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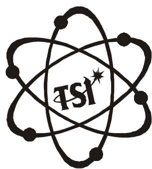

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Protective role of *centella asiatica* l. Extract against the genotoxic damage induced by megestrol acetate

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ABSTRACT

The genotoxicity study of megestrol acetate (MGA) was carried out on human lymphocytes using chromosomal aberrations (CA), mitotic index (MI) and sister chromatid exchanges (SCEs) as parameters. The effect of MGA was studied at 5, 10, 20 and 30μM of the culture medium. MGA was found to be genotoxic at 20 and 30 μM. The treatment of 30μM of MGA with 1.075×10^{-4}, 2.125×10^{-4} and 3.15×10^{-4} g/ml of *Centella asiatica* extract results in the significant reduction in CAs, MI and SCEs, suggesting a protective role of *C.asiatica* extract, during the megestrol acetate therapy.
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KEYWORDS

Megestrol acetate;
Genotoxicity;
Centella asiatica L.;
Chromosomal aberrations;
Sister chromatid exchanges.

INTRODUCTION

Megestrol acetate (MGA) is a synthetic progestin, used as oral contraceptives either singly or in combination with estrogens, in the treatment of breast and endometrial cancer[5]. MGA was tested by oral administration in mice, rats, dogs and monkeys. It produces nodular hyperplasia, and benign and malignant mammary tumours in dogs[11]. MGA plus ethinylestradiol was tested for carcinogenicity by oral administration to mice and rats. In mice, increased incidences of malignant mammary tumours were observed in animals of each sex, but no increase in tumour incidence was observed in rats[4]. MGA was reported negative in unscheduled DNA synthesis test using rat, hepatocytes; however, the presence of DNA adducts has been shown in rat liver *in vivo* and cultured human hepatocytes[9,25,21]. It has also been shown to induce micronucleus in rat liver *in vivo,* but has failed to cause chromosomal aberrations in human peripheral blood lymphocytes *in vitro*[16,2]. It induced chromosomal aberrations and sister chromatid exchanges in mice bone marrow cells at 16.25 and 32.50 mg/kg body weight[27,30,31]. The genotoxic effects of synthetic progestins can be reduced by the antioxidants[27,28,30,31,32] and natural plant products having antioxidant properties[26,27,28,30,31,32]. Prolonged use of oral contraceptives has been shown to develop various types of malignancies in human and experimental animals[5]. In this context the plant extract of some medicinal value can be use to ameliorate the possible genotoxic effects during the prolonged progestin therapy.

Centella asiatica L. belongs to the family Umbelliferae. It is found in swampy area of India, commonly found as a weed crop fields and other waste places throughout India upto an altitude of 600 meters. The crude extract of *C.asiatica* and the products derived from glycoside were used as oral infertility agents[23]. The extract of *C.asiatica* possess antioxidant[20], anti-inflammatory[7], immunodulating[12], antitumor[24], anti-proliferative[15], radio protective[8] and antigenotoxic properties[29]. The crude extract of

C.asiatica was shown to be non-toxic in normal human lymphocytes[24]. The objective of the present study was to study the antigenotoxic effect of *Centella asiatica* L plant extract against the genotoxic damage by megestrol acetate on cultured human lymphocytes.

EXPERIMENTAL

Chemicals

Megestrol acetate (CAs No: 595-33-5, Sigma); RPMI 1640 (Gibco), Fetal calf serum (Gibco), Phytohaemagglutinin (Gibco), Dimethylsulphoxide (E. Merk, India), Colchicine (Microlab), Hoechst 33258 (Sigma), 3% Giemsa solution in phosphate buffer (pH 6.8, E. Merck, India), Mitomycin C (Sigma), 5-Bromo-2-deoxyuridine (SRL, India), Antibiotic- antimycotic mixture (Gibco).

Extract preparation

Centella asiatica L. leaves were collected from the nursery of Forest Research Institute (FRI), Dehradun (U.A.) and were air dried and ground to fine powder. Extraction was performed by soaking samples (30 gm of dry weight) in 300 ml of acetone for 8-10 hr at 40-60^{0}C in soxhlet's apparatus. After filter, the excess of solvent was removed by rotatory evaporator[28,32]. The extract concentrations of 1.075×10-4, 2.127×10-4 and 3.15×10-4 g/ml of culture medium were established.

Human lymphocyte culture

Duplicate peripheral blood cultures were performed according to Carballo et al.[13]. Briefly 0.5 ml of heparinized blood samples were obtained from two healthy female donors, and were placed in a sterile culture tube containing 7 ml of RPMI 1640, supplemented with 1.5 ml of fetal calf serum, 0.1 ml of phytohaemagglutinin and 0.1 ml of antibiotic-antimycotic mixture. They were placed in an incubator at 37^{0}C for 24 hr. Dimethylsul phoxide (DMSO, 5µl/ml) and Mitomycin C(0.3µg/ml) were taken as negative and positive control, respectively.

Chromosomal aberration (CA) and mitotic index (MI) analysis

After 24 hr of the initiation of culture, the human lymphocytes were treated with megestrol acetate at 5, 10, 20 and 30 µM dissolved in dimethylsulphoxide and kept for another 48 hr at 37^{0}C in an incubator. 1 hr prior to harvesting 0.2 ml of colchicine (0.2µg/ml) was added to the culture flask. Cells were centrifuged at 1000 rpm for 10 min. The supernatant was removed and 5 ml of prewarmed (37^{0}C) 0.075 M KCl (hypotonic solution) was added. Cells were resuspended and incubated at 37^{0}C for 15 min. The supernatant was removed by centrifugation, and 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. The slides were stained in 3% Giemsa solution in phosphate buffer (pH 6.8) for 15 min. At least 300, metaphases were examined for the occurrence of different types of abnormality i.e. gaps, fragments and breaks. Criteria to classify the different types of aberrations were in accordance with the recommendations of Environmental Health Criteria 46 for Environmental Monitoring of Human Populations[6]. The mitotic index (MI) was scored as the number of metaphases among 1000 lymphocytes nuclei and expressed as a percentage.

Sister chromatid exchanges (SCEs) analysis

For SCE analysis, bromodeoxyuridine (BrdU, 10µg/ml) was added at the beginning of the culture. After 24 hr of the initiation of culture, the human lymphocytes were treated with megestrol acetate at final concentration of 5, 10, 20 and 30 µM dissolved in dimethyl sulphoxide and kept for another 48 hr at 37^{0}C in an incubator. Mitotic arrest was done 1 hr prior to harvesting by adding 0.2 ml of colchicine (0.2 g/ml). Hypotonic treatment and fixation were done in the same way as described for CAs analysis. The slides were processed according to Perry and Wolff[18]. The SCE average was taken from an analysis of the metaphase during second cycle of divisions.

Induction of chromosomal aberrations and sister chromatid exchanges in the presence of *Centella asiatica* L. extract

30 µM of megestrol acetate treatment was given with the three doses of *Centella asiatica* L. extract (i.e.) 1.07510^{-4}, 2.125×10^{-4} and 3.15×10^{-4} g/ml of culture medium to study the effect on chromosomal aberration, mitotic index and sister chromatid exchanges induced by megestrol acetate. Duplicate cultures were set for abnormal aberrations, mitotic indices and sister

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TABLE 1: Chromosomal aberrations (CAs) in human lymphocytes treated with megestrol acetate (MGA)

Treatment	Abnormal metaphases without gaps		Chromosome aberrations				MI %
	No.	Mean% ±SE	Gaps		Fragments and/or breaks		
			No.	%	No.	%	
MGA (µM)							
5	4	1.3±0.6	2	0.7	5	1.7	2.5
10	5	1.7±0.7	3	1.0	7	2.3	2.4
20	13	4.3±1.1[a]	7	2.3	16	5.3	2.1
30	17	5.7±1.3[a]	9	3.0	24	8.0	1.9
Untreated	2	0.7±0.4	1	0.3	2	0.7	2.8
Negative control (DMSO µl/ml)	2	0.7±0.4	1	0.7	2	0.7	2.8
Positive control (Mitomycin C, 0.3µg/ml)	26	8.7±1.6	13	4.3	33	11.0	1.1

[a]Significant difference with respect to untreated (P<0.01); MGA: Megestrol acetate; DMSO: Dimethylsulphoxide

TABLE 2: Effect of *Centella asiatica* L. extract on chromosomal aberrations in human lymphocytes treated

Treatment	Abnormal metaphases without gaps		Chromosome aberrations				MI %
	No.	Mean% ±SE	Gaps		Fragments and/or breaks		
			No.	%	No.	%	
MGA (µM)							
30	18	6.0±1.3[a]	9	3.0	26	8.7	1.9
MGA(µM)+ CAE (g/ml)	-	-	-	-	-	-	-
30+1.075×10^{-4}	12	4.0±1.1[b]	7	2.3	16	5.3	2.5
30+2.125×10^{-4}	9	3.0±0.9[b]	6	2.0	10	3.3	2.5
30+3.15×10^{-4}	8	2.7±0.9[b]	5	1.7	9	3.0	2.6
Untreated	2	0.6±0.4	1	0.3	2	0.6	2.7
CAE (g/ml)	-	-	-	-	-	-	-
1.075×10^{-4}	2	0.6±0.4	1	0.3	2	0.4	2.7
2.125×10^{-4}	3	1.0±0.15	2	0.6	3	1.0	2.7
3.15×10^{-4}	2	0.6±0.4	1	0.3	2	0.6	2.8

[a]Significant difference with respect to untreated (P<0.01); MGA: Megestrol acetate; DMSO: Dimethylsulphoxide

chromatid exchanges analysis, similarly as described earlier in the text.

Statistical analysis

Student "t" test was used for the analysis of CAs and SCEs.

RESULTS

In CAs analysis, with the treatment of megestrol acetate a dose dependent increase in the number of abnormal cells was observed. However, a significant increase was observed at 20 and 30 µM of megestrol acetate (TABLE 1). When 30 µM of megestrol acetate was treated with 1.075×10^{-4}, 2.125×10^{-4} and 3.15×10^{-4} g/ml of *Centella asiatica* L. extract separately, a significant decrease of abnormal cells was observed. However, the *C.asiatica* doses itself were not associated with the significance number of abnormal metaphases (TABLE 2). The mitotic index (MI) showed a reduction in the percentage of mitosis for all the doses of MGA assayed in the present study. The increase in the dosages of megestrol acetate was associated with the reduction in the MI (TABLE 1). The treatment of 30 M of megestrol acetate with 1.075×10^{-4}, 2.125×10^{-4} and 3.15×10^{-4} g/ml of *Centella asiatica* L. extract, separately, an increase in the percentage of mitosis was observed (TABLE 2). In SCE analysis, a clear dose dependent increase in SCEs/cell was observed with megestrol acetate treatment alone (TABLE 3). SCEs/cell was significantly increased at 20 and 30 µM of megestrol acetate as compared to untreated. The treatment of 30µM of megestrol acetate with 1.075×10^{-4}, 2.125×10^{-4} and 3.15×10^{-4} g/ml of *C.asiatica* L. extract, separately, a significant decreased in SCEs/cell was observed at each of the given dose. However, the *C.asiatica* doses alone were not associated with the significant increase in the SCEs/cell (TABLE 4).

TABLE 3: Sister chromatid exchange (SCEs) in cultured human lymphocytes exposed to megestrol acetate

Treatment	Cells scored	SCEs/cell (mean±SE)	Range
MGA (µM)			
5	50	2.32 ± 0.04	1 - 5
10	50	2.74 ± 0.05	1 - 5
20	50	5.76 ± 0.19[a]	2 - 7
30	50	6.54 ± 0.21[a]	2 - 7
Untreated	50	1.30 ± 0.01	0 - 5
Negative control (DMSO 5µl/ml)	50	1.74 ± 0.01	0 - 5
Positive control (Mitomycin C, 0.3µg/ml)	50	9.18 ± 0.27	2 - 10

[a]Significant difference with respect to untreated (P<0.05). MGA: Megestrol acetate; DMSO: Dimethyl sulphoxide

DISCUSSION

The results of the present study reveal that the megestrol acetate (MGA) was genotoxic of 20 and 30 µM. Our earlier study with megestrol acetate on mice bone marrow cells. Showed the genotoxicity of MGA at 16.25 and 32.50 mg/kg body weight[27,30,31]. The International Agency on Cancer (IARC), mainly on the

TABLE 4: Effect of *Centella asiatica* L. extract on sister chromatid exchanges (SCEs) induced by megestrol acetate

Treatment	Cells scored	SCEs/cell (mean±SE)	Range
MGA (μM)			
30	50	6.04 ± 0.20^{a}	2 - 7
MGA(μM) + CAE (g/ml)			
$30+1.075\times10^{-4}$	50	3.24 ± 0.10^{b}	1 - 6
$30+2.125\times10^{-4}$	50	3.02 ± 0.09^{b}	1 - 6
$30+3.15\times10^{-4}$	50	2.94 ± 0.06^{b}	1 - 5
Untreated			
CAE (g/ml)			
1.075×10^{-4}	50	1.84 ± 0.02	0 - 5
2.125×10^{-4}	50	1.86 ± 0.02	0 - 5
3.15×10^{-4}	50	1.54 ± 0.01	0 - 5

[a]Significant difference with respect to untreated (P<0.05). [b]Significant difference with respect to megestrol acetate (P<0.05). MGA: Megestrol acetate; CAE: *Centella asiatica* extract

basis of epidemiological studies classified steroidal estrogens and estrogen-progestins combinations among agents carcinogenic to human (Group 1), progestins as possibly carcinogenic (Group 2) and androgenic anabolic steroid, as probably carcinogenic (Group 2A)[1]. Carcinogenicity to humans of sex steroids has been evaluated, and is reported that high dose; of estrogen-progestin combinations can cause liver cancer among humans[5]. In a very recent "Multi Centre Study" on oral contraceptives and liver cancer came to the conclusion that the oral contraceptives may enhance the risk of liver carcinomas[1]. Concerning our study with megestrol acetate on mouse bone marrow cells, it was found to be genotoxic by generating free radicals[27,30,31]. Sister chromatid exchanges have been commonly used to evaluate cytogenetic responses to chemical exposure, and an excellent dose response relationship has been established for hundred of chemicals in wide variety of *in vivo* of *in vitro* short term experiments[10]. Chromosomal aberrations are the changes in chromosome structure resulting from a break or an exchange of chromosomal material. Most of the chromosomal aberrations observed in the cells are lethal, but there are many corresponding aberrations that are viable and can cause genetic effects, either somatic or inherited[22]. These events lead to the loss of chromosomal material at mitosis or due to the inhibition of accurate chromosome segregation at anaphase. SCE is generally a more sensitive indicator of genotoxic effects than structural aberrations[10]. There is a correlation between the carcinogenicity and SCE inducing ability of large number of chemicals[3]. In our present study with MGA, the treatment of *Centella asiatica* L. extract results in the reduction of the genotoxic damage, thereby possibly suggesting a protective role of the plant extract during the MGA therapy. As the plants extract is used as an alternative medicine it becomes necessary to detect one or more active principles present in the extract that are potentially useful for the mankind. The extract *C.asiatica* have certain bioactive terpene acid, such as, asiatic acid, madecassic acid and their respective glycoside, i.e. asiaticoside and madecassoside[19]. There are some phenolic compounds in the extract of *C.asiatica*, having the activity same as that of the α-tocopherol. These phenolic compounds probably scavenge free radicals and thus are responsible for the reduction in the genotoxic damage in the present study[17]. The potentiality of many carcinogens can be reduced by the use of anticarcinogens e.g. phytochemicals, but the knowledge of the specific mechanism of action of many phytoproducts or plant extract is still poor[14]. Medicinal herbs contain complex mixtures of thousands of components that can exert their action separately or in synergistic ways. Ascorbic acid is a well known antioxidant and has been reported to reduce the genotoxicity of megestrol acetate in mice bone marrow cells[27,30,31]. Phenolic compounds such as flavonoids present in the extract of *C.asiatica* L. have antioxidant potentially that may perform a protective role in ameliorating the genotoxic effect of megestrol acetate in the present study. The results of the present study suggests the protective role of *C.asiatica* L. extract against the genotoxic effect of MGA in cultured human peripheral blood lymphocytes. The isolation of the antioxidants and other compounds and suggesting their role may be the part of our future study but the traditional methods should be employed in using the plant extracts taking utmost care with regards to its concentration and duration treatments so that the extract may have the desired pharmacological effects without causing any toxicity.

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