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The Effect of Sustanon (Testosterone Derivatives) Taken by Athletes on the Testis of Rat

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Abstract

Intentional abuse of anabolic androgenic drugs (AASs) by athletes has been increased rapidly in many countries. Therefore, investigations are needed for studying the adverse effects of these drugs. Administration of three doses of Sustanon 250mg (3, 5 and 10mg/kg.B.wt) which includes four testosterone derivatives and taken by athletes as body builder androgenic drug has caused several biochemical, and histological alterations in the testes of rats. The biochemical changes included the increase of serum testosterone and malondialdehyde (MDA) levels while the histological effect included the degeneration of the germinal epithelium and the appearance of multinucleated giant cells and several variable sized vacuoles in a stage-like changes. The multinucleated giant cells appeared having a large number of apoptotic-like nuclei with marginated chromatin. This study has also showed a significant decrease of the epididymis sperm count. The later result has been confirmed by scanning electron micrographs. All these changes were observed in a dose related pattern.

Keywords: Sustanon, testosterone, testis.

1. Introduction

Testosterone plays an important role in male sexual differentiation (Hines, 2009), puberty (Mitchell *et al*., 2009), sexual behaviour (Yates *et al.*, 1999; Balthazart and Ball, 2010) and spermatogenesis (Sofikitis, 2008).

Anabolic-androgenic steroids (AASs) are the man-made derivatives of the male sex hormone testosterone (Hoffman and Ratamess, 2006). Testosterone is the main androgen (male sex hormone), cholesterol derived hormone (Saladin, 2004; Postlethwait and Hopson, 2006; Parr *et al*., 2010) that regulates male secondary sexual characteristics. Along with follicle-stimulating hormone (FSH), testosterone stimulates sperm production (Mader, 2010).

In recent years, the intentional abuse of anabolic androgenic drugs especially the testosterone derivatives by athletes have increased rapidly in many countries to become a serious negative phenomenon (Bin Bisher, 2009). Abusers and many athletes, especially in the power sports like bodybuilding and weight lifting, administer illegally high doses of these drugs during sport competitions (Hartgens and Kuipers, 2004). Unfortunately, according to recent report from Iraq, users of anabolic androgenic drugs gradually increased during the past decade (unpublished data).

The anabolic androgenic drugs are an important therapeutic target for the treatment of diseases such as hypogonadism (Mudali and Dobs, 2004; Seal, 2009), treat senile osteoporosis (Gooren, 2007), in conjunction with other hormones to promote skeletal growth in prepubertal boys with pituitary dwarfism (delay puberty) (Bagatell and Bremner, 1996; Yavari, 2009), and to treat some types of anemia such as Fanconi's anemia (Velazquez and Alter, 2004; Maravelias *et al*., 2005).Sustanon is a useful medical drug which possesses multiple clinical therapeutic benefits (Socas *et al.*, 2005). It consists of four different testosterone esters (testosterone propionate, testosterone phenylprpropionate, testosterone isocaproate and testosterone decanoate), which provides a continuous release of testosterone into the blood and producing a stable testosterone level for a long period of time extending from 3-4 weeks (Harvey *et al*., 2006).

The adverse effects caused by abusing anabolic androgenic drugs included cardiovascular disorders (Sader *et al*., 2001), liver dysfunction (Shahidi, 2001; Amsterdam *et al*., 2010), kidney disease (Mulronery *et al*., 1999), testicular problems (Feinberg *et al*., 1997; Socas *et al*., 2005), psychiatric and behavioural disorders in both sexes (Maravelias *et al.*, 2005) as well as other problems on human body (Mader, 2010). High testosterone rate induces oxidative stress by alteration of the balance between ROS production and antioxidant defences (Alonso-Alvarez *et al.*, 2007). The aim of the present work was to study the effect of sustanon 250 mg on the testis and sperm count.

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2. Materials and Methods

2.1. Experimental Animals

The present study was conducted using 28 mature male albino rats (*Rattus norvegicus*). All rats were healthy, weighing 200 - 270 gm. and 8-10 weeks old at the time when the experiment started. The animals were bred and housed in plastic cages (56 x 39 x 19 cm) bedded with wooden chips in groups of seven rats per cage in a room with controlled temperature of 24 ± 3 ^{o}C, in animal house of Biology Dept. /College of Science /Salahaddin University-Erbil-Iraq. The animals were kept in 12/12 hours light/dark schedule during the experimental study. The rats were fed with standard laboratory chow containing 0.5% NaCl, 22% protein and 4-6% dietary fat (Krinke, 2000) and allowed to drink water *ad libitum*.

2.2. Sustanon 250

Sustanon ampoules (manufactured by N.V. Organon Oss Inc. Holland) have been obtained from the local pharmacy in Erbil-Iraq. Each ampoule contains 1 mL of oily solution of Sustanon. According to the manufacturer, this 1 mL of Sustanon consists of four testosterone ester compounds which include testosterone propionate, testosterone phenylprpropionate, testosterone isocaproate and testosterone decanoate. During the present study, three doses of Sustanon have been selected which were 3, 5 and 10 mg/Kg of the animal body weight (b.wt.).

2.3. Experimental Design

The rats were divided randomly into four groups (first group served as control and the other groups as the treated groups). Each group consisted of seven rats per cage. Group 1: Control: was injected once a week with 0.1ml of corn oil intramuscularly (i.m.). Group 2: Sustanon (3 mg/kg b.wt. in corn oil: injected i.m., once a week with 0.1ml of 3 mg sustanon/kg b.wt. Group 3: Sustanon (5 mg/kg b.wt. in corn oil): were injected i.m., once a week with 0.1ml of 5 mg sustanon/kg b.wt. Group 4: Sustanon (10 mg/kg b.wt. in corn oil): was injected i.m., once a week with 0.1ml of 10 mg sustanon/kg b.w. The duration of the experiment was four weeks.

2.4. Biochemical Analysis

2.4.1. Determination of MDA

The level of serum MDA was determined spectrophotometrically by thiobarbituric acid (TBA) solution. In brief: 150µl of blood serum was added to the followings: 1ml trichloroacetic acid (TCA) 17.5%, 1ml of 0.66 % TBA, then mixed well by vortex, incubated in boiling water for 15 minutes, and then allowed to cool. One ml of 70% TCA was added and left to stand at room temperature for 20 minutes, centrifuged at 2000 rpm for 15 minutes, and the supernatant was taken out for scanning spectrophotometrically at 532nm (Guidet and shah, 1989).

2.4.2. Determination of Serum Testosterone

Serum testosterone was determined by using Mini VIDAS (Biomerieux, Italy). The assay principle combines an enzyme immunoassay competition method with a final fluorescent detection (Enzyme Linked Fluorescent Assay). The conjugate enzyme catalyzes the hydrolysis of this substrate into a fluorescent product (4-Methyl-umbelliferone), fluorescence was then measured at 450 nm. The intensity of the fluorescence is inversely proportional to the concentration of testosterone present in the sample. At the end of the assay, results were automatically calculated by the instrument in relation to the calibration curve stored in memory and then printed out.

2.5. Sperm Count

Sperms from the right cauda epididymis were released by cutting into 10 ml of normal saline in small petri dish and then minced by using manual glass homogenizer, using a light microscope (Axiostar plus microscope), and the number of sperms measured by using a hemocytometer. Sperm count was expressed as a number of sperm per milliliter (Elbetieha *et al*., 2008).

2.6. Histological Preparation

2.6.1. Paraffin Method

Testes were removed from the anesthetized animals, they immediately fixed in Bouin's fluid for 24 hours, followed by a dehydration using a series of graded ethanol in ascending concentrations (50%, 70%, 95%, and 100%), immersed in xylene for clearing, infiltrated in paraffin wax, and finally embedded in paraffin wax. Four micrometer thick paraffin sections were obtained by using rotary microtome (Bright, MIC) and stained by hematoxylin and eosin (H&E) (Bancroft *et al*., 1977). The specimens were examined and photographed under light microscope (digital binocular compound microscope 40x-2000x, built-in 3MP USB camera).

2.6.2. Plastic Method

Samples of testes ($\leq$1mm^3) were fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer pH 7.2 - 7.4 for 24 hours, washed by cacodylate buffer 0.1M, postfixed in 1% Osmium tetroxide for one hour, dehydrated through a graded series of ethanol (50%, 70%, 95%, and 100%), and then cleared in acetone for 15 minutes (twice), infiltrated with acetone plus resin mixture (1:1) for 1hours, then with acetone plus resin mixture (1:3) for 12 hours, and finally embedded in resin.

2.7. Scanning Electron Microscopy

Testes were fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer pH 7.2-7.4 for 24 hours. After washing by cacodylate buffer 0.1M, they postfixed in 1% Osmium tetroxide for two hours, and dehydrated in ethanol (50%, 70%, 85%, 100% and 100%), the samples were put in desiccator for air drying, after mounting they coated with gold by coating system (E5200 AUTO SPUTTER COATER) and then examined by SEM in Tehran University-Tehran-Iran (CamScan MV2300) and in Malaysia (ZEISS, super A, 55VP).

2.8. Statistical Analysis

All data were expressed as means ± standard error of mean (M ± SE) and statistical analyses were carried out using statistically available software of statistical package for social science (SPSS) version 11.5. One-way analysis of variance (ANOVA) was performed to test for significance followed by Duncan's multiple range comparison tests for comparisons between the groups. *P*

values ≤ 0.05 and 0.01 were considered statistically significant.

3. Results

The level of MDA was increased (p<0.05) dose dependently in the sustanon injected groups. In the lower dose of sustanon group, MDA (1.560 ± 0.1997 µmol/L) increased non-significantly while in the last two doses, it increased significantly (1.964 ± 0.4404 and 3.172 ± 0.3180 µmol/L respectively) when compared with control group (0.6367 ± 0.1289 µmol/L) (Fig 1).

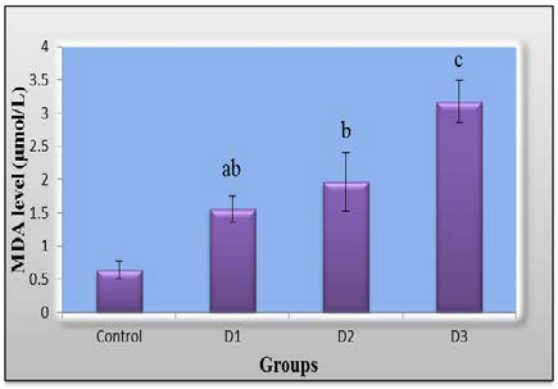

Figure 1. Effect of sustanon doses on MDA concentration.

The current study has shown that both last doses of sustanon caused significant increase (p<0.05) in the level of serum testosterone (3.434 ± 0.3401 and 4.540 ± 0.3835 ng/ml respectively) when compared with control group (1.640 ± 0.2261 ng/ml), while in the first dose, the level of serum testosterone (1.940 ± 0.2338 ng/ml) increased (but non-significantly) when compared with control group (Fig 2).

As shown in (Fig 3), Sustanon injected doses have caused significant decrease (p<0.05) of sperm count (2.142 ± 0.2743, 1.128 ± 0.2191 and 1.100 ± 0.2895 million/ml respectively) in comparison to control group (3.584 ± 0.1578 million/ml). Beside this, in both last two doses of sustanon injected rats, the numbers of multinucleated giant cells (1.813 ± 0.2277 and 3.375 ± 0.2869/seminiferous tubule respectively) were increased significantly (p<0.01) compared to control group (in which no such cells were detected) , while in the first dose, they (0.5000 ± 0.1291 / seminiferous tubule) increased non-significantly.

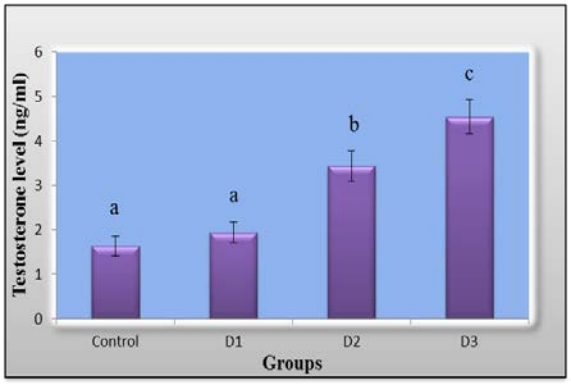

Figure 2. Effect of sustanon doses on serum testosterone level.

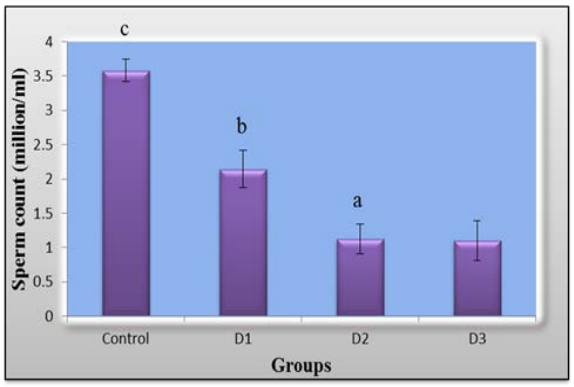

Figure 3. Effect of sustanon doses on sperm count

The histological structure of the testes of the control group showed normal germinal epithelium with well-formed spermatids (Fig 4A-D). In the lower sustanon dose treated group (3mg/kg wt), the most important histological changes were the absence of spermatids in the seminiferous tubules and the appearance of multinucleated giant cells and few vacuoles (Fig 4E and F).

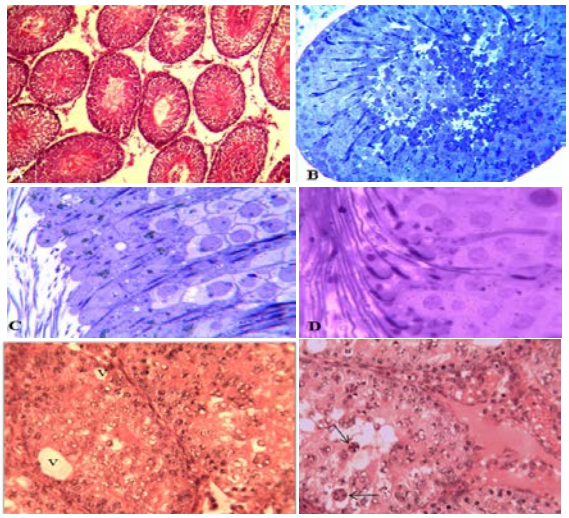

Figure 4. Sections through rat testes: A) control 100X,H&E, B) control 400X, Toluidine blue, C) and D) high magnification of the seminiferous tubule control 1000X, Toluidine blue, E) 3mg sustanon/kg b.wt showing few vacuoles (V), notice the large number of gonocytes and the absence of spermatids, 400X, H&E, F) same previous dose showing the appearance of multinucleated giant cells (arrows), 400X, H&E.

The number and size of vacuoles were increased in the second dose of sustanon (5mg/kg b.wt), while the number of gonocytes in the seminiferous tubules was decreased (Fig 5A & B). Higher numbers of multinucleated giant cells were seen in the testes of the last group (10mg/kg b.wt) (Fig 5C). The germinal epithelium in the seminiferous tubules appeared very thin (Fig 5D). The multinucleated giant cells appeared having a large number of apoptotic-like nuclei with marginated chromatin (Fig 5E & F). There was also a stage-like alteration in the higher dose of sustanon with respect to numbers and sizes of vacuoles and multinucleated giant cells and the thickness of the germinal layer.

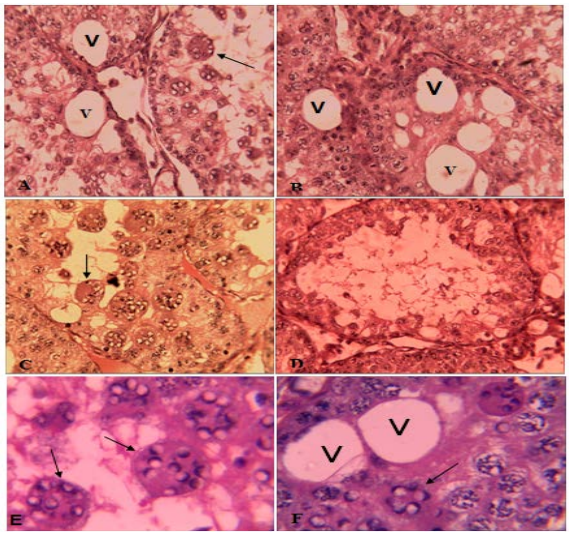

Figure 5. Sections through rat testes treated with sustanon: A) 5mg sustanon/kg b.wt treated rat testis showing increasing number of vacuoles (V), few multinucleated giant cells (arrows) are also seen, 400X, H&E, B) same dose with more vacuoles, 400X, (C-F): Section through the testes of 10mg/kg b.wt treated rats, H&E, C) high number of multinucleated cells, 400X, D) Little vacuoles and a thin layer germinal layer are seen, 400X, E) Number of multinucleated cells, notice they that contain apoptotic nuclei, 1000X, F) Number of multinucleated cells and few large sized vacuoles, 1000X.

The scanning electron micrographs of the testes of control rats showed seminiferous tubules with a large number of spermatids in the lumen (Fig 6A &B), while the seminiferous tubules of sustanon treated rats showed absolutely no spermatids, instead they contained large number of germinal epithelial cells, some tubules appeared having a thin germinal epithelium with empty lumen and a number of vacuoles (Fig 6C&D).

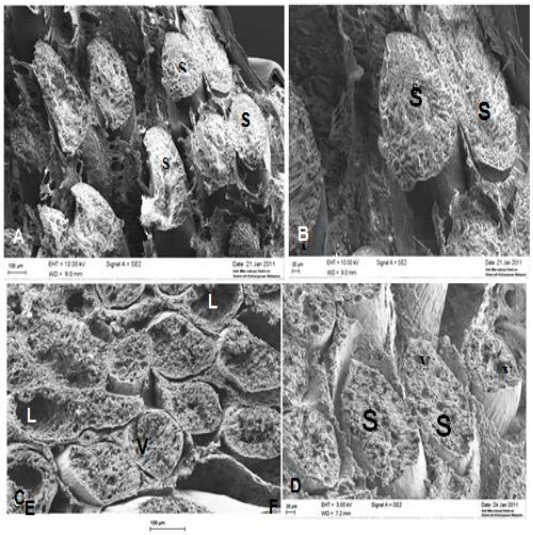

Figure 6. Scanning Electron micrograph sections through rat testes: A) & B) Control rat testis showing seminiferous tubules (S) containing large number of spermatids in the lumen, bar=100µm & 20µm respectively, C) & D) 10mg of sustanon/kg b.wt treated rat testis showing no spermatids and the presence of vacuoles, notice some seminiferous tubules appear empty (L), bar=100µm & 20µm respectively.

4. Discussion

Three different doses of sustanon, which have been chosen regarding the doses used by athletes, were administered to the rats in the current investigation. This study revealed that the higher doses of sustanon which used in this research caused a significant increase in the concentration of serum testosterone, while in the lower dose, the level of serum testosterone increased, but non-significantly when compared with control group. Increase or decrease of the concentration of serum testosterone in animals who received anabolic steroids depends on the period of AASs exposure, dosage use and type of AASs. This result is correlated with the findings of other investigators (Sanchis *et al.*, 1998; Muraoka, 2001; Shiono, 2001) who confirmed that taking testosterone and its derivatives caused elevation of the serum level of testosterone in rats. Not only testosterone derivatives could increase the level of serum testosterone, but giving other anabolic androgenic steroids which are used to increase blood testosterone levels for the purposes of increasing strength, lean body mass and sexual performance such as androstenedione and dehydroepiandrosterone were found to elevate testosterone levels (Bahrke and Yesalis, 2004). In contrast, Tahtamouni *et al*., (2010) showed that measuring total testosterone level in the serum of the control and treated groups indicated that injection of nandrolone decanoate caused a significant decrease in testosterone level in the treated animals compared with control group. Exogenous administered testosterone and its metabolite estrogen will suppress both GnRH productions by the hypothalamus and LH production by the pituitary gland and subsequently suppress testicular testosterone production (Dohle et al., 2003; Thabet et al., 2010). Also, high level of testosterone is needed inside the testis and this can never be accomplished by oral or parenteral administration of androgens. Suppression of testosterone production by Leydig cells results in a deficient spermatogenesis, as can be seen in men taking AASs (Dohle *et al*., 2003).

In the present study, the dose-dependent decrease in the cauda epididymal sperm count was observed in rats exposed to sustanon and indicated decreased spermatogenesis. As shown in the results, the sperm count decreased significantly in sustanon injected rats when compared with control group and decreasing the number of sperm was dose related. Sperm count decrease may occur due to increase free radical formation and undergoing apoptosis of somatic cells (Leydig and Sertoli cells) and the germ cells as a result of injection of AASs. Administration of testosterone propionate led to a significant elevation of oxidative stress (Aydilek *et al*., 2004). Subsequently, the targeted molecule becomes a free radical itself and initiates a cascade of events that can ultimately lead to cellular damage. The pathological roles of free radicals include lipid peroxidation, DNA damage and apoptosis (Kothari *et al*., 2010). Free radicals have the ability to directly damage sperm DNA by attacking the purine, pyrimidine bases and deoxyribose backbone as well as they can damage the sperm membrane (Tremellen, 2008). This supports the present results in which the level of serum MDA was increased dose dependently after sustanon administration, accompanied by dose dependent

biochemical and histological alterations. Testosterone administration has been found to cause oxidative stress through increasing the level of serum MDA level, thereby enhancing lipid peroxidation dose dependently (Sadowska-Krępa *et al*., 2011).

Decrease in the sperm count may be due to decreased level of intratesticular testosterone, because testosterone level is directly linked to spermatogenesis (Zirkin *et al*., 1989). Elkington, (1970) reported that testosterone converted in peripheral tissues to estradiol, subsequently, estradiol strongly suppresses the spermatogenesis.

It is also possible that the Sertoli cells might have been affected due to steroid injection and the other possibility might be due to its effect on the epididymal function (Bairy *et al*., 2010). The reduction in Sertoli cell number in treated rats with anabolic steroids could have resulted in a subsequent reduction in the number of spermatogonia leading eventually to a decrease in sperm count and testicular atrophy (Tahtamouni *et al*., 2010). Within the testes, the main target cells for toxicants that disrupt spermatogenesis are the somatic cells (Leydig and Sertoli cells) and the germ cells. In animal models, each of these cell types can be selectively targeted by specific toxicants, resulting in apoptosis (Reddy *et al*., 2009). Anabolic steroids such as nandrolone decanoate may cause loss of AR activity from Sertoli cells would lead to spermatogenic failure resulting in incomplete meiosis and collapse transition of spermatocytes to haploid round spermatids (Holdcraft and Braun, 2004).

O'donnell *et al*., (2001) revealed that exogenous testosterone administration caused significant decrease in sperm count and intratesticular testosterone concentration. Studies have reported that AASs use is strongly related to decreased sperm count, decreased sperm motility, abnormal sperm morphology (Ciocca, 2005; Brown, 2005; Doust *et al*., 2007).

The current work showed that multinucleated giant cells formed as a result of sustanon injection and they increased in number in dose dependent way, but the testes of control rats did not develop them. In the second and third doses of the present study, the multinucleated giant cells were increased significantly, while in the lower dose they increased non-significantly when compared with control group. Thabet *et al*., (2010) have also demonstrated that anabolic steroids caused formation of giant cells in the lumen of seminiferous tubules of rabbit. The multinucleated giant cells appeared to be one of the ultimate of testicular atrophy (Khattab, 2007). The findings suggested that the giant cells are formed as a result of the fusion of spermatids due to alterations in the intercellular bridges (Singh and Abe 1987) or failure of cytokinesis (Abdu, 2008).The most characteristics of these cells were their large size and the numerous nuclei they contain. The most important feature of the nuclei is the marginated chromatin material, a characteristic apoptosis feature (Goldsworthy *et al*.,1996).

Apoptosis which occurs in testis for regulation of germ cell population (Woolveridge *et al*.,1998; Giampietri *et al*.,2005) may be related to lowering of the level of testicular testosterone (Nandi *et al*.,1999; Richburg *et al*., 2000).The suppressing of testicular testosterone production, as mentioned previously, may be due to administration of exogenous testosterone (Thabet *et al*., 2010) and this may lead to the formation of more apoptotic germ cells. This may explain the dose dependent appearance of the multinucleated giant cells.

Depending on histological and ultrastructural study, the present work reported that intramuscular injection of male rats with different doses of sustanon for four weeks is deleterious to the structure of rat testes. After analyzing different sections of testes of control and treated animals by light microscope, clear differences were noted. The testes of sustanon injected groups showed histological and cytological changes including severe damage of the seminiferous tubules, degeneration of germinal epithelia, appearance of apoptotic nuclei, presence of multinucleated giant cells having an apoptotic nuclei, lack of spermatids in the seminiferous tubules and appearance of vacuoles which increase in size and number with elevated doses of sustanon. The scanning EM has confirmed these results. Other recent works have supported the present results (Naraghi *et al*., 2010; Thabet *et al*., 2010).

The present investigation showed a step-like stages of seminiferous tubules cells degeneration starting with the appearance of few apoptotic nuclei and vacuoles with small size, then followed by the appearance of multinucleated giant cells containing these apoptotic nuclei accompanied by larger size and number vacuoles and depletion of the germinal epithelium. Approximately similar stages were suggested by Anton (2003) after ligation of the efferent duct of the rat testis and he suggested a time dependence for this stepwise changes. Since the present work has a fixed duration for all groups, we propose a dose relation rather than time relation for these stages.

5. Conclusion

The present work illustrates that sustanon which contains testosterone derivatives taken by athletes has affected rat fertility through arresting spermatogenesis and degenerating the germinal epithelium in dose dependent stage-like pattern.

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