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Nandrolone Decanoate Administration to Male Rats Induces Oxidative Stress, Seminiferous Tubules Abnormalities, and Sperm DNA Fragmentation

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Abstract

The present study was conducted to evaluate the effects of Nandrolone Decanoate (an anabolic steroid) on the level of oxidative stress markers, sperm chromatin integrity, seminiferous tubules structure, and spermatogonia/Sertoli cell ratio in adult rat. Rats were divided into three groups: control, low (3mg/Kg) and high-dose (10mg/Kg) Nandrolone Decanoate-receiving groups. Seminal fluid analysis was performed, and the serum was used to evaluate testosterone level. Testicular oxidative stress markers were measured and routine histological preparation and androgen receptor immunochemistry was used to evaluate the effects of Nandrolone Decanoate injection on seminiferous tubules. Injection of Nandrolone Decanoate caused an increase in the production of thiobarbituric acid-reactive substances in the testes of treated rats. The level of sperm DNA fragmentation and the percentage of seminiferous tubules showing maturation arrest were also increased in treated animals. The absolute numbers of spermatogonia and Sertoli cells in the rats receiving Nandrolone Decanoate decreased significantly; however, the ratio of spermatogonia/Sertoli cells did not. Administration of anabolic steroids at supraphysiological doses leads to multiple pathological changes in the reproductive system of treated rats. Testosterone or its derivatives such as Nandrolone Decanoate are being abused commonly. Athletes, coaches, and physicians should be aware of their harmful side effects.

الملخص

تهدف الدراسة الحالية إلى تقييم آثار حقن Nandrolone Decanoate (واحد من الستيرويدات البنائية) على مستوى الجهد الاوكسيجيني، وسلامة المادة الوراثية للحيوانات المنوية، و شكل ومكونات الأنابيب المنوية، ونسبة الخلايا المنوية الأم للخلايا المساعدة (خلايا سرتولي) في الجرذان التي تم حقنها. تم تقسيم الجرذان إلى ثلاثة مجموعات: مجموعة الضبط و مجموعة حقنت بتركيز متدني من Decanoate Nandrolone (3مج/كغم) و مجموعة حقنت بتركيز عالي (10مج/كغم). توصلت الدراسة إلى أن الإستخدام المفرط لمركب Nandrolone Decanoate أدى إلى إفساد الحمض النووي للحيوانات المنوية و إلى إيقاف عملية تكوين الحيوانات المنوية في مراحل مختلفة. ولم يحدث أي تغيير في نسبة الخلايا المنوية الأم إلى خلايا سرتولي، ولكن العدد المطلق لهذه الخلايا قلَ بشكل كبير. وبالإضافة إلى ذلك، حقن الستيرويدات البنائية تسبب في زيادة إنتاج Acid Thiobarbituric دون حدوث أي تغيير في مستوى GSH في خصية الجراذين التي حقنت ب Nandrolone Decanoate. إن إساءة استعمال الستيرويدات البنائية بدأت تصبح مشكلة أساسية في مجال الصحة العامة وهو ما يعني ضرورة تنفيذ البرامج التعليمية لتوعية وتحذير المراهقين والمرشدين على حد سواء عن الآثار الجانبية والسلبية لهذه العقارات على صحة مستخدميها.

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1. Introduction

Anabolic-androgenic steroids (AAS) are synthetic compounds which are based on the structure of testosterone, and are used to treat various conditions such as reproductive system dysfunction, breast cancer and anemia (Thiblin and Petersson, 2005). Three basic modifications are made to the structure of testosterone to enhance deliverability and potency and slow down rate of degradation. The first of these modifications (class I) involves esterification of testosterone at the 17-β-hydroxy location (Hall and Hall, 2005). This modification, which is made to injectable AAS, slows down degradation but enhances androgenic properties (Hall and Hall, 2005).

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Nandrolone Decanoate (ND) is a class I AAS. The second modification (class II) results from alkylation at the 17-α-hydroxy position, which depresses hepatic degradation (Wilson, 1988). The third modification (class III) has been used to generate together oral and injectable formulations. One pattern of class III modifications results in an AAS with analogous properties to that of Class II AAS, but with a decreased or missing hepatic effect (Wilson, 1988; Hall and Hall, 2005).

AAS are taken by abusing athletes at supraphysiological doses; such doses are usually 10 to 100 fold the recommended therapeutic dose (Clark *et al.,* 1997). Traditionally, AAS have been abused in drug-use cycles of 6-14 weeks followed by a drug-free period to prevent building up tolerance to AAS (Karila *et al.,* 2004).

Defining the exact adverse effects of AAS abuse is very hard due to the lack of clinical trials which mimics AAS abuse by athletes (Hall and Hall, 2005). In addition, almost all major tissues in the body have androgen receptors, and thus AAS abuse affects almost all body systems (Karila *et al.,* 2004). Side effects of AAS abuse include liver failure, acne, a decrease in high-density lipoprotein (HDL) levels and hepatic adenomas (Boyadjiev et al, 2000; Hall and Hall, 2005), mood fluctuation, aggressive behavior, violence and suicide attempts (Clark *et al.,* 1997).

One of the most pronounced effects of AAS abuse is the negative impact on the hypothalamus-pituitary-gonadal axis (*Takahashi et al.,* 2004). In men abusing AAS, there is a noticeable reduction of serum testosterone level as well as FSH and LH (Jarow and Lipshultz, 1990). In addition, AAS abuse has been linked to reduced quality and quantity of semen which could be related to infertility (Torres-Calleja *et al.*, 2001). Long-term AAS administration results in reduction in testicular weight, testicular atrophy and abnormal morphology (Jarow and Lipshultz, 1990).

Although many studies have investigated the effects of testosterone suppression on spermatogenesis through sperm output evaluation (Torres-Calleja *et al.*, 2001), there are no reports on the effects of AAS abuse and thus testosterone suppression on Sertoli cells number and function, structural changes of the testis or sperm chromatin integrity. Hence, this work was conducted to evaluate the effects of administration of ND at supraphysiological doses on oxidative stress biomarkers and male reproductive system.

2. Experimental Procedure

2.1. Animals and treatments

Animal care, handling, and all of the experiments performed were approved by the Hashemite University Institutional Animal Care and Use Committee.

Forty-five adult Wister male rats (*Rattus norvegicus*), weighing between 150–300g (three to four months old), were randomly selected from the laboratory animal center at The Hashemite University (Zarqa-Jordan). The animals were housed individually in an air-conditioned room (12 hr dark/12 hr light) at 23 ± 2 ºC, and had free access to tap water and standard food pellet. After one week of acclimatization, the 45 animals were randomly divided into three groups of 15 each: Group 1 (control), receiving 10 mg/kg/wk of the vehicle peanut oil, Group 2 (low dose), receiving 3 mg/kg/wk of ND (N.V. Organon Oss Holland), and Group 3 (high dose), receiving 10 mg/kg /wk of ND Treatments were administered weekly via intramuscular injection (0.1ml) for 14 weeks. The doses of ND administered to rats and period of administration mimics one cycle of AAS abuse by athletes (Hall and Hall, 2005; Mirkhani *et al.,* 2005; Trenton and Currier, 2005).

2.2. Sample collection and processing

One week after the final drug administration, body weight was measured and the rats were killed rapidly under ether anesthesia followed by cervical dislocation. The following samples were collected and processed:

2.2.1. Serum

Blood was collected via heart puncture, centrifuged at 2,000 rpm for 10 min and the supernatant (serum) was stored at -20ºC. The serum was used to measure total serum testosterone which was determined using AxSYM ® Testosterone test (Abbott, USA). The test is based on Micropar t ical Enzyme Immunoassay (MEIA) technology for the quant itat ive determinat ion of total testosterone in serum (Gonzalez-Sagrado *et al.,* 2000) .

2.2.2. Sperm suspension

The left vas deferens of the rats from the three different groups was exposed and 1 cm of its distal portion was removed. The diffusion method was used to collect semen (Seed *et al.,* 1996). Sperm suspension was used to calculate sperm count and motility. Sperm samples were collected from distal region (cauda) of the left epididymis (Syntin and Robaire, 2001) and were used for evaluation of sperm morphology and chromatin integrity by acridine orange staining. For sperm morphology analysis, 20 µl of sperm suspension were smeared, air dried and stained with hematoxylin and eosin (H&E). 200 sperm per sample were evaluated for normal and abnormal sperm forms [such as angulated (bent) sperm, broken sperm (i.e., headless, tailless), short sperm, and coiled-tailed sperm]. Sperm DNA fragmentation was evaluated by acridine orange staining which was performed according to the method of Tejada *et al.* (1984) and Chohan *et al.* (2004). An average of 200 sperm cells was counted on each slide by the same examiner. Upon excitation, the monomeric acridine orange bound to double-stranded DNA fluoresces green. The aggregated acridine orange on single-stranded DNA fluoresce a spectrum of yellow-orange to red (Chohan *et al.,* 2004).

2.2.3. Right testis

The right testis was removed and cut in half. The first half was used to measure oxidative stress markers. Lipid peroxidation products were quant ified by the thiobarbituric acid (TBA) method (Ohkawa et al., 1979) and expressed as Thiobarbituric Acid-Reactive Substances (TBARS) Levels (nmoles/g protein). Total glutathione content (GSH) and oxidized glutathione (GSSG) were determined by the method of Anderson (1985) and the results were expressed as nmoles of GSH or GSSG/mg protein.

The second half of the right testis was used to analyse the effects of ND on the expression of androgen receptor

by immunoblotting. Briefly, the tissues were washed with cold PBS and then immersed in lysis buffer (10 mM Tris-HCl pH 7.5, 10 mM NaF, 2% SDS, 5 mM DTT, 2 mM EGTA). Equivalent amounts of protein were resolved by SDS-PAGE on 7.5% gel, transferred to nitrocellulose membrane and immunoblotted with rabbit polyclonal anti-androgen receptor (AR) primary antibody (1:2,000) (Sigma, USA). Blots are representative of at least two independent experiments. Left testis

The left testis was removed and weighed. The volume of testis prior to fixation was estimated using the immersion method (Plethysmometer, Ugo Basile, Italy). The tissue was fixed in 10 % neutral formalin fixative and embedded in paraffin. The tissue was then used either for routine H&E staining or for androgen receptor immunochemistry.

To study the process of spermatogenesis and on the basis of standard qualitative interpretation of H&E staining, testis sections were classified as follows: (i) Normal histology: almost all tubules showing elongating spermatids in each cross section, (ii) Early maturation arrest of spermatogenesis (EMA): most tubules showing spermatogenesis arrest at the level of primary of spermatocyte, with spermatids never observed, and (iii) Late maturation arrest (LMA) of spermatogenesis: most tubules showing spermatogenesis with only round spermatids being observed.

In order to determine the spermatogenic capacity of the male rat, the number of spermatogonia supported by one Sertoli cell was counted. Sertoli cells were identified by positive staining with rabbit polyclonal anti-androgen receptor (AR) primary antibody (1:200) (Sigma, USA). The primary/secondary antibody reaction was detected using high sensitivity streptavidin conjugated to horseradish peroxidase (HSS-HRP) and revealed by 3, 3 diaminobenzidine (DAB) chromogen. The sections were counterstained with Mayer's hematoxylin. A total of five seminiferous tubules per animal were randomly selected, and the number of spermatogonia and Sertoli cells were counted (Watanabe, 2005). All histological observations were carried out using bright field microscope (Nikon E800, Japan).

2.3. Statistical analysis

All samples were processed individually and measured in triplicate in the same assay. Data are stated as mean ± Standard Error of the Mean (SEM).When appropriate, data were analyzed by a one-way ANOVA test. A significant level of 0.05 was considered appropriate.

3. Results

The effects of ND administration on several parameters of serum, testis, and semen in male rats were investigated.

3.1. Effect of ND administration on body weight

The body weight for all animals in the three groups was measured before and after treatments (Fig. 1). After 14 weeks, the final body weight of animals in the three groups was not significantly different as compared to their weight at the beginning of the study, but the rate of weight gain was more in the control group; although not significant (p=0.582).

3.2. Effects of ND administration on testosterone level

Measuring total testosterone level in the serum of the control and treated groups indicated that injection of ND caused a significant decrease in testosterone level in both the low (5.3 ± 3.7 pg/ml) and the high-dose (60.1 ± 19.3 pg/ml) ND receiving groups (p=0.001) as compared to the control group (633.3 ± 210.1 pg/ml).

3.3. Effects of ND administration on sperm characteristics

The average sperm concentration of both groups receiving low (31.4 ± 5.9x10^6/ml) and high (44.7 ± 5.9x10^6/ml) doses of ND was significantly decreased as compared to that of the control group (116.0 ± 1.1 x10^6/ml) (p=0.001). 78.1 ± 2.7% of sperm extracted from the vas deferens of rats in the control group showed progressive forward motility. In contrast, treatment of rats with the low and high doses of ND caused a significant decrease (p=0.001) in the percentage of progressively motile sperm (39.2 ± 4.1% and 19.4 ± 3.1%, respectively).

The percentage of sperm with normal morphology was statistically decreased (p=0.001) in the low-and high-dose receiving groups (61.8 ± 4.7% and 59.4 ± 5.0%, respectively) as compared to the control group (92.9 ± 1.1%).

The effect of ND administration on sperm chromatin integrity was evaluated by acridine orange staining. 200 sperm per slide were counted, and the percentage of sperm with single-strand DNA (Red, yellow-orange/total x 100) was calculated (Fig. 2A). The percentage of sperm with defected DNA was significantly increased in the treated rats receiving low and high doses of ND (72.3% and 53.3%, respectively) as compared to the control group (19.7%) (p=0.001) (Fig. 2B).

3.4. Effects of ND administration on testes

The volume of the testes decreased significantly in animals which received low (0.81 ± 0.14 ml) and high doses (0.93 ± 0.02 ml) (p=0.001) of ND in comparison with the animals in the control group (1.78 ± 0.04 ml).

Injection of ND caused a significant increase in TBARS production in the testis of both the low and the high-dose receiving groups as compared to the control group (p=0.001) (Fig. 3). On the other hand, the testicular levels of both total glutathione (GSH) and oxidized glutathione (GSSG) were not significantly different (p=0.519 and 0.551, respectively) between treated and untreated groups (Table 1).

Table 1. Effect of Nandrolone Decanoate administration on testicular glutathione levels. Values are expressed as means ± SEM (n in each group=3-6)

Group	Total GSH (nmoles/mg protein)	GSSG (nmoles/mg protein)
Control	18.9 ± 1.2	2.4 ± 0.15
Low Dose	21.1 ± 2.3	2.1 ± 0.09
High Dose	19.3 ± 2.1	2.9 ± 0.35

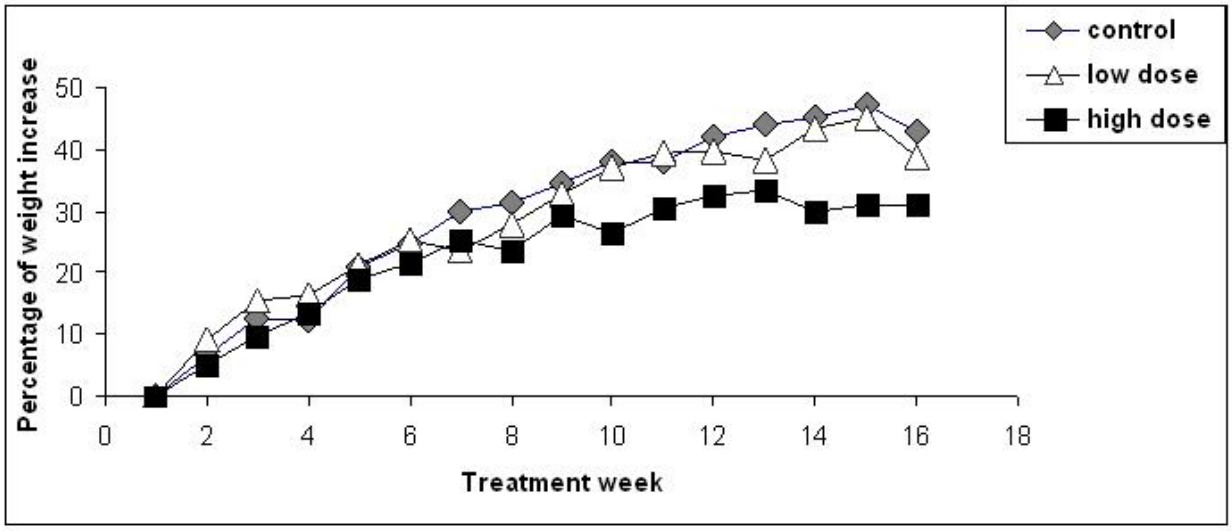

Figure 1. Effect of Nandrolone Decanoate administration on body weight. Body weight is expressed as (final body weight – initial body weight /initial body weight ×100). n=15 in each group.

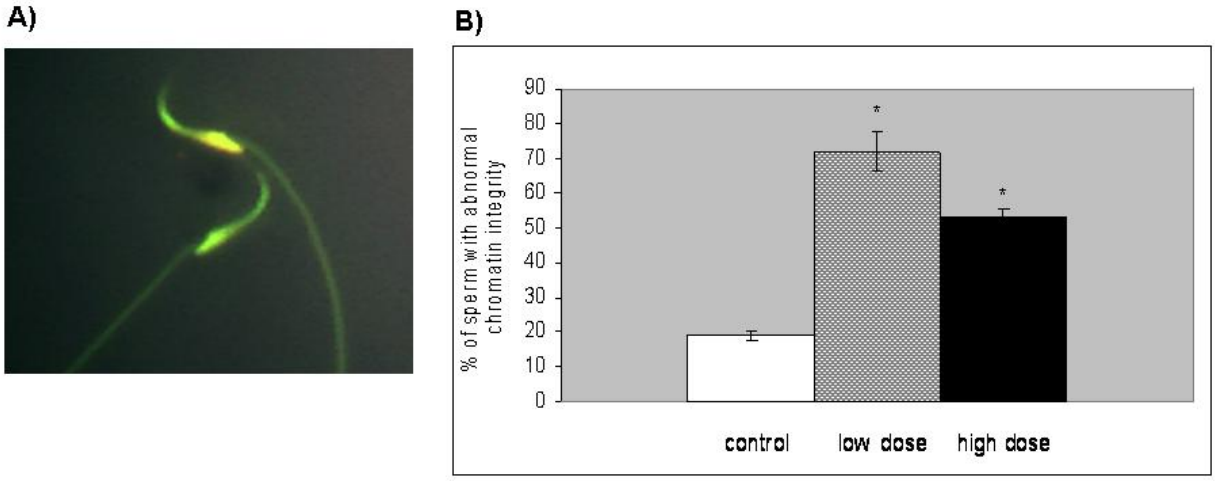

Figure 2. Effect of Nandrolone Decanoate administration on sperm chromatin integrity. A) Acridine orange test applied to rat sperm showing a sperm with intact chromatin (green) and a sperm with damaged chromatin (yellow-orange), B) The percentage of sperm with abnormal chromatin integrity was higher in treated groups as compared to the control group. (Values are expressed as mean ± SEM, n =15 in each group, *p=0.001).

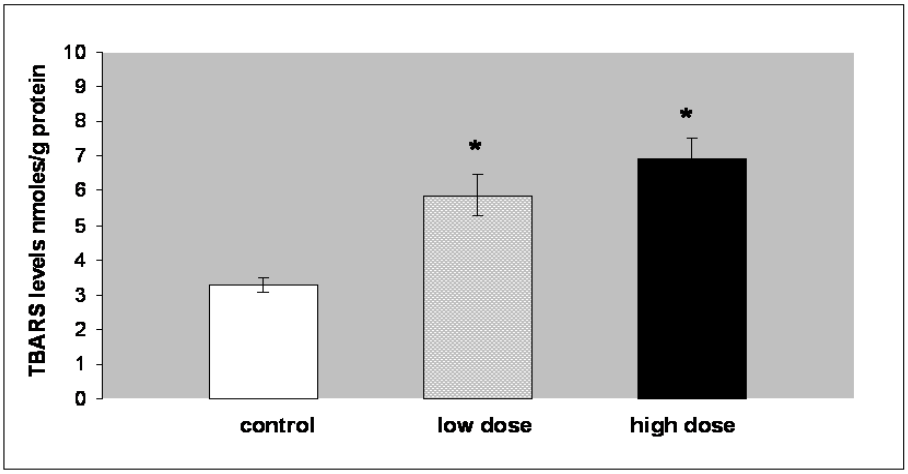

Figure 3. Effect of Nandrolone Decanoate administration on testicular TBARS levels. Both low and high-dose receiving groups had a higher level of TBARS as compared to the control group. (Values are expressed as mean ± SEM, n = 6-9 in each group, *p=0.001).

After analyzing different cross sections of testes of control and treated animals, obvious differences were noted. One of these differences was atrophy (low number of germ cells) in the seminiferous tubules (ST) of treated rats (receiving low or high doses of ND) with large focal areas, and a noticeable decreased accumulation of spermatozoa within the lumen (hypospermatogenesis) (Fig. 4B) as compared to ST of control rats (Fig. 4A). Also, some ST in the testes of treated rats showed abnormal architecture of the seminiferous epithelium (Fig. 4C), and maturation arrest (early or late) (Fig. 4D and E, respectively). In addition to the abnormal patterns of spermatogenesis mentioned, normal seminiferous tubules were also found in cross sections of testes of treated rats. A summary of spermatogenesis patterns in control and treated rats is shown in Table (2).

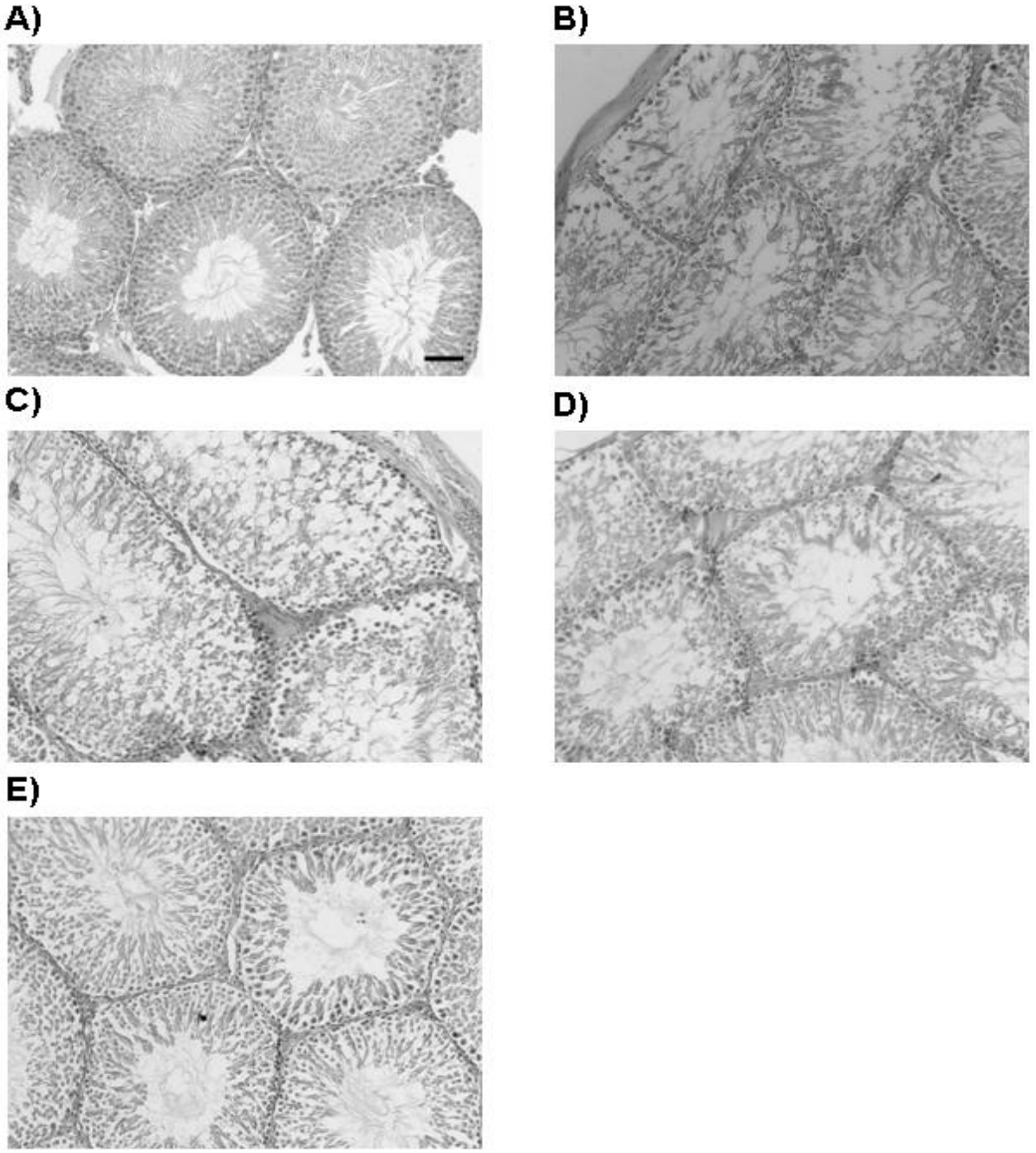

Figure 4. Histological appearance of representative examples of seminiferous tubules (ST) of control and treated rats stained with hematoxylin and eosin. A) A cross section of ST from control rat showing normal architecture of germinal epithelium, i.e., multiple layers of germ cells ranging from spermatogonia, 1^o spermatocytes, 2^o spermatocytes, round and elongated spermatids and sperm in the lumen, B) A cross section of ST from a low-dose treated rat displaying atrophic germinal epithelium (2-3 layers of germ cells) and almost lacking sperm in the lumen, C) A cross section of ST from a low-dose treated rat showing abnormal organization of the germinal epithelium, D) A cross section of ST from a low-dose treated rat showing early maturation arrest in which germ cells differentiation stops at 1^o spermatocytes, E) A cross section of ST from a high-dose treated rat displaying round spermatids as the last stage of germ cell differentiation (late maturation arrest). Scale bar = 50 μm.

Table 2. Different patterns of spermatogenesis observed in control and Nandrolone Decanoate -receiving rats. Values represents mean of percentages ± SEM, n in each group=7, *: p=0.001

Group	% of ST showing normal spermatogenesis	% of ST showing maturation Arrest		% of ST showing hypo-spermatogenesis
		Early	Late	
Control	94.1 ± 0.26	4.3 ± 0.3	1.2 ± 0.1	0.4 ± 0.3
Low Dose	21.1 ±1.2*	44.3 ± 1.2*	20.7 ± 1.8*	13.9 ± 1.5*
High Dose	20.7 ± 2.1*	30.0 ± 1.1*	33.7 ± 1.8*	15.6 ± 1.2*

Finally, we examined the pathological changes that might have occurred to spermatogonia and Sertoli cells in the testes of treated rats. Immunohistochemical localization of AR expression within seminiferous tubules was restricted to nuclei of Sertoli cells (Fig. 5A). Spermatogonia in seminiferous tubules of control rats were closely packed and the nuclei were darkly-stained, and the nuclei of Sertoli cells were located regularly in the periphery of the tubules (Fig. 5A). Seminiferous tubules of treated rats (receiving either low or high doses of ND) showed abnormal organization of spermatogenic cells (Fig. 5B), and the number of layers of germ cells was reduced. In addition, the morphology, nuclear size and position of Sertoli cells were changed in the STs of treated rats (Figure 5B). The number of spermatogonia in the testes of rats in both low and high-dose receiving groups was significantly decreased as compared to the control (p=0.001) (Fig. 5C). In addition, there were fewer Sertoli cells in the seminiferous tubules of ND-treated animals than in those of the control rats (p=0.001) (Fig. 5C), which was confirmed by analyzing the level of AR expression by immunoblotting of extracted testicular proteins (Fig. 5D). Quantification of normalized AR levels in treated animals relative to that of controls (lane 1,2) revealed that extracts from both low- (lane 3) and high-dose receiving groups (lane 4) showed a reduction (32% and 47%, respectively) in AR expression as compared to extracts of control animals (Fig. 5D). However, the ratio of spermatogonia to Sertoli cells in the treated rats was not significantly different from that of the control group (p=0.287).

4. Discussion

One of the most prominent effects of AAS is the negative impact on the pituitary-gonadal axis (Aubert *et al.,* 1985; Takahashi *et al.,* 2004).AAS stimulates hypogonadotrophic hypogonadism coupled with decreased serum testosterone concentrations (Harkness *et al.*, 1975; Schurmeyer *et al.*, 1984; Jarow and Lipshultz, 19908). In the current study, total serum testosterone level in treated rats was lower than in control animals, which is in accord with what was previously reported.

The dysfunction accounting for these abnormalities is supposed to be steroid-induced suppression of gonadotrophin production; it was reported that there is a noticeable depression of serum FSH and LH in men abusing AAS (Harkness *et al.*, 1975; Clerico *et al.*, 1981). This in turn results in a condition of impaired spermatogenesis including oligozoospermia to azoospermia, abnormalities of sperm motility and morphology (Ramaswamy *et al.*, 2000; Torres-Calleja *et al.*, 2001; Takahashi *et al.*, 2004). Suppression of sperm output is attributed to the degree of inhibition of germ cell development which is related to the degree of FSH, LH and testosterone suppression (O'Donnell *et al.*, 2001). This could explain decreased sperm concentration, motility and normal morphology induced by long-term ND administration to rats. Also, it could explain the maturation arrest noted in the ST of treated rats (Table 2).

Since little information is available on the effects of 17-β-alkylated steroid treatment on oxidative stress markers, we aimed to investigate whether prolonged treatment of rats with low or high doses of ND modified oxidative stress markers through studying TBARS production as a result of lipid peroxidation and redox status of glutathione (GSH). Figure 3 shows that prolonged administration of ND induced a significant increase (p=0.001) in TBARS levels which serves as an index of extended lipid peroxidation. However, this treatment did not modify testicular levels of GSH in treated rats (Table 1). To our knowledge, this is the first time that the effects of ND treatment on oxidative stress biomarker levels have been studied. AAS seek to maximize the anabolic effects and overcome the catabolic pathways thus increasing anabolic pathways, so the possibility of oxidative stress condition could increase (Saborido *et al.*, 1993; Molano *et al.*, 1997).

It was shown previously that prolonged administration of Stanazolol (17-α-alkylated AAS) provoked dysfunction of the mitochondria respiratory chain complexes and mono-oxygenase systems; it would be possible that these alterations were accompanied by an increased reactive oxygen species (ROS) generation (Saborido *et al.*, 1993; Molano *et al.*, 1997; Pey *et al.*, 2003).

One more supposed source of free radicals generation is cytochrome P450 cholesterol side-chain cleavage enzyme isoforms of the steroidogenic pathway during their catalytic cycles, these isoforms have been shown to release ROS under basal conditions especially with uncoupled substrates (Chang *et al.*, 1995). Consequently, metabolism of high doses of ND by cytochrome P450 mono-oxygenases would have increased greatly the production of ROS resulting in a state of oxidative stress and up-regulation of the activity of the antioxidant enzymes such as SOD and glutathione peroxidase (GPx), gluthatione reductase (GR) and catalase (Georgiou *et al.*, 1987; Diemer *et al.*, 2003; Pey *et al.*, 2003; Chen *et al.*, 2005). However, the mechanism through which the intramuscular injection of ND could be associated with free radical production is unidentified at present.

On the other hand, there was no change on GSH level (Table 1), which might be due to the body using other antioxidant defense mechanisms such as the activities of

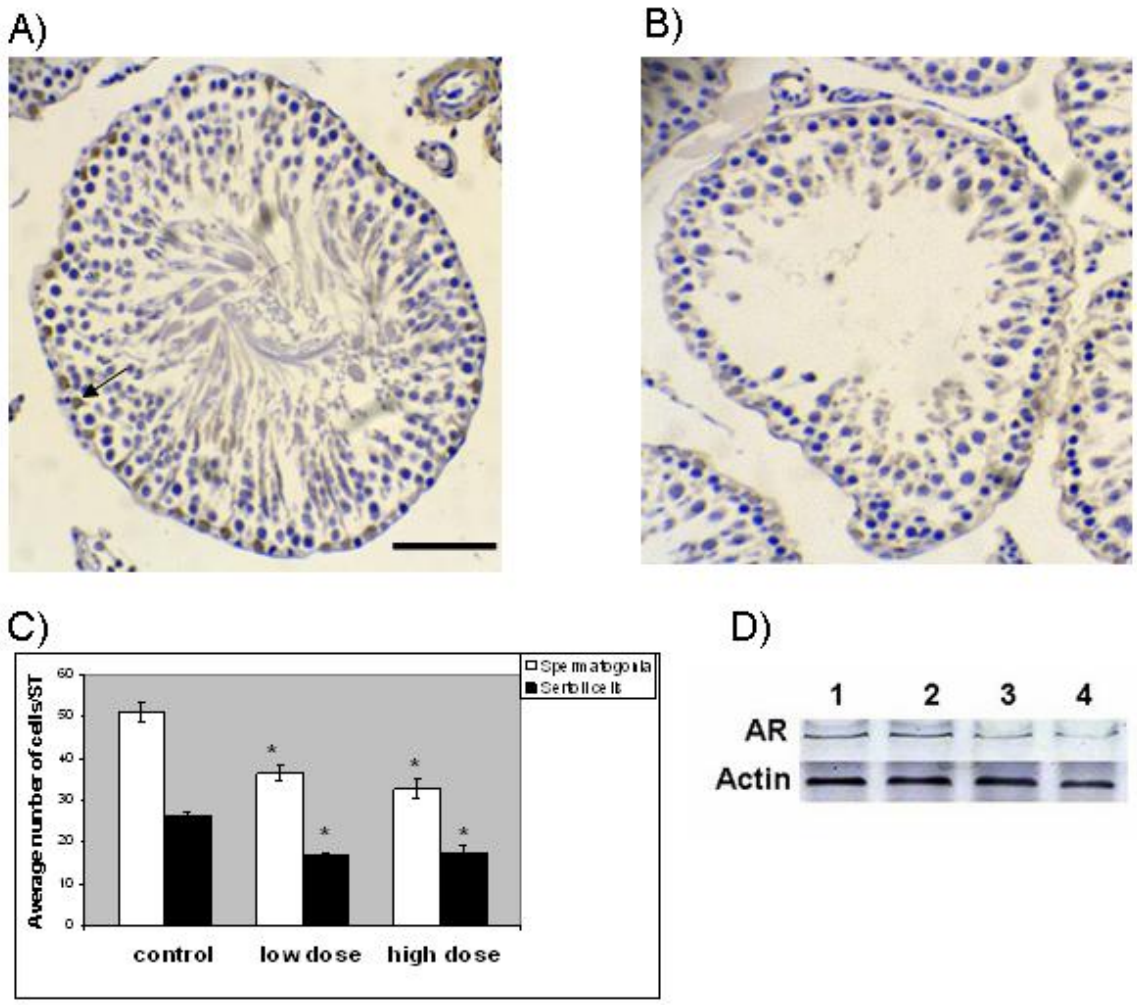

Figure 5. Effect of Nandrolone Decanoate administration on spermatogonia and Sertoli cells. A) A representative example of a cross section of a ST from control rat immunostained for androgen receptor, the outermost layer shows darkly stained spermatogonia and evenly placed Sertoli cells (arrow), B) A representative example of a cross section in ST of a low-dose treated rat immunostained for androgen receptor, showing reduced number of germ cells, and misplaced Sertoli cells. Scale bar = 50 µm. C) The number of spermatogonia and Sertoli cells in the ST of treated rats was significantly reduced as compared to the control group, values are expressed as mean ± SEM, n=5, **p*=0.001, D) Immunoblot analysis for AR levels. Testis lysates from control (lanes1, 2), low (lane 3) and high-dose receiving rats (lane 4) where immunoblotted with anti-AR antibody.

several scavenging enzymes [SOD, GPx, GR, catalase and hemoxygenase-1 (HO-1)] (Georgiou *et al.*, 1987; Diemer *et al.*, 2003; Chen *et al.*, 2005).

To our knowledge there is no available data that evaluates the effect of ND on sperm chromatin integrity. Sperm DNA fragmentation could be due to several reasons such as deficiency in recombination during spermatogenesis (Bannister and Schimenti, 2004). Exposure of mature spermatozoa to excessive levels of ROS produced by immature sperm during migration from the seminiferous tubules to the epididymis could also lead to fragmented DNA (Ollero *et al.*, 2001). In addition, abnormal spermatid maturation could result in DNA-fragmented sperm.

Around 80-90% of the weight of each testis consists of tightly packed ST (Greenspan and Gardner, 1994). The major supporters of spermatogenesis process are the Sertoli cells which take up a volume of around 17–19% of the seminiferous tubules of adult rats. Sertoli cells are the only somatic cells in direct connection to germ cells (Mruk and Yan, 2004).

Androgen action in the testis, as in other tissues, is mediated through androgen receptor (AR) transcriptional activation (Bremner *et al.*, 1994). Inside Sertoli cells, testosterone is selectively bound to the androgen receptor and activation of the receptor will result in initiation and maintenance of the spermatogenic process and inhibition of germ cell apoptosis (Dohle *et al.*, 2003). In testes, ARs are expressed in the somatic Leydig, peritubular myoid and Sertoli cells as well as to rete testis, the epithelial cells of the epididymis, and prostate (Vornberger *et al.*, 1994; Bilinska *et al.*, 2005).

AR expression is maintained by endogenous testicular androgens; withdrawal of testosterone is known to lead to disruption of spermatogenesis (Kerr *et al.,* 1985). After long-term hypophysectomy and elimination of residual testosterone, spermatogenesis rarely proceeds beyond meiosis, with very few round spermatids observed and elongated spermatids nearly non-existing (Franca *et al.,* 1998). In the current work, injection of male rats with low or high doses of ND resulted in reduction of testosterone, which caused maturation arrest at the primary

spermatocyte level (Fig.4D), and at the spermatid level (Fig.4E).

In addition, it was found that loss of AR activity from Sertoli cells would lead to spermatogenic failure resulting in incomplete meiosis and collapse to transition of spermatocytes to haploid round spermatids (Birgner et al., 2008; Holdcraft and Braun, 2008).In the current study, the immunoexpression of AR was scored as the number of positive nuclei within the boundaries of STs (Fig. 5A) and by immunoblotting (5D), and it was found that ND-administration caused a reduction in the number of Sertoli cells expressing AR (Fig.5C), which in turn could explain the maturation arrest observed in treated animals (Table 2) and testicular atrophy (Fig.4B).

Sertoli cells play an important role in organizing the somatic cell lineages and in determining the structure of the testis (McLaren, 2000); they also support a finite number of germ cells, and thus their number determines the spermatogenic capacity of the adult (Orth *et al*., 1988; Sharpe, 1994; McLachlan *et al.,* 1996). Our results indicate an AAS-induced reduction in Sertoli cell number (Fig.5C) which might be due to a structural response of Sertoli cells to deprivation of testosterone (Watanabe, 2005). The reduction in Sertoli cell number in treated rats could have resulted in a subsequent reduction in the number of spermatogonia (Fig.5C) leading eventually to a decrease in sperm count.

There are no reports on the effects of testosterone suppression on spermatogonia cell differentiation. In addition, the quantitative structural changes of the testis caused by AAS abuse received little or no attention. The current study reports a number of abnormalities in the architecture of the seminiferous tubules of treated rats (Table 2) namely atrophy in the ST (Fig. 4B), abnormal organization of the germinal epithelium (Fig. 4C), and maturation arrest (Fig. 4D and E).

Injection of male rats with low or high doses of ND caused a reduction in testicular volume as compared to control rats. Estimation of testicular volume is a good indicator of testicular atrophy, as evident in Fig. 4B. The decrease in testis volume might be a consequence of reduction in seminiferous tubules length (Noorafshan *et al.,* 2005), or could have resulted from a negative feedback on the hypothalamic-pituitary axis with consequent testicular atrophy (Dohle *et al.,* 2003). On the other hand, and as reported by others (Takahashi *et al.*, 2004), body weight of the experimental animals did not differ from controls over the route of treatment (Fig.1).

One final observation from the current work is that in some of the situations studied (effects of ND administration on TBRAS levels, motility and morphology of sperm, number of Sertoli cells and degree of late maturation arrest in ST), the actions of ND were directly receptor and dose dependent; the more drug injected the more adverse the side effect. When ND acts in a receptor mediated mode, we can assume that the high concentration of ND injected to rats can overcome the fact that AR has low affinity to ND (Saartok *et al.,* 1984). In contrast, the effects of ND injection on level of sperm DNA fragmentation, serum testosterone concentration, sperm concentration, and the degree of ST early maturation arrest were receptor- and dose-independent. This observation about ND action could provide some evidence that ND has two distinct modes of actions, receptor-dependent and independent. However, both of ND modes of action in the testicular tissue may be closely linked (Rommerts, 1998).

Androgen deficiency may have serious consequences in men, and this condition requires diagnosis and appropriate treatment. When administered properly, androgens are safe; however, when testosterone or its derivatives (such as AAS) are abused at supraphysiological doses, they may cause considerable harm. AAS are commonly used in our society, and physicians should be aware of their physiological effects. The present work reports that intramuscular injection of male rats with commonly used anabolic androgenic steroid ND (3 or 10 mg\kg) for 14 weeks was deleterious to the structure of rat testes. These effects included testicular atrophy, maturation arrest in seminiferous tubules, severe depletion of the absolute number of spermatogenic and Sertoli cells, and marked suppression of sperm count. In addition, ND administrations caused testosterone depression, enhanced lipid peroxidation, as well as severe fragmentation in the DNA of sperm of treated rats. Although the concentration of ND which was administered to male rats is comparable to what is injected by AAS abusers, we are aware that caution should be taken when such results are extrapolated from animal to man.Acknowledgements

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