Protective effect of silymarin on the testes of rats treated with anabolic androgenic steroid: A biochemical, histological, histochemical and immunohistochemical study

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Abstract

**Background:** Many harmful effects have been reported for the abuse of testosterone with supraphysiologic doses on the male genital system.

**Objective:** To study the possible protective effect of silymarin in testosterone induced testicular changes.

**Materials and Methods:** Eighty adult male albino rats were randomly divided into four groups; untreated rats (controls), rats treated with silymarin, rats treated with testosterone and rats treated with testosterone and silymarin. Relative testicular weight, serum testosterone and FSH were estimated in addition to histological, histochemical and immunohistochemical assessment.

**Results:** Serum testosterone was significantly higher in testosterone treated rats compared with the controls and rats treated with testosterone plus silymarin. Serum FSH was significantly lower in testosterone treated rats as well as rats treated with silymarin plus testosterone compared with controls. There was a significant increase in caspase-3 expression between testosterone treated rats and the other studied groups (P<0.001). There was a significant increase in P53 expression between the testosterone treated rats and the control groups (P<0.001 for each), and also between the testosterone treated rats and rats treated with testosterone and silymarin (P=0.004). P53 expression was also significantly different between rats treated with testosterone plus silymarin and the control groups (P=0.012 for each).

**Conclusion:** Oral concomitant treatment with silymarin was effective in attenuating testosterone- induced testicular damage in adult male albino rats.

**Keywords:** Testosterone, testis, apoptosis, caspase, P53

Introduction

Androgens play an important role in male reproductive organs development, and are of major importance in puberty, fertility, and sexual function. Leydig cells secrete intra-testicular testosterone which is a steroid hormone that has a great importance in the development of the male phenotype and regulation of reproduction as it is essential for spermatogenesis [1,2].

Anabolic-androgenic steroids (AAS) are natural and synthetic compounds that are analogues to the male sex hormones. Their medical utilization is limited and they are only used with great caution in some disorders such as poor growth, osteoporosis, hypogonadism and anemia [3]. AAS in supraphysiological doses can enhance athletic performance. However, these compounds could be abused by athletes to increase their muscle mass by increasing protein synthesis [4]. On the other hand, AAS abuse can lead to some harmful side effects such as liver failure and decrease high density lipoprotein levels, acne vulgaris, gynaecomastia, cholestasis, and renal failure [5,6]. AAS also might cause side effects in the reproductive systems, both in human and experimental animals, disturbing the regular endogenous
production of testosterone and gonadotrophins [7].

Sustanon is one of AAS, which is used to treat several cases in andrology such as male hypogonadism and infertility [8]. It is characterized by a unique structure and properties compared with the other AAS drugs in the market as it is composed of a mixture of four different testosterone esters [9]. Many harmful effects have been reported for Sustanon abuse on the male reproductive health as high level of testosterone induces oxidative stress by alteration of the balance between reactive oxygen species (ROS) production and antioxidant defenses [10]. Free radicals have many harmful effects including lipid peroxidation, DNA damage and apoptosis [11].

Lately, much attention has been paid on using plant-derived materials including various forms of extracts as an antioxidant source to eliminate excessive ROS [12-14]. Silymarin, the active components of milk thistle extract, is a flavonoid polyphenolic compound extracted from Silybummarianum seeds that has long been extensively used in patients suffering from liver disease. It has also other biological activities as anti-inflammatory, antioxidant and anti-cancer properties both in in-vitro and in-vivo studies [15-18].

Some studies have been performed on the protective effects of silymarin on male reproduction but have not been completed yet and the mechanism of its action on sex hormones has not been clearly examined [19]. In addition, its possible protective effects against anabolic steroids related testicular adverse effect has no previous attention.

The harmful effects of Sustanon on testes have been confirmed in several studies; however to the best of our knowledge, no single work has studied its possible protective effect in Sustanon induced testicular changes. So, this study aimed to assess the possible protective effect of Silymarin in Sustanon induced testicular changes in rats.

Materials and methods
This experimental study was approved by the Ethical Committee of Research, Faculty of Medicine with conformity to institutional and national guidelines for the care and use of animals. Eighty adult male albino rats were used (weight 135-150 g). They were caged individually and they were randomly divided into 4 equal groups (n=20 each) as follows:

• Group 1. Untreated control rats fed on ordinary chew plus saline (the vehicle).
• Group 2. Rats treated by silymarin orally (20 mg/kg/day).
• Group 3: Rats injected with IM testosterone (Sustanon®
Organon, India) (10 mg/kg/week).
• Group 4. Rats treated by silymarin (20 mg/kg/day) orally and IM Sustanon (10 mg/kg/week).

Sustanon® 250 mg ampule is one of AAS that is composed of an oily mixture of four testosterone esters that provide a permanent release of testosterone into the blood for length level from 3-4 weeks (30 mg testosterone propionate, 60 mg testosterone phenylpropionate, 60 mg testosterone isocaproate and 100 mg testosterone decanoate).

Following treatment for 8 weeks, all rats were sacrificed and their testes were removed immediately and weighed for their relative weight (testes weight/body weight X100). Also, blood samples were collected for determination of serum follicle stimulating hormone (FSH) and total testosterone hormone (T).

Biochemistry
Quantitative measurement of both total testosterone [20] and FSH [21] was done using IMMULITE analyzer (Catalog number: LKTW1).

Histopathology, histochemistry and Immunohistochemistry studies
The removed testes were immediately excised and fixed in 10% neutral buffered formalin. Paraaffin sections (5μm) were prepared for processing the histological, histochemical and immunohistochemical studies. For histology, the sections were stained with haematoxylin and Eosin (Hx & E). Leydig cells in interstitial connective tissue were counted under 400X in five distinct microscopic fields for each rat. For histochemical studies, Periodic acid Schiff’s (PAS) reaction was used for demonstration of polysaccharides [22].

For immunohistochemistry using Caspase-3 and P53 antibodies, deparaffinized, rehydrated sections were heated in a microwave oven in 10 mmol/L citrate buffer (pH 6) for antigen retrieval. The sections were immersed in 0.3% H2O2 in methanol to block endogenous peroxidase and were pre-incubated with normal mouse or rabbit serum to block nonspecific binding of antibodies. The sections were then incubated with primary antibody. The avidin-biotin-peroxidase–complex method was used for detection, with diaminobenzidine tetrachloride as the chromogen. The primary antibodies used were rabbit polyclonal Caspase-3 antibody (CPP32) (1:100; Thermo Fisher Scientific Co., USA) and rabbit polyclonal P53 antibody (ab131442) (1:100; abcam, USA). As a negative control for immunostaining, the second antibody was used without the first antibody for each protein. Immunostained germ cells were identified in a minimum of 1000 germ cells examined in the testes by light microscopy at 400X in 5 random fields. For Caspase-3 both nuclear and cytoplasmatic staining were observed, however nuclear staining only was used in scoring. Only nuclear staining was detected for P53. For scoring of both markers the number of stained germ cell nuclei was expressed relative to the total number of germ cell nuclei in the seminiferous tubules in each testis and then was expressed as percentages. This percentage is then converted to grades namely (0) no staining, (1) 0-5%, (2) 6-50% and (3) >50% [23].

Statistical analysis
Statistical analysis was done using SPSS statistical software package version 21 (SPSS Inc., Chicago, IL, USA). The results were presented as mean and standard deviation (SD) for quantitative data, frequencies and percent for qualitative data. Chi-square test was used for comparing qualitative variables.
between groups. ANOVA test followed by post-hoc tests was used for intergroup comparison of quantitative variables. Correlation between quantitative variables was presented by Pearson correlation co-efficient \( P \) value <0.05 was set as statistically significant.

**Results**

**Relative testicular weight and hormones**

There was significant decreases in testicular weights in testosterone treated rats compared with the controls whereas the mean testis weight in testosterone and silymarin treated rats showed nonsignificant difference compared with the controls. Serum testosterone was significantly higher in testosterone treated rats compared with the controls and rats treated with testosterone plus silymarin. Serum FSH was significantly lower in testosterone treated rats as well as the rats treated with silymarin plus testosterone compared with the controls (**Table 1**).

**Histopathological examination**

Light microscopic examination of Hx & E stained sections of testes of the control group (**Figure 1A**) and controls treated with silymarin (**Figure 1B**) revealed normal histological appearance of the seminiferous tubules, germ cells with sperm formation and interstitial Leydig cells. In testosterone treated rats (**Figure 1C**), the testes showed degeneration, disorganization, reduced number of mature sperms, necrotic debris, decreased spermatogenic cells, decreased spermatogenic layers with reduced diameter in most seminiferous tubules and atrophy/hyalinization in some of them with dilated interstitial spaces and apparently reduced number of randomly distributed Leydig cells. In rats that received testosterone and silymarin (**Figure 1D**), the testes showed relatively normal histological structure of semineferous tubules.

**Table 1. Comparison of the four studied rat groups (mean±SD).**

<table>
<thead>
<tr>
<th></th>
<th>Untreated controls</th>
<th>Controls+silymarin</th>
<th>Rats on testosterone</th>
<th>Rats on testosterone+silymarin</th>
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<tbody>
<tr>
<td><strong>Relative testicular weight (%)</strong></td>
<td>2.35±0.4</td>
<td>2.28±0.2</td>
<td>1.98±0.1</td>
<td>2.34±0.2</td>
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<td>P1</td>
<td></td>
<td>0.274</td>
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<td>P2</td>
<td></td>
<td></td>
<td>&lt;0.001*</td>
<td>0.348</td>
</tr>
<tr>
<td>P3</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001*</td>
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<tr>
<td><strong>Serum Testosterone (ng/ml)</strong></td>
<td>7.75±0.4</td>
<td>7.68±0.4</td>
<td>10.84±1.9</td>
<td>9.6±1.4</td>
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<tr>
<td>P1</td>
<td></td>
<td>0.818</td>
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<td>P2</td>
<td></td>
<td></td>
<td>&lt;0.001*</td>
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<tr>
<td>P3</td>
<td></td>
<td></td>
<td></td>
<td>0.002*</td>
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<tr>
<td><strong>Serum FSH (U/ml)</strong></td>
<td>2.59±0.42</td>
<td>2.64±0.3</td>
<td>1.1±0.4</td>
<td>1.31±0.3</td>
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<tr>
<td>P1</td>
<td></td>
<td>0.633</td>
<td>&lt;0.001*</td>
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<tr>
<td>P2</td>
<td></td>
<td></td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
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<tr>
<td>P3</td>
<td></td>
<td></td>
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<td>0.061</td>
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<tr>
<td><strong>Leydig cell count</strong></td>
<td>20.5±1.5</td>
<td>20.6±1.6</td>
<td>12.8±1.6</td>
<td>18.8±1.4</td>
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<tr>
<td>P1</td>
<td></td>
<td>0.839</td>
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<tr>
<td>P3</td>
<td></td>
<td></td>
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<td>&lt;0.001*</td>
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*Significant at \( P < 0.05 \)

P1 comparison with untreated controls

P2 comparison with control rats treated with silymarin

P1 comparison with rats treated with testosterone.
appearance of most seminiferous tubules and Leydig cells. Leydig cell count was significantly different in testosterone treated rats compared with the controls and significantly different in testosterone plus silymarin received rats from the two control groups and testosterone only received group (Table 1).

**Histochemical observations**
The testes of the untreated control group ([Figure 2A]) and rats treated with silymarin ([Figure 2B]) showed normal distribution of PAS positive reaction in the tunica albuginea and intertubular connective tissue, while the testes of the rats that received testosterone showed reduced staining affinity with interstitial edema ([Figure 2C]). In rats that received testosterone and silymarin ([Figure 2D]), the PAS positive material showed normal distribution in the testis.

![Image](image_url)

**Immunohistochemical observation**
Immunohistochemical staining for Caspase-3 was represented in ([Figure 3]). The grades of staining for the untreated control group ranged from 0 to 2 and from 0 to 1 for the silymarin treated rats, while for the testosterone treated rats the grades ranged from 2 to 3 and from 0 to 1 for testosterone plus silymarin treated rats. There was a significant difference in Caspase-3 expression between testosterone treated rats and all other studied groups (P<0.001).

Immunohistochemical staining for P53 was represented in ([Figure 4]). Both control groups showed negative staining (Grade 0). The grades of staining for the testosterone treated group ranged from 0 to 3, and from 0 to 1 for the testosterone and silymarin treated group. There was a significant difference in P53 expression between the testosterone treated group and the control groups (P<0.001 for each), and also between the testosterone treated group and the group treated with testosterone and silymarin (P=0.004). P53 expression was also significantly different between the group treated with testosterone and silymarin and the control groups (P=0.012 for each).

**Discussion**
Lately, the deliberate abuse of anabolic androgenic drugs especially testosterone derivatives by athletes have increased rapidly world-wide. Meanwhile, their reproductive effects include many hazards as some researchers found that the anabolic steroids treated rats showed reduction in the number of spermatogonia leading to decreased sperm count and
Sprague-Dawley rats, one injected with testosterone and the other with anabolic steroids for six weeks. Also, De Souza and another with an anabolic steroids for six weeks. Also, De Souza reported similar results in Wistar rats that were treated with testosterone esters mixture.

There was significant increases in serum testosterone and significant decreases in serum FSH in testosterone treated rats. Some studies confirmed that administration of testosterone and its derivatives causes elevated of serum testosterone. Also, these hormonal changes can be explained by that exogenous administration of testosterone suppresses both GnRH and LH production and subsequently suppress testicular testosterone production. However, Al-Alwany reported that when testosterone was injected to three groups of albino rats in doses of (0.05, 0.1 and 0.2) mg/kg/day for six weeks there was a significant increase in the mean levels of FSH hormone. They explained that the increase in testosterone level of athletes can cause increase secretion of FSH from hypothalamus pituitary gland.

The histopathological changes detected in testosterone treated rats was similar to that observed in other study after administration of three different doses of testosterone to three groups of rats weekly for four weeks and also on studying the histological changes in rat testes after injection of repeated weekly administration of two doses of testosterone for two months. However, Mutalip et al. reported that testicular histology of rats treated with testosterone or stanozolol showed slight changes. Other studies reported severe depletion of Leydig cells when treated by AAS and a significant decrease in the number and size of the interstitial cells of Leydig compared with controls. This all goes with the results of this study.

There was a reduction in PAS positive reaction in the testosterone treated group of rats compared with the controls. Similar observations were reported by many observers in the testes of mice intoxicated with different factors. It was postulated that the decrease in proteins could be attributed to disruption of lysosomal membranes under the effect of various toxicants liberating their hydrolytic enzymes with marked lysis of the target materials.

In apoptosis, caspases play a crucial role in the transduction of apoptotic signals in cells that are destined to die. This expression may be related to testosterone related harmful propagation of DNA damage. Thus, it serves as a checkpoint control mechanism and protects the cell from harmful propagation of DNA damage.

The significant increase in caspase-3 immunohistochemical staining of germ cells compared with the controls was also observed by Kim et al. in the testes of rats treated with testosterone and this was explained as that germ cell apoptosis resulted from a reduced intratesticular testosterone concentration is caspase-3 activation dependent.

Absence of P53 immunohistochemical expression in germ cells of controls is similar to that found by other researchers. This expression may be related to testosterone related testicular damage; as p53 can be highly expressed in testes under cellular stress as its intracellular concentration increase to a level that can be detected. Silymarin has also been reported to have antioxidant, anti-inflammatory and immunomodulatory effects. The current study showed that rats that received testosterone and silymarin had significantly better results than that of testosterone only.
treated rats regarding all variables examined except for FSH level that showed nonsignificant difference. It is worth mention that all these studied variables are significantly different compared with the controls except for the relative testicular weight and caspase-3 immunohistochemical expression.

It is known that testosterone administration causes oxidative stress [10]. So the improved variables detected in this study could be attributed to both antioxidants and inhibitory effect of free radicals formation [49]. In their study, Moshtaghion et al. [50] showed that silymarin-treated animals were protected from varicocele-induced testicular atrophy and these animals showed a significant increase in the percentage of seminiferous tubules with positive tubular differentiation, repopulation, and spermiogenesis indices.

It is concluded that oral treatment with silymarin is effective in attenuating testosterone-induced biochemical and histopathological damage in adult male albino rats. These facts could present an approach for managing AAS abuse.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions

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