

# Determination of DNA damage and telomerase activity in stanozolol-treated rats

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**Abstract.** Anabolic androgenic steroids (AAS) are performance-enhancing drugs commonly abused by athletes. Stanozolol is a synthetic testosterone-derived anabolic steroid. Although it is well known that AAS have several side-effects, there are only few toxicological studies available on the toxic effects and mechanisms of action of stanozolol. The aim of this study was to investigate the genotoxic effects of stanozolol and to determine its effects on telomerase activity in Sprague-Dawley male rats. For this purpose, 34 male rats were divided into 5 groups as follows: i) the control group (n=5); ii) the propylene glycol (PG)-treated group (n=5); iii) the stanozolol-treated group (n=8); iv) the PG-treated group subjected to exercise (n=8); and v) the stanozolol-treated group subjected to exercise (n=8). PG is used as a solvent control in our study. Stanozolol (5 mg/kg) and PG (1 ml/kg) were injected subcutaneously 5 days/week for 28 days. After 28 days, the animals were sacrificed, and DNA damage evaluation (comet assay) and telomerase activity assays were then performed using peripheral blood mononuclear cells (PBMCs). Telomerase activity was measured by using the TeloTAGGG Telomerase PCR ELISA PLUS kit. The results of this study revealed that stanozolol treatment induced DNA damage, while exercise exerted a protective effect. Stanozolol treatment without exercise stimulation was

associated with a significant increase in telomerase activity in the PBMCs.

## Introduction

Anabolic androgenic steroids (AAS) are synthetic testosterone derivatives, which are widely abused by athletes for enhancing performance and psychological well-being (1,2). These drugs have been abused for many years by athletes and teenagers for the purpose of performance enhancement (3,4).

The chemical structure of steroids, dosage and usage frequency are the main factors responsible for the long-term side-effects of AAS. At supraphysiological doses, several side-effects have been reported due to the use of AAS. These effects include the disruption of lipid metabolism, high blood pressure, cardiac disorders, testicular atrophy, erectile dysfunction, impotence, decreased sex hormone levels, menstrual irregularities, clitoral enlargement, peliosis hepatis, neoplasia, cholestasis, jaundice, acne, alopecia, aggressiveness, depressive symptoms, manic symptoms and psychosis (5). According to worldwide testing data, it has been demonstrated that testosterone, nandrolone, methandienone and stanozolol are the most commonly abused steroids (6).

Stanozolol is a synthetic 17 $\alpha$ -alkylated derivative of testosterone, with slow hepatic degradation (7). It is medically used for the treatment of anemia and hereditary angioedema (8). Stanozolol has the largest anabolic/androgenic ratios (9). It is known that stanozolol abuse causes a wide range of adverse effects (10). The 'Association of Racing Commissioners International' classified stanozolol as a class III drug (11). According to the '2016 Prohibition List' prepared by the World Anti-Doping Agency (WADA), stanozolol is classified as class S1.1a and it has been prohibited both in and out of competition (12). However, due to the widespread usage of stanozolol, its metabolites have been found to be present in 50% of positive doping cases (10,13). 3'-Hydroxystanozolol, 4 $\beta$ -hydroxystanozolol and 16 $\beta$ -hydroxystanozolol are the main three metabolites of stanozolol. The acceptable urinary concentrations of stanozolol and/or its metabolites are restricted to a maximum of 2 ng/ml (13). The consumption steroid hormone

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**Abbreviations:** AAS, anabolic androgenic steroids; EB, ethidium bromide; LMA, low melting agarose; NMA, normal melting agarose; PBMCs, peripheral blood mononuclear cells; SD, standard deviation; WADA, World Anti-Doping Agency

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drugs is associated with toxicity, mutagenicity, genotoxicity and cancerogenesis, depending on several factors, including genetic and epigenetic modifications (14). Torres-Bugarín *et al* reported micronucleus (MN) frequency increase in buccal mucosa cells from bodybuilders who abused AAS (15). However, Hana *et al* indicated that testosterone synthetic derivatives had no genotoxic effects on mouse germ cells (16). Controversial results have been obtained regarding the genotoxic effects of steroids (17,18).

Telomeres play a key role in cell aging and DNA damage mechanisms. The loss of telomere function causes several adverse effects, such as genomic instability, aneuploidy and even cancer. It has been reported that tumor tissues express elevated levels of telomerase compared to normal tissues (19). Several parameters can affect telomere and telomerase functions, including oxidative stress, DNA damage, dietary supplementations and environmental/occupational exposures (20,21). The inhibition of telomerase activity is known to be associated with genomic instability, and plays an important role in DNA damage mechanisms and cancer development. It has been demonstrated that the expression of telomerase enzyme protects cells from DNA-damaging events, and it has been shown that this protective function is independent from its telomere synthesis function (22).

In this study, we investigated the DNA-damaging potential of stanozolol and its effects on telomerase activity in stanozolol-treated Sprague-Dawley rats. The role of exercise in DNA damage and telomerase activity was also assessed, as the main target population of stanozolol users are usually physically active individuals.

## Materials and methods

**Animal Study.** A total of 34 Sprague-Dawley male rats were divided into 5 groups as follows: i) the control group (n=5); ii) the propylene glycol (PG)-treated group (n=5); iii) the stanozolol-treated group (n=8); iv) the PG-treated group subjected to exercise (n=8); and v) the stanozolol-treated group subjected to exercise (n=8) (Table I). The mice were injected with 1 ml/kg PG as a solvent control. Stanozolol (5 mg/kg) was injected subcutaneously for 28 days for 5 days per week. Swimming was selected as a mode of exercise. The mice were subjected to exercise (swimming) 1 week prior to the commencement of the treatments so that the animals may be able to adapt. During the 28 days of the experiment, the animals subjected to 20 min of swimming for 5 days per week. After 28 days, the animals were lightly anesthetized and euthanized by cervical dislocation. Peripheral blood samples were collected from the tail vein of the rats for the determination of telomerase activity and DNA damage by comet assay. Peripheral blood mononuclear cells (PBMCs) were isolated by using Histopaque 107 (MP Biomedicals LLC, Solon-Ohio, USA). Heparin-treated blood was diluted with an equal volume of PBS and layered carefully over density gradient media and centrifuged at 200 x g for 3 min at 4°C. During centrifugation, cells migrate differentially and layers are formed. PBMCs were retrieved from just above the boundary between plasma and Histopaque. Comet assay and telomerase activity assays were all performed using the PBMCs. Cell viability was examined by the trypan blue exclusion assay before performing comet assay and the viability results were >90% for each sample (data not shown).

Table I. Experimental groups in this study.

Animal groups <sup>a</sup>	No. of rats	Procedures undertaken
Group I	5	No injection, no exercise
Group II	5	Subcutaneous injection of 1 ml/kg PG
Group III	8	Subcutaneous injection of 5 mg/kg stanozolol
Group IV	8	Subcutaneous injection of 1 ml/kg PG, exercise 20 min/day, 5 day/week (swimming)
Group V	8	Subcutaneous injection of 5 mg/kg stanozolol, exercise 20 min/day, 5 day/week (swimming)

<sup>a</sup>Group I, control; group II, PG; group III, stanozolol; group IV, PG treatment and exercise; group V, stanozolol treatment and exercise; PG, propylene glycol.

Rat care, handling, and all of the experimental procedures employed were in accordance with the instructions of the Ethics Committee on Animal Experimentation of Istanbul University, HADYEK and approved with no. 2013/100.

**Comet assay.** The alkaline comet assay was performed as previously described by Singh *et al* (23) with slight modifications, as described in Ozcagli *et al* (24). Each microscopic slide was covered with 0.65% normal melting agarose (NMA). Histopaque density gradient centrifugation was used for lymphocyte isolation. The isolated cells were mixed with 0.65% low melting agarose (LMA) and embedded on NMA-covered slides. The slides were incubated overnight in cold lysing solution (1% Triton X-100 and 10% DMSO added just before use in 2.5 M NaCl, 100 mM Na<sub>2</sub>EDTA, 10 mM Tris, pH 10) at 4°C. Subsequently, an electrophoresis step was performed with (1 mM Na<sub>2</sub>EDTA and 300 mM NaOH, pH 13) fresh solution following alkali exposure to 20 min for DNA unwinding. The electrophoresis step was conducted at 1.6 V/cm for 20 min (300 mA) at 4°C. Prior to ethidium bromide (EB) dye staining, the alkali conditions were neutralized with tris buffer (0.4 M Tris, pH 7.5). All these steps were conducted under dimmed light and the slides were evaluated by one slide reader, blindly. One hundred cells per slide were analyzed under a fluorescence microscope equipped with an excitation filter of 546 nm and a barrier filter of 590 nm (Olympus BX53; Olympus Corp., Tokyo, Japan). The results were evaluated using the Comet Assay IV image analysis system (Comet Assay IV; Perceptive Instruments Ltd., Suffolk, UK) in order to score DNA damage. Tail intensity data was selected for the evaluation of DNA damage.

**Telomerase activity assay.** The determination of telomerase activity in the PBMCs was performed quantitatively using the teloTAGGG telomerase PCR ELISA PLUS kit (Roche Diagnostic GmbH, Mannheim, Germany). The kit protocol was followed for telomerase activity assessment as previously described (25). A total of 2x10<sup>5</sup> cells were transferred to a PCR tube for sample preparation for telomerase activity assay. Following centrifugation at 3,000 x g for 10 min, the supernatant was carefully removed and the pellets were collected. The assay

Table II. Expression of tail intensity and telomerase activity (%) in the peripheral blood mononuclear cells from the rats in the experimental groups.

Assay	Exposure <sup>a</sup> group	No. of rats	Mean ± standard deviation	Median	Kruskal-Wallis
Tail intensity	Group I	5	4.0±0.5	4.3	$\chi^2=10.56$ df=4 P=0.032
	Group II	5	4.8±2.8	3.9	
	Group III	8	5.8±1.6	0.5	
	Group IV	8	5.1±1.7	4.85	
	Group V	8	3.5±0.6	3.6	
Telomerase activity (%)	Group I	5	100.0±19.4	93.3	$\chi^2=24.65$ df=4 P<0.001
	Group II	5	474.9±358.2	597.0	
	Group III	8	1,851.4±319.2	1,909.2	
	Group IV	8	193.4±27.9	196.5	
	Group V	8	328.7±183.1	240.0	

<sup>a</sup>Group I, control; group II, PG; group III, stanozolol; group IV, PG treatment and exercise; group V, stanozolol treatment and exercise.

principle was consisting of two steps. The first step included the addition of telomeric repeats (TTAGGG) to the 3' end of biotin-labelled primers and product amplification by PCR. The second step consisted of ELISA reaction.

**Statistical analysis.** The mean, standard deviation (SD) and the median were used for the expression of tail intensity. Telomerase activity was expressed in proportion of the control group (%). The non-parametric Kruskal-Wallis test was applied for comparing the results of comet assay. The same test was also applied for the comparison of expressed telomerase activity. Non-parametric post-hoc comparisons were assessed using Dunn's test. Box and whisker plots were used for the graphical representation of the data. IBM SPSS Statistical software 21.0 (IBM SPSS, Armonk, NY, USA) was used for statistical analysis. A value of P<0.05 was set for accepting (or rejecting) the null hypothesis and was considered to indicate a statistically significant difference.

## Results

The results of tail intensity and the telomerase activity (%) in the experiential groups (exposed and controls) are shown in Table II. The analysis revealed that tail intensity differed significantly between groups (P=0.032), while differences in telomerase activity were also significant (P<0.001). It can be seen that the highest values were observed in group III for both comet assay and telomerase activity assessment (5.8±1.6 and 1851.4±319.2, respectively) in comparison to the other groups (I, II, IV and V). All other values ranged between 3.5±0.6 (group V) to 5.1±1.7 (group IV) for tail intensity, while the lowest telomerase activity (%) was 100±19.4 (group I).

According to the results of the pair wise comparison of tail intensity based on Dunn's test, was observed that the cells from the animals in group V exhibited significantly lower levels of tail intensity compared to those from the animals in group IV (P=0.025) and group III (P=0.002). Our results revealed that telomerase activity (%) differed significantly

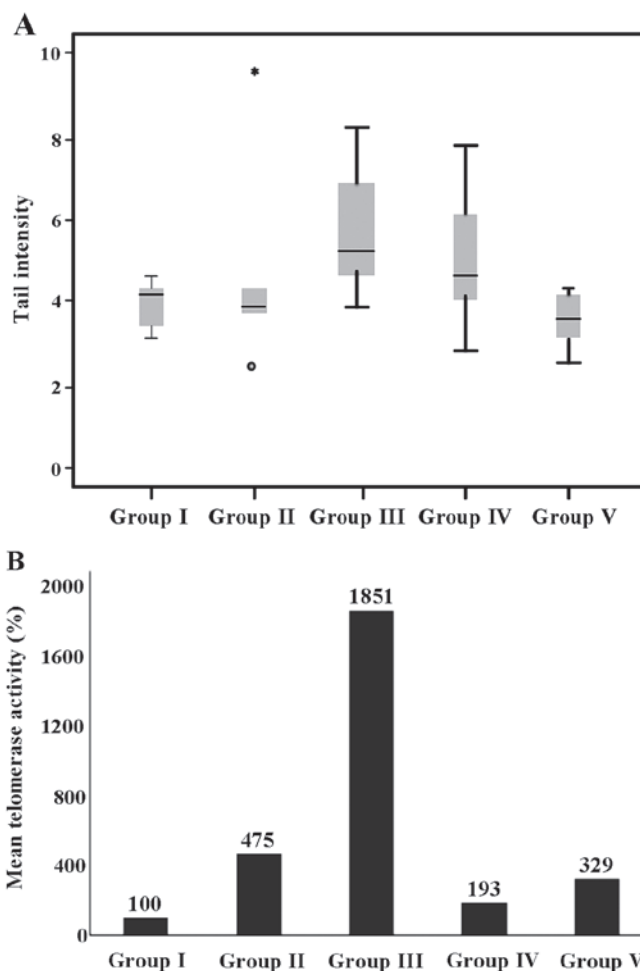


Figure 1. (A) Tail intensity and (B) telomerase activity (%) of stanozolol and propylene glycol (PG)-exposed groups. Group I, control; group II, PG; group III, stanozolol; group IV, PG treatment and exercise; group V, stanozolol treatment and exercise. Extreme outliers are indicated by an asterisk (\*) and circle (o).

when group III was compared with the other groups (group I, P<0.001; group II, P=0.016; group IV, P<0.001; and group V,

$P=0.012$ ). Group I exhibited a significant difference compared with the other groups apart from group IV ( $P=0.121$ ). Group II did exhibit a significant difference compared to group V ( $P=0.292$ ).

The expression of tail intensity is also presented in a box and whisker plot (Fig. 1A), while the telomerase expression (%) is presented in a bar chart (Fig. 1B).

## Discussion

Apart from causing several adverse health effects, the DNA-damaging effects of different AAS have been studied using various techniques. Stanozolol is a widely abused steroid, particularly for the purposes of doping and improving physical status. Very limited information is available regarding the DNA-damaging effects of stanozolol, while a positive correlation between exposure to nandrolone and DNA damage has been determined (14,26,27). In previous studies, a significant increase in DNA damage was determined in blood, liver, bone marrow, brain and testicle cells in experimental animals exposed to high doses of nandrolone (14,28). In addition, in a review, Boettcher *et al* reported increased DNA damage with trenbolone treatment in zebrafish embryos via comet assay and the micronucleus test; however, this increase was not observed with high-dose trenbolone treatment (29 and refs therein). Our results are in accordance with the findings of these studies, demonstrating the DNA-damaging effects of stanozolol. Although exercise has been defined as a key element of a healthy lifestyle, many controversial results have also been published (30-32). Exercise-induced DNA damage has been documented in several studies in physically active/overtrained individuals or even in experimental animals by several techniques (33-35). According to our search, there was only one study available in the literature investigating stanozolol and exercise-induced genotoxicity. Martins *et al* demonstrated that the administration of anabolic steroids (decadurabulin and winstrol) induced chromosomal damage, which was not associated with physical activity (36). The results of our study indicated increased DNA damage in the exercise-exposed control group (PG and exercise group); however, stanozolol played a protective role in exercise-induced DNA damage in the stanozolol-exposed groups.

It is well-documented that telomerase activity plays a role in DNA damage and repair mechanisms. In addition, several pathways have been defined regarding the mechanisms of the effects of AAS on telomerase activity (20,37,38). Nourbakhsh *et al* demonstrated that testosterone and androstenedione both increased cell viability by upregulating telomerase activity in the OVCAR-3 cell line. This upregulation was triggered by the PI3K/Akt pathway. Quantitative PCR analyses demonstrated that the hTERT mRNA levels were significantly increased with exposure to testosterone and androstenedione (37). Zhou *et al* demonstrated that the testosterone derivative, estrogen, increased telomerase activity via the mitogen-activated protein kinase (MAPK) pathway in endometrial cancer cells (39). Geier *et al* reported significantly increased telomerase activity and gene expression with testosterone and dihydrotestosterone (DHT) treatment (40). In addition, androgen exposure has previously been reported to increase telomerase activity by higher TERT mRNA

levels in PBMCs (38). According to our search, there was no study available in the literature on the effects of stanozolol on telomerase activity. There is limited evidence regarding the genotoxicity of stanozolol, while there are several studies available on the effects of other AAS. We demonstrated that stanozolol induced telomerase activity and this increase may be associated with DNA damage.

The effects of exercise on telomerase activity primarily depend on the amount of exercise and cell types. Increased telomerase enzyme activity in skeletal muscles with exercise has been shown by Ludlow *et al* (20). Exercise may reduce telomere shortening-associated aging; however, extreme amounts of exercise have been reported to shorten telomeres. Furthermore, the effects of exercise on telomeric genes and miRNA regulation provides insight into the mechanistic association between exercise, telomeres and health (41). Mosallanezhad *et al* demonstrated significantly increased levels of telomerase activity and improved telomere length in sedentary women who performed high-intensity interval training (42). In this study, we observed a decrease in telomerase activity in stanozolol-treated rats subjected to exercise. Our results are in line with those of previous findings regarding the effects of exercise on telomerase activity.

In conclusion, in this study, an increase in DNA damage was detected with exercise; however, it was observed that exercise did not have a significant DNA-damaging effect in the steroid treated group. Therefore, the preventive role of stanozolol should be taken into consideration for the interpretation of these results for physically active or overtrained individuals. There is a need for further studies in order to clarify the preventive role of stanozolol in physical activity in terms of DNA damage. Thus, the effects of exercise warrant further clarifications for the determination of the genotoxic effects of stanozolol and the mechanisms responsible for stanozolol-induced DNA damage. The DNA-damaging effects of stanozolol may be mediated through alterations in telomerase activity. However, further studies are required in order to fully elucidate the association between stanozolol use, exercise and DNA damage.

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