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ORIGINAL ARTICLE

Chromosomal Damage Induced by Androgenic Anabolic Steroids, Stanozolol and Trenbolone, in Human Lymphocytes

Tanveer Beg, Yasir Hasan Siddique and Mohammad Afzal

Human Genetics and Toxicology Laboratory, Department of Zoology, Aligarh Muslim University, Aligarh (UP), 202 002, India.

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ABSTRACT

Stanozolol and trenbolone, commonly used as performance enhancing and bodybuilding drugs, were studied for their genotoxic effect on human lymphocyte chromosomes using chromosomal aberrations (CAs) and sister chromatid exchanges (SCEs) as parameters, both in the presence and absence of metabolic activation (S9 mix). This experiment was aimed at finding the dosage at which these two steroids are genotoxic enough to cause chromosome damage. They were studied at 1, 10, 20, 40, 60 mM respectively, and were found to be similarly genotoxic at 40 and 60 mM, in the presence as well as absence of S9 mix. The results suggest a strong genotoxic effect of both steroids *in vitro* in human lymphocytes.

Keywords: Stanozolol, Trenbolone, Androgens, Genotoxicity, Chromosomal aberrations, Sister chromatid exchanges, Human lymphocytes.

Introduction

Naturally occurring anabolic steroids are synthesized in the testis, ovary and adrenal gland from cholesterol via pregnenolone. Anabolic steroids bind to specific receptors present especially in reproductive tissue, muscle and fat[16]. Stanozolol is a synthetic androgenic anabolic steroid, similar to the naturally occurring androgen testosterone. It is used in the treatment of anemia and hereditary angioedema, which causes episodes of swelling of the face, extremities, genitals, bowel wall and throat[7]. Stanozolol may decrease the frequency of these attacks. It is one of the anabolic steroids commonly used by athletes and bodybuilders, although there is no clear evidence that anabolic steroids enhance overall athletic performance[5].

Like stanozolol, trenbolone is also a synthetic steroid, usually used by veterinarians on livestock as a growth promotant in animal husbandry[19]. To increase its effective half-life, trenbolone is not used in an unrefined form, but is rather administered as trenbolone acetate or trenbolone enanthate. Trenbolone is then produced as a metabolite by the reaction of these compounds with androgen receptor. No trenbolone compounds have been approved by the Food and Drug Administration, USA, for human use, due to a lack of clinical applications and considerable negative side effects. However, bodybuilders use the drug illegally to increase body mass and strength more effectively than by weight training alone.

The extensive use of these two steroids, legally and illegally, by humans and their ability to act as genotoxic agents, as suggested by earlier studies using mammalian systems, was a good enough reason for us to investigate their genotoxic potential using cultured human lymphocytes.

Corresponding Author

Mohammad Afzal, Human Genetics and Toxicology Laboratory, Department of Zoology, Aligarh Muslim University, Aligarh (UP), 202 002, India.
E-mail: afzal_amu@yahoo.com

Materials and methods

Chemicals and reagents

Stanozolol (Sigma); Trenbolone (Sigma); RPMI-1640 (Gibco); Fetal calf serum (Gibco); 0.1 ml Antibiotic-antimycotic mixture (Gibco); Phytohaemagglutinin-M (Gibco); Colchicine (Microlab); Hoechst 33258 stain (Fluka); 3% Giemsa solution in phosphate buffer, pH 6.8 (Merck); 5-Bromo-2-deoxyuridine, BrdU (Sigma); Dimethylsulphoxide, DMSO (Merck); Mitomycin C (Merck); Cyclophosphamide, CP (Sigma). For S9 mix preparation, Swiss albino healthy rats (Wistar strain) and 0.1% Phenobarbital were used.

Lymphocyte culture and chromosomal aberration analysis

Peripheral blood cultures were done in duplicate[3]. Heparinized blood (0.5 ml) samples were taken from healthy female donors, and were placed in sterile tubes containing 7 ml of RPMI-1640, supplemented with 1.5 ml of fetal calf serum and 0.1 ml of phytohaemagglutinin, and incubated at 37°C for 24 hours. Stanozolol and trenbolone, both dissolved in dimethyl sulphoxide (DMSO), were added separately to different cultures at the doses of 1, 10, 20, 40 and 60 μM. DMSO (5 ml/ml) served as negative control. For metabolic activation experiments, liver S9 fraction was freshly prepared as per standard procedure[14]. 0.5 ml of S9 mix was added along with each treatment after 24 hours of the commencement of culture. Negative control was also given 0.5 ml of S9 mix.

Two hours before harvesting, 0.2 ml of colchicine (0.2 mg/ml) was added to the culture tubes. Cells were centrifuged at 800-1000 rpm for 10 min. The supernatant was removed and 5 ml of pre-warmed (37°C) 0.075 M KCl hypotonic solution was added. Cells were resuspended and incubated at 37°C for 15 min. The supernatant was removed by centrifugation and 5 ml of fixative (methanol: glacial acetic acid, 3:1) was added. The fixative was removed by centrifugation and the procedure was repeated twice. The slides were stained in Giemsa solution in phosphate buffer for 15 min. At least 300 metaphases were analyzed for different types of chromosome breakage frequencies. Classification of aberrations was done according to the guidelines of the International Programme on Chemical Safety, WHO, Geneva, for the study of genetic defects in human population.

Sister chromatid exchange analysis

For SCE analysis, BrdU (10 mg/ml) was added at the beginning of the culture. After 24 hours, same treatments were given as for CA analysis. Two hours before harvesting, 0.2 ml of colchicine (0.2 mg/ml) was added, followed by hypotonic treatment and fixation and processing of slides[18] was done as for CA analysis. Slides were stained for 20 min in 0.05% (w/v) Hoechst 33258 solution, rinsed with tap water and placed under a UV lamp for 90 min covered with Sorensen's buffer (pH 6.8) and stained with Giemsa solution in phosphate buffer for 15 min. The sister chromatid exchange average was taken from an analysis of 50 metaphases.

Statistical analysis

Student's t-test was used for calculating the statistical significance in CAs and SCEs. The level of significance was tested using standard statistical tables[23].

Results and discussions

Results

The number of abnormal cells with CAs was insignificantly low at treatment doses of 1, 10, 20 mM, respectively, for both steroids being tested for genotoxicity, in the presence as well as absence of metabolic activation i.e. S9 mix (Tables 1 and 2). A dramatic increase in genotoxic damage was noticed when the dosage was increased to 40 and 60 mM. At this dosage level, the abnormal cells more than doubled, although the dose-dependent increase in CAs was small from 40 to 60 mM.

SCE frequency in human lymphocytes treated separately with stanozolol and trenbolone (Tables 3 and 4), again with and without S9 mix, followed a pattern similar to the one observed for CAs. At 1, 10 and 20 mM, the SCE frequency remained quite low and showed little increase. But as the dosage increased to 40 mM and then 60 mM, a more than three fold increase in SCEs per cell was observed. Again, the dose-dependent rise in SCE frequency was small going from 40 to 60 mM.

Discussion

The use of stanozolol in sports is illegal and banned by the International Association of Athletics Federations. It has a large oral bioavailability due to a C_{17} alpha-alkylation which allows the hormone to survive first pass liver metabolism. High doses of stanozolol could exert a proliferative effect on liver cells[2]. Acute overdosage can produce nausea and gastrointestinal upset. Chronic use of stanozolol can cause menstrual irregularities and virilization in women and impotence, premature cardiovascular disease and prostatic hypertrophy in men. Precocious prostatic cancer has been described after long term anabolic steroid abuse[20]. Cases where hepatic cancers have been associated with anabolic steroid abuse have been reported[17]. Also, androgen ingestion by a pregnant mother can cause virilization of a female fetus[4].

Table 1: Stanozolol induced CAs in human lymphocytes.

Treatment (without S9 mix)	Abnormal cells (% ± SE)	Break-up of CAs					Treatment (with S9 mix)	Abnormal cells (% ± SE)	Break-up of CAs				
		Gaps	CTB	CSB	CTE	DIC			Gaps	CTB	CSB	CTE	DIC
Stanozolol (mm)							Stanozolol (mM)						
1	1.00 ± 0.57	3	2	1	-	-	1	1.00 ± 0.57	1	2	1	-	-
10	1.33 ± 0.66	3	2	2	-	-	10	1.00 ± 0.57	2	2	1	-	-
20	1.67 ± 0.73	4	2	2	-	-	20	1.33 ± 0.66	2	2	2	-	-
40	4.00 ± 1.13[b]	8	9	3	-	-	40	4.33 ± 1.17[b]	9	9	4	-	-
60	5.33 ± 1.29[b]	12	11	5	-	-	60	5.00 ± 1.25[b]	10	10	5	-	-
Untreated	0.67 ± 0.47	1	1	1	-	-	Untreated	0.67 ± 0.47	1	2	-	-	-
Negative control (DMSO, 5 ml/ml)	1.00 ± 0.57	1	2	1	-	-	Negative control (DMSO, 5 ml/ml)	1.00 ± 0.57	1	2	1	-	-
Positive control (Mitomycin C, 0.3 mg/ml)	8.33 ± 1.59[a]	14	17	12	6	2	Positive control (CP, 0.5 x 10^{-5} M)	7.67 ± 1.53[a]	12	14	6	4	2

[a]Significant difference with respect to untreated (P<0.05).
[b]Significant difference with respect to untreated (P<0.01).
SE: Standard Error.

Table 2: Trenbolone induced CAs in human lymphocytes.

Treatment (without S9 mix)	Abnormal cells (% ± SE)	Break-up of Cas					Treatment (with S9 mix)	Abnormal cells (% ± SE)	Break-up of CAs				
		Gaps	CTB	CSB	CTE	DIC			Gaps	CTB	CSB	CTE	DIC
Trenbolone (mM)							Trenbolone (mM)						
1	0.67 ± 0.47	1	2	-	-	-	1	0.67 ± 0.47	2	2	-	-	-
10	1.00 ± 0.57	1	2	1	-	-	10	0.67 ± 0.47	2	2	1	-	-
20	1.33 ± 0.66	2	2	2	-	-	20	1.00 ± 0.57	2	2	1	-	-
40	3.33 ± 1.03[b]	9	6	4	-	-	40	3.67 ± 1.08[b]	8	7	4	-	-
60	4.33 ± 1.17[b]	10	8	5	-	-	60	4.00 ± 1.13[b]	9	8	4	-	-
Untreated	0.67 ± 0.47	1	1	-	-	-	Untreated	0.67 ± 0.47	2	2	-	-	-
Negative control (DMSO, 5 ml/ml)	1.00 ± 0.57	1	2	1	-	-	Negative control (DMSO, 5 ml/ml)	0.67 ± 0.47	1	1	1	-	-
Positive control (Mitomycin C, 0.3 µg/ml)	9.33 ± 1.67[a]	16	18	10	5	1	Positive control (CP, 0.5 x 10^{-5} M)	8.00 ± 1.56[a]	15	17	11	6	2

[a]Significant difference with respect to untreated (P<0.05).
[b]Significant difference with respect to untreated (P<0.01).
SE: Standard Error.

Table 3: SCE frequency in human lymphocytes treated with Stanozolol.

Treatment (without S9 mix)	SCEs/Cell (Mean ± SE)	Treatment (with S9 mix)	SCEs/Cell (Mean ± SE)
Stanozolol		Stanozolol	
1 mM	2.12 ± 0.18	1 mM	2.46 ± 0.20
10 mM	2.36 ± 0.22	10 mM	2.56 ± 0.22
20 mM	2.74 ± 0.24	20 mM	2.82 ± 0.26
40 mM	6.82 ± 0.64[b]	40 mM	6.24 ± 0.62[b]
60 mM	7.22 ± 0.66[b]	60 mM	7.62 ± 0.68[b]
Untreated	1.24 ± 0.12	Untreated	1.28 ± 0.14
Negative control (DMSO, 5ml/ml)	1.16 ± 0.10	Negative control (DMSO, 5ml/ml)	1.22 ± 0.12
Positive control (Mitomycin C, 0.3 µg/ml)	10.22 ± 1.02[a]	Positive control (CP, 0.5 x 10^{-5} M)	10.86 ± 1.12[a]

[a]Significant difference with respect to untreated (P<0.05).
[b]Significant difference with respect to untreated (P<0.01).
SE: Standard Error.

Table 4: SCE frequency in human lymphocytes treated with Trenbolone.

Treatment (without S9 mix)	SCEs/Cell (Mean ± SE)	Treatment (with S9 mix)	SCEs/Cell (Mean ± SE)
Trenbolone		Trenbolone	
1 mM	2.06 ± 0.14	1 mM	2.02 ± 0.12
10 mM	2.18 ± 0.18	10 mM	2.10 ± 0.14
20 mM	2.42 ± 0.20	20 mM	2.26 ± 0.18
40 mM	6.24 ± 0.64[b]	40 mM	6.02 ± 0.60[b]
60 mM	6.88 ± 0.66[b]	60 mM	6.88 ± 0.66[b]
Untreated	1.02 ± 0.08	Untreated	1.98 ± 0.14
Negative control (DMSO, 5ml/ml)	1.96 ± 0.12	Negative control (DMSO, 5ml/ml)	1.88 ± 0.10
Positive control (Mitomycin C, 0.3 µg/ml)	9.96 ± 0.96[a]	Positive control (CP, 0.5 x 10^{-5} M)	9.72 ± 0.92[a]

[a]Significant difference with respect to untreated (P<0.05).
[b]Significant difference with respect to untreated (P<0.01).
SE: Standard Error.

Trenbolone compounds have a binding affinity for the androgen receptor thrice as high as that of testosterone. Once metabolized, the drugs have the effect of increasing nitrogen uptake by muscles, leading to an increase in the rate of protein synthesis. They also have the secondary effects of stimulating appetite, reducing the amount of fat being deposited in the body, and decreasing the rate of catabolism. Some short term side effects include insomnia, high blood pressure and increased aggression and libido. However, women suffer virilization effects even at small doses[29].

The only legitimate therapeutic indications for such anabolic steroids are in the replacement of male sex steroids in men who have androgen deficiency, e.g. due to loss of both testes, in the treatment of certain rare forms of aplastic anemia which are or may be responsive to anabolic androgens, and in certain countries to counteract catabolic states, e.g. after major trauma.

This experiment implies that the synthetic anabolic steroids, stanozolol and trenbolone, have the potential to cause genotoxic damage in human lymphocytes *in vitro* at higher dosage both in the presence and absence of S9 mix. Changes in chromosome structure due to a break or a swapping of chromosomal material are termed as CAs. Most of the CAs in cells are lethal, but many of them are also viable and can cause genetic effects, either somatic or inherited[27]. These events can lead to the loss of chromosomal material at mitosis or to the inhibition of exact chromosome segregation at anaphase. The result of these changes is cell lethality[28]. In our experiment, we came across significant differences compared with control in the CA frequency at 40 and 60 mM, with or without S9 mix. SCE is usually a more sensitive indicator of genotoxic effects than CA[28]. There is a correlation between the carcinogenicity and SCE inducing ability of many chemicals. Moreover, the SCE induction mechanism is heterogeneous and very different from the mechanism of CA induction[8]. Androgenic steroids display teratogenic effects in all species that have been studied so far, and do so in a very predictable and consistent way[13]. Various psychological and physiological effects have been reported in both males and females among frequent users of androgens[15]. There is little, if any, information available on the exact reasons for the genotoxic behavior of stanozolol and trenbolone. However, the present study is concurrent with the studies performed on synthetic steroids such as cyproterone acetate, ethynodiol diacetate, chlormadinone acetate, medroxyprogesterone acetate, norgestrel and megestrol acetate that induced CAs and SCEs with or without metabolic activation system[1,10,21,22,23,25,26].

Conclusion

The outcome of this investigative experiment shows that stanozolol and trenbolone have the potential to be genotoxic and cytotoxic, especially at 40 and 60 mM, with or without metabolic activation, in cultured human lymphocytes. The evaluation of these genotoxicity tests is a useful tool for determining the toxic effects of potentially genotoxic chemicals, leading to identification of such carcinogenic agents. It is advisable to use the steroids studied here at their lowest effective dosage so that the risk to public health could be minimized. The risk of damage to human genetic material is very likely at higher doses of these drugs.

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