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Antioxidant Effect of ECG on Testosterone Propionate Induced Chromosome Damage

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Abstract: The aim of this experiment was to find the appropriate dosage at which Testosterone Propionate (TP) is genotoxic enough to cause significant chromosome damage. TP was examined at 10, 20 and 40 μM, respectively and found to be significantly genotoxic at 20 and 40 μM, only in the presence of metabolic activation. Epicatechin Gallate (ECG) proved to be an effective antioxidant by reducing genotoxic damage. The results imply a strong genotoxic effect of TP *in vitro* on human lymphocytes and also a relevant antigenotoxic role of ECG in ameliorating steroid induced genotoxicity.

Key words: Testosterone propionate, epicatechin gallate (ECG), antioxidants, tea androgens, genotoxicity, chromosomal aberrations, sister chromatid exchanges, human lymphocytes

INTRODUCTION

Synthetic anabolic steroids are based on the principal male hormone testosterone and bind to receptors in reproductive tissues, muscles and fat (Matsumoto *et al*., 2008). Testosterone Propionate (TP) is a very strong androgenic compound having a high anabolic effect. Users of this compound gain powerful strength fast without excessive gain in body weight. TP is the acetate form of trenbolone and hence its effect lasts only for a short time leading to the need for frequent administration. TP has considerable androgenic side effects both in men and women. Athletes using this compound often report headaches, nasal bleeding, high blood pressure, oily skin with acne and partly an enormous increase in aggressive behavior (Pope *et al*., 2000; Chance *et al*., 2000; http://www. testosteronepropionate.com/ Testosterone Propionate-profile.html).

The treatment with androgens has been reported to render Cerebellar Granule Cells less vulnerable to oxidative stress-induced apoptosis by potentiating antioxidant defences (Ahlbom *et al*., 1999). Androgen ingestion by a pregnant mother can cause virilization of a female fetus (Dewhurst and Gordon, 1984). 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 (International Programme on Chemical Safety, 1993). The steroids have been reported to be genotoxic in human lymphocytes *in vitro* and in mouse bone-marrow cells (Dhillon *et al*., 1994; Hundal *et al*., 1997; Joosten *et al*., 2004; Siddique and Afzal, 2004a-c, 2005a; Siddique *et al*., 2005a, b, 2006a). The genotoxic effects of steroids can be reduced by the use of antioxidants and natural plant products (Ahmad *et al*., 2004; Siddique and Afzal, 2005b; Siddique *et al*., 2005b, 2006b, 2007a-c, 2008a, b; Beg *et al*., 2007). The earlier studies are concerned with the estrogens and synthetic progestins. The present study deals with the androgen that is used in the treatment of various hormonal diseases and for boosting muscle growth in patients and also in body-building (International Programme on Chemical Safety, 1993).

TP has been shown to induce cell transformation in a dose-related manner in Syrian Hamster Embryo (SHE) cells (Tsutsui *et al*., 1995). TP does not reduce the toxic effect of anticancer drugs on hemopoietic pluripotent stem cells (Bogliolo *et al*., 1982). Treatment with TP for a long duration causes development of leiomyosarcomas in the vas deferens or uterus of Golden Syrian hamsters (Hudson *et al*., 1998). Apart from these reports of TP toxicity in hamsters, TP has been reported to reduce the toxicity of thiamphenicol in male Sprague-Dawley rats (Maita *et al*., 2004). TP also produces a direct toxic effect in heart cell cultures neonatally in rat (Welder *et al*., 1995).

Such reports of TP toxicity in experiments conducted on hamsters, rats and mice, prompted us to test TP's toxic potential using cultured human lymphocytes in the presence as well as absence of metabolic activation (using S9 mix).

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Epicatechin gallate (ECG), a catechin, is a polyphenolic antioxidant plant metabolite whose largest source in the human diet is from various teas (including white tea, green tea, black tea and Oolong tea) derived from the tea-plant *Camellia sinensis* (Balentine *et al*., 1998). The health benefits of catechins have been studied extensively in humans and in animal models. Reduction in atherosclerotic plaques (Chyu *et al*., 2004) and in carcinogenesis was seen *in vitro* (Mittal *et al*., 2004) and *in vivo* models. Epigallocatechin-3-gallate (EGCG), a related compound, is an antioxidant that helps protect the skin from UV radiation-induced damage and tumor formation (Katiyar *et al*., 2007). Green tea catechins have also been shown to possess antibiotic properties due to their role in disrupting a specific stage of the bacterial DNA replication process (Gradisar *et al*., 2007). White tea typically contains higher level of catechins (http://findarticles.com/p/articles/mi_mo854/is_9_26/ai_n18616257).

In this study, we also observed the antioxidant activity of ECG when it was used in order to reduce genotoxic damage due to TP, both in the presence and absence of metabolic activation.

MATERIALS AND METHODS

Chemicals and reagents: Testosterone propionate (Sigma-Aldrich); Epicatechin gallate (Sigma-Aldrich); RPMI-1640 (Gibco); Fetal calf serum (Gibco); 0.1 mL Antibiotic-antimycotic mixture (Gibco); Phyto-haemagglutinin-M (Gibco); Colchicine (Microlab); Hoechst 33258 stain (Fluka); 3% Giemsa solution in phosphate buffer, pH 6.8 (Merck); 5-Bromo-2-deoxyuridine, BrdU (Sigma-Aldrich); Dimethylsulphoxide, DMSO (Merck); Mitomycin C, (Merck); Cyclophos-phamide (Sigma-Aldrich).

Lymphocyte culture and chromosomal aberration analysis: Peripheral blood cultures were done in duplicate (Carballo *et al*., 1993). 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 h.

Testosterone propionate dissolved in dimethyl sulphoxide (DMSO) was added separately to different cultures at 10, 20 and 40 μM DMSO (5 μL mL^{-1}) served as negative control. For metabolic activation experiments, 0.5 mL of S9 mix was given with each treatment. S9 mix was prepared according to the standard protocol of Maron and Ames (1983). S9 mix was given for 6 h after which cells were collected by centrifugation and washed in prewarmed media to remove the traces of S9 mix and drugs. Two hours before harvesting, 0.2 mL of colchicine (0.2 μg mL^{-1}) 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 (International Programme on Chemical Safety, 1985).

Sister chromatid exchange analysis: For SCE analysis, BrdU (10 μg mL^{-1}) was added at the beginning of the culture. After 24 h, same treatments were given as for CA analysis. Two hours before harvesting, 0.2 mL of colchicine (0.2 μg mL^{-1}) was added, followed by hypotonic treatment and fixation and processing of slides (Perry and Wolff, 1974) 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.

Treatment of TP with ECG: Each genotoxic dose of TP was treated with the appropriate dose of ECG. 20 and 40 μM each of TP was treated with 20, 30 and 40 μM of ECG in the presence of S9 mix. This was followed by CA and SCE analysis as described.

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 (Fisher and Yates, 1963).

RESULTS AND DISCUSSION

TP was not genotoxic in the absence of metabolic activation (Table 1, 3). A significant increase in CAs was observed in the presence of metabolic activation at 20 and 40 μM of TP in a dose-related manner. ECG proved to be effective in reducing the number of abnormal cells with

Table 1: CAs induced by TP in cultured human lymphocytes and the antioxidant effect of ECG on CA frequency, without S9 mix

Treatment (without S9 mix)	Abnormal cells (% ±SE)	CAs Gaps	CTB	CSB	CTE	DIC
TP (μM)						
10	3 (1.00±0.57)	2	3	-	-	-
20	4 (1.33±0.66)	3	3	1	-	-
40	7 (2.33±0.87)	5	5	2	-	-
Untreated	2 (0.67±0.47)	1	2	-	-	-
Negative control (DMSO, 5 μL mL^{-1})	3 (1.00±0.57)	2	3	-	-	-
Positive control (Mitomycin C, 0.3 μg mL^{-1})	34 (11.33±1.82[a])	18	21	15	2	1

[a]Significant difference with respect to untreated ($p<0.05$), SE: Standard Error, CTB: Chromatid Break, CSB: Chromosome Break, CTE: Chromatid Exchange, DIC: Dicentric Chromosome

Table 2: CAs induced by TP in cultured human lymphocytes and the antioxidant effect of ECG on CA frequency, with S9 mix

Treatment (without S9 mix)	Abnormal cells (% ±SE)	CAs Gaps	CTB	CSB	CTE	DIC
TP (μM)						
10	4 (1.33±0.66)	3	4	-	-	-
20	12 (4.00±1.13)[b]	10	10	2	-	-
40	22 (7.33±1.50)[b]	13	15	7	-	-
ECG (μM)						
20	4 (1.33±0.66)	3	3	1	-	-
30	3 (1.00±0.57)	2	3	-	-	-
40	2 (0.67±0.47)	1	1	1	-	-
ECG+TP						
20+20	7 (2.33±0.87[c])	5	6	1	-	-
20+40	17 (5.67±1.34[c])	8	12	5	-	-
30+20	5 (1.67±0.74[c])	4	4	1	-	-
30+40	15 (5.00±1.26[c])	7	10	5	-	-
40+20	3 (1.00±0.57)	2	2	1	-	-
40+40	12 (4.00±1.13)	11	9	3	-	-
Untreated	3 (1.00±0.57)	1	2	1	-	-
Negative control (DMSO, 5 μL mL^{-1})	3 (1.00±0.57)	2	2	1	-	-
Positive control (CP, 0.3 μg mL^{-1})	38 (12.67±1.92[a])	19	26	17	4	3

[a]Significant difference with respect to untreated ($p<0.01$), [b]Significant difference with respect to untreated ($p<0.05$), [c]Significant with respect to TP ($p<0.05$), SE: Standard Error, CTB: Chromatid Break, CSB: Chromosome Break, CTE: Chromatid Exchange, DIC: Dicentric Chromosome

CAs induced by TP at the tested dosage. A dose-dependent decrease in number of abnormal metaphases was observed when 20 and 40 μM of TP was treated with 20, 30 and 40 μM of ECG. The selected dosage of ECG was not genotoxic itself (Table 2). During SCE analysis, a significant increase in SCEs was observed at 20 and 40 μM of TP while a significant decrease in SCEs was observed when 20 and 40 μM of TP was treated with 20, 30 and 40 μM of ECG. The selected dosage of ECG itself did not induce SCEs significantly enough and hence was not found genotoxic (Table 4).

The result of the present study reveals that TP is genotoxic only in the presence of metabolic activation (S9 mix+NADP). The first step may involve aromatic hydroxylation catalyzed by cytochrome p450 as in the case of other steroids (Fishman, 1983). Cytochrome p450 in liver S9 fractions plays an important role in activating promutagens to proximate and/or ultimate mutagens (Maron and Ames, 1983; Guengerich and Shimada, 1991). Testosterone in human body can be converted to both estrogen (aromatization) as well as dihydrotestosterone. Estrogen is the main metabolite for many side-effects in the human body and it has also been reported for genotoxicity in various *in vitro* and *in vivo* experimental models (Dhillon *et al*., 1994; Hundal *et al*., 1997; Joosten *et al*., 2004; Siddique *et al*., 2005a).

Table 3: SCEs induced by TP in cultured human lymphocytes and the antioxidant effect of ECG on SCE frequency, without S9 mix

Treatment (without S9 mix)	SCEs/Cell (Mean±SE)
TP (μM)	
10	2.36±0.24
20	2.42±0.20
40	2.76±0.23
Untreated	1.24±0.10
Negative control (DMSO, 5 μL mL^{-1})	1.18±0.09
Positive control (Mitomycin C, 0.3 μg mL^{-1})	10.22±1.20[a]

[a]Significant difference with respect to untreated ($p<0.01$), SE: Standard Error

Table 4: SCEs induced by TP in cultured human lymphocytes and the antioxidant effect of ECG on SCE frequency, with S9 mix

Treatment (without S9 mix)	SCEs/Cell (Mean±SE)
TP (μM)	
10	2.56±0.22
20	5.84±0.46[b]
40	7.81±0.62[b]
ECG (μM)	
20	1.74±0.33
30	1.77±0.37
40	1.94±0.45
ECG+TP	
20+20	2.88±0.26[c]
20+40	2.74±0.24[c]
30+20	2.54±0.22[c]
30+40	4.98±0.36[c]
40+20	4.22±0.32
40+40	3.98±0.30
Untreated	1.22±0.10
Negative control (DMSO, 5 μL mL^{-1})	1.20±0.10
Positive control (CP, 0.3 μg mL^{-1})	11.48±1.42[a]

[a]Significant difference with respect to untreated ($p<0.01$), [b]Significant difference with respect to untreated ($p<0.05$), [c]Significant with respect to TP ($p<0.05$), SE: Standard Error

In present study, TP was found to increase CAs and SCEs at 20 and 40 μM. CAs are changes in chromosome structure resulting from a break or an exchange of chromosomal material. Most of the CAs observed in the cells are lethal but there many other aberrations that are viable and can cause genetic effects either somatic or inherited (Swierenga *et al*., 1991). SCE is generally a more sensitive indicator of genotoxic effects than structural aberrations (Tucker and Preston, 1996). There is a correlation between the carcinogenicity and SCE inducing ability of large number of chemicals (Gebhart, 1981). The

ready quantifiable nature of SCEs with high sensitivity for revealing toxicant-DNA interaction and the demonstrated ability of genotoxic chemicals to induce significant increase in SCEs in cultured cells has resulted into this endpoint being used as an indicator of DNA damage in blood lymphocytes of individuals exposed to genotoxic carcinogens. The above genotoxic endpoints are well known markers of genotoxicity and any reduction in the frequency of the genotoxic endpoints gives an indication of the antigenotoxicity of a particular compound (Albertini *et al*., 2000). Increase of chromosomal aberration has been associated with the increase in the incidence of formation of various carcinomas (Hagmar *et al*., 1994, 1998).

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 (Ahmad *et al*., 2004; Siddique and Afzal, 2005b; Siddique *et al*., 2005b, 2006b, 2007a-c, 2008a, b; Beg *et al*., 2007). In this study, ECG reduces genotoxicity of TP in human lymphocytes. The reduction in genotoxic damage may be due to the possibility of the prevention of metabolic activation of TP by ECG. The selected dosage of ECG 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 (Martelli *et al*., 2003) and this higher concentration may lead to genotoxic damage and may further increase the possibility of the development of various types of cancers (Albertini *et al*., 2000).

CONCLUSION

Epicatechin gallate reduces genotoxic damage when used at the highest tested dosage, thereby giving a strong indication of its protective role in reducing the genotoxicity induced by testosterone propionate.

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