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Evaluation of genotoxic potential of synthetic progestin ethynodiol diacetate in human lymphocytes *in vitro*

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The genotoxicity study of a synthetic progestin ethynodiol diacetate, used as oral contraceptives, was carried out on human lymphocyte chromosomes using sister chromatid exchanges (SCEs), replication index (RI) and chromosomal aberrations (CAs) as parameters. The study was carried out in the presence as well as in the absence of metabolic activation (S9 mix). The aim of the present study is to achieve a precise characterization of the genotoxic activity of ethynodiol diacetate and to establish the value of cytogenetic assays in order to determine the effect of the drugs, at therapeutic doses, to settle an improved risk assessment. Ethynodiol diacetate was studied at three different concentrations (50, 100 and 150 µg/ml of peripheral blood lymphocyte culture) and was found non-genotoxic in the absence of metabolic activation (S9 mix). But in the presence of S9 mix ethynodiol diacetate increases SCE ($P < 0.03$) and CA ($P < 0.005$) frequencies and inhibits lymphocyte proliferation ($P < 0.03$) at 150 µg/ml. The results suggest a genotoxic and cytotoxic effect of ethynodiol diacetate in human peripheral blood cultures *in vitro*.

SYNTHETIC progestins are widely used as oral contraceptives in addition to their use in the treatment of various menstrual disorders, various types of cancers, and in hormonal replacement therapy. For contraception, these are either used alone or in combination with estrogens. Progestins, like estrogens, diffuse easily across the cell membranes and bind to highly specific, soluble receptor proteins in the cytoplasm. These receptors are members of a large family of proteins that act as receptors for a wide range of hydrophobic molecules, including other steroid hormones, e.g. thyroid hormones and retinoids. The steroid receptor complex modifies the expression of specific genes by binding to control elements in DNA[1,2]. Ethynodiol diacetate is used either as single entity drug or in combination with estrogen, such as ethinyloestradiol or mestranol in oral contraceptives[3]. However, studies conducted on the mutagenic activity of various contraceptives and synthetic progestins are contradictory. A significant increase in the number of lymphocytes with DNA migration in alkaline comet assay and frequency of sister chromatid exchanges (SCEs) per metaphase were observed in oral contraceptive users as compared with their age-mat-

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ched untreated controls[4]. Norethisterone acetate, a commonly used progestin was found to induce chromosomal aberrations and sister chromatid exchanges in human lymphocytes *in vitro*, both in presence and absence of metabolic activation[5]. Norethisterone induced chromosomal aberrations in mouse bone marrow *in vivo* but did not produce significant level of micronuclei[6]. Chromosomal abnormalities have been also reported in women taking oral contraceptives[7]. Different progestins failed to produce genotoxic effects in human lymphocytes *in vitro*[8,9] and also in Ames salmonella/microsome assays[5,10]. Ethynodiol diacetate increased the incidence of benign liver tumours in male mice and of mammary tumours in castrated male mice and produced benign mammary tumours in male rats when tested orally. Oral administration of ethynodiol diacetate plus mestranol to mice increased incidence of pituitary tumours which was observed in animals of each sex. Ethynodiol diacetate plus ethinyloestradiol induced increase of pituitary tumours in animals of each sex and of malignant tumours of connective tissues of the uterus. In rats, malignant mammary tumours were produced in animals of each sex[11]. Percutaneous application of ethynodiol diacetate disturbed spermatogenesis and caused atrophy of Leydig cells and the accessory genital organs[12].

Duplicate peripheral blood cultures were prepared[13]. Briefly, 0.5 ml of heparinized blood samples were obtained from two healthy female donors and were placed in a sterile flask containing 7 ml of F-10 medium (Gibco) supplemented with 1.5 ml fetal calf serum (Gibco) and 0.1 ml phytohemagglutinin-M (Gibco). 50, 100 and 150 μg/ml of ethynodiol diacetate (CAS No.: 297-76-7 Wyeth Lab) dissolved in dimethylsulphoxide (DMSO, 5 μl/ml E. Merck, Mumbai, India) was added at the beginning of the culture and then incubated for 72 h at 37°C. Normal, negative and positive control cultures were grown under identical conditions. For metabolic activation experiments, liver S9 fraction (S9 mix) was prepared from Swiss albino healthy rats (wistar strain) as per standard procedures of Maron and Ames[14]. The S9 fraction was enhanced by addition of 5 μM NADP and 10 μM glucose-6-phosphate just before use. About 1 ml of S9 mix was given along with each dose of ethynodiol diacetate (i.e. 50, 100 and 150 μg/ml) at the beginning of the culture. Normal, negative and positive controls were also given 1 ml of S9 mix for metabolic activation experiment and incubated at 37°C for 72 h. These doses were selected considering that at 400 μg/ml, inhibition of cell growth (MI = 0.7 ± 0.3) was observed in the absence of S9 mix.

For sister chromatid exchange (SCE) analysis, bromodeoxyuridine (BrdU, 10 μg/ml; Sigma) was added at the beginning of the culture, cells were harvested after 72 h of incubation at 37°C. Two hours before harvesting, 0.2 ml of colchicine (0.2 μg/ml) was added to the culture flask. Cells were centrifuged at 800–1000 rpm for 10 min. The supernatant was removed and 5 ml of prewarmed (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 fixation of cells was done by slowly adding 5 ml of Carnoy's fixative (methanol : glacialacetic acid; 3 : 1). The fixative was removed and the procedure was repeated twice. The slides were processed according to Perry and Wolff with some modifications[15]. Slides were stained for 20 min in a 0.05% (w/v) Hoechst 33258 (Sigma) solution, rinsed with tap water and placed under a near UV lamp for 90 min, covered with Sorensen's buffer, and stained with a 3X Giemsa solution in phosphate buffer (pH 6.8) for 15 min. The SCE average was taken from an analysis of the metaphases during the second cycle of divisions.

For cell cycle analysis 100 metaphases per culture were examined. Each metaphase was classified as being in the first (M_1), second (M_2) or third and further (M_{3+}) divisions[16]. The replication index (RI) was calculated as follows:

$$\mathrm{RI} = \frac{(\%\,\text{of cells in}\,M_1) + 2(\%\,\text{of cells in}\,M_2) + 3(\%\,\text{of cells in}\,M_{3+})}{100}.$$

For the analysis of structural CA, parallel cultures were carried out without BrdU for 72 h. Mitotic arrest was initiated 2 h prior to harvesting by the addition of 0.2 ml of colchicine (0.2 μg/ml). Hypotonic treatment and fixation were performed in the same way as in SCEs analysis. The slides were stained in 3% Giemsa solution in phosphate buffer (pH 6.8) for 15 min. 300 well-spread metaphases were examined for the occurrence of different types of structural CAs. Gaps were excluded in the calculation of chromosome breakage frequencies. Chromatid exchange configurations and dicentric chromosomes were scored as two (2) breaks. The criteria to classify the different types of aberrations were in accordance with the recommendation of EHC 46 for Environmental Monitoring of Human Populations[17].

The means of frequencies of SCEs were statistically analysed by the Kruskal–Wallis test, comparisons between groups were done by the Dunn procedure. Differences between the values of abnormal cells and cell cycle kinetics were analysed by the χ^2 test. The level of significance was tested from standard statistical tables of Fisher and Yates[18].

Ethynodiol diacetate neither induced SCEs nor CA, nor affected the cell growth kinetics at significant level in the absence of metabolic activation (Tables 1 and 2). However in the presence of S9 mix, the data on the frequencies of SCE and cell cycle kinetics in human lymphocytes treated with ethynodiol diacetate are presented in Table 1. A significant increase in the values of SCEs/cell was observed at 150 μg/ml ($P < 0.03$). At 50 and 100 μg/ml the values of SCEs/cell were not significant. Cyclophosphamide (positive control) showed a significant value at $P < 0.01$.

Table 1. Frequency of SCE and RI in cultured human lymphocytes exposed to ethynodiol diacetate[a]

Group	SCEs/cell (mean ± SE)	RI	Group	SCEs/cell (mean ± SE)	RI
Without S9			With S9		
Ethynodiol diacetate			Ethynodiol diacetate		
50 µg/ml	7.35 ± 0.54	1.78	50 µg/ml	8.23 ± 0.71	1.77
100 µg/ml	8.63 ± 0.68	1.76	100 µg/ml	9.72 ± 0.89	1.73
150 µg/ml	9.32 ± 0.73	1.74	150 µg/ml	15.77 ± 0.92†	1.52**
Normal	5.24 ± 0.13	1.80	Normal	5.72 ± 0.14	1.82
Negative control DMSO (5 µl/ml)	5.46 ± 0.15	1.79	Negative control DMSO (5 µl/ml)	5.02 ± 0.12	1.81
Positive control mitomycin C (0.04 mg/ml)	32.86 ± 1.24‡	1.48*	Positive control cyclophosphamide (4 mg/ml)	35.52 ± 1.32‡	1.46*

[a]A total of 100 second-division metaphases of each group were analysed for SCE.
*$P < 0.005$; **$P < 0.03$ vs normal χ^2 test; †$P < 0.03$, ‡$P < 0.01$ vs control Kruskal–Wallis test; DMSO, Dimethylsulphoxide.

Table 2. Structural chromosomal aberrations (CAs) in human lymphocytes treated with ethynodiol diacetate without S9 mix[a]

Treatment	Cells scored	Abnormal cells (%)	Total structural CA					Total breaks without gaps (%)
			Gaps	CTB	CSB	CTE	DIC	
Ethynodiol diacetate								
50 µg/ml	300	12 (4)	9	9	5	–	–	14 (4.67)
100 µg/ml	300	15 (5)	12	12	6	–	–	18 (6)
150 µg/ml	300	16 (5.34)	16	16	6	–	–	23 (7.67)
Normal	300	8 (2.67)	7	4	3	–	–	7 (2.34)
Negative control DMSO (5 µl/ml)	300	9 (3)	7	5	3	–	–	8 (2.67)
Positive control mitomycin C (0.04 mg/ml)	300	120 (40)*	43	50	29	3	1	87 (29)

[a]Metaphases showing only gaps were not scored as abnormal.
*$P < 0.001$ vs normal χ^2 test.
CTB, Chromatid break; CSB, Chromosome break; CTE, Chromatid exchange; DIC, Dicentric; DMSO, Dimethylsulphoxide.

The cell proliferation kinetics, which is the average number of times that cells have undergone DNA replication, showed a significant difference between the culture exposed to 150 µg/ml of ethynodiol diacetate and the normal control at $P < 0.03$. The cytostatic effect was evidenced by a decrease of M_2 (48%) cells and M_3 (2%) cells and by the increment of cells in M_1 (50%).

Table 3 shows types and values of structural abnormalities in human lymphocytes in the presence of S9 mix. The number of abnormal cells was significant at 150 µg/ml of ethynodiol diacetate ($P < 0.005$). The aberrations observed were mostly chromatid and chromosome breaks. On the other hand, the increase of abnormal cells in the cultures exposed to cyclophosphamide (positive control) was significant at $P < 0.001$.

The results of the present investigation reveals that ethynodiol diacetate is not potent enough to cause genotoxic damage in human lymphocytes in the absence of S9 mix at the dose which was found to be genotoxic in the presence of S9 mix. The experimental evidence suggests that the metabolic activation of ethynodiol diacetate and possible conversion of it to reactive species is responsible for its genotoxicity. Metabolism of estrone 3,4-quinone produces free radicals in human breast cancer cells (MCF-7) and these are responsible for chromosomal DNA damage[19]. Induction of DNA repair was reported in primary rat hepatocytes exposed to synthetic progestins[20]. Synthetic progestins like megesterol acetate and chlormadinone acetate also shows the formation of DNA adducts in primary cultures of human hepatocytes[21]. Certain synthetic steroids have been reported to be mutagenic in the Ames tester strains by generating the active oxygen species in the system[22]. The SCEs are the cytological manifestation of interchanges between DNA replication products at apparently homologous loci. SCEs have been commonly used to evaluate cytogenetic responses to chemical exposure, and an excellent dose–response relationship has been established for hundreds of chemicals in a wide variety of *in vitro* and *in vivo* short term experiments[23]. Even though the mechanism of SCE formation is not yet fully determined, it seems that SCEs could be the consequence of errors in DNA replication, probably at the replication fork itself[24]. The interpretation of SCE results as indicative of genotoxic effect is based on either doubling of the SCE frequency compared with controls, or a statistically significant increase at any dose[25]. In this study of chromosome instability, represented by SCE frequencies in the presence of S9 mix (Table 1), exhibition of an increase could possibly be related to the interaction between genetic material and the reactive species or

Table 3. Structural chromosomal aberrations (CAs) in human lymphocytes treated with ethynodiol diacetate in presence of S9 mix[a]

Treatment	Cells scored	Abnormal cells (%)	Total structural CA					Total breaks without gaps (%)
			Gaps	CTB	CSB	CTE	DIC	
Ethynodiol diacetate								
50 μg/ml	300	17 (5.67)	10	20	6	–	–	26 (8.67)
100 μg/ml	300	21 (7.00)	15	24	8	1	–	34 (11.34)
150 μg/ml	300	31 (10.34)*	28	34	13	2	1	53 (17.67)
Normal	300	8 (2.67)	6	5	2	–	–	7 (2.34)
Negative control DMSO (5 μl/ml)	300	7 (2.34)	7	5	1	–	–	6 (2.00)
Positive control cyclophosphamide (4 mg/ml)	300	130 (43.34)**	45	57	32	4	2	101 (33.67)

[a]Metaphases showing only gaps were not scored as abnormal.
*$P < 0.005$; **$P < 0.001$ vs normal χ^2 test.
CTB, Chromatid break; CSB, Chromosome break; CTE, Chromatid exchange; DIC, Dicentric; DMSO, Dimethylsulphoxide.

modified form of ethynodiol diacetate. This behaviour could be related to the mechanisms of SCE formation, since SCE production requires DNA synthesis and its detection requires replication in presence of BrdU[25]. Our results show significant increase in SCEs frequency at 150 μg/ml in the presence of S9 mix.

CA are changes in chromosome structure resulting from a break or an exchange of chromosomal material. Most of the CAs observed in cells are lethal, but there are many corresponding aberrations that are viable and can cause genetic effects, either somatic or inherited[26]. These events can lead to the loss of chromosomal material at mitosis or to the inhibition of an accurate chromosome segregation at anaphase. The outcome is cell lethality[23]. In the present work, we found significant differences compared with control in the frequency of cells with CA in lymphocyte cultures exposed to 150 μg/ml of ethynodiol diacetate in the presence of S9 mix.

The exposure to the highest dose produced a delay in the cell cycle resulting in reduction of the number of cells in second division and an increase of those in the first division. These results show an important cytotoxic effect with a decrease of M_2 cells, prolonging the cell cycle as a cytostatic agent.

SCE is generally a more sensitive indicator of genotoxic effects than structural aberrations[23]. There is a correlation between the carcinogenicity and SCE-inducing ability of a large number of chemicals[27]. Moreover, the mechanism leading to the formation of SCE are heterogeneous and fundamentally different from those that cause structural chromosome aberrations[27].

The results obtained in this study suggest a genotoxic and cytotoxic effect of ethynodiol diacetate in the presence of S9 mix at 150 μg/ml in human peripheral blood cultures *in vitro*. The measurement of cytogenetic alterations *in vitro* is considered as an initial step in the risk assessment procedures for genotoxic agent. It is, therefore, advisable to be careful of the potential hazards of these drugs. This demands the lowest possible use of effective and acceptable doses of these drugs so as to minimize any potential risk. Otherwise they may become capable of attacking the genetic material.

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Earthquake sequence in and around Bhavnagar, Saurashtra, western India during August–December 2000 and associated tectonic features

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An earthquake sequence started in Bhavnagar in eastern Saurashtra on 9 August 2000. Temporary field observatories were established immediately and 132 earthquakes of M (magnitude) 0.5–3.8 were recorded from 9 August 2000 to 15 December 2000. Among these, five earthquakes were of $M \geq 3$, with maximum magnitude of 3.8. The damages were disproportionately high considering the magnitudes of the events. This may be partly explained as due to presence of loose alluvium/poorly consolidated alluvium. Locations of 43 events were obtained from the data collected by the field observatories. The events were confined to an area of 6 km × 4 km, which is located in and around the southeastern part of Bhavnagar town and had focal depths ranging between 1 and 4 km. This earthquake sequence exhibits a doublet pattern (a pair of mainshock–aftershock sequences) with two mainshocks occurring on 10 August 2000 (M : 3.6) and 12 September 2000 (M : 3.8). Successive occurrence of mainshock–aftershock sequences is also known as earthquake swarm of the second kind. Past seismicity of the region shows that the eastern part of Saurashtra is more active than the western part. According to historical records, an earthquake of magnitude 7 occurred near Bhavnagar in February 1705. Earthquake swarm activity also occurred in and around Paliyad (60 km north of Bhavnagar) during July–August 1938 for about two months, with four earthquakes of $M \geq 5$ and maximum magnitude of 6.0. The north-south trending western margin Cambay basin fault passes close to the Bhavnagar area. Further, the east-west trending Shihor fault has offset the western margin Cambay fault near Bhavnagar. The earthquake activity in this area appears to be associated with these two fault systems.

SAURASHTRA region forms a vital geodynamic part of the western continental margin of India, and falls in the seismic zone-III of the Zoning Map of Bureau of Indian Standards. The region as a whole and the area around Bhavnagar in particular has been tectonically unstable, as revealed by the earthquakes that have taken place in the

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