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Cytogenetic evaluation of hormonal drug fludrocortisone in human lymphocyte chromosomes *in vitro*

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Abstract: *Fludrocortisone is a potent steroidal drug with many therapeutic activities. The cytogenetic effects of this drug were assessed in human lymphocyte culture. The parameters used were sister chromatid exchanges (SCE) and chromosomal aberrations (CA). A wide range of concentrations i.e. 25µg, 50µg and 100µg/ml of the drug were used to see the genotoxic effects in the absence as well as in the presence of microsomal activation system (S9 mix) at different exposure durations (i.e., 24, 48 and 72hr). The drug did not induce any significant increase in chromosomal aberration frequency at any of the concentrations, nor was it effective at any of the exposure times either in the absence or in the presence of S9 mix. It was concluded that fludrocortisone does not have any genotoxic effect in human lymphocytes at wider ranges of concentrations and it is safe to be used as medicine.*

Key words: *Fludrocortisone, Chromosomal aberration, Sister chromatid exchange, Human lymphocyte.*

Introduction

Steroidal hormones play an important role in growth, development, differentiation, metabolic regulation and homeostatic processes. These hormones and their synthetic derivatives are being extensively used for therapeutic purposes (Ahmad *et al.,* 2000a, 2001). In certain circumstances, these are administered as life saving molecules. Many hormonal drugs have been studied for their teratogenic (Mitchel *et al.,* 1983; Lukic and Bajektarovic, 1987), carcinogenic (Lang and Redmann, 1979; Rosenfield *et al.,* 1983), mutagenic and clastogenic (Wheeler *et al.,* 1986; Singh *et al.,* 1994; Ahmad *et al.,* 2000b, 2003) potentials. Steroid hormones, being smaller molecules, diffuse through the cell membrane and after conjugating with specific receptor proteins in the cytoplasm of target cell, pass through the nuclear pore to form steroid-receptor protein-DNA complex. This complex alters the gene expression of many cellular processes including enzyme synthesis (Rubin, 1982; Thomas and Thomas, 1993). Some of these molecules have been found to bind with isolated DNA (Blackburn *et al.,* 1974) and induce fragmentation (Yamafuji *et al.,* 1971). Although sex steroids have been studied widely and several of these have been found to be genotoxic (Singh *et al.,* 1994; Ahmad *et al.,* 2000b; Ahmad *et al.,* 2002), but only few other types of steroid hormones have been studied despite being used extensively as medicine. Reports on the mutagenic effects of non-sex steroids are controversial. Some of these molecules do not exhibit mutagenicity in Ames/*Salmonella* test system but are clastogenic in mammals (Bali *et al.,* 1990; Islam *et al.,* 1991; Singh *et al.,* 1994; Shadab *et al.,* 2002). A few of such drugs inhibit DNA synthesis in rapidly dividing liver cells *in vitro* at low dose concentration (Loeb *et al.,* 1973). In the present study we investigated the effect of fludrocortisone on human chromosomes in lymphocyte cultures *in vitro* for CAs and SCEs. Fludrocortisone is a potent steroidal drug with many therapeutic activities. It is used to cure chronic adrenal insufficiency, congenital adrenogenital syndromes and also displays anti -inflammatory effects.

Materials and Methods

Chromosome aberration analysis: Metaphase chromosome analysis for the detection of CAs was performed according to conventional technique (Moorehead *et al.,* 1960). Lymphocyte cultures were set up by adding 0.5 ml of heparinized whole blood, from adult and healthy donors (who were not occupationally exposed to mutagens) to 4.5 ml of TC-199 medium (Flow Laboratories) supplemented with 15% fetal calf serum (Gibco), antibiotics (penicillin and streptomycin 100 IU/ml each; Hoechst) and L-glutamine (ImM; Gibco). Lymphocytes were stimulated to divide by adding 0.1 ml of phytohaemagglutinin-M (PHA-M; Microlab). Cultures were incubated at 37 ºC in the presence of 5% CO_2 for 72 hr in the dark. Preliminary screening of the drug with various concentrations was undertaken for determining their influence on the mitotic index, while fludrocortisone (Wyeth lab, India), at a final concentration of 25, 50 and 100 µg /ml, respectively was added for 24, 48 and 72 hr of duration by adding the steroid after 0, 24 and 48 hr in reverse order after the initiation of cultures. Dimethyl sulphoxide (DMSO 5µg/ml; SRL Mumbai, India) was added to the cultures kept simultaneously for 72 hr of incubation as negative control. For positive control cyclophosphamide (CP $1x10^{-7}$M) was added to the culture for 24, 48 and 72hr of incubation. The cells were shaken after every 12 hr in experiments to avoid unsynchrony in cell proliferation.

In the metabolic activation experiment, 6 hr to 30 hr old cultures were given treatments of 25, 50 and 100 µg/ml concentrations of the drug in the presence of rat liver S_9 microsomal fraction. Liver S_9 fraction was prepared from healthy albino rats induced with phenobarbital and it was stored in liquid nitrogen until use. S_9 mix was freshly prepared for use. The cells were collected after centrifugation and the pellets were washed

Table – 1: Chromosomal aberrations in human lymphocytes following fludrocortisone treatment.

Treatment (µg/ml)	Duration (hr)	Metaphase scanned	Percent aberration metaphase		Types of aberration (%)			Abberation/ Cell ± SE
			Including gap	Excluding gap	Chromatid	Chromosome	Total	
Fludrocortisone								
25	24	300	3.66	3.66	3.33	0.00	3.33	0.03±0.00
	48	300	3.00	3.00	3.00	0.00	3.00	0.03±0.01
	72	300	4.00	2.66	2.66	0.66	3.32	0.03±0.01
50	24	300	4.33	4.33	4.00	0.66	4.66	0.05±0.02
	48	300	4.33	3.66	3.00	1.00	4.00	0.04±0.02
	72	300	4.66	4.33	3.33	1.33	4.66	0.05±0.02
100	24	300	5.33	5.00	3.00	0.66	3.66	0.04±0.01
	48	300	5.00	3.66	2.66	1.00	3.66	0.04±0.01
	72	300	5.00	3.33	2.66	1.00	3.66	0.04±0.01
Control								
Normal	72	300	5.33	3.00	2.00	1.00	3.00	0.03±0.01
DMSO (5µg/ml) (-ve control)	72	300	4.33	2.66	1.33	2.00	3.33	0.03±0.01
CP ($1x10^{-7}$M)	24	300	14.00	13.00	16.33	5.00	21.33*	0.21±0.03
(+ve Control)	48	300	28.33	27.00	26.00	20.00	46.00*	0.46±0.05
	72	300	31.66	26.33	30.00	17.00	47.00*	0.47±0.08

CP: Cyclophosphamide; DMSO: Dimethyl sulphoxide; SE: Standard error; ***Significant at p< 0.05.

twice in the prewarmed medium to remove the chemical and the S_9 mix. The parallel cultures receiving the same dose of the drug for similar treatment durations but without S_9 mix were also set simultaneously for comparison. Colchicine (0.20µg/ml; Microlab) was added to the cultures prior to harvesting. The cells were collected by centrifugation (10min; 1200 rpm). Hypotonic treatment (0.075M KCl) was given for 10-12 minutes at 37ºC and the cells recollected by centrifugation were fixed in methanol: acetic acid (3:1). Preparation of slides, staining and scanning was done under code. A total of 300 well-spread metaphases were analyzed per treatment per duration for all types of chromatid and chromosome type of aberrations. In case of cultures with metabolic activation only 200 well-spread metaphases were analyzed. Three independent reproductions of each experiment were carried out.

Sister chromatid exchange analysis: Analysis of SCEs was carried out following the fluorescent plus Giemsa techniques (Perry and Wolff, 1974; Wolff and Perry, 1974; Latt *et al.,* 1977; Azfer and Afzal, 1996). The cells were exposed to 5-bromo-2-deoxyuridine (BrdU 2µg/ml; Sigma) solution after 24 hr of the initiation of cultures. The test compounds were added together with the BrdU. To minimize photolysis, remaining part of the cultures was maintained in the dark for 48 hr.

For SCE analysis in the presence of metabolic activation system, the drug along with S_9 mix was added into the cultures after 48 hr of its initiation and re-incubated at 37ºC. After 90 minutes of this pulse treatment the cells were washed twice to remove the traces of the steroid and the liver metabolites. Finally, the cell pellets were re-suspended in fresh medium supplemented with fetal calf serum, PHA-M and BrdU and put to culture for another 24 hr in the dark at 37ºC. For comparison, parallel cultures without S_9 mix were also set simultaneously. Hoechst 33258 stains (0.5 µg/ml; Sigma) was used along with 10% Giemsa stain for differential staining, and 50 well spread metaphase cells were scanned per concentration and the number of exchanges scored. Here again, three independent reproductions of each experiment were carried out.

Results

Chromosomal aberrations: Fludrocortisone did not show any significant increase in the frequency of CAs at any of the concentrations (i.e. 25, 50 and 100 µg/ml), nor was it effective at any exposure time (i.e. 24, 48 and 72 hr) (Table 1). The maximum aberrations observed were 4.66%, which were recorded for 50 µg/ml concentration at 24 and 72 hr of incubation was non significant by higher than normal control (i.e. 3.00%). The data recorded for CP (positive control) were 21.33%, 46.00% and 37.00% at 24, 48 and 72 hr of incubations, respectively and were highly significant at p<0.05 as compared to the normal control values.

Even in the presence of metabolic activation, the drug was unable to induce any clastogenic effect on the human lymphocytes (Table 2). The percentage of CAs were 4.00 %, 5.00 % and 4.50 % at 25, 50 and 100µg/ml of concentration respectively, which were non significant as compared to normal control (i.e. 4.50 %), whereas for CP the value was 33.50 %

Table – 2: Chromosomal aberrations in human lymphocytes after fludrocortisone treatment with and without metabolic activation**.**

Treatment (µg/ml)	Metabolic activation	Metaphase scanned	Percent aberration metaphase		Types of aberration (%)			Abberation/ Cell ± SE
			Including gap	Excluding gap	Chromatid	Chromosome	Total	
Fludrocortisone								
25	$-S_9$	300	3.66	3.66	3.33	0.00	3.33	0.03±0.00
	$+S_9$	200	4.00	3.50	4.00	0.00	4.00	0.04±0.01
50	$-S_9$	300	4.33	4.33	4.00	0.66	4.66	0.05±0.02
	$+S_9$	200	5.00	4.50	4.00	1.00	5.00	0.05±0.02
100	$-S_9$	300	5.33	5.00	3.00	0.66	3.66	0.04±0.01
	$+S_9$	200	5.66	5.33	3.50	1.00	4.50	0.05±0.02
Control								
Normal	$-S_9$	300	5.33	3.00	2.66	1.00	3.66	0.04±0.01
	$+S_9$	200	6.00	2.00	3.50	1.00	4.50	0.04±0.01
DMSO (5µg/ml)	$-S_9$	300	4.33	2.66	1.33	2.00	3.33	0.03±0.01
(-ve Control)	$+S_9$	200	4.52	2.50	1.50	2.50	4.00	0.04±0.01
CP ($1x10^{-7}$ M)	$-S_9$	300	14.00	13.00	16.33	5.00	21.33*	0.21±0.03
(+ve Control)	$+S_9$	200	20.00	18.00	21.00	12.50	33.50*	0.34±0.04

CP: Cyclophosphamide; DMSO: Dimethyl sulphoxide; SE: Standard error; * Significant at p< 0.05.

Table – 3: Sister chromatid exchange (SCE) in human lymphocytes after fludrocortisone exposure *in vitro*.

Treatment (µg/ml)	Duration (hr)	No. of metaphases	Total SCE	Range	SCEs/Cell ± SE
Fludrocortisone					
25	48	50	67	0-4	1.34±0.11
50	48	50	103	0-6	2.06±0.15
100	48	50	80	0-4	1.60±0.11
Control					
Normal	48	50	79	0-5	1.58±0.12
DMSO (5µg/ml) (-ve control)	48	50	78	0-5	1.56±0.12
CP ($1x10^{-7}$M) (+ve Control)	48	50	215	2-11.	4.30±0.72*

CP: Cyclophosphamide; DMSO : dimethyl sulphoxide; SE: Standard error; * Significant at p< 0.05

Table – 4: Sister chromatid exchange (SCE) in human lymphocytes after fludrocortisone exposure with metabolic activation (S_9 mix).

Treatment (µg/ml)	Duration (hr)	No. of metaphases	Total SCE	Range	SCEs/Cell ± SE
Fludrocortisone					
25	48	50	104	0-6	2.08±0.16
50	48	50	115	1-8	2.30±0.20
100	48	50	112	1-7	2.24±0.18
Control					
Normal	48	50	105	0-6	2.10±0.16
DMSO (5µg/ml) (-ve control)	48	50	117	0-7	2.34±0.15
CP ($1x10^{-7}$ M) (+ve control)	48	50	321	2-12	6.42±0.96

CP: Cyclophosphamide; DMSO: Dimethyl sulphoxide; SE: Standard error; * Significant at p< 0.05

which was highly significant at p<0.05 compared to the normal control values.

Sister chromatid exchanges: The results of SCE in human lymphocytes after the treatment of fludrocortisone are presented in (Table 3). The mean SCE frequencies of the drug at 25, 50 and 100 µg/ml concentrations were 1.34± 0.11, 2.06 ± 0.15 and 1.60 ± 0.11 respectively. These values were non significant, compared to normal control (1.58 ± 0.12). The value

observed for positive control was 4.30 ± 0.72 which was highly significant at $p<0.05$ compared to the normal control value.

Fludrocortisone did not prove to be effective in enhancing the SCE frequency in the presence of S_9 mix (Table 4) at any of the concentrations. The mean frequency values were 2.08 ± 0.16, 2.30 ± 0.20 and 2.24 ± 0.18 at 25, 50 and 100 μg/ml concentrations, respectively. All these values were non significant as compared to the normal control (i.e. 2.10+0.16) value.

Discussion

The induction of CAs and SCEs are well known cytogenetic markers and are used for the assessment of genotoxic property of occupational agents (Nagalakshmi *et al.,* 1995). Clastogenic chemicals may lead to many occupational diseases including cancer. Particularly, a chemical inducing SCE is supposed to be carcinogenic. Varieties of steroidal drugs, which are being used therapeutically, have been studied previously for their genotoxic effects (Bali *et al.,* 1990; Singh *et al.,* 1994; Shadab *et al.,* 1999). A number of them have been found to be mutagenic, carcinogenic and clastogenic (Bishun and Williams, 1977). Diethylstilbestrol, an estrogen, induces SCE in metabolically competent hepatoma cell lines but not in fibroblasts (Hill and Wolff, 1983; Buenaventura *et al.,* 1984). Diethylstilbestrol and estradiol induce C-mitoses, polyploidies and micronuclei in human peripheral lymphocytes *in vitro*, but no structural chromosomal aberrations and SCEs (Glatt *et al.,* 1979; Banduhn and Obe, 1985). Estrogens and their synthetic analogs, diethylstilbestrol, dienestrol, hexestrol, β-estradiol, ethinyl estradiol and estriol are reported to cause mitotic arrest and aneuploidy in Chinese hamster cells *in vitro* (Wheeler *et al.,* 1986; Hundal *et al.,* 1997). Estradiol-17β, estriol, estrone and ethynyl estradiol enhance SCE rates in cultured CHO cells in a dose related manner (Kochhar; 1988). Anovlar 21, an oral contraceptive which is a combination drug containing the oestrogen `ethinyloestradiol' and the progestin `norethisterone acetate', induces pulverization and stickiness in mouse bone marrow cells in a dose dependant manner, however, this drug is unable to induce significant increase in the frequencies of micro-nuclei (Shyama *et al.,* 1991). Mestranol, a widely used estrogen, is again clastogenic in human lymphocytes and mouse bone marrow cells but has no positive effect in Ames/*Salmonella*/ S_9 test system (Dhillon *et al.,* 1994). Estradiol-17β induces structural chromosomal aberrations and SCEs in human lymphocyte chromosomes *in vitro* (Ahmad *et al.,* 2000a)

Glucocorticoids and mineralocorticoids, which have not been widely studied for genotoxic effects, also show contradictory results. Hydrocortisone was reported to suppress DNA synthesis in hepatoma cells by inhibiting the incorporation of thymidine, thus, ultimately inhibiting cell proliferation (Loeb *et al.,* 1973). Hydrocortisone was again found to be genotoxic in human lymphocytes and mouse bone marrow by inducing SCEs, CAs and micronuclei but did not have any effect in Ames/*Salmonella* assay (Bali *et al.,* 1990; Shadab *et al.,* 1999). Another glucocorticoidal medicine, dexamethasone induces chromatid and chromosome type of aberrations, and high frequency of SCE in human lymphocytes *in vitro* (Ahmad *et al.,* 2000a). It was also found to be capable of attacking the genetic material in mouse bone marrow *in vitro*, whereas it did not show any increase in His^+ revertants in Ames/ *Salmonella* assay both in the presence as well as in the absence of S_9 mix.

Cytogenetic evaluation of fludrocortisone, as reported in this study, for the first time using CA and SCE analysis in human lymphocyte cultures showed that fludrocortisone could not induce a significant increase in chromosomal aberrations and sister chromatid exchanges at any dose and time duration. This may be due to the inability of fludrocortisone to generate free oxygen radicals and to form hydrogen peroxide as well as superoxide and hydroxy radicals, as has been observed in the case of certain synthetic steroids (Islam *et al.,* 1991). It may be concluded that fludrocortisone does not have any toxic effect on human lymphocyte chromosomes at wide range of concentrations, and thus it can be concluded that it is safe to be used as medicine.

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