabet NS, Abelrazek EM, Ghazy EM, Elballal SS. Effect of the anabolic steroids, boldenone undecylenate on reproductive performance of male rabbits. J Reprod Infertil 2010; 1:8–17.

56 Tousson E, El-Moghazy M, Massoud A, Akel A. Histopathological and immunohistochemical changes in the testes of rabbits after injection with the growth promoter boldenone. Rep Sci 2012; 19:253–259.

57 Meseguer M, Martınez-Conejero JA, Enrique O'Connor J, Pellicer A, Remoh J, Garrido N. The significance of sperm DNA oxidation in embryo development and reproductive outcome in an oocyte donation program: a new model to study a male infertility prognostic factor. Fertil Steril 2008; 89:1191–1199.

58 Hall RC, Hall RW. Abuse of supraphysiologyic doses of anabolic steroids. South Med J 2005; 98:550–555.

59 Dohle GR, Smit M, Weber RFA. Androgens and male fertility. World J Urol 2003; 21:341–345.

60 Tousson E. Histopathological alterations after a growth promoter boldenone injection in rabbits. Toxicol Ind Health 2016; 32:299–305.

61 Yahyaei B, Nouri M, Matmir H. Healing effects of Ziziphusjujubahydroalcoholic extract with exercise training on histopathological changes of male wistar rats testicular tissue in response to Boldenone steroid administration. Jorjani Biomed J 2018; 6:12–21.

62 Sadeghi M, Daloiii AA, Ziaolhagh SJ. Effect of 6 weeks of resistance training and boldenone supplementation on 5-alpha reductase and aromatase gene expression in testes tissue of male wistar rats. Horizon Med Sci 2017; 23:199–205.

63 Groot MJ, Biolatti B. Histopathological effects of boldenone in cattle. J Vet Med A Physiol Pathol Clin Med 2004; 51:58–63.

64 Bueno A, Carvalho FB, Gutierres JM, Lhamas CL, Brusco I, Oliveira SM, *et al.* Impacts of dose and time of boldenone and stanazolol exposure in inflammatory markers, oxidative and nitrosative stress and histopathological changes in the rat testes. Theriogenology 2019; 1:101–108.

65 Soma LR, Uboh CE, Guan F, McDonnell S, Pack J. Pharmacokinetics of boldenone and stanozolol and the results of quantification of anabolic-androgenic steroids in race horses and non race horses. J Vet Pharmacol Ther 2007; 30:101–108.

66 Tousson E, Ali EMM, Ibrahim W, Mansour MA. PCNA as a molecular biomarker for spermatogenesis in PTU-induced hypothyroidism of rats. Reprod Sci 2011; 18:679–686.

67 Ali E, Tousson E, Abdel Daim H. Effect of short and long term growth promoter boldenoneundecylenate treatments on antioxidant enzyme activity and oxidative stress markers in rabbit muscles. Pharmacologia 2013; 7:576–581.

68 Tousson E, Zahran F, Shalapy MA. Oral supplementation of aqueous moringa and ginkgo leaf extracts abates oxidative stress and testicular injury associated with boldenone injection in rats. Biomed Nurs 2016; 2:3.