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RESEARCH ARTICLE

Reproprotective Effects of Moderate Exercise in Male Rats Doped with Testosterone Enanthate

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Abstract

A total of forty adult Sprague-Dawley male rats were used to study the ameliorative effects of moderate exercise on reproductive function in male rats doped with a high dose of anabolic steroids. Rats were randomly assigned into 4 groups; sedentary, sedentary doped (I/M 25 mg/kg BW per week), moderately exercised (20 minutes/day for five days every week along one month) and moderately exercised doped groups. The results clarified that the moderate exercise significantly increased sperm motility (91.67%±1.67); live sperm and sperm count percentages (93.33%±1.67 & 115.33%±7.51 respectively) and lowered sperm abnormalities (11.0±3.61). On contrary, doping induced via testosterone administrations in both sedentary and moderately exercised rats significantly decreased the previously mentioned sperm parameters (63.33%±3.33, 53.33%±3.33 & 65.33%±9.45 respectively) while increasing sperm abnormalities (53.33±11.50); although amelioration via exercise was noticed in these parameters. Serum free testosterone levels were significantly increased with both sedentary doped or moderately exercised doped groups. Moreover, superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) activities were significantly elevated with moderate exercise (52.05±6.67, 39.25±7.09 and 69.17±7.69 respectively). While, malondialdehyde (MDA) levels were increased with doping of male rats (86.5±9.99). Furthermore, StAr gene expression was significantly ($P \leq 0.01$) up-regulated with moderate exercise and significantly ($P \leq 0.01$) down-regulated with doping of male rats. In addition, HSD17B3 gene expression was significantly ($P \leq 0.01$) up-regulated in both moderately exercise and moderately exercise doped groups. On contrary, HSD17B3 gene expression was significantly ($P \leq 0.01$) down-regulated in sedentary doped group Overall, these results suggest a beneficial and protective effect of moderate treadmill exercise on male fertility especially in steroid abuse models.

Keywords: Testosterone enanthate; Doping; StAr; HSD17B3; Moderate exercise

Introduction

Various synthetic derivatives of testosterone have been developed, Anabolic Androgenic steroids (AAS), mainly to promote skeletal muscle growth (anabolic effect) and to potentiate the development of male sexual characteristics (androgenic effects). Illegally, these drugs are regularly self-administered by power lifters and bodybuilders to augment their performance. Anabolic Androgenic steroids abuse has been reported to be associated with different side effects including both endocrine and metabolic [1]. Yet, their potent effect in the male reproductive function

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remains not fully understood. Exercise refers to any planned moderate activity which can be aerobic exercise, resistance training or combination [2]. Moderate exercise is also a proper approach for promoting good health [3]. The need to design appropriate exercise program to attain maximal benefit at the lowest levels of risk has been recommended [4]. Several exercise regimen parameters including the type and intensity of moderate activity governs its final beneficial or detrimental effects [5]. Yet, the full extent of these effects remains understudied. In males, a moderate exercise regime possesses a potential positive effect on our bodily physiological systems, particularly, the gonads and eventually fertility [6].

Androgens are essential for a proper development of male reproductive organs, puberty, fertility and sexual function in males. They have been implicated as having an important role in many vital processes other than regulating the reproductive function [7-9]. Testosterone (T) and 5α-dihydrotestosterone (DHT) are considered the main androgens, testosterone is synthesized primarily by the Leydig cells in the testes and can be converted into DHT in a reaction catalyzed by5-alpha-reductase enzyme [10, 11] are vital for accurate spermatogenesis [12]. 5α-dihydrotestosterone is considered the most active form of endogenous androgens, although less abundant; it is more biologically active than testosterone [13, 14]. Androgens mediate their different actions via binding to the androgen receptor (AR), a single nuclear receptor [15]. The AR is a member of the steroid hormone group including the estrogen receptor (ER), glucocorticoid receptor (GR), progesterone receptor (PR) and mineralocorticoid receptor (MR) [15, 16]. Other types of androgens can be synthesized locally in the zona reticulata and zona fasciculata of the adrenal cortex including dehydroepiandrosterone (DHEA) and androstenedione [17].

The synthesis of testosterone in Leydig´s cell is controlled by stimulation of the release of the luteinizing hormone (LH) from the pituitary gland in response to gonadotropin-releasing hormone (GnRH) release from the hypothalamus [18]. The LH initiates the process of steroidogenesis by promoting the steroidogenic acute regulatory protein (StAR) expression that enhances the inner mitochondrial uptake of cholesterol [18, 19]. This cholesterol is used to produce pregnenolone that is converted to DHEA, that produces androstenediol and androstenedione that produces testosterone via reaction catalyzed by cholesterol side chain cleavage P450 (CYP11A1), 3-β-hydroxysteroid dehydrogenase, 17-α-hydroxylase/17, 20-lyase P450 (CYP17A1), and 17-β hydroxysteroid dehydrogenase type III (HSD17-β3) [20-23]. Once produced, circulating testosterone is mostly bounded to serum sex hormone-binding globulin (SHBG) and albumin [24].

The main objective of the current work was to study the effect of doping with anabolic steroids such as high dose of testosterone enanthate on reproductive performance in adult male rat and the ameliorative effect of moderate exercise on such model through assessment of semen parameters, testosterone level in serum, the activity of antioxidant and lipid peroxidation markers and gene expression of StAr and HSD3B2 in testicular tissue of male rats.

Materials and Methods

Experimental animals and design

A total of forty adult Sprague-Dawley male rats weighing about 300±20 g were used. All experimental procedures were approved by the institutional Animal Care and Use Committee of Zagazig University under approval number (ZU-IACUC/2/F/119/2019). The rats were adapted for two weeks before initiating the experiment. During this period the rats were adapted for the treadmill training. They were housed at 24 oC and were fed on standard pelleted feed ad-libitum with free access to water.

Experimental design

After acclimatization period, rats were randomly assigned into four groups (10 rats/group); Sedentary group; rats were treated with 1 mL vehicle /kg BW per week for one month and kept in a stationary treadmill for 20 minutes/day for five days/week for one month. sedentary doped group, rats were treated with an intramuscular injection of 25 mg

testosterone enanthate (sigma Aldrich)/ kg BW per week for a month as [25], moderately exercised group, rats received a training session using a treadmill lasted for 20 minutes/day for five days/week for one month [26]. The treadmill speed was maintained at 14 m/min with no inclination [27] and moderately exercised doped group, the rats were treated with testosterone enanthate as described in the sedentary doped group and receive moderate exercise as described in the moderately exercised group. The exercise protocol was initiated daily at 9:30 am.

Sampling

Rats were sacrificed via cervical dislocation twenty-four hours following the last exercise session. The collected blood samples were left to clot then centrifuged at 3000 rpm for 15 min. Serum was separated and immediately stored at -20 oC until further biochemical analysis. Testes were immediately excised and approximately 30 mg of testicular tissue from each rat were immersed into liquid nitrogen before transferring to be stored at -80 oC for subsequent gene expression analysis. Another 0.5 gm of testicular tissue was homogenized, centrifuged at 4000 rpm for 15 min and the supernatants were separated in dry falcon tubes and stored at -20 oC for determination of antioxidant enzyme activities.

Semen evaluation and sperm parameters

The caudal part of epididymis was removed and transferred into a clean Petri dish containing normal saline maintained at 37 °C. The epididymis was cut to allow diffusion of its content into warm saline. The individual sperm motility was subjectively evaluated using the high power of a light microscope. The live/dead ratio was evaluated using eosin nigrosine stain. The sperm cell concentration was evaluated using a hemocytometer. The aforementioned stained smears were examined under oil emersion lens for estimating the percentage of sperm abnormalities in 100 spermatozoa. All procedures were applied according to the previously described methods [28-30].

Biochemical and hormonal measurement

The activities of Superoxide dismutase (SOD), Glutathione peroxidase (GPx) and Catalase (CAT) activity, lipid peroxidation marker (Malondialdehyde; MDA) concentration and the concentration of serum free testosterone were measured as previously reported in [31-34] using the commercially available ELISA kit from MyBioSource (San Diego, CA, United States) according to the manufacturer's instructions.

Real time PCR

The real time analysis has been applied according to the methods previously reported [31, 33]. Briefly, total RNA was extracted from 30 mg of rat testis with Trizol reagent (ThermoFisher Scientific; Waltham, MA, United States) according to manufacturer's instructions. Two-step real-time PCR was used to evaluate gene expression. In brief, cDNA synthesis using a HiSenScript™ RH (-) cDNA Synthesis Kit (iNtRON Biotechnology Co., South Korea) was carried out in a Veriti 96-well thermal cycler (Applied Biosystems, Foster City, CA) and then followed by Real time PCR using 5x HOT FIRE Pol EvaGreen qPCR Mix Plus (Solis BioDyne, Tartu, Estonia). All primers were synthesized by Sangon Biotech (Beijing, China) as shown in Table 1. The real time PCR cycling conditions were consisted of initial denaturation at 95 °C for 12 min, followed by 40 cycles of denaturation at 95 °C for 30 sec, annealing at 60 °C for 30 sec, and extension at 72 °C for 30 sec. The relative expression level of the target genes was normalized to that of the housekeeping Gapdh, and the relative fold changes in gene expression were calculated based on the $2^{-\Delta\Delta CT}$ comparative method [35].

Table 1: Oligonucleotide primers sequences used for real time PCR

	Forward primer (5′–3′)	Reverse primer (5′–3′)	Accession No
StAr	CCCAAATGTCAAGGAAATCA	AGGCATCTCCCCAAAGTG	NM_031558.3
HSD17B3	AGTGTGTGAGGTTCTCCCGGTACCT	TACAACATTGAGTCCATGTCTGGCCAG	NM_054007.1
Gapdh	GGCACAGTCAAGGCTGAGAATG	ATGGTGGTGAAGACGCCAGTA	NM_017008.4

StAr; the steroidogenic acute regulatory protein, HSD17B3; 17β-Hydroxysteroid dehydrogenase 3 and Gapdh; Glyceraldehyde 3-phosphate dehydrogenase

Statistical analysis

The data was presented as mean ± standard error of the mean (SEM). The statistical significance was evaluated by one-way ANOVA and a post hoc Duncan's multiple range test using statistical package of SPSS (version 18.0 for Windows). $P < 0.05$ was considered statistically significant. Figures were generated using GraphPad Prism 8 software (San Diego, CA, United States).

Results

Effect of doping and/or moderate exercise on semen parameters

Doping in sedentary rats was associated with significant ($P< 0.01$) reduction in sperm motility percent (63.33±3.33), live ratio percent (53.33±3.33) and sperm count (65.33±9.45) as well as a significant elevation in abnormality percent (53.33±11.50) compared to sedentary rats (86.67±1.67, 88.33±1.67, 108.33±6.03 and 13.33±2.08) or moderately exercised rats (91.67±1.67, 93.33±1.67, 115.33±7.51 and 11.0±3.61) correspondingly. Moreover, application of moderate exercise in doped rats improved the semen parameter (78.33±1.67, 66.67±4.41, 80.33±5.13 and 32.67±3.51) compared to doped sedentary group, but it failed to restore the control level (Table 2).

Effect of doping and/or moderate exercise on serum free testosterone level

A significant ($P< 0.01$) increase in the serum level of free testosterone (ng/ml) was recorded in doped rats either with (18.38 ± 1.36) or without (17.23 ± 2.08) moderate exercise compared to sedentary rats (7.18 ± 0.34) or moderately exercised rats (8.68 ± 1.01) (Table 3).

Table 2: Effect of doping and/or moderate exercises on semen parameters

Treatment	Sperm motility%	Live ratio%	Sperm Count (sperm cell concentration x 125 x 104)	Sperm abnormalities%
Sedentary	$86.67±1.67^{a}$	$88.33±1.67^{a}$	$108.33±6.03^{a}$	$13.33±2.08^{c}$
Sedentary + Doping	$63.33±3.33^{c}$	$53.33±3.33^{c}$	$65.33±9.45^{c}$	$53.33±11.50^{a}$
Moderate exercise	$91.67±1.67^{a}$	$93.33±1.67^{a}$	$115.33±7.51^{a}$	$11.0±3.61^{c}$
Moderate exercise + Doping	$78.33±1.67^{b}$	$66.67±4.41^{b}$	$80.33±5.13^{b}$	$32.67±3.51^{b}$
P-value	< 0.001	< 0.001	< 0.001	< 0.001

Mean values with different superscript letters in the same column are statistically significant from each other at level **(p<0.05).**

Table 3: Effect of doping and/or moderate exercises on free testosterone level.

Treatment	Testosterone free (ng/ml)
Sedentary	$7.18 ± 0.34^{b}$
Sedentary + Doping	$17.23 ± 2.08^{a}$
Moderate exercise	$8.68 ± 1.01^{b}$
Moderate exercise + Doping	$18.38 ± 1.36^{a}$
P-value	< 0.01

Mean values with different superscript letters in the same column are statistically significant from each other at level **(p<0.05).**

Effect of doping and/or moderate exercise on antioxidant activity and lipid peroxidation markers in testicular tissue

moderate exercise was associated with a significant (P< 0.01) increase in the concentrations of SOD (U/g tissue), GPx (µmol NADPH/g tissue) and CAT (µmol H_2O_2decomposed/g tissue) and a significant decrease in MDA (nmol/g tissue) (52.05±6.67, 39.25±7.09, 69.17±7.69and 31.67±5.86) compared to sedentary -non doped rats (30.37±2.67, 29.62±2.43 56.77±5.58and 41.0±15.09). On contrary, doping significantly (P< 0.01) decreased the concentrations of SOD, GPx and CAT and significantly increased the MDA (13.35±2.89, 15.45±3.38, 35.33±8.69 and 86.5±9.99) compared to sedentary rats. Moderate exercise of doped rats significantly (P< 0.01) increased the level of SOD and GPx but not CAT as well as significantly decreased the MDA (21.30±3.96, 24.33±3.66, 34.65±5.05and 59.17±20.05) compared to sedentary doped rats (Table 4).

Table 4: Effect of doping and/or moderate exercises on antioxidant and lipid peroxidation markers in testicular tissues

Treatment	Antioxidant			Lipid peroxidation
	SOD (U/g tissue)	GPx (µmol NADPH/g tissue)	CAT (µmol H_2O_2decomposed/g tissue)	MDA (nmol/g tissue)
Sedentary	30.37±2.67^{b}	29.62±2.43^{b}	56.77±5.58^{a}	41.0±15.09^{c}
Sedentary + Doping	13.35±2.89^{d}	15.45±3.38^{d}	35.33±8.69^{b}	86.5±9.99^{a}
Moderate exercise	52.05±6.67^{a}	39.25±7.09^{a}	69.17±7.69^{a}	31.67±5.86^{c}
Moderate exercise + Doping	21.30±3.96^{c}	24.33±3.66^{c}	34.65±5.05^{b}	59.17±20.05^{b}
P-value	< 0.001	< 0.001	< 0.001	< 0.001

Mean values with different superscript letters in the same column are statistically significant from each other at level (p<0.05).

Effect of doping and/or moderate exercise on the relative expression of StAr and HSD17β3 in testicular tissues of male rats

Figure 1 shows that the relative expression of StAr and HSD17β3 was significantly (P≤0.01) up-regulated in moderately exercised rats compared to the sedentary group. On contrary, doping significantly (P≤0.01) down-regulated their expression level compared to sedentary rats. Application of moderate exercise in doped rats significantly up-regulated the relative expression of HSD17B3 (P≤0.01) but not StAr compared to the doped rats without exercise.

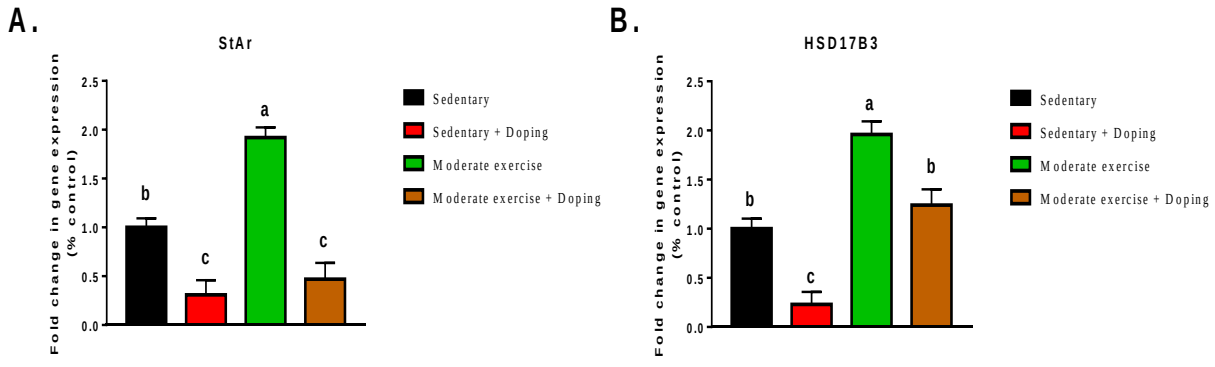

Figure 1: Effect of doping and/or moderate exercises on mRNA expression of (A) StAr and (B) HSD17B3 in testicular tissues of male rats.

Discussion

The results obtained have clearly demonstrated that moderately exercised groups had a highly significant increase in sperm motility, sperm live and sperm count percentages. On contrary, doping induced by testosterone administrations significantly reduced the sperm motility, sperm viability and sperm cell concentration and increased the sperm cell abnormalities. Based on the contraception of the oxidative indicators in doped rats, the underlined mechanism may include overproduction of free radicals and oxidative stress. Aziz *et al*. [36] have reported a negative correlation between reactive oxygen species production and sperm morphology.

Contradictory to our results, the findings of Arisha and Moustafa [32] observed that following intensive exercised in rodents, major reductions in sperm count, motility and viability ($p < 0.05$). This might be due to the prolonged duration exercise protocol, non the less, swimming exercise is more stressful than treadmill training. Moreover, sperm abnormalities percentages were significantly elevated with steroid abuse in male rats. Overall, several sperm parameters were clearly impacted by the level of oxidative stress [37]. Also, the levels of ROS correlate with sperm motility negatively [38]. The free radical-induced oxidative stress contributes significantly in producing and increasing abnormal sperm and decreasing sperm count and transformation and fragmenting sperm DNA [39]. These changes in sperm DNA result in infertility. In this regard, incubation of spermatozoa under high oxygen pressure reduces the rate and motility of sperm; however, adding catalase to the culture medium prevents this effect [40]. The results obtained showed that testosterone free levels were significantly increased in sedentary doped or moderately exercised doped groups. On the other hand, moderate exercise itself, although a tendency present, did not significantly increased the level of free testosterone.

The results obtained may be attributed to a significant increase in the level of leptin. Isidori *et al.* [41] reported, in men, a correlation between the circulating levels of leptin with total and free testosterone, however still there is a need for further investigation.

Kraemer *et al.* [42] reported a marked increase in testosterone levels in men before, during, and after heavy exercise. Interestingly, the mechanisms underlying the exercise-induced increase in testosterone levels are not fully understood, although the intensity of exercise might be a contributing factor. In male rats, Lu *et al.* [43] referred to a partial role of direct simulation and LH-independent effect of lactate on testosterone secretion in the testis, which can markedly improve testosterone levels during exercise.

The results obtained, in this study, clarified that CAT, SOD and GPx activities were significantly elevated in moderately exercised male rats. On contrary, the lower levels of these antioxidant markers were present with doping in male rats. This oxidative status was associated with an increased production of ROS or decreased antioxidant activities and elevated lipid peroxidation in leydig cells and germ cells [44].

Lipid peroxidation via oxidation of polyunsaturated fatty acids is induced by ROS [45]. Measurement of the MDA level, an end product of lipid peroxidation, indicates the extent of oxidative damage. Moreover, our results revealed that malondialdehyde levels were significantly increased with doping of male rats and decreased in moderately exercised groups of male rats. Interestingly, intensive swimming in rats increased lipid peroxidation and reduced the SOD, CAT and GPx activity [32, 46].

Testicular leydig cells are responsible for the biosynthesis of androgens. Several endocrine disruptors can potentially impact biosynthesis and metabolic activation of testosterone and results in sexual dysfunction, infertility or even sterility. The aforementioned endocrine disruptors directly suppressthe enzymatic activities of CYP11A1, CYP17A1, HSD3B and HSD17B3 in leydig cells resulting in a reduction in testosterone production [47]. Regarding StAr gene expression, it was significantly up-regulated ($P \leq 0.01$) in moderately exercised group of rats. On

contrary, StAr gene expression was significantly down-regulated ($P \leq 0.01$) in doped male rats. In addition, HSD17B3 gene expression was significantly up-regulated ($P \leq 0.01$) in moderately exercised rats. On contrary, HSD17B3 gene expression was significantly down-regulated ($P \leq 0.01$) with doping of male rats. These changes in gene expression levels might be associated with a free radical associated damage of essential cellular elements, receptor activity disturbance, proliferation inhibition, cell death or immunity perturbation [48] following steroid abuse. On the other hand, moderate exercise could increase the antioxidant enzymes activity and catalyze the decomposition of ROS [49].

Conclusions

Following an appropriate moderate exercise regime can be helpful for maintaining fertility even under conditions of individual abuse of steroids.

Conflict of Interest

The authors declare no conflicts of interest, financial or otherwise.

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الملخص العربي

التأثيرات الوقائيه للتدريب المعتدل علي التناسل في ذكور الفئران التي تتعاطي هرمون الذكورة

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تم استخدام مجموعه مكونة من ٤٠ من ذكور الفئران البالغين، لدراسة تأثير تناول المنشطات مثل التستوستيرون إينونثات بجرعة عالية وكذلك لدراسة مدى قدره النشاط البدني على تحسين اداء الجهازالتناسلي في ذكور الفئران التي تتعاطي هرمون التستوستيرون. تم تقسيم الفئران الي مجموعه الفئلان المستقره و المتعاطيه و المدربه و المتعاطيه المدربه. وقد أظهرت النتائج أن التمرين الجسدى قد زاد بشكل كبير من حركة الحيوانات المنوية(91.67%±1.67)، ونسبة الحيوانات المنوية الحية والنسب المئوية لعدد الحيوانات المنوية(115.33%±7.51 & 93.33%±1.67) وكذلك أدى الى انخفاض تشوهات الحيوانات المنوية(11.0±3.61). على العكس من ذلك ، أدى تعاطي المنشطات مثل الجرعات العاليه من التستوستيرون في كل من الفئران المستقرة والممارسة للرياضة بشكل معتدل إلى انخفاض معاملات الحيوانات المنوية بشكل كبير (63.33%±3.33, 53.33%±3.33 & 65.33%±9.45) مع زيادة تشوهات الحيوانات المنوية (53.33±11.50)؛ على الرغم من التحسن من خلال ممارسة الرياضة الذى لوحظ. وكذلك تحسنت بشكل ملحوظ مستويات هرمون تستوستيرون مع كل من المنشطات و/ أو ممارسة رياضة معتدلة. علاوة على ذلك ، فقد تحسنت بشكل ملحوظ أنشطة انزيمات ديسموتاز الفائق الأكسيد (SOD) والجلوتاثيون بيروكسيديز (GPx) والكاتالاز (CAT) من خلال التمرين المعتدل لدى الفئران الذكور (52.05±6.67, 39.25±7.09& 69.17±7.69). في حين حدث زيادة فى مستويات المالون داى الدهايد مع المنشطات(86.5±9.99) في الفئران الذكور. بالنسبة إلى التعبير الجيني لـ StAr ، فقد تم زيادة له ($P\leq0.01$) في التمرين المعتدل، كما انخفض ($P\leq0.01$) مع تعاطي المنشطات من الذكور. بالإضافة إلى ذلك، ازداد التعبير الجيني لـ HSD17B3 ($P\leq0.01$) في كل من التمرين المعتدل والتمرين المعتدل ومجموعة المنشطات. على العكس من ذلك ، انخفض التعبير الجيني HSD17B3 ($P\leq0.01$) في كل من مجموعة الفئران المستقرة والفئران المنشطة.