December 2008 Volume 4 Issue 3

Trade Science Inc.

Natural Products

An Indian Journal

Full Paper

NPAIJ, 4(3), 2008 [210-215]

Antigenotoxic effect of EGCG against androgenic steroids stanozolol and trenbolone

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Received: 8th August, 2008 ; Accepted: 13th August, 2008

ABSTRACT

A catechin, EGCG, was studied for its antigenotoxic effect on CAs and SCEs induced in human lymphocytes by androgenic steroids, Stanozolol and Trenbolone. The steroids induced CAs and SCEs at 40 and 60μM and EGCG was used at 20 and 30 μM along with both steroids separately. EGCG was found to reduce significantly the genotoxicity caused by both steroids, with and without metabolic activation, but the antigenotoxic potential of EGCG was higher in the presence of metabolic activation system.

KEYWORDS

Epigallocatechin gallate (EGCG);
Catechins;
Antioxidants;
Stanozolol;
Trenbolone;
Genotoxicity;
Chromosomal aberrations;
Sister chromatid exchanges.

INTRODUCTION

Epigallocatechin-3-gallate (EGCG), a compound closely related to Epicatechin gallate (ECG), is a catechin and polyphenolic antioxidant plant metabolite found in abundance in various types of tea, derived from the tea plant *Camellia sinensis*[1]. It helps protect the skin from ultraviolet radiation-induced genotoxic damage and tumor formation[2]. Stanozolol is a synthetic steroid similar to the naturally occurring androgen called testosterone. It is used in the treatment of many disorders such as anemia and hereditary angioedema[3]. Athletes and bodybuilders commonly use this anabolic steroid for performance enhancement[4]. Its large oral bioavailability is due to a C17 alpha-alkylation principle which allows the hormone to survive the first pass through liver metabolism. At high dosage, stanozolol could exert a proliferative effect on liver cells[5]. Precocious prostate cancer has been reported after a long term steroid abuse[6]. Hepatic cancer has also been linked to anabolic steroidal abuse[7]. Trenbolone is a synthetic steroid used frequently by veterinarians on livestock as a promoter of growth in animal husbandry[8]. Trenbolone compounds have not yet been approved by the Food and Drug Administration, USA for use of humans due to their considerable negative side effects, although bodybuilders use the drug illegally to increase body mass and strength. Cases of prostate and hepatic cancers have been associated with long term anabolic steroid abuse[6, 7]. Trenbolone compounds increase nitrogen uptake by muscles after metabolization, leading to increased rate of protein synthesis[9]. EGCG was studied for its possible antigenotoxic effect on the CAs and SCEs induced by Stanozolol and Trenbolone, in the presence as well as absence of metabolic activation system in human lymphocytes *in vitro*.

EXPERIMENTAL

Chemicals

Stanozolol (CAS No.: 10418-03-8, Sigma-Aldrich); Trenbolone (CAS No.: 10161-33-8, Sigma-

Aldrich); Sodium phenobarbitone (Sigma-Aldrich); Colchicine (Microlab); Dimethyl sulphoxide (Merck); Epigallocatechin-3-gallate (CAS No.: 989-51-5, Sigma-Aldrich); RPMI 1640 (GIBCO, Invitrogen); Phytohaemagglutinin-M (GIBCO, Invitrogen); Antibiotic-antimycotic mixture (GIBCO, Invitrogen); Fetal serum - calf (GIBCO, Invitrogen); 5-bromo-2-deoxyuridine (Sigma-Aldrich); Hoechst 33258 stain (Sigma-Aldrich); Giemsa stain (Merck); Mitomycin-C (Sigma-Aldrich); Cyclophosphamide (Sigma-Aldrich); NADP (SRL).

Human lymphocyte culture

Duplicate peripheral blood cultures were conducted according to Carballo et al.[10]. Briefly, 0.5 ml of the heparinized blood samples was obtained from a healthy female donor and was placed subsequently in a sterile flask containing 7 ml of RPMI 1640, supplemented with 1.5 ml of fetal calf serum and 0.1 ml of phytohaemagglutinin. These flasks were placed in an incubator at 37^0C for 24 hours. Untreated culture and also negative and positive controls were run simultaneously.

Chromosomal aberration analysis

Stanozolol, at 40 and 60 μM concentrations respectively, was dissolved in dimethylsulphoxide and was added later after 24 h. The cells were cultured for another 48 h at 37^0C keeping them in an incubator. For metabolic activation experiments, 0.5 ml of S9 mix dose was added to the stanozolol treatment. S9 mix was prepared from the liver of healthy rats (Wistar strain) as per standard procedure of Maron and Ames[11]. The S9 fraction so obtained was enhanced by addition of 5 μM of NADP and 10 μM of glucose-6-phosphate just before the use to make the S9 mix. The S9 mix without NADP was also given with each of the tested dose of stanozolol. An amount of 0.2 ml of colchicine (0.2 μg/ml) was added to the culture flask, 1 h prior to harvesting. Cells were centrifuged at 1000 rpm for 10 min. The supernatant was removed and 5 ml of prewarmed (37^0C) 0.075 M KCl (hypotonic solution) was added. Cells were resuspended and incubated at 37^0C for 15 min. The supernatant was removed by centrifugation, and, subsequently 5 ml of chilled fixative (methanol: glacial acetic acid, 3:1) was added. The fixative was removed by centrifugation and the procedure was repeated twice. To prepare slides, 3-5 drops of the fixed cell suspension were dropped on clean slides and air-dried. The slides were stained in 3% Giemsa solution in phosphate buffer (pH 6.8) for 15 min. Three hundred metaphases were examined for screening the presence of different types of abnormality. Criteria to classify different types of aberrations were in accordance with the recommendation of Environmental Health Committee 46 for Environmental Monitoring of Human Populations[12].

Sister chromatid exchange analysis

For sister chromatid exchange analysis, bromodeoxyuridine (BrDU, 10 μg/ml) was added at the beginning of the culture. After 24 h, stanozolol at final concentrations of 40 and 60 μM, earlier dissolved in dimethylsulphoxide, was added and kept for another 48 h at 37^0C in an incubator. For metabolic activation experiments, 0.5 ml of S9 mix with and without NADP was given along with each of the tested dose. Mitotic arrest was attempted, 1 h prior to harvesting by adding 0.2 ml of colchicine (0.2 μg/ml). Hypotonic treatment and fixation were done in the same way as described for chromosomal aberration analysis. The slides were processed according to Perry and Wolff[13], and Afzal and Azfer[14]. The sister chromatid exchange induction was analysed from 50 plates of second division mitoses per dose.

A similar method was followed for CA and SCE analysis using Trenbolone (at 40 and 60 μM) in a separate experiment.

Chromosomal aberration analysis in human lymphocytes treated with Stanozolol in the presence of EGCG

After 24 h of incubation of human lymphocyte culture, Stanozolol (at 40 and 60 μM) was administered with 20 and 30 μM of EGCG respectively and kept for 48 h at 38^0C in the incubator. Prior to 1 h of harvesting, 0.2 ml of colchicine (0.2 μg/ml) was added to the culture flasks. Hypotonic treatment, fixation and processing of slides were done as described earlier in the text. About three hundred metaphases were examined for the occurrence of different types of abnormality i.e. gaps, break and exchanges. The criteria to classify different types of aberrations were in accordance with the rec-

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TABLE 1: Antimutagenic effect of EGCG on CAs induced by stanozolol in cultured human lymphocytes without S9 mix

Treatment (μM)	Abnormal cells (% ± SE)	Chromosomal Aberrations Gaps	CTB	CSB	CTE	DIC
Stanozolol						
40	13(4.33±1.17)[a]	9	10	5	1	-
60	16 (5.33±1.29)[a]	12	12	7	1	-
EGCG						
20	3 (1.00±0.57)	4	4	1	-	-
30	4 (1.33±0.66)	3	3	1	-	-
Stanozolol+EGCG						
40+20	8 (2.67±0.93)[b]	7	7	4	1	-
60+20	10(3.33±1.04)[b]	8	10	6	2	-
40+30	5 (1.67±0.74)[b]	4	4	2	-	-
60+30	7 (2.33±0.87)[b]	6	7	3	1	-
Untreated	3 (1.00±0.57)	2	1	1	-	-
Negative control (DMSO, 5 μl/ml)	2 (0.67±0.47)	1	1	1	-	-
Positive control (Mitomycin C, 0.3 μg/ml)	42 (14.00±2.00)[a]	22	30	16	5	4

Significant difference: [a]P<0.01 with respect to untreated; [b]P<0.05 with respect to stanozolol

TABLE 2: Antimutagenic effect of EGCG on CAs induced by stanozolol in cultured human lymphocytes with S9 mix

Treatment (μM)	Abnormal cells (% ± SE)	Chromosomal Aberrations Gaps	CTB	CSB	CTE	DIC
Stanozolol						
40	15(5.00±1.26)[a]	10	11	5	2	-
60	17(5.67± 1.34)	12	12	6	3	-
EGCG						
20	3 (1.00 ± 0.57)	3	3	1	-	-
30	5 (1.67 ± 0.74)	2	2	1	-	-
Stanozolol+EGCG						
40+20	8 (2.67 ± 0.93)[b]	7	7	3	1	-
60+20	11 (3.67± 1.09)[b]	10	11	4	2	-
40+30	4 (1.33 ± 0.66)[b]	3	3	2	-	-
60+30	5 (1.67 ± .74)[b]	4	5	2	1	-
Untreated	3 (1.00 ± 0.57)	2	1	1	-	-
Negative control (DMSO, 5 μl/ml)	3 (1.00 ± 0.57)	3	2	1	-	-
Positive control (CP, 0.5×10^{-5} M)	45 (15.00±2.06)[a]	27	33	15	6	3

Significant difference: [a]P<0.01 with respect to untreated; [b]P<0.05 with respect to stanozolol

TABLE 3: Antimutagenic effect of EGCG on CAs induced by trenbolone in cultured human lymphocytes without S9 mix

Treatment (μM)	Abnormal cells (% ± SE)	Chromosomal Aberrations Gaps	CTB	CSB	CTE	DIC
Trenbolone						
40	11 (3.67 ± 1.09)[a]	9	10	3	1	-
60	13 (4.33 ± 1.17)[a]	10	10	4	1	-
EGCG						
20	2 (0.67 ± 0.47)	1	1	1	-	-
30	3 (1.00 ± 0.57)	2	1	1	-	-
Stanozolol+EGCG						
40+20	6 (2.00 ± 0.81)[b]	4	5	2	-	-
60+20	8 (2.67 ± 0.93)[b]	6	6	2	1	-
40+30	4 (1.33 ± 0.66)[b]	3	4	1	-	-
60+30	6 (2.00 ± 0.81)[b]	5	5	2	1	-
Untreated	2 (0.67 ± 0.47)	1	2	1	-	-
Negative control (DMSO, 5 μl/ml)	2 (0.67 ± 0.47)	1	1	1	-	-
Positive control (Mitomycin C, 0.3 μg/ml)	37 (12.33 ± 1.90)[a]	19	25	15	3	1

Significant difference: [a]P<0.01 with respect to untreated; [b]P<0.05 with respect to trenbolone

ommendation of EHC 46 for Environmental Monitoring of Human Population[12].

Sister chromatid exchange analysis in human lymphocytes treated with Stanozolol in the presence of EGCG

For sister chromatid exchange analysis, bromo deoxyuridine (BrdU, 10 μg/ml) was added at the beginning of the culture. After 24 h of the initiation of culture, 40 and 60 μM of Stanozolol separately and along with 20 and 30 μM of EGCG were treated and kept for 48 h at 37^{0}C in the incubator. Mitotic arrest was done one hour prior to harvesting by adding 0.2 ml of colchicines (0.2μg/ml). Hypotonic treatment and fixation were performed in the same way as described earlier in the text. The slides were processed according to Perry and Wolff[13]. Sister chromatid exchange average was taken from an analysis of about fifty second division metaphases.

A similar method was followed for CA and SCE analysis in human lymphocytes treated with Trenbolone (at 40 and 60 μM along with EGCG at 20 and 30 μM) in a separate experiment.

Statistical analysis

Student's two tailed *t*-test was used for the analysis of chromosomal aberrations and sister chromatid exchanges. The level of significance was tested from standard statistical tables of Fisher and Yates[15].

RESULTS

EGCG proved its worth as an antimutagenic agent by substantially reducing the CAs induced by Stanozolol in cultured human lymphocytes in the absence as well as presence of metabolic activation. EGCG proved to be more effective in reducing chromosome damage when applied in the presence of metabolic activation system (TABLES 1 and 2). Trenbolone induced CAs were observed to have a lower frequency of occurrence when treated with EGCG both in the absence

TABLE 4: Antimutagenic effect of EGCG on CAs induced by trenbolone in cultured human lymphocytes with S9 mix

Treatment (μM)	Abnormal cells (% ± SE)	Chromosomal Aberrations				
		Gaps	CTB	CSB	CTE	DIC
Trenbolone						
40	12 (4.00 ± 1.13)[a]	9	9	4	1	-
60	15 (5.00 ± 1.26)[a]	12	13	7	2	-
EGCG						
20	3 (1.00 ± 0.57)	2	3	1	1	-
30	4 (1.33 ± 0.66)	3	3	2	1	-
Stanozolol+EGCG						
40+20	6 (2.00 ± 0.81)[b]	4	4	2	1	-
60+20	9 (3.00 ± 0.98)[b]	7	8	3	2	-
40+30	3 (1.00 ± 0.57)[b]	2	3	2	1	-
60+30	5 (1.67 ± 0.74)[b]	4	4	1	1	-
Untreated	3 (1.00 ± 0.47)	1	2	1	-	-
Negative control (DMSO, 5 μl/ml)	2 (0.67 ± 0.47)	1	2	1	-	-
Positive control (CP, 0.5×0^{-5} M)	40 (13.33 ± 1.96)[a]	21	28	13	4	2

Significant difference: [a]P<0.01 with respect to untreated; [b]P<0.05 with respect to trenbolone

TABLE 5: Antimutagenic effect of EGCG on SCEs induced by Stanozolol in cultured human lymphocytes with and without S9 mix

Treatment (μM)	SCEs/Cell (Mean ± SE)	Treatment (μM)	SCEs/Cell (Mean ± SE)
Stanozolol (without S9)		Stanozolol (with S9)	
40	6.89 ± 0.66[a]	40	7.07 ± 0.67[a]
60	7.73 ± 0.69[a]	60	7.95 ± 0.71[a]
EGCG		EGCG	
20	2.75 ± 0.30	20	2.88 ± 0.32
30	2.83 ± 0.31	30	2.94 ± 0.33
Stanozolol + EGCG		Stanozolol + EGCG	
40+20	3.12 ± 0.33[b]	40+20	3.10 ± 0.31[b]
60+20	4.47 ± 0.45[b]	60+20	4.43 ± 0.43[b]
40+30	2.72 ± 0.32[b]	40+30	2.98 ± 0.33[b]
60+30	4.06 ± 0.42[b]	60+30	4.11 ± 0.40[b]
Untreated	2.12 ± 0.23	Untreated	2.27 ± 0.26
Negative control (DMSO, 5 μl/ml)	2.02 ± 0.21	Negative control (DMSO, 5 μl/ml)	2.17 ± 0.23

Significant difference: [a]P<0.01 with respect to untreated; [b]P<0.05 with respect to trenbolone

and presence of S9 mix, with an almost similar pattern in the ability of EGCG to reduce genotoxicity, both without and with metabolic activation (TABLES 3 and 4). When SCEs were induced using Stanozolol and Trenbolone as toxic agents both in the absence and presence of metabolic activation system and EGCG was again used as the ameliorating antimutagenic agent, a very similar pattern was again observed in the antigenotoxic potential of EGCG, with only slight differences between the observations in the cases of absence and presence of S9 mix (TABLES 5 and 6). Thus

TABLE 6: Antimutagenic effect of EGCG on SCEs induced by trenbolone in cultured human lymphocytes with and without S9 mix.

Treatment (μM)	SCEs/Cell (Mean ± SE)	Treatment (μM)	SCEs/Cell (Mean ± SE)
Trenbolone (without S9)		Trenbolone (with S9)	
40	6.34 ± 0.59[a]	40	6.52 ± 0.61[a]
60	7.11 ± 0.63[a]	60	7.32 ± 0.64[a]
EGCG		EGCG	
20	2.55 ± 0.26	20	2.62 ± 0.27
30	2.67 ± 0.29	30	2.78 ± 0.29
Trenbolone + EGCG		Trenbolone + EGCG	
40+20	4.04 ± 0.41[b]	40+20	4.14 ± 0.42[b]
60+20	4.52 ± 0.48[b]	60+20	4.44 ± 0.46[b]
40+30	3.92 ± 0.38[b]	40+30	3.98 ± 0.40[b]
60+30	4.38 ± 0.43[b]	60+30	4.27 ± 0.43[b]
Untreated	2.02 ± 0.22	Untreated	2.17 ± 0.23
Negative control (DMSO, 5 μl/ml)	1.96 ± 0.20	Negative control (DMSO, 5 μl/ml)	2.10 ± 0.21

Significant difference: [a]P<0.01 with respect to untreated; [b]P<0.05 with respect to trenbolone

EGCG, by itself, was observed to be non genotoxic when tested for genotoxicity in all experiments performed, with or without metabolic activation (TABLES 1-6).

DISCUSSION

Natural plant products have been reported to reduce genotoxic effect of steroids in various in vitro and in vivo models. The genotoxic effects of steroids can be reduced by the use of antioxidants and natural plant products[33,34,27,35,36,37,38,39,40,41]. In this study, EGCG reduces genotoxicity induced by Stanozolol and Trenbolone, in the presence as well as absence of metabolic activation system in human lymphocytes. EGCG was more effective in reducing genotoxic damage in the presence of metabolic activation. The reduction in genotoxic damage may be due to the possibility of the prevention of metabolic activation of Stanozolol and Trenbolone by EGCG. The selected dosage of EGCG is potent enough to reduce genotoxicity. The concentrations studied here are higher than those of commonly used steroids. The higher concentration may be reached in some clinical conditions[28] and this higher concentration may lead to genotoxic damage and may further increase the possibility of the development of various types of cancers[31]. EGCG reduced the genotoxic damage induced by the steroids/mutagens, by the highest tested dosage i.e. 20 and 30 μM, thereby giving a clear

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indication of its protective role.

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[16]. 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[17]. In our experiment, we came across significant differences compared with control in the CA frequent at 40 and 60 μM, with or without S9 mix. SCE is usually a more sensitive indicator of genotoxic effects than CA[17]. 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[18]. Androgenic steroids display teratogenic effects in all species that have been studied so far, and do so in a very predictable and consistent way[19]. Various psychological and physiological effects have been reported in both males and females among frequency users of androgens[20]. 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[21,22,23,24,25,26,27]. The International Agency on Cancer (IAC), mainly on the basis of epidemiological studies classifies steroidal estrogen progestin combinations among agents carcinogenic to humans (Group 1), progestins as possibly carcinogenic (Group 2) and androgenic anabolic steroids, as probably carcinogenic (Group 2A)[28].

An increase in the frequency of chromosomal aberrations in peripheral blood lymphocytes is associated with an increased overall risk of cancer[29,30]. The readily quantifiable nature of sister chromatid exchanges with high sensitivity for revealing toxicant-DNA interaction and the demonstrated ability of genotoxic chemicals to induce significant increase in sister chromatid exchanges in cultured cells has resulted in this endpoint being used as indicator of DNA damage in blood lymphocytes of individuals exposed to genotoxic carcinogens[31]. The above genotoxic endpoints are well known markers of genotoxicity and any reduction in the frequency of these genotoxic endpoints gives us indication of the antigenotoxicity of a particular compound[31]. Many products protect against xenobiotics either by inducing detoxifying enzymes or by inhibiting oxidative enzymes[32].

ACKNOWLEDGMENTS

We are grateful to the Chairman, Department of Zoology, AMU, Aligarh, and our supervisor (M.Afzal) for encouraging research into new areas and also for providing the necessary laboratory facilities for this purpose.

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