Potential Toxic Effect of Steroids Abuse on Reproductive System of Adult Male Albino Rats

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Abstract

Introduction: The abuse of anabolic-androgenic steroids (AAS) has recently emerged as a major form of substance abuse worldwide. Chronic AAS use suppresses the function of the hypothalamic-pituitary-gonadal axis, which may lead to hypogonadism and thus infertility. Aim of the work: This study was performed to evaluate the potential effects of methandrostenolone (dianabol), nandrolone decanoate (nandrolone) separately and in combination on reproductive system of adult male albino rats and their recovery response. Material and Methods: 72 adult male albino rats were divided into 4 groups: group I; control group, group II; treated with dianabol only, group III; treated with nandrolone only, group IV; treated with both nandrolone and dianabol. Treatments were given for 8 weeks followed by 4 weeks recovery. Hormonal level assay [Testosterone, Follicle Stimulating Hormone (FSH) and Lutenizing Hormone (LH)], semen analysis, histopathological and immunohistochemical examinations were performed. Results: There was a highly significant decrease in the mean values of serum testosterone, FSH, LH and all the seminal parameters except for the abnormal forms. Testicular and epididymal structural abnormalities and decreased cytochrome p450scc immunoreaction were observed. The recovery period showed partial improvement in all parameters. Conclusion: AAS adversely affect the reproductive system with partial improvement on their withdrawal. Recommendation: It is recommended to raise awareness of athletes, teenagers and grown-ups about the adverse effects of AAS and to limit their misuse and self-administration.

Keywords: testis- epididymis- anabolic steroids- Methandrostenolone, Nandrolone decanoate.

Introduction

Doping is defined as use of drugs or other substances for performance enhancement, which has become an important topic in nearly every sport (¹). The abuse of anabolic androgenic steroids (AAS) have brought great attention world-wide. Many young adolescents abuse AAS to improve their
physical fitness and appearance (2). Anabolic androgenic steroids may be endogenous occurring naturally within the body (e.g. testosterone, androstenediol and dihydroepiandrosterone) or exogenous synthetic products of testosterone (3).

Testosterone is a steroid hormone which can be presented either free or bound to plasma proteins. It is the natural male hormone produced by the testis. It is also produced by females but in lesser amounts. Testosterone is responsible for the androgenic (masculinizing) and anabolic (tissue building) effects throughout male adolescence and adulthood (4).

Anabolic steroids are synthetic products of testosterone. They are divided into two main groups: a group with alkylation of 17-α position with ethyl or methyl group and the other with esterification of 17-β-OH group. These modifications enable these chemical compounds to have prolonged physiological effects up to several months (5).

Generally, the preferred effects of AAS are increase in muscle size and strength and also, reduced muscle damage, increase in protein synthesis, glycogen storage and decrease body fat percentage. AAS also increase the erythropoiesis, bone mineral density, hemoglobin and hematocrit levels (6). Adverse side effects of AAS include hypogonadism, cardiac and hepatic dysfunction and alteration of blood lipid levels (7). In addition, the AAS abuse may lead to mood alterations, aggression (8) and may cause dependence (9).

Methandrostenolone (dianabol) is a 17-alpha alkylating anabolic steroid. It was originally established by John Ziegler and released in the US. It was used as a helping agent to muscle growth by body builders (10). Nandrolone decanoate is a non-alkylating anabolic steroid was developed by Organon and released under trade name of Deca-durabolin in U.S. It had a wide range of usage including pre and post-operative building lean mass, osteoporosis, advanced breast cancer, burns and ulcers, but now in the U.S this drug is used for treating anemia only. Despite this, it became one of the most widely distributed anabolic steroid in the world (11).

From these points of view, this study was performed to evaluate the potential effects of methandrostenolone (dianabol), nandrolone decanoate (nandrolone) either separately or in combination on the reproductive system of adult male albino rats and their recovery response. This evaluation occurs through assessment of testosterone, LH and FSH levels, seminal analysis, histopathological and immunohistochemical study.

Materials and methods

Chemicals

Methandrostenolone (dianabol), a light yellow crystalline powder that was purchased from Sigma Aldrich (St. Louis, MO, USA).

Nandrolone decanoate (nandrolone), the powder obtained from Sigma Aldrich (St. Louis, MO, USA).
Experimental animals

72 adult male albino rats (150-200 g weight; 50-60 day age) were used in this study. They were recruited from the Animal House, Faculty of Medicine, Zagazig University. They were kept under suitable conditions and fed on a balanced diet and water ad-libitum. The study was performed in accordance with institutional guidelines for the care and use of experimental animals and approved by the Ethical Committee of Zagazig University (Egypt).

Experimental design

The rats were divided into 4 groups as follow:

**Group I** (control group) (36 rats): which were subdivided into 3 equal subgroups: Subgroup (IA) (negative control group): 12 rats received only regular diet and water to determine the basic values of performed tests. Subgroup (IB) (vehicle control group): 12 rats received normal saline 0.9% Nacl (solvent of dianabol) (1ml/day) by oral gavage once daily for 8 weeks, then saline was stopped for the following 4 weeks; the withdrawal or recovery period. Subgroup (IC) (vehicle control group):12 rats received 1ml of corn oil (solvent of nandrolone) by intraperitoneal injection once weekly for 8 weeks, then corn oil was stopped for the following 4 weeks; the recovery period.

**Group II** (dianabol treated group) (12 rats): which were subdivided into 2 equal subgroups, Subgroup (IIA): 6 rats received 50mg/kg body weight (1/20 of LD50) in 1ml of normal saline by oral gavage once daily for 8 weeks. Subgroup (IIB): 6 rats received 50mg/kg body weight (1/20 of LD50) in 1ml of normal saline by oral gavage once daily for 8 weeks then dianabol was stopped for the following 4 weeks; the withdrawal or recovery period (12). The LD50 of dianabol for rats is larger than 1 gm/kg body weight orally (13).

**Group III** (nandrolone treated group) (12 rats): which were subdivided into 2 equal subgroups, Subgroup (IIIA): 6 rats received 20 mg/kg body weight nandrolone (1/27 of LD50) in 1ml of corn oil by intraperitoneal injection once weekly for 8 weeks. Subgroup (IIIB): 6 rats received 20 mg/kg body weight nandrolone (1/27 of LD50) in 1ml of corn oil by intraperitoneal injection once weekly for 8 weeks then nandrolone was stopped for the following 4 weeks; the withdrawal or recovery period (14). The LD50 of nandrolone is larger than 566 mg/kg body weight by intraperitoneal injection (15).

**Group IV** (dianabol and nandrolone or stack treated group) (12 rats): which were subdivided into 2 equal subgroups, Subgroup (IVA): 6 rats received both nandrolone and dianabol at the same doses of the above groups for 8 weeks. Subgroup (IVB): 6 rats received both nandrolone and dianabol for 8 weeks then both drugs were stopped for the following 4 weeks; the withdrawal or recovery period.

The rats were sacrificed at 8\textsuperscript{th} and 12\textsuperscript{th} week by intraperitoneal injection of thiopental (50 mg/kg) (16). Venous blood samples were collected from the retro-orbital plexus of the rats using capillary glass tubes in serum separator tubes and samples were allowed to clot for 30 minutes before
centrifugation for 15 minutes to separate the serum. Testosterone, LH and FSH hormone in serum samples were measured by rat enzyme-linked immunosorbent assay (ELISA) Kit assay (17,18).

**Seminal analysis**

Spermatozoa collection was done as described by Blandau and Jordan (19). The fresh semen was obtained from each rat by cutting the tail of epididymis and carefully squeezing it. Then the semen was put in a clean Petri dish and incubated at 37ºC for half an hour for liquefaction to proceed to the following examinations:

**Progressive motility:** it was estimated according to Bearden and Flyquary (20).

**Epididymal sperm viability:** it occurred by mixing a drop of semen with an equal drop of eosin-nigrosin stain, films were spread on clean and grease free slides. Two hundred sperms were counted per rat using light microscope and the number of live (unstained) and dead (stained) sperms were estimated among the two hundred sperms. Then the viability percentage was calculated (21).

**Sperm count:** it was assessed according to Blazak et al. (22) & Assayed et al. (23).

**Epididymal sperm abnormalities:** the description of the sperm abnormal forms observed in this study was done according to Mori’s classification (24).

**Histopathological study**

**Haematoxylin and Eosin (H&E) stain:** the testes and epididymis were dissected. Specimens for light microscopy were fixed in 10% saline formalin and processed to prepare 5-μm-thick paraffin sections for H&E stain (25).

**Immunohistochemical study**

Immunohistochemical staining for localization of Cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc) was carried out by means of the avidin biotin complex (ABC) method (Dako ARK™, Peroxidase, Code No. K3954, Dako, Glostrup, Denmark) following the manufacturer’s instructions. Wax was removed from the paraffin sections and then hydration of the sections was performed. To expose target proteins, antigen retrieval was performed using citrate buffer (pH 6.0) microwaved for 8-15 minutes. Tissues were blocked in 3% bovine serum albumin at room temperature for 30 minutes. Then, they were incubated with the specific primary antibody overnight (4 ºC); anti cytochrome p450scc antibody (rabbit polyclonal antibody; ABS236; dilution 1/8000; Sigma Aldrich (St. Louis, MO, USA). Endogenous peroxidase was quenched by incubation in 10% H₂O₂ in phosphate buffered saline (PBS) (pH 7.4). Detection was done using biotinylated secondary antibodies and labeled horseradish peroxidase, followed by colorimetric detection by 3, 3’-diaminobenzidine (DAB). Tissues were counterstained with haematoxylin and prepared for mounting. Negative control sections were incubated with PBS instead of the primary antibody. Stained slides were examined by light microscopy. A brown coloration appeared at the antigen site (26).
Morphometric study

The Leica QWin 500 image analyzer computer system (Leica Ltd, Cambridge, UK) was used. The data analyzed by Leica QWin 500 software with the aid of a digital camera connected to an optical microscope (Olympus, Tokyo, Japan). Number of cells positive for cytochrome p450scs immune reactions was estimated. Ten non-overlapping fields were randomly chosen and analysed per each animal in each group. The measurements were performed by an investigator who was unaware of the experiment.

Statistical analysis

The collected data were expressed as Mean± SD. The statistical analysis was done by Epi-info statistical package program version 6.04d, January 2001. According to the type of data, the following tests were used to test differences for significance; Differences between multiple means (quantitative variables) were compared by ANOVA test, Followed by LSD, paired data by paired t test. P value was set at >0.05 for non-Significant results, <0.05 for significant results and <0.001 for high significant result.

Results

Biochemical findings, semen analysis, histopathological and immunohistochemical results of the negative and both vehicle control groups were within normal values and there were no statistically significant differences between them (p>0.05). So the results of the negative control group were used for comparison with those of steroids treated groups.

Hormonal level assay results

As regarding treated groups, by the end of 8th week there was a highly significant decrease in testosterone, FSH and LH levels in group IIA (dianabol), group IIIA (nandro lone) and group IVA (stack) in comparison with the control group with the least levels seen in group IVA (stack) which was treated with both drugs. The least significant difference (LSD) among the above groups revealed that there was a highly significant difference between all treated groups and control group. There was a non-significant difference between group IIA and group IIIA. There was a significant difference between group IIA and group IVA for testosterone and FSH, while it was non-significant for LH. There was a significant difference between group IIIA and group IVA for testosterone while it was non-significant for LH and FSH, table (1).

After 4 weeks of recovery there was still a significant decrease in the values of testosterone and FSH, but not in LH in all groups as compared to the control group. The least significant difference (LSD) among the above groups revealed that there was a highly significant difference between all the treated groups and the control group except for the LH that was non-significant. There was a non-significant difference between group IIB (dianabol) and group IIB (nandro lone). There was a significant difference between group IIB and group IVB (stack) for testosterone and FSH, while it was
non-significant for LH. There was a highly significant difference between group IIIB and group IVB for testosterone while it was non-significant for LH and FSH, table (2).

By comparing different parameters of mean values of the treated groups after 8 weeks of steroid administration with those after 4 weeks of recovery there was a significant increase in the values of testosterone, LH and FSH in the recovery period, Tables (3,4&5).

**Semen analysis results**

As regarding treated groups, by the end of 8th week there was a highly significant decrease in all seminal parameters {sperm count (10⁶/mm3), percent of sperm motility, sperm viability} except for abnormal forms which showed significant increase in group IIA (dianabol), group IIIA (nandrolone) and group IVA (stack) as compared to the control group with the least levels seen in group IVA. The least significant difference (LSD) among the treated groups revealed that there was a highly significant difference between all the treated groups and the control group. There was a non-significant difference between group IIA and group IIIA and a significant difference between group IIA and group IIIA when compared with group IVA, Table (1).

**Table (1): Statistical comparison between mean values of testosterone (ng/ml), FSH (mIU/ml), LH (mIU/ml) and semen analysis in group IA, group IIA, group IIIA and group IVA after 8 weeks of steroid administration using ANOVA (analysis of variance) test & least significant difference test (LSD):**

<table>
<thead>
<tr>
<th>Group (n=6)</th>
<th>Variable</th>
<th>Group I (A) (–ve control)</th>
<th>Group IIA (dianabol)</th>
<th>Group IIIA (nandrolone)</th>
<th>Group IVA (stack)</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD</td>
<td>Testosterone: (ng/ml)</td>
<td>1.43 ± 0.03bcd</td>
<td>0.80 ± 0.1 ad</td>
<td>0.74 ± 0.17 ad</td>
<td>0.59 ± 0.13 abc</td>
<td>98.97</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td></td>
<td>FSH: (mIU/ml)</td>
<td>0.12 ± 0.02 bcd</td>
<td>0.06 ± 0.01 ad</td>
<td>0.06 ± 0.01 a</td>
<td>0.05 ± 0.01 ab</td>
<td>59.93</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td></td>
<td>LH: (mIU/ml)</td>
<td>0.07 ± 0.0011 bcd</td>
<td>0.06 ± 0.005 a</td>
<td>0.06 ± 0.007 a</td>
<td>0.05 ± 0.008 a</td>
<td>11.69</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td></td>
<td>Count: (10⁶/mm³)</td>
<td>120.15 ± 1.29 bcd</td>
<td>99.8 ± 4.69 ad</td>
<td>94.3 ± 8.15 ad</td>
<td>68 ± 6.27 abc</td>
<td>143.52</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td></td>
<td>Motility: (%)</td>
<td>85.62 ± 1.05 bcd</td>
<td>63 ± 12.29 ad</td>
<td>62.9 ± 11.4 ad</td>
<td>48.5 ± 8.83 abc</td>
<td>26.13</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td></td>
<td>Viability: (%)</td>
<td>82.46 ± 0.99 bcd</td>
<td>40.7 ± 3.97 ad</td>
<td>37.58 ± 4.27 ad</td>
<td>32.36 ± 5.23 abc</td>
<td>328.6</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td></td>
<td>Abnormal forms: (%)</td>
<td>9.29 ± 0.21 bcd</td>
<td>53.44 ± 4.4 ad</td>
<td>57.46 ± 6.18 ad</td>
<td>70.96 ± 4.59 abc</td>
<td>363.5</td>
<td>&lt;0.001**</td>
</tr>
</tbody>
</table>
After 4 weeks of recovery, there was still a significant increase in abnormal forms and a significant decrease in all other seminal parameters in all groups as compared to the control group. The least significant difference (LSD) among the treated groups revealed that there was a significant difference between all the treated groups and the control group. There was a non-significant difference between group IIB (dianabol) and group IIIB (nandrolone). There was a significant difference between group IIB and group IVB (stack) for all the parameters except for the sperm viability that was non-significant. Also there was a significant difference between group IIIB and group IVB for all the parameters except the abnormal forms that was non-significant, Table (2).

Table (2): Statistical comparison between mean values of testosterone (ng/ml), FSH (mIU/mL), LH (mIU/mL) and semen analysis in group (I A) , group (IIB), group (IIIB) and group IVB (stack) after 4 weeks of recovery using ANOVA (analysis of variance) test & least significant difference test (LSD):

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group (n=6)</th>
<th>Group I(A) (–ve control)</th>
<th>Group IIB (dianabol)</th>
<th>Group IIIB (nandrolone)</th>
<th>Group IVB (stack)</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD</td>
<td></td>
<td>1.43 ± 0.03 bcd</td>
<td>1.01 ± 0.03 ad</td>
<td>1 ± 0.03 ad</td>
<td>0.86 ± 0.05 abc</td>
<td>494.6</td>
<td>&lt;0.001 **</td>
</tr>
<tr>
<td>Testosterone: (ng/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSH: (mIU/ml)</td>
<td></td>
<td>0.11 ± 0.02 bcd</td>
<td>0.09 ± 0.01 ad</td>
<td>0.08 ± 0.01 a</td>
<td>0.07 ± 0.01 ab</td>
<td>27</td>
<td>&lt;0.001 **</td>
</tr>
<tr>
<td>LH: (mIU/ml)</td>
<td></td>
<td>0.07 ± 0.01</td>
<td>0.06 ± 0.005</td>
<td>0.07 ± 0.006</td>
<td>0.07 ± 0.008</td>
<td>1.65</td>
<td>&gt; 0.05 NS</td>
</tr>
<tr>
<td>Count: (10⁶/mm)</td>
<td></td>
<td>121.5 ± 1.18 bcd</td>
<td>110.3 ± 2.87 ad</td>
<td>106.8 ± 6.58 ad</td>
<td>97.3 ± 4.67 abc</td>
<td>53.49</td>
<td>&lt;0.001 **</td>
</tr>
<tr>
<td>Motility: (%)</td>
<td></td>
<td>85.64 ± 0.88 bcd</td>
<td>75.7 ± 4.62 ad</td>
<td>77.8 ± 3.65 e ad</td>
<td>69.5 ± 7.62 abc</td>
<td>18.99</td>
<td>&lt;0.001 **</td>
</tr>
<tr>
<td>Viability: (%)</td>
<td></td>
<td>82.96 ± 1.97 bcd</td>
<td>69.5 ± 8.32 a</td>
<td>73.29 ± 5.05 ad</td>
<td>66.15 ± 3.95 ac</td>
<td>18.51</td>
<td>&lt;0.001 **</td>
</tr>
<tr>
<td>Abnormal forms: (%)</td>
<td></td>
<td>9.41 ± 0.29 bcd</td>
<td>28.9 ± 0.99 acd</td>
<td>35 ± 3.46 ab</td>
<td>33.1 ± 3.93 ab</td>
<td>193.44</td>
<td>&lt;0.001 **</td>
</tr>
</tbody>
</table>

SD: Standard Deviation. **: highly significant (<0.001). NS: Non significant (> 0.05). n: Number of rats in each group a = significant with group I(A) b= significant with group II c= significant with group III d = significant with group IV.

By comparing different seminal parameters of mean values of treated groups after 8 weeks of steroid administration with those after 4 weeks of recovery, there was a significant decrease in abnormal forms and a significant increase in the rest of seminal parameters in the recovery period, Tables (3, 4 & 5).
Table (3): Statistical comparison between mean values of testosterone (ng/ml), FSH (mIU/mL), LH (mIU/mL) and semen analysis at 8 weeks of dianabol administration and after 4 weeks of recovery in group II (dianabol) using Paired t-test:

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group (n=6)</th>
<th>Group II (dianabol)</th>
<th>Paired t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IIA 8 weeks</td>
<td>IIB 4 weeks recovery</td>
<td></td>
</tr>
<tr>
<td>Testosterone: (ng/ml)</td>
<td>0.80 ± 0.1</td>
<td>1.01 ± 0.03</td>
<td>5.45</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>FSH: (mIU/ml)</td>
<td>0.06 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>5.04</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>LH: (mIU/ml)</td>
<td>0.057 ± 0.005</td>
<td>0.064 ± 0.005</td>
<td>2.69</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>Semen analysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Count: (10^6/mm³)</td>
<td>100.8 ± 4.69</td>
<td>110.3 ± 2.87</td>
<td>6.86</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Motility: (%)</td>
<td>63 ± 12.29</td>
<td>75.7 ± 4.62</td>
<td>3.52</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>Viability: (%)</td>
<td>45.7 ± 3.97</td>
<td>69.5 ± 8.32</td>
<td>6.67</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Abnormal forms: (%)</td>
<td>53.44 ± 4.4</td>
<td>28.9 ± 0.99</td>
<td>17.31</td>
<td>&lt;0.001**</td>
</tr>
</tbody>
</table>

SD: Standard Deviation.  **: highly significant (<0.001).  *: significant (< 0.05).  n: Number of rats in each group.

Table (4): Statistical comparison between mean values of testosterone (ng/ml), FSH (mIU/mL), LH (mIU/mL) and semen analysis at 8 weeks of nandrolone administration and after 4 weeks of recovery in group III (nandrolone) using Paired t-test:

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group (n=6)</th>
<th>Group III (nandrolone)</th>
<th>Paired t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IIIA 8 weeks</td>
<td>IIIB 4 weeks recovery</td>
<td></td>
</tr>
<tr>
<td>Testosterone: (ng/ml)</td>
<td>0.74 ± 0.17</td>
<td>1 ± 0.03</td>
<td>5.21</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>FSH: (mIU/ml)</td>
<td>0.06 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>4.63</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>LH: (mIU/ml)</td>
<td>0.06 ± 0.007</td>
<td>0.07 ± 0.006</td>
<td>6.13</td>
<td>&lt;0.001**</td>
</tr>
</tbody>
</table>

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Table (5): Statistical comparison between mean values of testosterone (ng/ml), FSH (mIU/mL), LH (mIU/mL) and semen analysis at 8 weeks of dianabol and nandrolone administration and after 4 weeks of recovery in group IV (stack) using Paired t-test:

<table>
<thead>
<tr>
<th>Group (n=6)</th>
<th>Group IV (stack)</th>
<th>Paired t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variable</td>
<td>IVA 8 weeks</td>
<td>IVB 4 weeks recovery</td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone: (ng/ml)</td>
<td>0.59 ± 0.13</td>
<td>0.86 ± 0.05</td>
<td>8.44</td>
</tr>
<tr>
<td>FSH: (mIU/ml)</td>
<td>0.05 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>8.57</td>
</tr>
<tr>
<td>LH: (mIU/ml)</td>
<td>0.05 ± 0.008</td>
<td>0.07 ± 0.008</td>
<td>3.42</td>
</tr>
<tr>
<td>Count: (10^6/mm³)</td>
<td>68 ± 6.27</td>
<td>97.3 ± 4.67</td>
<td>10.43</td>
</tr>
<tr>
<td>Motility: (%)</td>
<td>48.5 ± 8.83</td>
<td>69.5 ± 7.62</td>
<td>8.57</td>
</tr>
<tr>
<td>Viability: (%)</td>
<td>32.36 ± 5.23</td>
<td>66.15 ± 3.95</td>
<td>14.72</td>
</tr>
<tr>
<td>Abnormal forms: (%)</td>
<td>70.96 ± 4.59</td>
<td>33.1 ± 3.93</td>
<td>21.07</td>
</tr>
</tbody>
</table>

SD: Standard Deviation. **: highly significant (<0.001). *: significant (< 0.05).

Histopathological results

Testis
Histological results of H&E-stained sections of testes of the control groups revealed parts of seminiferous tubules with regular basement membrane, separated by narrow interstitium and containing interstitial cells. Myoid cells were also observed. The stratified germinal epithelium was formed of spermatogonia, primary spermatocytes and spermatids, the lumen was filled with sperms. Sertoli cells were observed between the spermatogenic cells ([Figure 2a]). H&E-stained sections of the treated groups showed seminiferous tubules containing widely separated germinal cells with darkly stained nuclei and vacuolated cytoplasm. The lumen of the tubules had scanty amount of sperms and sloughed germ cells. The interstitium was filled with acidophilic hyaline material, congested blood vessels with thick wall and interstitial cells with pyknotic nuclei. ([Figure 2b: dianabol treated group (IIA), d: nandrolone treated group (IIIA) and f,g: stack treated group (IVA).] H&E-stained sections of testes of the recovery subgroups showed the seminiferous tubules with different germ cell stages with some darkly stained nuclei and some separation between them. Formed sperms were seen in their lumina. Interstitial cells were seen in the interstitium. ([Figure 2c: dianabol recovery group (IIB), e: nandrolone recovery group (IIIB) and h: stack recovery group (IVB).]

Immunohistochemical stained sections for cytochrome p450scc showed positive cytoplasmic immunoreactions of interstitial cells of Leydig in control group ([figure 3a]). Few positive cytoplasmic immunoreactions were seen in the treated groups ([figure 3b: group (IIA), d: group (IIIA) and f: group (IVA)]. Increased positive cytoplasmic immunoreactions in the recovery groups compared with the treated group ([figure 3c: group (IIB), e: group (IIIB) and g: group (IVB).

Epididymis

Histological results of H&E-stained sections of the epididymis of the control group showed that the tubules were lined by pseudo-stratified columnar epithelium with brush border and surrounded by a thin layer of smooth muscle. The lumen was filled with abundant amount of sperms ([figure 4a]). H&E-stained sections of the treated groups showed widely separated tubules with reduced epithelial height and cellular vacuolations. The lumen had scanty amount of sperms and the interstitium showed acidophilic hyaline material, extravasated blood and cellular infiltration. ([Figure 4b&c: dianabol treated group (IIA), e: nandrolone treated group (IIIA) and g&h: stack treated group (IVA).] H&E-stained sections of the epididymis of the recovery groups revealed normal pseudo-stratified columnar epithelium with preserved height and brush border, minimal cytoplasmic vacuolations and some darkly stained nuclei. Moderate amount of sperms were seen in the lumen ([figure 4d: group (IIB), f: group (IIIB) and i: group (IVB).]
Figure 1. A photomicrograph of sections of sperms from the semen of an adult albino rat of different groups. **a**: control group. Normal shape of head and tail. **b,c**: group (IIA). (b) Bifid tail (circle). (c) detached heads (→), bent neck (blue circle) and banana shaped head (black circle). **d,e**: group (IIIA). (d) Amorphous head (black circle). (e) sperm without head (black circle) and a sperm with bent tail (black arrow). **f,g,h,i**: group (IVA). (f) abnormal hookless head, bent tail (black arrow), (g) thick middle piece (black arrow), (h) double headed sperm (circle) and detached head (black arrow), (i) detached head (black arrow). **Scale bar: 10 µm.**
Figure 2. A photomicrograph of sections in the testes of adult male rats of different groups. a: control group. Seminiferous tubules with regular basement membrane (black arrow), narrow interstitium (I),
interstitial cells (circle) and myoid (arrow head). Spermatogonia (g), primary spermatocytes (p) and spermatids (Sd). Sertoli cells (blue arrow) are observed between the spermatogenic cells and the lumen is filled with abundant amount of sperms (S). b: group (IIA). c: group (IIB). d: group (IIIA). e: group (IIIB). f,g: group (IVA). h: group (IVB). Widely separated germinal cells (black arrow), darkly stained nuclei (arrow head), vacuolated cells (V), acidophilic hyaline material (*), interstitial cells with pyknotic nuclei (white circle) and scanty sperms (S). Sloughed germ cells (black circle) in the lumen of the tubule. Degenerated scanty disarranged germ cells (G) and congested blood vessels (black circle) with thick wall (green arrow). Scale bar: 25 µm.

Figure 3. Histological results of Immunohistochemically stained sections for cytochrome p450scc showed positive cytoplasmic immunoreactions of some interstitial cells of Leydig a: in control group. Few positive cytoplasmic immunoreactions in b: group (IIA), d: group (IIIA), f:

**Figure 4.** A photomicrograph of sections in the epididymis of adult male rats of different groups. 

**a:** control group. Pseudo-stratified columnar epithelium with brush border (black arrow), smooth muscle (M). The lumen is filled with abundant amount of sperms (s). 

**b,c:** group (IIA). 

**d:** group (IIB). 

**e:** group (IIIA). 

**f:** group (IIIB). 

**g,h:** group (IVA). 

**i:** group (IVB). Widely separated tubules (thick black arrows), cellular vacuolations (V). Acidophilic hyaline material (*), scanty amount of sperms (S). Decreased epithelial height (blue arrow). Brush border (black arrow). Darkly stained nuclei (arrow head), extravasated blood and infiltrations (circle). Scale bar: 25 µm.

**Morphometric results**
Our statistically analyzed results for number of cells positive for cytochrome p450scc immune reactions revealed significant decrease in all treated groups by the end of 8th week and after 4 weeks recovery when compared with control group. By comparing different parameters of mean values of the treated groups after 8 weeks of steroid administration with those after 4 weeks of recovery there was a significant increase in anti-cytochrome p450scc immune-stained cells in the recovery period (Table 6).

Table (6): Statistical comparison between mean values of number of anti-cytochrome p450scc immune-stained cells in different groups using ANOVA (analysis of variance)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Group IA</th>
<th>Group IIA</th>
<th>Group IIB</th>
<th>Group IIIA</th>
<th>Group IIIB</th>
<th>Group IVA</th>
<th>Group IVB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD</td>
<td>11.36±1.844</td>
<td>5.38±.760&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.41±1.299&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>4.77±1.059&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.08±1.722&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>1.98±.980&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.71±1.117&lt;sup&gt;ad&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>= significant as compared to group IA  
<sup>b</sup>= significant as compared to group IIA  
<sup>c</sup>= significant as compared to group IIIA  
<sup>d</sup>= significant as compared to group IVA

Discussion

Androgens exert their biological effects through androgen receptor (AR) present in the Leydig cells, Sertoli cells, peritubular myoid cells, epithelial cells of the epididymis and the prostate (27). It is also present in non-reproductive tissues including bone, skeletal muscle, brain, liver, kidneys and adipocytes (28).

Anabolic Androgenic steroids (AAS) are effective in increasing physical performance but they have adverse side effects that can threaten health (29). The adverse effects of anabolic androgenic drugs misuse included cardiovascular disorders (30), liver dysfunction (31), kidney disease (32), testicular problems (3), psychiatric and behavioral disorders in both sexes (33).

The current study was carried out for 12 weeks (8 weeks of steroids administration and 4 weeks for recovery). The eight weeks period chosen in this work coincides with the spermatogenic period in rats which is approximately 48-56 days (34).
The results of the present study showed a highly significant decrease in the mean values of serum testosterone, LH and FSH by the end of the 8th week of AAS administration as compared to those of the control group. With the least levels seen in the stack treated group and no significant difference was found between dianabol and nandrolone treated groups. These results coincides with those of Daniel and Marilyn (35); Urhausen et al. (36). Also, Purkayastha and Mahanta (37) found that the change in LH was delayed starting to give a significant difference after 30 days of AAS treatment.

High doses of exogenous androgen, in the form of AAS, cause interaction between AAS and AR in the cells. AAS-receptor complex affects the hypothalamic-pituitary-gonadal axis by negative feedback resulting in the reduced levels of LH and FSH and in turn testosterone decrease, beside the the local suppressive effects of excess androgens on the testis. This condition is known as hypogonadotrophic hypogonadism state, which is recognized to be a reversible condition (5, 38&39). Moreover, stacking of AAS not only causes strong inhibition of the gonadal functions but also they affect the behavioral measures (35).

By the end of the 4th week withdrawal period, the mean values of serum testosterone, LH and FSH of the treated groups were significantly higher than those recorded by the end of the 8th week of AASs administration, indicating that recovery was occurring but not complete compared to those of the control groups, where these mean values still significantly lower than those of the control group, except for LH that showed non-significant difference with the control. These results coincide with those of Karbalay-Doust (5) who reported that normal hormonal function usually recovers after discontinuation, but sometimes the condition is irreversible.

On the other hand, Boyadjiev et al. (40) found that the process of complete recovery of the hypogonadal state took more than 10 months. While, others stated that FSH and LH suppression sustained for a period of one year after AAS withdrawal (41). Hijazi, et al. (42) found that the levels of testosterone, LH and FSH took 14 months to recover to normal levels. This conflict could be explained by variation among individuals in the time needed for full recovery depending on the dose and duration of AAS use (43).

The present study showed a high significant reduction in the mean values of all the seminal parameters (sperm count (10^6/mm^3), percent of sperm motility, sperm viability) except for the abnormal forms which showed significant increase in the treated groups by the end of 8th weeks as compared to the control group. These results were in line with other researchers who found that any disturbing factor affecting the axis of neuro-hormonal signals can affect spermatogenesis and thus male fertility (5,44&45). The suppression in sperm output was attributed to the degree of inhibition of the germ cells development, which is closely related to the degree of testosterone, FSH and LH suppression (46). In addition, the increased ROS produced by AAS lead to fragmentation of DNA of treated rats’ sperms (27). DNA damage and apoptosis prevent sperm maturation with the resultant azoospermia (47). The most frequent abnormalities were in body builders using more than one type of AAS (48).

By the end of 4th week of recovery period, the mean values of all the seminal parameters of the recovered groups were significantly higher than those recorded by the end of the 8th week.
of steroids administration, except for abnormal forms which were significantly lower, indicating that recovery was occurring but not complete when compared with those of the control groups. These results coincide with others who found that there is a degree of improvement in the seminal parameters after AAS discontinuance; however, these levels were not completely normalized even after 14 weeks (5,40). According to most reports, sperm quality tends to recover spontaneously within 4–12 months after discontinuation. However, the negative effect on semen quality may persist for longer periods (38).

In the current study, the histological affection of testis was in the form of seminiferous tubules containing widely separated germinal cells with darkly stained nuclei and vacuolated cytoplasm. The lumen of the tubules had scanty amount of sperms and sloughed germ cells. These findings were supported by Daniel and Marilyn (35); Naraghi et al., (49) & Rahil et al., (39). It is clear that any toxicants or oxidative stress within the testis can directly affect the germ cells or the somatic cells (Leydig and Sertoli cells) leading to disruption of spermatogenesis process (50).

ASS misuse leads to decrease in FSH level (39). This can enhance the Sertoli cells to secrete proteins that initiate the apoptosis signaling cascade in germ cells which are expressed as dark nuclei with vacuolations around them and the resultant decrease or absence of sperms in the lumen of some tubules (51). It has also been described that apoptosis in the germ cells may be due to the free radical formation and the Fas-signaling system that is activated by exogenous toxicants as AAS (32,52).

Sloughing of germ cells in the lumen of seminiferous tubules and their detachment from the basement membrane seen in the current work was in line with others, who attributed these changes to rapid disruption of the Sertoli-germ cells interaction as a result of AAS (52).

In this work, the interstitial tissue showed acidophilic hyaline material and congested thickened blood vessel walls. This was maximal in the stack-treated group. Similar changes were reported by Mark et al. (53) who mentioned that AAS can increase arterial thickening as part of their pro-atherogenic effect. The increased breakage of blood capillaries and their leakage leads to interstitial edema followed by damage of the interstitial tissue and formation of the acidophilic hyaline material. This damage in the interstitial tissue may also affect the Leydig cells and so the production of testosterone (45).

The interstitial cells in this study appeared with pyknotic nuclei. Mesbah et al. (52) clarified that Leydig cells, which is known to have LH receptors stimulating them to produce testosterone, have a high risk for the exogenous toxicants due to the close relationship between them and the blood vessels. On referral to the use of AAS, they not only lead to Leydig cell morphological alterations, but also decrease in their number leading to depletion in LH receptors. This is augmented by the decrease in peripheral LH by exogenous testosterone administration leading to reduction in testosterone (38).

The affection of Leydig cells was confirmed immunohistochemically in our study by significant decrease in the number of anti-cytochrome p450scc immune-positive cells in the treated groups and exaggerated in the stack treated group. However, the number of immune-positive cells began to increase in the recovery period. Cytochrome P450scc enzyme is presented in
Leydig cells and other steroid forming cells. The conversion of cholesterol to pregnenolone by this enzyme represents the initiation of steroidogenesis (54). This suggested that reduced testosterone level in our study was due to decreased expression of these enzymes in Leydig cells (55). However, the reduced cytochrome P450scc was also explained by decreased availability of its substrate (cholesterol) which is affected by the reduction in FSH and LH level (56).

Regarding the epididymis in the present study, some tubules were widely separated and showed decreased epithelial height and cellular vacuolations with little amount of spermatozoa in their lumina. Others showed accumulation of hyaline material and cellular infiltration in between. These findings were supported with those of Mesbah et al (44) & Fauser (57).

Testosterone plays a vital role in maintaining the epididymal structure and function, where they have found that epithelial cell proliferation decreased after testosterone diminution (58). Androgen deprivation in the epididymis induces a wave of apoptosis along the epididymis; which could explain the presence of pyknotic nuclei and vacuolations (59).

Spermatozoa that enter the epididymis are nonfunctional gametes and the epithelial cells of the epididymis are responsible for their maturation (60). Unfortunately, epididymis can be a target for different toxicants or any change in the environment that may affect epididymal sperm maturation and hence the fertility of individuals (61). Moreover, the epididymis can protect spermatozoa from oxidative stress by eliminating ROS and by secreting antioxidant enzymes into the lumen of the epididymis. So, any insult causing oxidative stress leads to deficiency in the enzyme system and affect the integrity of spermatozoa (62).

In the present work, stoppage of the drug revealed an observed improvement but not complete in the histological structure of both the testis and epididymis, where some cells still show pyknotic nuclei, but some sperms were seen in the tubular lumen and normal cells appeared in the interstitium. This was in line with Hijazi et al. (42) who attributed the recovery of the germinal epithelium to the beginning of restoration of testosterone, FSH and LH after drug withdrawal that helps to perform normal spermatogenesis.

However, Noorafshan et al. (63) found that there were still structural changes in rat testis after stopping the drug for the same period of administration which was 14 weeks. After AAS discontinuation, Leydig cells tend to proliferate but remain below the normal counts, even after longer periods (38). On the contrary, Hickson et al. (64) found complete recovery after stoppage of AAS.

In conclusion, dianabol and nandrolone have an undesirable effect on the reproductive system and can inversely affect the fertility in men. They affect the pituitary gonadal axis hormones, the semen quality, the histological structure of the testis and the epididymis and their stacking has a synergistic effect. Incomplete recovery can occur after stoppage of their administration. So, it is recommended to stop unnecessary use AAS especially in large doses for long periods.

REFERENCES


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