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Antigenotoxic Effect of Genistein and Gingerol on Genotoxicity Induced by Norethandrolone and Oxandrolone in Cultured Human Lymphocytes

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Abstract: Norethandrolone and Oxandrolone were studied for their genotoxic effect on human lymphocyte chromosomes using chromosomal aberrations (CAs) and sister chromatid exchanges (SCEs) as parameters and subsequently Genistein and Gingerol were used as antigenotoxic agents to ameliorate the genotoxicity induced by the steroids. This experiment was aimed at finding the dosage at which these two steroids are genotoxic enough to cause chromosome damage. They were studied at 5, 10, 20, 30 and 40 μM, respectively and were found to be significantly genotoxic at 30 and 40 μM. Genistein and Gingerol proved to be equally effective in reducing genotoxic damage at appropriate doses. The results suggest a strong genotoxic effect of both steroids *in vitro* in human lymphocytes and also a significant antigenotoxic action of Genistein and Gingerol against steroid induced genotoxic damage.

Key words: Norethandrolone, oxandrolone, genistein, gingerol, androgens, genotoxicity, chromosomal aberrations, sister chromatid exchanges, human lymphocytes

INTRODUCTION

Genistein is an anticarcinogenic isoflavone found in soybean seeds in the form of glycosides. It has been demonstrated that genistein has a great number of biological activities in animal cells including weak estrogenic effect, inhibition of different kinases (including tyrosine kinase), antioxidant properties and modulation of cell proliferation and transformation (Muster *et al*., 1997). Epidemiological and animal model studies have shown a relationship between a diet high in soyfoods and a gamut of beneficial effects with regard to health, including reduced incidence of breast and prostate cancers, cardiovascular disease and post-menopausal ailments (Dixon and Ferreira, 2002). Several studies suggest that genistein exerts a protective effect against lipid peroxidation of low and high density lipoproteins (LDL and HDL) (Patel *et al*., 2001; Ferretti *et al*., 2004). Genistein is synthesized from the flavonone naringenin in plants through a ring migration reaction catalyzed by the cytochrome P450 enzyme isoflavone synthase (IFS). IFS genes have recently been cloned from many plant species and now genistein can be produced in non-legumes through recombinant DNA technique (Dixon and Ferreira, 2002).

The key constituents of Ginger (*Zingiber officinale*) include volatile oils, oleoresin (Gingerol, shogaol), phenol (Gingerol, zingerone), vitamins and minerals. Ginger has medicinal purposes (Huang *et al*., 1990). It has carminative, diaphoretic and antispasmodic properties, reduces clotting, cholesterol and blood pressure, is anti-inflammatory, helpful in nausea and indigestion.

Naturally occurring anabolic steroids are synthesized in the testis, ovary and adrenal gland from cholesterol via pregnenolone. Synthetic anabolic steroids are based on the principal male hormone testosterone (Haynes and Murad, 1985). They bind to receptors in reproductive tissue, muscles and fat (Mooradian *et al*., 1987). Norethandrolone and Oxandrolone are oral anabolic-androgenic steroids (AAS), synthesized and approved under the brand names Nilevar and Anavar, respectively. They have moderate progestagenic activity and share liver toxicity issues common to 17-alkylated steroids. Acute overdosage can produce nausea and gastrointestinal upset. Chronic use of Norethandrolone can lead to excessive androgens: menstrual irregularities and virilization in women and impotence, premature cardiovascular disease and prostatic hypertrophy in men (International Programme on Chemical Safety, 1993).

Precocious prostatic cancer has been described after long term anabolic steroid abuse (Roberts and Essenhigh, 1986). Cases where hepatic cancers have been associated with anabolic steroid abuse have been reported (Overly *et al*., 1984). Also, androgen ingestion by a

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pregnant mother can cause virilization of a female fetus (Dewhurst and Gordon, 1984). Oxandrolone is used in the treatment of anemia and hereditary angioedema, which causes episodes of swelling of the face, extremities, genitals, bowel wall and throat (Gannon, 1994) and also prescribed for a number of medical disorders causing involuntary weight loss, in order to promote muscle regrowth and in treating cases of osteoporosis. 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, b, c; Siddique and Afzal, 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, b, c; Beg *et al*., 2007; Siddique *et al*., 2008a, b). The above studies are concerned with the estrogens and synthetic progestins. The present study deals with the androgens that are 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).

The use of these two steroids by humans and their ability to act as genotoxic agents, as suggested by earlier studies was an ample reason for us to examine their genotoxic potential using cultured human lymphocytes. In this study, we primarily analyse the antigenotoxic effect of Genistein and Gingerol separately on the frequency of CAs and SCEs induced by Norethandrolone and Oxandrolone respectively, *in vitro* in human lymphocytes.

MATERIALS AND METHODS

Time and place of study: The study was conducted from April-September, 2007, in the Human Genetics and Toxicology Laboratory, Department of Zoology, AMU, Aligarh (UP), India.

Chemicals and reagents: Norethandrolone; Oxandrolone; Genistein (Sigma); Gingerol (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).

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. Norethandrolone and Oxandrolone, both dissolved in dimethyl sulphoxide (DMSO), were added separately to different cultures at 5, 10, 20, 30 and 40 μM. DMSO (5 μl mL^{-1}) served as negative control.

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 (1985) WHO, Geneva, for the study of genetic defects in human population.

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.

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

Both Genistein and Gingerol proved to be equally effective in reducing the number of abnormal cells with CAs induced by Norethandrolone and Oxandrolone at all their tested doses. A significant increase in CAs was observed at 30 and 40 μM of both norethandrolone and oxandrolone (Table 1, 2). The dose-dependent increase in CAs was not much from 30 to 40 μM.

A dose-dependent decrease in number of abnormal metaphases was observed when the genotoxic doses of norethandrolone were treated with 30 and 40 μM of

Table 1: Ameliorative action of genistein and gingerol on CAs induced by norethandrolone in cultured human lymphocytes

Treatments	No. of abnormal cells (%±SE)	Total CAs				
		Gaps	CTB	CSB	CTE	DIC
Norethandrolone (μM)						
5	7 (2.33±0.87)	2	4	3	-	-
10	8 (2.67±0.93)	3	5	3	-	-
20	10 (3.33±1.04)	4	6	3	-	-
30	21 (7.00±1.47)[b]	8	13	7	-	-
40	27 (9.00±1.65)[b]	11	18	6	-	-
Genistein (μM)						
30	4 (1.33±0.66)	1	2	2	-	-
40	5 (1.67±0.74)	2	3	2	-	-
Genistein + Norethandrolone						
30 + 30	15 (5.00±1.26)[c]	5	9	6	-	-
30 + 40	21 (7.00±1.47)[c]	8	13	8	-	-
40 + 30	11 (3.67±1.09)[c]	4	7	4	-	-
40 + 40	17 (5.67±1.34)[c]	6	11	6	-	-
Gingerol (μM)						
20	3 (1.00±0.57)	1	2	1	-	-
30	4 (1.33±0.66)	2	3	1	-	-
Gingerol + Norethandrolone						
20 + 30	13 (4.33±1.18)[c]	5	8	5	-	-
20 + 40	18 (6.00±1.37)[c]	6	10	8	-	-
30 + 30	9 (3.00±0.98)[c]	3	5	4	-	-
30 + 40	11 (3.67±1.09)[c])	4	7	4	-	-
Untreated	3 (1.00±0.57)	2	2	1	-	-
Negative control (DMSO, 5 μl mL^{-1})	3 (1.00±0.57)	1	2	1	-	-
Positive control (Mitomycin C, 0.3 μg mL^{-1})	60 (20.00±2.31[a])	28	45	20	4	2

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

Table 2: Ameliorative action of genistein and gingerol on CAs induced by Oxandrolone in cultured human lymphocytes

Treatments	No. of abnormal cells (%±SE)	Total CAs				
		Gaps	CTB	CSB	CTE	DIC
Oxandrolone (μM)						
5	5 (1.67±0.74)	3	4	1	-	-
10	7 (2.33±0.87)	3	4	3	-	-
20	10 (3.33±1.04)	4	6	4	-	-
30	15 (5.00±1.26)[b]	7	10	5	-	-
40	19 (6.33±1.41)[b]	9	15	4	-	-
Genistein (μM)						
30	3 (1.00±0.57)	1	2	1	-	-
40	4 (1.33±0.66)	2	3	1	-	-
Genistein + Oxandrolone						
30 + 30	10 (3.33±1.04)[c]	3	6	4	-	-
30 + 40	14 (4.67±1.22)[c]	4	9	5	-	-
40 + 30	7 (2.33±0.87)[c]	2	5	2	-	-
40 + 40	11 (3.67±1.09)[c]	3	8	3	-	-
Gingerol (μM)						
20	2 (0.67±0.47)	1	1	1	-	-
30	3 (1.00±0.57)	1	2	1	-	-
Gingerol + Oxandrolone						
20 + 30	8 (2.87±0.93)[c]	3	6	2	-	-
20 + 40	12 (4.00±1.13)[c]	5	10	2	-	-
30 + 30	6 (2.00±0.81)[c]	2	4	2	-	-
30 + 40	9 (3.00±0.98)[c]	2	6	3	-	-
Untreated	3 (1.00±0.57)	1	2	1	-	-
Negative control (DMSO, 5 μl mL^{-1})	3 (1.00±0.57)	1	2	1	-	-
Positive control (Mitomycin C, 0.3 μg mL^{-1})	48 (16.00±2.12)[a]	22	38	10	5	1

[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 Oxandrolone ($p<0.05$), SE: Standard Error, CTB: Chromatid Break, CSB: Chromosome Break, CTE: Chromatid Exchange, DIC: Dicentric Chromosome

Table 3: Ameliorative action of genistein and gingerol on SCEs induced by Norethandrolone in cultured human lymphocytes

Treatments	SCEs/Cell (Mean±SE)
Norethandrolone (μM)	
5	0.79±0.35
10	1.07±0.58
20	1.33±0.64
30	4.88±1.30[b]
40	5.96±1.53[b]
Genistein (μM)	
30	1.35±0.67
40	1.39±0.68
Genistein + Norethandrolone	
30 + 30	3.22±1.12[c]
30 + 40	4.12±1.27[c]
40 + 30	3.01±1.03[c]
40 + 40	3.52±1.23[c]
Gingerol (μM)	
20	1.10±0.57
30	1.28±0.62
Gingerol + Norethandrolone	
20 + 30	3.02±1.14[c]
20 + 40	3.78±1.26[c]
30 + 30	2.92±1.08[c]
30 + 40	3.22±1.21[c]
Untreated	1.00±0.56
Negative control (DMSO, 5 μl mL^{-1})	1.33±0.66
Positive control (Mitomycin C, 0.3 μg mL^{-1})	13.22±2.02[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 Norethandrolone ($p<0.05$), SE: Standard Error

Table 4: Ameliorative action of genistein and gingerol on SCEs induced by Oxandrolone in cultured human lymphocytes

Treatments	SCEs/Cell (Mean±SE)
Oxandrolone (μM)	
5	0.75±0.32
10	1.08±0.56
20	1.26±0.60
30	4.36±1.18[b]
40	5.22±1.24[b]
Genistein (μM)	
30	1.37±0.68
40	1.42±0.69
Genistein + Oxandrolone	
30 + 30	3.32±1.10[c]
30 + 40	4.02±1.16[c]
40 + 30	3.04±1.06[c]
40 + 40	3.64±1.12[c]
Gingerol (μM)	
20	1.12±0.56
30	1.32±0.58
Gingerol + Oxandrolone	
20 + 30	3.02±1.06[c]
20 + 40	3.72±1.25[c]
30 + 30	2.78±0.98[c]
30 + 40	3.34±1.02[c]
Untreated	1.00±0.56
Negative control (DMSO, 5 μl mL^{-1})	1.33±0.66
Positive control (Mitomycin C, 0.3 μg mL^{-1})	14.26±1.96[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 Oxandrolone ($p<0.05$), SE: Standard Error

genistein and 20 and 30 μM of gingerol (Table 1). The selected dosage of genistein and gingerol was not genotoxic itself (Table 1, 2). Similar dose-dependent decrease in number of abnormal metaphases was observed when the genotoxic doses i.e., 30 and 40 μM of oxandrolone was treated with 30 and 40 μM of genistein and 20 and 30 μM of gingerol, respectively (Table 2).

For sister chromatid exchange analysis, a significant increase in SCEs/cell was observed at 30 and 40 μM of norethandrolone and oxandrolone, respectively (Table 3, 4). A significant decrease in SCEs/cell was observed when 30 and 40 μM of norethandrolone was treated with 30 and 40 μM of genistein and 20 and 30 μM of gingerol (Table 3). Similar trend was observed when 30 and 40 μM of oxandrolone was treated with 30 and 40 μM of genistein and 20 and 30 μM of gingerol, respectively (Table 4). The selected dosage of gingerol and genistein did not induce SCEs/cell significantly as compared to the untreated (Table 3, 4).

DISCUSSION

Genistein is particularly effective in quenching free radicals produced by toxic agents and protects cells against oxidative damage especially with respect to DNA (Foti *et al*., 2005; Lee *et al*., 2000). It is a common precursor in the biosynthesis of antimicrobial phytoalexins and phytoanticipins in legumes and an important nutraceutical molecule. It is also capable of inhibiting lipoprotein oxidation *in vitro* and suppressing formation of plasma lipid oxidation products *in vivo*. Extracts of Ginger (including Gingerol) have antioxidant activity through scavenging of superoxide and hydroxyl radicals and by inhibiting lipid peroxidation (Kikuzaki and Nakatani, 1993). It is also antibacterial, antifungal and used for common cold (Bode *et al*., 2001). The pungency of Ginger is due to Gingerol which is an alcohol of oleoresin and the aroma is due to its oil (Hasenöhrl *et al*., 1998). Gingerol is the major pharmacologically active component inducing apoptosis (Lee and Surh, 1998; Lee *et al*., 1998) in cancer cells. The results of the present study reveal that the selected dosages of genistein and gingerol are not genotoxic *per se* but reduced the genotoxic damage caused by oxandrolone and norethandrolone in human lymphocytes *in vitro*. The International Agency on Cancer (IAC), mainly on the basis of epidemiological studies classifies steroidal estrogens and 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) (Martelli *et al*., 2003).

An increase in the frequency of chromosomal aberrations in peripheral blood lymphocytes is associated with an increased overall risk of cancer (Hagmar *et al*., 1994, 1998). 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 (Albertini *et al*., 2000). 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 antigen toxicity of a particular compound (Albertini *et al*., 2000). Many plant products protect against xenobiotics either by inducing detoxifying enzymes or by inhibiting oxidative enzymes (Morse and Stoner, 1993).

The protective effect observed in the present study i.e., significant reduction in the frequency of cells with chromosomal damage and sister chromatid exchanges may be due to the direct action of the compounds (i.e., Genistein and Gingerol).

The outcome of this experiment shows that Norethandrolone and Oxandrolone have the potential to be genotoxic and cytotoxic, especially at 30 and 40 μM, in cultured human lymphocytes and their genotoxicity is reduced significantly on applying Genistein and Gingerol separately, at appropriate dosage. 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. The effectiveness of Genistein and Gingerol as antimutagenic agents is an attribute that can be effectively used in making anticancer drugs.

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