The interaction effects of resistance training and sustanon abuse on liver antioxidant activities and serum enzymes in male rats

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Abstract: *Background:* Anabolic–androgenic steroids (AAS) are synthetic drugs derived from testosterone, the uncontrolled usage of which may lead to serious side effects. Previous studies have shown that resistance training (RT) is the main exercise modality practiced by AAS abusers. Thus, this work was carried out to evaluate the hepatotoxic effects of sustanon (Su) as an example of AAS in trained male rats. *Methods:* Rats were divided into sedentary/non-Su, sedentary/Su, RT/non-Su, and RT/Su. Su-administration groups received Su 10 mg/kg intramuscularly once a week for 8 weeks. In the 8-week RT, the rats climbed a vertical ladder 3 days/week. *Results:* After Su administration, the mean values of serum parameters related to hepatic function were within normal ranges. Superoxide dismutase, glutathione peroxidase, and glutathione reductase activities were higher ($P < 0.05$) in the liver of Su-treated rats. Chronic exercise alone did not change any of the above parameters. *Conclusions:* The present findings suggest that the 8-week injection of Su, either with or without concurrent RT upregulation of enzymatic antioxidant activities and RT, did not attenuate the increase of enzymatic activities due to the Su administration. Furthermore, Su abuse in this dose did not make any severe liver damage.

Keywords: resistance training, anabolic–androgenic steroids, antioxidant, sustanon, drugs

Introduction

Sustanon (Su) is an oil-based injectable anabolic–androgenic steroid (AAS) typically containing four different testosterone esters (testosterone propionate, testosterone phenylpropionate, testosterone isocaproate, and testosterone decanoate), which provides a continuous release of testosterone into the blood and produces a stable testosterone level for a prolonged period extending for 3–4 weeks [1, 2].

AASs are a group of synthetic compounds structurally related to testosterone, which are pharmacologically important for treatment of hypogonadism, impotence, delayed puberty, muscle wasting, diaphragm atrophy, osteoporosis, types of anemia, endometriosis and fibrocystic breast disease, alcohol hepatitis, wound and burn healings, and finally, renal failure [3–7]. In addition to their therapeutic uses, AASs are also taken in high doses by athletes, bodybuilders, and youths to enhance muscle mass or physical endurance [8]. Uncontrolled usage of AASs may lead to serious side effects, such as cardiovascular disorder (particularly enlargement of the left ventricle), which can lead to a sudden death, acute hepatitis and jaundice, testicular dysfunction, which leads to infertility, hypertension, and behavioral disorders [9, 10]. Abusing AAS by many bodybuilders, athletes, and the youth is a serious health phenomenon that has recently increased rapidly [11, 12].

Liver is a key organ actively involved in numerous metabolic and detoxifying functions. AASs are rapidly metabolized in the liver [12]. The adverse effects of AASs on the liver include transient serum enzyme elevations, an acute cholestatic syndrome, chronic vascular injury (peliosis hepatis), and hepatic tumors including adenomas and hepatocellular carcinoma [9, 13]. Moreover, high AAS rate induces oxidative stress in liver by alteration of

the balance between reactive oxygen species (ROS) production and antioxidant defenses [14]. Oxidative stress occurs when the production of reactive species, derived largely from oxygen and nitrogen, exceeds degradation by the antioxidant defense system. The ensuing damage to DNA, protein, and lipid has been implicated in cardiovascular and pulmonary diseases, diabetes, neurodegenerative disorders, and some cancers [15, 16].

It is well established that regular exercise can influence a large number of physiological factors (hemodynamics, blood pH, body temperature, etc.), which, in turn, may affect the pharmacokinetics of numerous drugs. Furthermore, previous studies have shown that it can reduce oxidative stress by upregulating the antioxidant system [15–17]. Thus, the concurrence of exercise training and anabolic steroid consumption could be expected to modify the potential hepatotoxicity of these compounds given that resistance training (RT) is the main exercise modality practiced by AAS abusers [4]. However, to date, information about the effects of Su treatment and simultaneous RT on liver injury has been scarce. It is of interest to investigate the effects of an RT protocol, Su administration on liver enzymes, and changes of antioxidant status.

Materials and Methods

Rats and experimental design

Forty male Wistar rats (weighing approximately 250 g) were obtained from Anistito Pastor (Karaj, Iran). They were housed in collective cages at 22–24 °C on a 12:12-h light–dark cycle, with free access to tap water and food (standard chow for rodents – Purina). Rats care, handling, and all of the experimental procedures were carried out in accordance with the Ethics Committee on Animal Experimentation of University of Guilan. After the adaptation period (7 days), the rats were randomly divided into four experimental groups derived from two interventions: RT (sedentary vs. RT) ($n = 20$) and Su administration (non-Su vs. Su) ($n = 20$). Each specific intervention (i.e., sedentary/non-Su, sedentary/Su, RT/non-Su, and RT/Su) was carried out in groups of 10 rats and the experimental period lasted 8 weeks.

RT protocol

The RT protocol was adapted from Hornberger and Farrar [18]. Initially, the rats were adapted to the RT protocol, which required the rats to climb a vertical ladder (110 cm high, 18 cm wide, with 2 cm grid steps) with weights attached to their tails. At the top of the ladder was a $20 \times 20 \times 20$ cm chamber that served as a shelter during the period of rest between a series of climbs. The size of the ladder induced the rats to perform 8–12 movements per climb. When necessary, a stimulus with tweezers was applied to the rat's tail to initiate the movement. This procedure was repeated until the rats would voluntarily climb the ladder for three consecutive times, without stimulus.

The training protocol was started 3 days after the adaptation period. The first training session consisted of four to eight ladder climbs while progressively carrying heavier loads. The initial climb consisted of carrying a load that was 75% of the rat's body weight. At the top of the ladder, the rats reached the housing chamber and were allowed to rest for 120 s. Upon successful completion of this load, an additional 30-g weight was added to the load apparatus. This procedure was successively repeated until a load was reached with which the rat could not climb the entire length of the ladder. Failure was determined when the rat could not progress up the ladder after three successive gentle stimuli to the tail. The highest load successfully carried along the entire length of the ladder was considered to be the rat's maximal carrying capacity.

Subsequent training sessions consisted of four to nine ladder climbs. During the first four ladder climbs, the rats carried 50%, 75%, 90%, and 100% of their previous maximal carrying capacity, respectively. During subsequent ladder climbs, an additional 30-g load was progressively added until the rat's new maximal carrying capacity was achieved.

Su administration

Su ampoules (manufactured by N.V. Organon Oss Inc., Holland) have been obtained from the local pharmacy in Guilan, Iran. Each ampoule contains 1 mL of oily solution of Su (250 mg Su per mL). Each ampoule dissolved in 100 mL olive oil. Each rat was given 10 mg/kg body weight of Su suspension once a week by intramuscular (IM) injection in the gluteus (alternating the lateral side each week) for 8 weeks. This dose is comparable with the dose that has been reported as being frequently used by athletes. The non-Su-administered groups were injected with olive oil as vehicle [10].

Tissue collection and preparation

After completion of the 8-week exercise program, rats were not exercised for 48 h and received the last steroid dose 5 days before they were sacrificed. Rats were anesthetized with ketamine–xylazine and sacrificed by cannulation of the abdominal aorta. Blood samples were then collected by cardiac puncture according to the method of Hoff and Rlatg [19] and centrifuged, and serum was frozen at −20 °C for later analysis. Livers were rapidly excised, weighed and washed with cold saline and frozen in liquid nitrogen, and stored at −80 °C for further analysis.

Serum analyses

The activities of the serum enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) were assayed using routine enzymatic methods (Pars Azmoon, Tehran, Iran) on an automated chemistry analyzer (Mindray Bs-380, China).

Liver homogenate preparation for antioxidant activity

An amount of 1 mL of homogenization buffer (0.1 M phosphate buffer, pH 7.4 containing 1 mM ethylenediaminetetraacetic acid, and 0.005% butylated hydroxytoluene) per 100 mg of tissue was added. Then, liver tissue samples were homogenized. After homogenization, samples were centrifuged at 8,000 rpm for 10 min and the resulting supernatant was used for the estimation of enzymatic antioxidant activity.

Estimation of superoxide dismutase (SOD)

SOD activity was examined in the supernatant by the spectrophotometric method described by Winterbourn et al. [20]. The principle of the assay was based on the ability of SOD to inhibit the reduction of nitro-blue tetrazolium (NBT). Briefly, the reaction mixture contained 2.7 mL of 0.067 M phosphate buffer (pH 7.8), 0.05 mL of 0.12 mM riboflavin, 0.1 mL of 1.5 mM NBT, 0.05 mL of 0.01 M methionine, and 0.1 mL of enzyme samples. Uniform illumination of the tubes was wrapped by an aluminum foil box under a 15 W fluorescent lamp for 10 min. A control without the enzyme source was included. The absorbance was measured at 560 nm. One unit of SOD was defined as the amount of enzyme required to inhibit the reduction of NBT by 50% under the specific conditions. The SOD activity was expressed as nM/μg protein content/min of the tissue homogenate for each group.

Estimation of glutathione peroxidase (GPx)

GPx was estimated by the method of Rotruck et al. [21]. Briefly, the reaction mixture contained 0.2 mL of 0.4 M phosphate buffer (pH 7.0), 0.1 mL of 10 mM sodium azide, 0.2 mL supernatant in 0.4 M phosphate buffer (pH 7.0), 0.2 mL reduced glutathione, and 0.1 mL of 0.2 mM hydrogen peroxide. The contents were incubated for 10 min at 37 °C, and 0.4 mL of 10% trichloroacetic acid was added to stop the reaction and centrifuged at 3,200 × *g* for 20 min. The supernatant was assayed for glutathione content using Ellman's reagent (19.8 mg 5,5′-dithiobisnitrobenzoic acid in 100 mL of 0.1% sodium nitrate). The activities were expressed as μg of glutathione consumed/min/mg protein.

Estimation of glutathione reductase (GR)

GR activity was measured by the method of Mohandas et al. [22], in which the following reaction is implicated:

$$NADPH + H^+ + GSSG \rightarrow NADP^+ + 2GSH.$$

In the presence of GR, oxidized glutathione undergoes reduction and simultaneously NADPH is oxidized to $NADP^+$. Enzyme activity is quantified at room temperature by measuring the disappearance of NADPH/min at 340 nm spectrophotometrically.

Statistical analysis

The obtained data were analyzed using SPSS 19.0 J (SPSS Japan, Tokyo, Japan) with advanced modules. Initially, the statistical analysis was done both by Kolmogorov–Smirnov normality test and homoscedasticity. A two-way analysis of variance test was used to evaluate the two main effects of RT and Su treatment and the interaction between them. When a significant F value was obtained, a Tukey's post-hoc test was performed. The results were expressed as mean ± standard deviation. $P < 0.05$ was considered to be statistically significant.

Results

All of the RT rats successfully completed 8 weeks of training. *Figure 1* shows that the RT/Su and RT/non-Su groups increased their maximal carrying capacity by the same amount during the training period. Nevertheless, there was no significant difference in their increased maximal carrying capacity between these groups in the training period ($P > 0.05$) *(Fig. 1)*.

Table I shows the final body and liver weights of the rats during the experimental period. There were no significant differences in final body and liver weights between the groups ($P > 0.05$). This fact suggests that abnormal retention of fluids was not produced as a consequence of the Su administration and that the rats in the RT groups adapted to the stress of physical exercise and remained healthy.

Measurements of enzymatic antioxidant activity in the liver homogenate are reported in *Fig. 2*. There were no differences in antioxidant activity (SOD, GPx, and GR) in both sedentary and trained rats ($P > 0.05$) *(Fig. 2)*. However, a significant main effect was observed for Su treatment ($P < 0.05$) on SOD, GPx, and GR activities, so that the enzymatic activities measured in the Su-administration groups were significantly higher than those determined in the respective untreated groups ($P < 0.05$). In no case, there was an interaction between RT and Su administration.

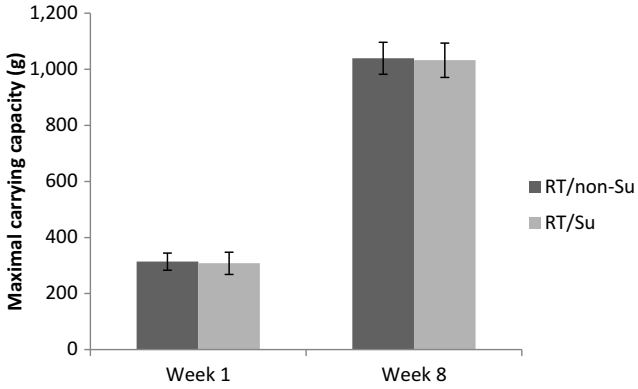

Fig. 1. The non-sustanon resistance training and sustanon resistance training groups' maximal carrying capacity at weeks 1 and 8. Values are expressed as mean ± standard deviation ($n = 10$)

The results of biochemical analysis of the blood serum are listed in *Table II*. Neither the RT nor the administration of Su modified significantly the mean values of AST, ALT, and ALP ($P > 0.05$).

Discussion

The use of AASs as ergogenic aids accompanied by exercise training and RT is the main exercise modality practiced by AAS abusers [4]. Since exercise is known to affect a large number of physiological factors and the liver is the main target organ for gastrointestinal effects of AAS, the effect has been studied individually and in combination of variables, RT, and steroid treatment in this investigation.

The main finding of this study is that IM injection of Su at supraphysiological dose (10 mg/kg BW) for 8 weeks induced a significant increase ($P < 0.05$) in the activities of the antioxidant enzymes, such as SOD, GPx, and GR, and did not marked elevation in levels of ALT, AST, and ALP in blood serum. Simultaneous realization of resistance exercise training did not alter the effects of Su administration. The present results support the previous study [14] reporting that AAS abuse for 8 weeks, either with or without concurrent exercise training, can increase the activities of the antioxidant enzymes in liver tissue.

A remarkable increase in the activities of SOD, GPx, and GR in liver from both sedentary and trained Su-treated rats means that oxidative stress occurred to some extent. How abuse of Su could be associated with an excessive free radical production is still unknown, but the two available hypotheses support the concept that AAS could lead to an increase in the production of oxygen free radicals in liver. One hypothesis involves the mitochondrial electron transport chain dysfunction. A continuous and prolonged abuse of AAS provokes a decrease in

Table I Effect of resistance training (RT) and sustanon (Su) treatment on body and liver weights in male rats

	Su		Non-Su		*P* values		
Parameter	RT	Sedentary	RT	Sedentary	RT	Su	RT × Su
Body weight (g)	423.19 ± 16.74	432.88 ± 21.86	420.66 ± 19.405	430.47 ± 23.08	0.206	0.582	0.863
Liver weight (g)	12.50 ± 1.37	12.97 ± 1.22	12.44 ± 1.16	12.72 ± 1.47	0.384	0.712	0.821

Values are expressed as mean ± standard deviation ($n = 10$)

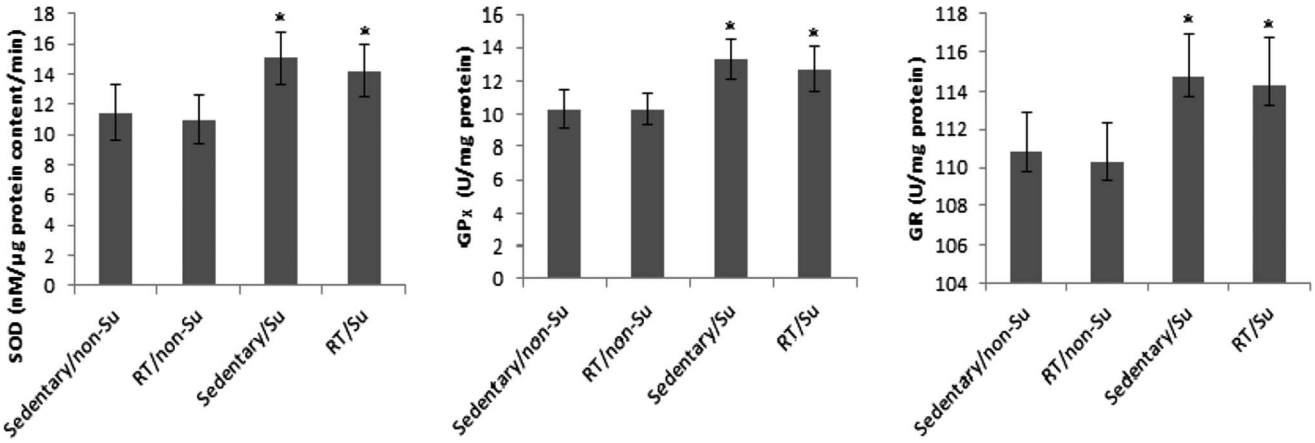

Fig. 2. Effects of resistance training (RT) and sustanon (Su) treatment on rat liver antioxidant enzyme activities. SOD, superoxide dismutase; GPx, glutathione peroxidase; GR, glutathione reductase. Values are expressed as mean ± standard deviation ($n = 10$). *Statistically significant difference compared with non-Su groups

Table II Effect of resistance training (RT) and sustanon (Su) treatment on serum parameters in male rats

	Su		Non-Su		*P* values		
Parameters	RT	Sedentary	RT	Sedentary	RT	Su	RT × Su
ALT (U/L)	46.20 ± 10.41	47.50 ± 11.75	40.10 ± 12.97	42.30 ± 12.52	0.646	0.179	0.702
AST (U/L)	120.7 ± 21.09	119.1 ± 17.22	115 ± 18.55	117.40 ± 16.94	0.946	0.531	0.735
ALP(U/L)	102 ± 16.11	103.5 ± 17.78	96.5 ± 14.33	93.7 ± 20.75	0.908	0.179	0.702

Values are expressed as mean ± standard deviation ($n = 10$). ALT: alanine aminotransferase; ALP: alkaline phosphatase; AST: aspartate aminotransferase

the activity of the mitochondrial respiratory chain complexes as well as degenerative changes of the mitochondria [23, 24]. Thus, dysfunction of this chain could result in overproduction of ROS exceeding antioxidant defenses. A dysfunction of cytochrome P450 (CYP) oxidase systems is another hypothesis used to explain an increase in free radicals. It was shown that hepatic CYP isoforms release ROS during their catalytic cycles contributing significantly to the total cellular production of reactive oxygen in rat's liver even under basal conditions [25]. Therefore, metabolism by CYP monooxygenases of the continuous and prolonged abuse of Su would increase greatly the production of ROS and the resulting oxidative stress.

The activity of antioxidant enzymes remained unchanged in the liver of trained rats, and no significant changes were observed in the levels of these factors between sedentary and trained rats. Therefore, it is indicated that the increase of antioxidant activity was not due to the resistance exercise training. This observation is consistent with previously reported results [14, 26–28]. However, there are conflicting reports of detecting decrease [29] or increase [29–31] in the liver antioxidant activity in response to exercise training. The reason for these apparent discrepancies is not clear, but it could be related to the differences in the intensity and/or duration of the exercise sessions employed in the aforementioned studies. A single bout of intense and/or prolonged exercise can lead to an acute oxidative stress in the liver [16, 31–33]; therefore, exercise stimulus throughout the training period could activate the synthesis of antioxidant enzymes as a long-term strategy to cope with the encountered oxidative stress during exercise sessions. The RT session used in this experiment likely possesses minimal oxidative stress to the liver, probably owing to its high-intrinsic antioxidant capacity.

In this study, classical serum parameters (ALT, AST, and ALP) were not increased in the liver of either sedentary or trained rats after Su treatment. Our results are in agreement with previous studies showing that prolonged administration of high doses of AAS results in minor and infrequent alterations of these parameters [14, 34, 35]. In this regard, experimental evidence obtained in controlled studies indicated that conventional biochemical liver tests would not always reflect liver abnormalities, particularly at the initial stages, and it should be kept in mind that the liver function tests, in spite of their name, would not measure the liver function in any quantitative sense; they are concerned rather with severe liver damage [14, 24, 36].

On the other hand, previous studies have indicated that hepatotoxic effects of steroids were associated with 17α-alkylation of the molecules [9]. The 17α-alkylated steroids prevent deactivation by the first-pass metabolism, sterically hindering oxidation of the 17β-hydroxyl group. Therefore, biochemical structure could be related to hepatotoxicity, in addition to the obvious fact that 17α-alkyl steroids are mainly taken orally compared with 17β-hydroxyl steroids (injectable testosterone) in high dosages, which potentially damage liver cells due to the high steroid load (first-pass effects) [5, 6]. The Su is an oil-based injectable testosterone blend and it has a slow absorption rate into the blood stream, so that the liver experiences a low concentration of the drug compared with the substance taken orally.

A limitation of this study was that we did not evaluate oxidative stress markers and histopathological parameters. In addition, using several doses of Su was considered to be more appropriate in this study. With regard to the observed undesirable effects of Su, it is greatly recommended to investigate the side effects of Su and its optimal dose in future human studies on people who take Su. On the other hand, the strong point of this study was its sample size. A total of 10 rats in each group would decrease the rate of error.

According to the results of this study, it can be concluded that 8-week IM injection of Su in supra-physiological dose, either with or without concurrent RT upregulation of enzymatic antioxidant activities and resistance exercise training, did not attenuate the production of ROS due to the Su administration. Furthermore, Su abuse in this dose did not make any severe liver damage because the liver function tests did not cause any significant increase. These data support the finding that liver function tests do not always reflect liver abnormalities particularly at the initial stages. Overall, our results suggest that the development of hepatic oxidative stress caused by Su abuse is not attenuated by RT.

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Authors' contribution: HA designed the study. HA and SR prepared the manuscript. SR gathered data and searched the literature. HG analyzed data. All authors read and approved the final form.

Conflict of interest: The authors declare no conflict of interest regarding this paper.

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