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Androgenic Anabolic Steroidal-Based Effects on the Morphology of Testicular Structures of Albino Rats

M. Mazhar Hijazi,[1] M. Ahmed Azmi,[2*] Asghar Hussain,[3] S.N.H. Naqvi,[4] Rehana Perveen[4] and Safia Hijazi[1]

[1]*Department of Anatomy, Umm Al Qura University, Makkah, Saudi Arabia*
[2]*Department of Physiology, Al-Tibri Medical College, Isra University Campus, Karachi-75040*
[3]*Department of Physiology, Quetta Institute of Medical Sciences, Quetta, Baluchistan*
[4]*Department of Pharmacology, Institute of Pharmaceutical Sciences, Baqai Medical University, Karachi*

Abstract.- This study was aimed to identify the changes in the fine structure of the testis following chronic administration of *testoviron.* Thirty male adult albino rats of *Wistar* strain were taken in control or untreated group and thirty rats in the treated group. The animals of this group were medicated with intramuscular injection of testoviron at a dose of 1.143mg / rat in the gluteal region. The body weight and relative testicular weight of each animal was noted. One week after the last injection, the rats were sacrificed and their testes were removed for *microtomy*. Each testis was also observed for its size, shape, color, and consistency before microscopic examination. Three micron thick H & E stained sections were prepared to observe the quantitative and qualitative changes in the different parameters of testicular tissues. The histological sections of the testis revealed that there is a time related effects over spermatogenesis, resulting in the decrease in the relative weight of the testis and the number of seminiferous tubules along with the decrease in the number and the diameter of the interstitial cell nuclei in the treated animals. These finding were further confirmed by using Scanning electron microscopy. These results suggests that androgenic anabolic steroids produce toxic effects on the male reproductive tract, particularly the testicular tissues including the degenerative damage in the germ cells and Leydig cells that can affect spermatogenesis. We concluded that synthetic androgenic anabolic steroids affect fertility parameters and can cause testicular atrophy.

Keywords: Androgenic anabolic steroid, testicular structures, testoviron, albino rats, fertility parameters.

INTRODUCTION

Anabolic steroids or anabolic androgenic steroids (AAS) are a class of steroid hormones closely related to the hormone testosterone capable of initiating increase in muscle mass and physical strength. However, these drugs can cause serious side effects such as changes in cholesterol level (increased low density lipoprotein and decreased high-density lipoprotein), acne, hypertension, structural damage of liver and left ventricle of heart, if used for long term basis or in excessive doses. Some of these effects can be mitigated by exercise or by taking supplement drugs (Dony *et al*., 1985; Medras and Tworowska, 2001; Katznelson *et al*., 2006).

Many research publications appear each year in scientific literature related to the history of development, characteristics and adverse effects of androgenic-anabolic steroids. Some researchers in this regard reported the effects of AAS (*e.g.*, oxandrolone) on the reproductive development of male rats as well as on the reproductive functions of young and mature stallions (Squires *et al*., 1982; Groket *et al*., 1992; Nagata *et al*., 1999). Farrell *et al*. (2003) reported the effects of pubertal AAS on the reproductive and aggressive behaviour in male rats. Similar studies related to the effects of AAS in athletic persons and its early potent effects on muscle mass, strength and functional performance in older men have also been reported (Hartgens and Kuipers, 2004; Schroeder *et al*., 2005). Moretti *et al*. (2007) reported the structural effects on sperms and aneuloidies studies in case of spermatogenesis recovery after the use of androgenic anabolic steroids.

Correlation of different parameters of testicular tissues such as in case of relative testicular weights, counts, diameters and thickness of seminiferous tubules as well as interstitial cells nuclear counts and their diameter have also been reported (Daalgard *et al*., 2002; Dohle *et al*., 2003;

* Corresponding author: azmiahmed@hotmail.com
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Noorafshan *et al*., 2005; Mesbah *et al*., 2007; Shittu *et al*., 2009).

In the present study, we examined the quantitative changes in the seminiferous tubules in relation to the number, diameter and thickness as well as the interstitial cells nuclear count and their nuclear diameter before and after the treatment with the drug (testoviron). In addition we have also studied the harmful effects of this drug on the male reproductive system particularly on different testicular tissues of albino rats, using light and scanning electron microscopy.

MATERIALS AND METHODS

Experimental animals

Male adult Wistar albino rats (200 – 250 g) from Charles River Breeding Laboratories, Brooklyn Massachusetts, USA, were used. They were kept in cages (1-2 rats per cage) in the experimental room of the animal house of Baqai Medical University, Karachi and were maintained on well balanced laboratory diet. They were also allowed to give water freely one week prior to the start of the experimental study. The animals were then weighed and put under observation for assessment of the state of their health on the basis of weight gain or loss during the experimental procedure. Studies were carried out in two groups of animals (30 animals per group). Animals of group A were kept as control (un-treated) and group B animals were treated with injection of testoviron intra-muscularly for fourteen weeks (treated group). All experimental procedures used here were approved by the Ethics committee of Baqai Medical University, Karachi.

Administration of drugs

Testoviron (Schering AG. Pharmaceutical Co., Federal Republic of Germany) was administered intramuscularly in the gluteal region for fourteen weeks at a dose of 400 mg/70 kg according to the method of Gillman (2006). Each 1 ml of testoviron depot contains 25 mg testosterone propionate and 110 mg testosterone enanthate (which together is equal to about 100 mg testosterone) in oily solution. The drug was administered intramuscularly at a dose of 0.2 ml / rat in the animals of group 'B' (treated group). Animals of group 'A' were not treated and kept as control (un-treated). In each group, the body weight before and after the scarifice of animals as well as the relative weight of the testis of each animal was also calculated.

Procedure adopted

Anesthetized rats were placed on a dissection board and all blood was withdrawn from the heart by intracardiac puncture (Allen *et al*., 2001) and then sacrificed (Inuawa and Williams, 1996). A midline incision was made and skin flaps were turned aside to expose scrotal sac for proper illumination. The testes were then examined grossly for any obvious changes in their color, size, shape and consistency. The spermatic cords were clamped by an artery forceps and a cut was given one inch above the upper pole of testis with the help of scissors. The testis was taken out from the scrotal sac and the surface of each testis was wiped gently with a piece of gauze moistened in normal saline. Each testis was also observed for its size, shape, color and consistency before microscopic examination. Each testis was then weighed with the help of Sartorius balance.

Processing of testes for histological study

The testes were fixed in Bouin's fixative for 24 h. They were cut longitudinally into two halves and fixed again in fresh Bouin's fluid for another 24 h (McDonald and Scothorne, 1988). Tissues were dehydrated in ascending grades of alcohol, cleared in xylene-I and xylene-II and then embedded in paraffin at 58ºC.

Four to six µm thick sections were cut, stretched at 42ºC, dried on a hot plate at 37ºC for 24 h and were stained with hematoxylin and eosin (H&E) according to Bancroft and Stevens (1990).

Each section of the testicular tissue was examined under light microscope. Ten observations were taken in each group of animal and the experimental procedure was replicated three times making a total of 30 animals per group. Different parameters such as tubular count, diameter of tubules, thickness of germinal epithelium, interstitial cells nuclear count and their diameter were recorded. In addition the layers and number of

spermatogonia, spermatocytes and spermatids were also studied.

Scanning electron microscopy (SEM)

The tissues for SEM were processed according to McDonald and Scothorne (1988), then they were placed over the cover slip and immediately frozen to -30°C and then coated upto 300° with gold and on a accelerating voltage of 5 KV. After that the images of section were taken for detailed study of structural changes.

Statistical analysis

Data are presented as the means±SEM for 30 rats per group. Statistical analyses were performed by using a pair wise comparison analysis according to Walpole *et al*. (1998) and the values of $P<0.001$ were calculated for significance or otherwise (Table I).

Table I.- Pair wise comparison of statistical analysis of different testicular structures.

Parameters	Un-treated (Mean±S.E.)	Treated (Mean±S.E.)
Relative wt. of testis (mg)	496.90±21.22	376.40±16.01^{NS}
Seminiferous tubules		
Count	16.62±0.50	24.10±0.49**
Diameter (µm)	265.08±3.63	214.76±1.49*
Thickness of germinal epithelium (µm)	95.30±2.27	67.75±2.12*
Interstitial cell nuclei		
Count	13.30±0.75	7.00±0.58*
Diameter (µm)	4.02±0.01	3.09±0.08*

RESULTS

The testis were found to be oval in shape, grayish red in color with smooth glistening surface. The size was found almost same in each animal (Table II). The surface was irregular, grayish white in color. No abnormal nodule, growth or cyst was found.

Control testes

The rat testicular tissue showed seminiferous tubules with an obliterated narrow lumen occupied by spermatozoa (Fig. 1a). A single layer of spermatogonia was also found along with basement membrane of seminiferous tubules with large spheroidal nucleus containing condensed chromatin and a relatively smaller cytoplasm containing 3-4 spermatogonia, thus showed mitotic activities in this group of animals. Spermatocytes particularly the primary were seen two layer thick as compared to the secondary spermatocytes. Also the spermatids were seen 4-5 layers thick in the different stages of spermatogenesis. All the layers of spermatogonia *i.e.* primary and secondary spermatocytes, spermatids and spermatozoa showed that the spermatogenesis was complete (Fig. 1b).

As far as the arrangement and orientation of germ cells in the tubules is concerned, most of the spermatogonia were found of type-A. There was also a layer of old spermatocytes in the pachytene stage followed by a solid layer of spermatids and spermatozoa in the lumen. These findings showed that the tubules were in the stage-VIII of the cycle. In some tubules, young spermatids and immature spermatocyte lost their bundle arrangements so that their head caps reached to its maximum size. These observations closely showed the stage-VII of the cycle of seminiferous epithelium. As there was more crowding of spermatogenic cells and more thickening of germinal epithelium, so this arrangement showed the presence of all layers of spermatogenic epithelium (Fig. 1c). The Leydig cells were polyhedral in shape. Their nucleus containing coarse chromatin granules and a distinct nucleolus. The cytopolasm contained numerous lipid droplets that appeared in vacuoles and also their nuclear diameter seen normal as no changes have been noticed in their nuclear outlines (Fig. 1d). In addition, scanning electron microscopy has also been done on control rat testis to observe the number of seminiferous tubules present with in 50 µm and 100 µm covered area (Fig. 2a, b).

The spermatids were 1-2 layers thick indicating severe atrophy when compared with control animals. In most of the spermatogonia, mitotic division stopped and in primary spermatocytes meiosis was partly or completely interrupted at the pachytene stage. Mitosis and meiosis both were altered and metaphase was not achieved in most of the primary spermatocytes. The spermatocytes were one layer thick in most of

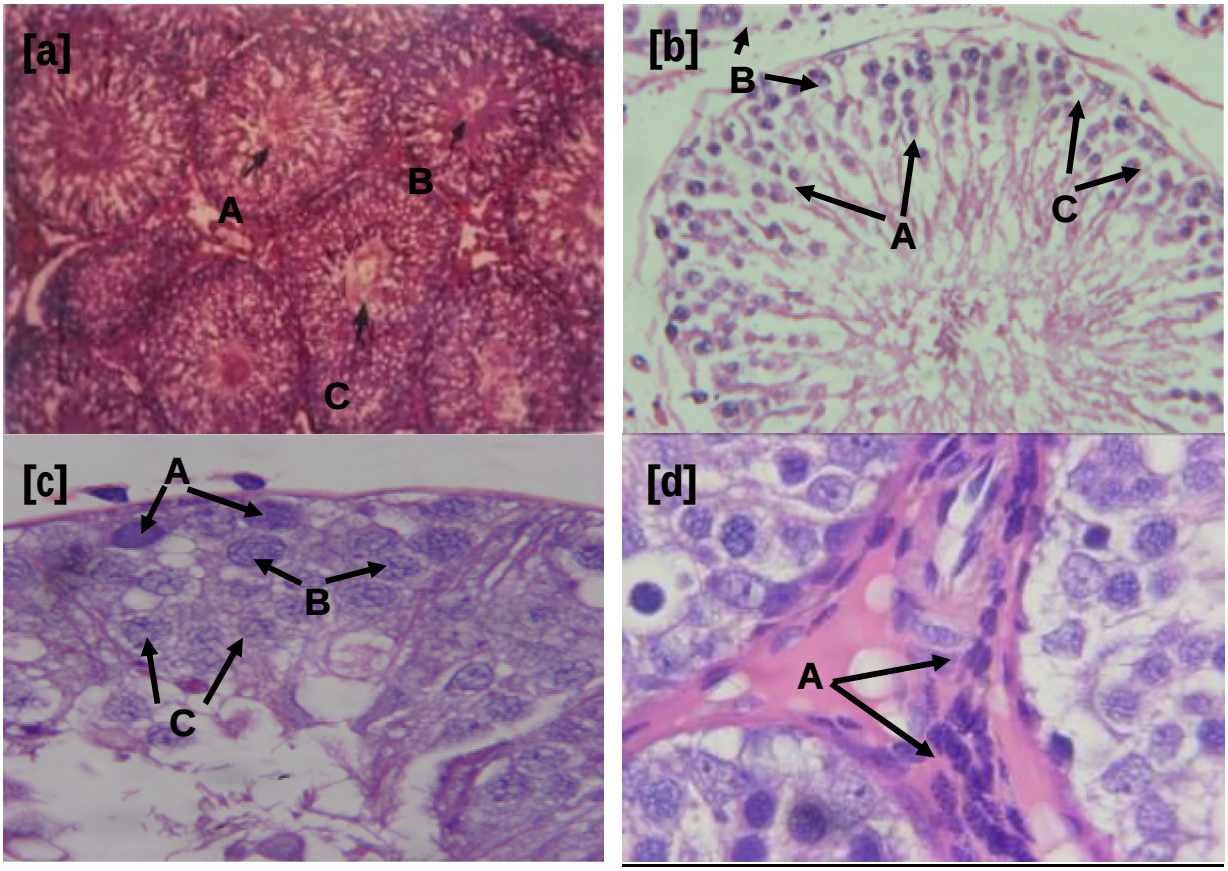

Fig. 1. Histological structure of control rat testicular tissues. **[a]** Showing count of seminiferous tubules with narrow lumen (A, B, C) filled with spermatozoa. **[b]** Showing diameter of seminiferous tubules filled with spermatozoa containing 4-5 layer of spermatids (A), spermatogonia (B) along the basement membrane and (C) two layer thick spermatocytes. **[c]** Showing thickness of seminiferous tubules containing spermatogonia (A), primary spermatocytes (B), thick layer spermatids (C) in stage VIII of the cycle. **[d]** Showing interstitial space between the seminiferous tubules containing normal Leydig cells (A).

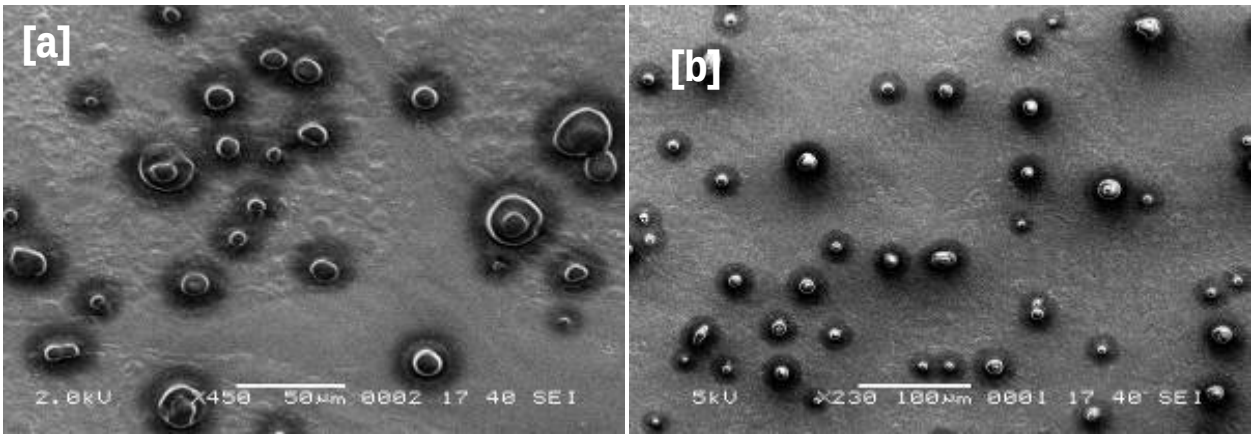

Fig. 2. Scanning electron microscopy (SEM) of control rat testicular tissue showing number of seminiferous tubules present **[a]** With in 50 µm covered area x 450. **[b]** With in 100 µm covered area x 230.

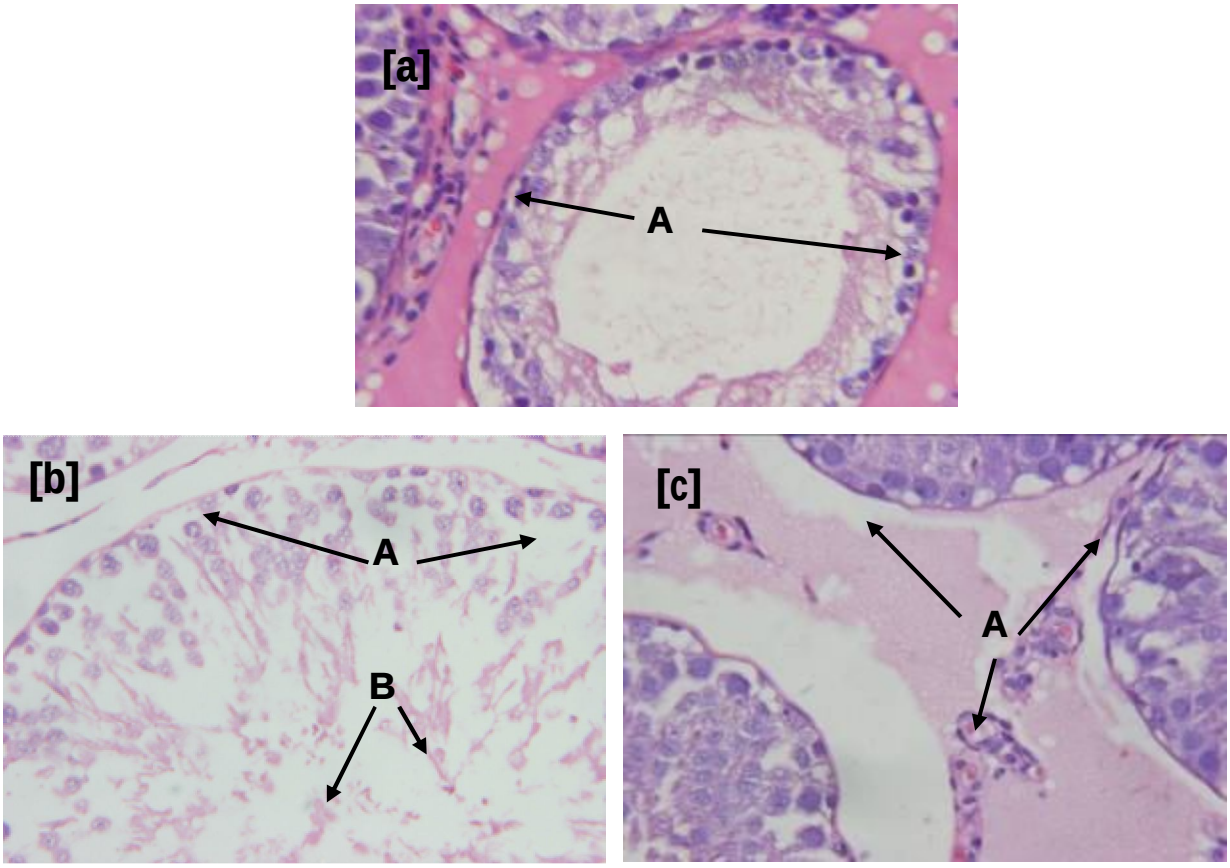

Fig. 3. Histological structure of rat testicular tissues treated with testoviron for fourteen weeks. **[a]** Showing the seminiferous tubules with wide lumen, absence of spermatozoa and markedly reduced thickness of germinal epithelium (A). **[b]** Showing decreased number of spermatocytes (A), with degenerative changes and reduced number of spermatozoa (B). **[c]** Showing decreased number and diameter of interstitial cells and wide space (A) seen between the seminiferous tubules.

the tubules, having greatly increased size of cytoplasm and larger nuclei as shown in (Fig. 3a).

Regressive changes were seen in the tubular epithelia that affected both the germinal and Sertoli cells. Some evidences of spermatogenesis and spermiogenesis were equally seen in some of the seminiferous tubules with expanded intracellular spaces. Moreover, there were abnormal luminal dilatation and gradual reduction in epithelial heights of the tubules. However, immature germ cells with variable nuclear sizes and irregular profiles associated with atrophic changes were seen in some seminferous tubules. Basement membranes of most of the tubules were distorted and ruptured, the vacuoles between the germinal epithelium appeared more in numbers and extensive. The lumen contained slough without spermatozoa (Fig. 3b). There was degeneration of interstitial space with marked widening of Interstitial space with area of necrosis. There were less Leydig cells, blood vessels, and fibroblasts were observed and the interstitial spaces were wider (Fig. 3c). In some of the treated animal's interstitium showed a great abundance of lipid droplets in the Leydig's cells. Scanning electron microscopy shows decreased number of spermetocytes and spermatozoa, with degenerative spermetocytes (Fig. 4a, b).

DISCUSSION

AAS can exert strong effect on human physiology that may be beneficial for athletic performance. When this drug is used at high doses frequently by athletes for improving their athletic

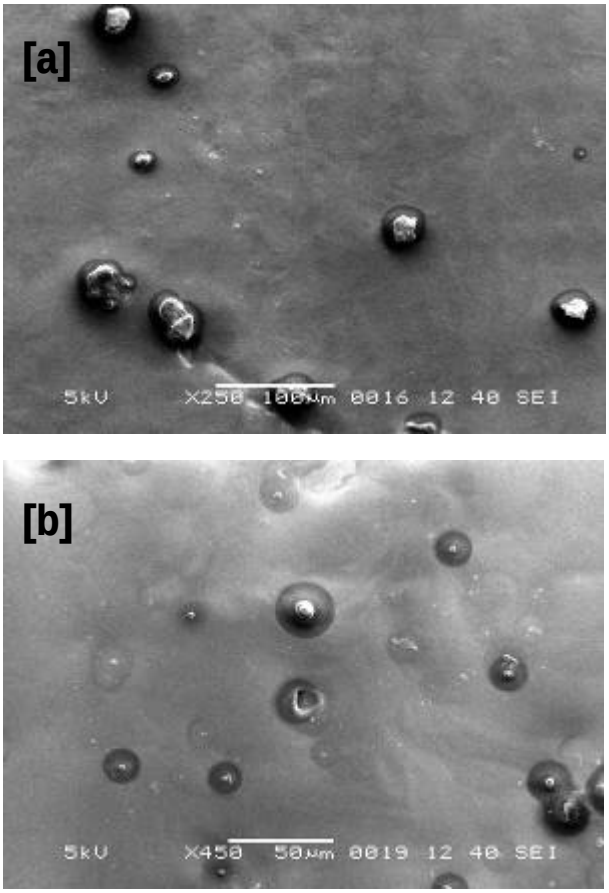

Fig. 4. Scanning electron microscopy (SEM) of rat testicular tissue treated with testoviron for fourteen weeks showing decreased number of seminiferous tubules present **[a]** With in 50 µm cover area. **[b]** With in 100 µm area.

ability, physical appearance and muscle mass, many undesirable side effects of these steroids regarding the male reproductive functions have been noticed (Noorafshan *et al.*, 2005). Because of the lack of data on the effects of anabolic steroids on the reproductive system, the present study was carried out to evaluate the effects of testoviron on the behaviour, body weight of the animals, gross appearance and relative weight of the testes. In addition the morphological and histological changes in the spermatogenic cells with reference to their number and diameter, the number of seminiferous tubules and the thickness of germinal epithelium as well as the interstitial cells nuclear counts and diameter were also evaluated.

Table II.- Changes in Body weight, testicular weight and quantitative findings in the testicular structures.

Observation	Group-A (Un-treated)								Group-B (Treated or Chronic)							
	Body weight (gms)		Wt. of testis (mg)	Quantitative testicular changes					Body weight (gms)		Wt. of testis (mg)	Quantitative testicular changes				
				Seminiferous tubule			Interstitial cells nuclei					Seminiferous tubule			Interstitial cells nuclei	
	I	F	Rel	Count	Dia (µm)	Thkn (µm)	Count	Dia (µm)	I	F	Rel	Count	Dia (µm)	Thkn (µm)	Count	Dia (µm)
1	192	228	566	19.31	280.10	100.60	12.00	3.97	201	225	411	22.00	219.50	69.30	7.00	3.40
2	205	238	367	16.20	277.30	86.80	16.00	4.01	200	248	371	23.80	209.20	59.00	6.00	3.22
3	196	230	495	14.80	244.80	89.60	14.00	3.99	196	250	374	22.80	209.40	69.80	10.00	3.00
4	190	240	456	16.20	254.20	103.20	13.00	3.98	198	235	382	24.90	211.90	75.10	8.00	3.00
5	203	245	479	17.80	266.60	97.30	11.00	4.01	199	255	359	25.00	212.90	57.40	7.00	2.90
6	200	220	525	15.80	258.90	86.30	13.00	4.01	205	240	379	25.50	219.65	64.00	9.00	3.21
7	198	215	530	17.00	261.95	106.60	18.00	4.00	200	240	387	24.00	209.60	66.60	6.00	3.01
8	210	250	468	18.90	272.40	88.60	14.00	3.90	208	258	358	21.50	217.82	67.20	4.00	3.09
9	220	249	521	15.20	258.10	98.80	12.00	4.14	201	245	392	26.00	221.40	69.40	5.00	3.11
10	222	255	562	15.00	276.50	95.30	10.00	4.05	210	256	351	25.50	216.21	79.70	8.00	3.00

I, Initial body wt; F, Final body wt; Rel, Relative wt. of testis; Dia, Diameter; Thkn, Thickness.

Numerous studies on androgenic anabolic steroids have shown that it has potent toxicity on male sexual organs particularly testis by causing the disturbances of spermatogeresis in albino rats (Lee and Kims, 1985; Shittu *et al*., 2006; Abel *et al*., 2008). When rats were examined before sacrifice, the weight was gained in the animals of group 'B' probably due to the anabolic effects of this drug testoviron or from water retention in the body and decreased high density lipoprotein (Wagner, 1989; Smith and Perry, 1992; Pope and Katz, 1994). Regarding the relative weight of the testis, the testoviron–treated group showed the significant reduction as compared to control group. The differential changes observed in the testicular weights of the animals have been correlated with the seminiferous tubular profiles of the testis for the different groups in other studies (Squires *et al*., 1982; Noorafshan *et al*., 2005; Mesbah *et al*., 2008). It has also been reported that the mean average weight gain in the treated and control group of animals were not significantly different (Mesbah *et al*., 2007; Shittu *et al*., 2006). However significant increased gain in weight was noted in the testoviron – treated group of animals as compared to control group probably due to the anabolic effect of this drug.

During present study the partial arrest of spermatogenesis as indicated by the decrease in the number and layers of spermatogonia, spermatocytes, spermatids and spermatozoa in animals of group 'B' was observed when compared with the age matched control group. These changes occurred in the stage VIII of the cycle of seminiferous epithelium that may be attributed to the action of androgenic anabolic steroids on the dividing spermatogenic cells. As most of the AAS and high doses of testosterone intake exert an inhibitory effect on the hypothalamo–hypophyseal testicular axis with a resultant suppression in the normal testicular function which may further lead to a reduction in the testosterone production. Similar findings on decreased spermatogenesis and also on testicular atrophy have been reported by some researchers (Heller and Clermont, 1963; Feinberg *et al*., 1997). The count of seminiferous tubules per field area was significantly increased in animals of treated group as compared to control. This increase may be attributed due to the loss of germ cells, thus resulting in the shrinkage of the tubules. Our observations closely reflected to the conclusion drawn by Squires *et al*. (1982) who reported that all the anabolic steroid treatment reduced the number of developing germ cells, thus resulting in the increase number of seminiferous tubules. The findings related to the seminiferous tubules were also re-confirmed through scanning electron microscopy (SEM). The diameter of the seminiferous tubules and the thickness of the germinal epithelium on the other hand were also decreased significantly in the testoviron–treated group. As compared to the un-treated group, the seminiferous epithelium of the treated animals was disrupted with broad spaces between the cellular components showing the presence of copious vacuoles frequently associated with degenerating germ cells as reported by Mesbah *et al*. (2008). They also reported that there was disruption of the seminiferous epithelium with broad spaces between the cellular components and the testicular atrophy with shrinkage, resulting in the decreased diameter of the seminiferous tubules. Previous study by Pope and Katz (1994) also showed reduction in the length of the seminiferous tubules, as a result there was a reduction in the weight of the testis. This significant decrease in the thickness of germinal epithelium may be attributed to the injury caused by AAS treatment which results in the cessation of mitosis and meiosis. Consequently, the two layers of spermatocytes have been reduced to a single layer in thickness and a 4–5 layers thick zone of spermatids get reduced to 2–3 layers and so only few spermatozoa could be demonstrated in the tubular lumen. These findings are consistent with the findings of Mesbah *et al*. (2008) who reported that this may be due to the reduction in the germ cell population and maturation or it could be due to the effects of AAS on the Sertoli cells which are responsible for the hormonal, nutritional and physical support. Drugs such as AAS's that disrupt the functions of Sertali cells can effectively reduced their supportive roles, resulting in the eliminaltion of large number of germ cells via apoptosis (Skinner *et al*., 1985).

Another study in this regard showed that major intra-testicular changes occurred during

AAS's therapy resulting a decrease in the size and diameter of the seminiferous tubules as well as the size of the testes (Heller and Clermont, 1963). These findings confirm the results of Noorafshan *et al*. (2005) who reported that the volume and weight of the testis was also reduced significantly ($P<0.01$). Similar findings were also presented in the literature by Fainber *et al*. (1997) who also reported that reduction in the testicular weight was noted even after the withdrawal of hormonal injection for few weeks (Fainber *et al*., 1997).

A significant decrease in the number and size of the interstitial cells of Leydig ($P<0.01$) and depletion of intact cells in the testoviron – treated group of animals were noted when compared with that of control animals. These observation were consistent with the previous findings (Groket *et al*., 1992; Fainber *et al*., 1997) who reported the severe depletion of Leydig cells when treated by AAS's. The results of our study confirms the finding observed by Mesbah *et al*. (2008), which showed the less number of Leydig cells, blood vessels, fibroblasts and wider interstitial spaces. On the basis of present study, we concluded that administration of AAS compounds have a clear effect. Administration of testoviron has an obvious effect on testicular structures including the degenerative changes in the germ cells and Leydig cells. These are accompanied by the changes appeared in the seminiferous tubular parameters and testicular atrophy. Therefore, this study shows major adverse effects in rat testicular tissue and this may be true in the case of male reproductive organs of athletes and those who abuse AAS compounds. So the AAS's compounds like testoviron should be used with caution as short intermittent therapy, if desired for better spermatogenic cycle and improve overall fertility processes.

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Table II.- Changes in Body weight, testicular weight and quantitative findings in the testicular structures.

Observation	Group-A (Un-treated)								Group-B (Treated or Chronic)							
	Body weight (gms)		Wt. of testis (mg)	Quantitative testicular changes					Body weight (gms)		Wt. of testis (mg)	Quantitative testicular changes				
				Seminiferous tubule			Interstitial cells nuclei					Seminiferous tubule			Interstitial cells nuclei	
	I	F	Rel	Count	Dia (µm)	Thkn (µm)	Count	Dia (µm)	I	F	Rel	Count	Dia (µm)	Thkn (µm)	Count	Dia (µm)
1	192	228	566	19.31	280.10	100.60	12.00	3.97	201	225	411	22.00	219.50	69.30	7.00	3.40
2	205	238	367	16.20	277.30	86.80	16.00	4.01	200	248	371	23.80	209.20	59.00	6.00	3.22
3	196	230	495	14.80	244.80	89.60	14.00	3.99	196	250	374	22.80	209.40	69.80	10.00	3.00
4	190	240	456	16.20	254.20	103.20	13.00	3.98	198	235	382	24.90	211.90	75.10	8.00	3.00
5	203	245	479	17.80	266.60	97.30	11.00	4.01	199	255	359	25.00	212.90	57.40	7.00	2.90
6	200	220	525	15.80	258.90	86.30	13.00	4.01	205	240	379	25.50	219.65	64.00	9.00	3.21
7	198	215	530	17.00	261.95	106.60	18.00	4.00	200	240	387	24.00	209.60	66.60	6.00	3.01
8	210	250	468	18.90	272.40	88.60	14.00	3.90	208	258	358	21.50	217.82	67.20	4.00	3.09
9	220	249	521	15.20	258.10	98.80	12.00	4.14	201	245	392	26.00	221.40	69.40	5.00	3.11
10	222	255	562	15.00	276.50	95.30	10.00	4.05	210	256	351	25.50	216.21	79.70	8.00	3.00

I, Initial body wt; F, Final body wt; Rel, Relative wt. of testis; Dia, Diameter; Thkn, Thickness.