Primary Genetic Damage Induced by Oral Contraceptive Pills "Anovlar 1" on Human Chromosomes

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ABSTRACT

This work aims at disclosing the capability of the oral contraceptive drug "Anovlar 1" in inducing primary genetic damage in human chromosomes. In order to achieve such a purpose *In Vivo* induction of sister chromatid exchanges after treatment with the drug, was studied employing human lymphocytes. Five different concentrations that correspond to 0.5; 1; 2; 5; and 10 folds of the daily therapeutic dose were used. Bromodeoxyuridine substituted DNA was stained by FPG technique. The obtained results showed that "Anovlar 1" is a positive inducer of primary genetic damage, since a concentraction response relationship was achieved.

INTRODUCTION

Genotoxins are agents specifically producing genetic alterations at sub toxic exposure levels which result in organisms with altered hereditary characteristics. Depending upon the developmental stage of an individual, a genotoxin can exert teratogenic effect or cause mutations not only in somatic but also in germinal cells.

Mutational damage results in situation where not only an exposed person has the possibility of deleterious effects but also his progeny generation upon generation.

A cytogenetic technique currently in wide use is the analysis for SCEs. This phenomenon was originally pbserved by Taylor in 1958, but analysis for SCEs on a routine basis only became possible following the development of simple staining techniques that differentiate sister chromatids. SCE analysis appears to be a very good screening tool, for evaluation of primary genetic damage induced by contaminants and / or pollutants.

The ideal genetic assay for occupational monitoring would be rapid, inexpensive, highly objective, and predictive. One technique, SCE detection, appears to have most of these desired properties.

The SCE method using human lymphocytes recovered from test populations is currently undergoing numerous trial studies, and the results appear promising. Early studies on hospital patients receiving chemotherapeutic alkylating agents showed increased SCE. (Lambert *et. al.*, 1978) Another pilot study with actual occupational exposures demonstrated a significant elevation of SCEs in a population of petroleum workers. The former results were not surprising, since many chemotherapeutic drugs are suspect human mutagens and carcinogens on the basis of *in vitro* studies (Matheson *et. al.*, 1978). The significant facts derived from these studies are that human populations are

amenable to sensitive and rapid assessment with the technique. Particular features of these studies appear encouraging, however. The studies showed that (1) analysis of SCEs is rapid and not subject to the same spontaneous background variability as conventional chromosome analysis; (2) blood samples could be collected, transported over long distances, and grown in culture under relatively uniform conditions; and (3) effects from low-level occupational exposures can be detected with a degree of sensitivity unequaled by conventional cytogenetic analysis.

Analysis of SCE in human cells may also play a role in establishing the dose received, or at least in identifying affected individuals following accidental exposure to known genotoxic substances. For example, the target site (chromosome) does on a somatic cell basis may be assessed by degree of the increase in SCE in workers located at various distances from the primary release site. This analysis is rapid and sensitive and can be completed very shortly after the actual exposure has occurred (about 2-3 days). Based on this information, workers requiring immediate attention or long-term follow-up can be identified. There are probably no other available tests for genetic monitoring which more closely meet the requirements of a human dosimeter than does the sister chromatid exchange technique (Brusick, 1986).

MATERIALS AND METHODS

As previously mentioned (A1-Ayoubi, 2003), the oral contraceptive pills "Anovlar 1" are produced by Schering AG Company in the from of tablets, each one is composed of Ethinyl estradiol (0.05 ,g) and Northestrone acetate (1mg). The following concerntrations per Liter medium were chosen and tested:

1- 0.42 □g ethinyl estradiol + 8.5 □g Norethisterone acetate.
2- 0.85 □g ethinyl estradiol + 17 □g Northisterone acetate.
3- 1.70 □g ethinyl estradiol + 34 □g Northisterone acetate.
4- 4.25 □g ethinyl estradiol + 85 □g Northisterone acetate.
5- 8.5 □g ethinyl estradiol + 170 □g Northisterone acetate.

The above mentioned concentrations correspond to 0.5; 1; 2; 5; and 10 folds of the daily therapeutic dose.

In vitro induction of sister chromatid exchanges in human lymphocytes.

Heparinized venous blood was collected from normal healthy adults. Human karyotyping medium purchased from GIBCO (USA) was used in this assay. In order to study the frequency of the sister chromatid exchange in human chromosomes in response to Anovlar-1 100 ug BrdU were added 8 hr before the treatment of cultur with the drug. The cultures were incubated in tightly sealed tubes at 37 C for 72 h Before harvesting at 70 hr 0.1 ml colcemid was added to each culture ar. incubation was continued for 2 hr.

Preparation of metaphase chromosomes

The method described by Schwarzacher (1974) was used as follows : The cultures were contrifuged for 8 min at 1200 rpm, the supernatant was discarded and the cell pellet was resuspended with last drop of supernatant, then about 8 ml of prewormed (37C) hypotonic (0.075 M KCl) were added, allowed to stand for 10 min at 37C, centrifuged for 8 min at 1200 rpm, the cell pellet was fixed for 1 hr in about 8 mL freshly prepared fixative fluid (3 parts methanol : 1 part glacial acetic acid) and centrifuged . The cell pellet was fixed three times; 20 min each.

Slide staining:

Staining was performed by the method of Goto *et. al.* (1976). The slides were stained with 50 mg / ml of Hoechest 33258 dye in distilled water, pH 7.0 for 10 min (protected from light) . The slides were then rinsed in water, and covered by a layer of Mc Ilvaines buffer [add 18 ml of solution A (1.92% citric acid) to 82 ml of solution B (2.42% disodium phosphate A) and adjust the pH to 7.0 or 7.5 with further mixing], mounted by cover slip and subjected to light with intensity <= 400nm, at a distance of about 2 inches for 20 min. during this time, slides were placed on a wormer tray at 50 °C. The slides then were rinsed in distilled water and immersed in 4% Giemsa dye, rinsed again in water and allowed to dry for subsequent light microscope analysis.

Screening of slides and analysis:

Scanning slides for metaphase spreads was conveniently accomplished with a 25 X magnification objective, and analysis was with a 100X objective. For control of bias, all prepared slides were coded prior to scoring. There are two ways for counting sister chromatid exchange frequncies i.e., (1) from the microscope images of second division cells, (2) the cells may be photographed and SCE frequencies are counted from the microscope images. An interstial exchanged segment was counted to be 2 SCEs.

Usually, wide ranges of SCE values were encountered specially in treated cells, and then the analysis of variance using F-test was applied. To evaluate the differences in mean SCE frequencies between treated and control groups, Duncun's multiple range test was used (Snedecor, 1958).

RESULTS

Table (1) shows the averages of sister chromatid exchanges obtained after cytological examination of human lymphocytes treated with the different concentrations of the oral contraceptive "Anovlar 1".

In the control group the average was found to be 2.2 per cell. It increased after treatment to be ranged from 3.46 to 12.42 .

Analysis of variance using F-test showed that the tested concentrations were proven to be positive in inducing significant increases in sister chromatid exchanges using Duncant's multiple range test (Table, 2) 12 mean differences were shown to be significant while three were not. However, a concentration response relationship (Figure, 1) was observed, giving an evidence that the

tested concentrations were proven be positive in causing primary DNA damage (Figures 2-5).

Table 1. Averages of sister chromatid exchanges in human chromosomes after treatment with "Anovlar 1".

Conc.	X + S.E.	Range
Control	2.2 ± 0.2	0 – 4
1-	3.46 ± 0.3	2 – 6
2-	* 4.82 ± 0.24	3 – 8
3-	* 7.28 ± 0.42	3 – 10
4-	* 8.64 ± 0.63	5 – 12
5-	* 12.42 ± 1.2	6 - 18

* Significant at 0.05 level of probability.

Table 2. Duncan's multiple range test for mean differences of SCEs.

Conc.	X	X-Xc	$X-X_1$	$X-X_2$	$X-X_3$	$X-X_4$
5	12.42	*10.22	* 8.96	*7.60	*5.14	*3.78
4	8.64	*6.44	*5.18	*3.78	1.36	
3	7.28	*5.08	*3.82			
2	4.82	*2.62	1.36			
1	3.46	1.26				
Control	2.20					

* Significant at 0.05 level of probabiliy.

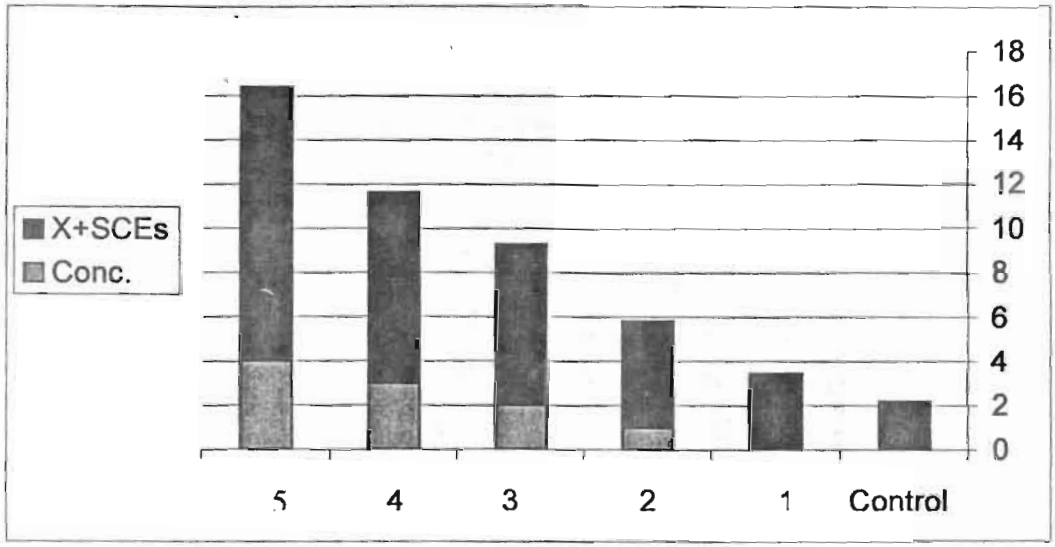

Figure 1. Relationship between the tested concentrations and SCEs after treatment with the tested drug.

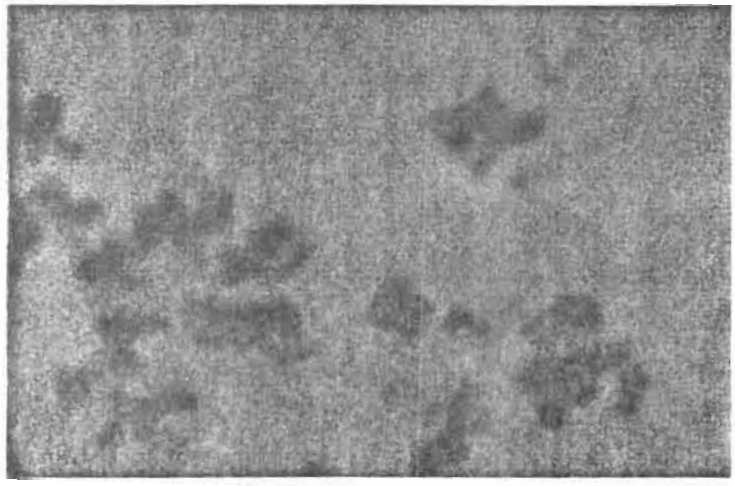

Figure 2. Photomicrograph of metaphase stage showing SCEs after treatment of human chromosomes with "Anovlar 1" (concentration No. 2).

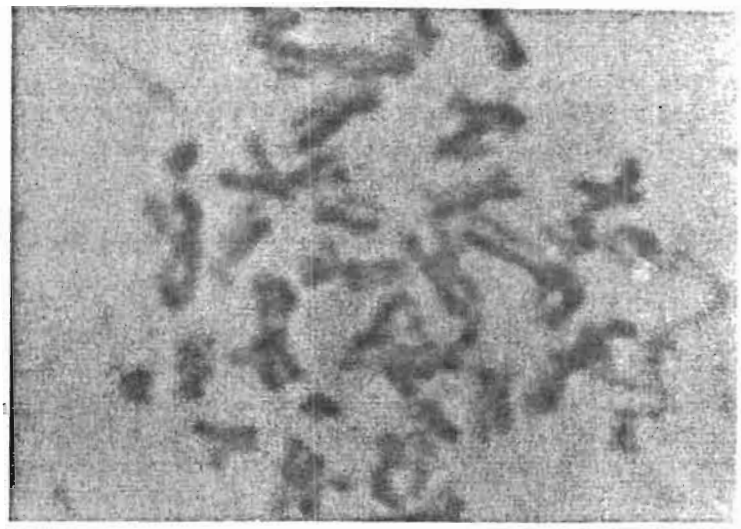

Figure 3. Photomicrograph of metaphase stage showing SCEs after treatment of human chromosomes with "Anovlar 1" (concentration No. 3).

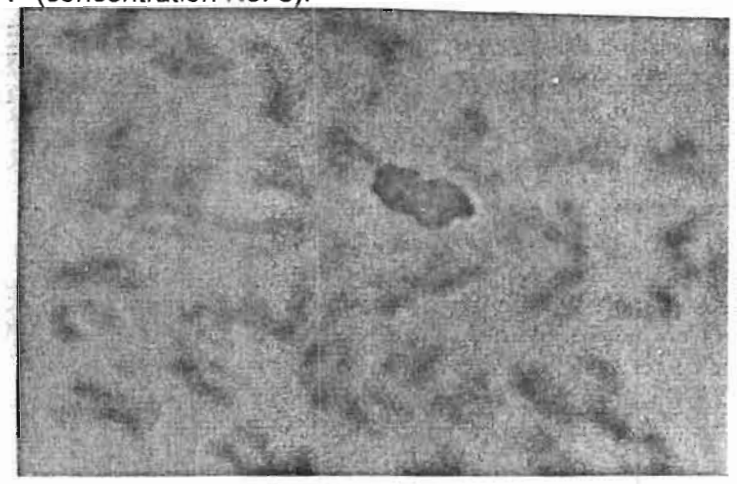

Figure 4. Photomicrograph of metaphase stage showing SCEs after treatment of human chromosomes with "Anovlar 1" (concentration No. 4).

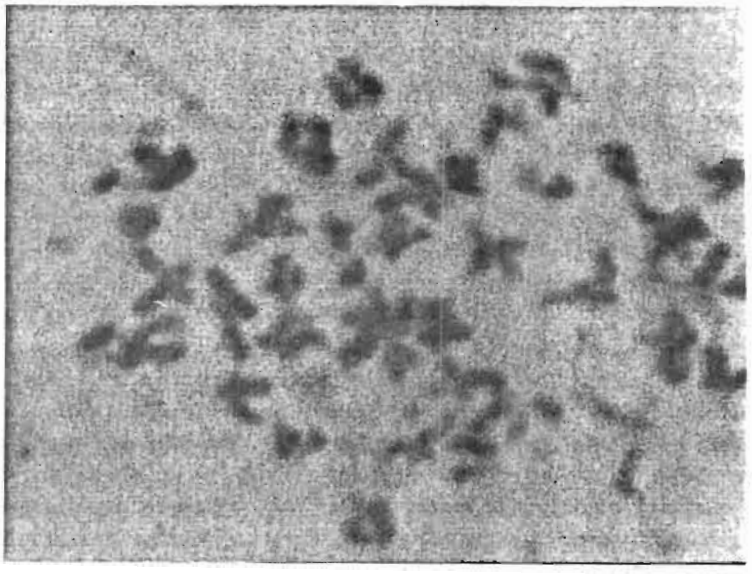

Figure 5. Photomicrograph of metaphase stage showing SCEs after treatment of human chromosomes with "Anovlar 1" (concentration No. 5).

DISCUSSION

One of man's concerns about his surrounding environment is the pollutive impact of various physical and chemical agents released at high levels as a result of recent intensive industrilization. Consequently, scientists designed several techniques in order to monitor, model, and to assess pollutats in an ecosystem. (Seehy, 1989).

Environmental biologists concentrated their efforts to elucidate clearly the potentiality of such pollutants to induce harmful effects on the biological systems. Several tests were recommended for achieving valid results, these tests include battery type tests, where test organisms are of different organization complexity and from different groups, i.e., microorganisms, plants, and animals.

Sister chromatid exchanges, (SCEs) represent exchange of DNA between replication products at homologous points. At metaphase, these represent symmetrical exchanges between sister chromatids at identical loci, which can only be visualized if the sister chromatids can be distinguished either by radioactive labelling or differential staining following incorporation of 5-bromodeoxyuridine. Although several models for the origin of SCEs have been proposed (e.g., Painter, 1980), the molecular basis of their formation has not been elucidated. These may represent some repair processes associated with DNA replication. Agents which induce chromosomal aberrations in an s-

dependent manner are also very efficient in inducing SCEs in contrast to the S-independent agents which are poor inducers of SCEs, so it has already been shown in several model systems that lesions leading to SCEs and to chromosonal aberrations are essentially different phenomena (Lukic & Barjacktarovic, 1987, and Seehy, 2003). While chromosomal aberrations are connected with disturbed cellular mechanisms, possibly resulting in cell death, SCEs do not present an obstacle to cell survival. This is probably the reson why they are more interesting in studies of mutagenic processes and intrinsic cellular processes. However, it is still believed that they are genetically neutral (Wolff et al., 1974), but some uneven exchange might lead to a deletion, insertion or frame-shift mutation.

According to Seehy (1989), the following three criteria were used to call on increase of sister chromatid exchange significant or relevant. First : In a rank test the value of P shoul be < 0.05; Second : The difference between the mean of SCEs of the matching negative control and the experiment in question must be 1.5 or more; and Third : A dose – reponse relationship should prevail.

The stafistical analysis carried out for the obtained results showed that the tested concentrations were proven to be positive in causing significant increases of SCEs and a concentration response relationship was achieved.

The present study revealed that "Anovlar 1" has mutagenic activity upon human genome, since the tested drug was capable in causing primary DNA damage.

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الملخص العربي

أقراص منع الحمل أنوفلار – ١

تحدث ضرر وراثي أولي على كروموسومات الإنسان

دجاته يحيى الأيوبى

كلية التربية للبنات – وراثة

الرياض – المملكة العربية السعودية

هدف البحث الحالي لدراسة القدرة لعقار منع الحمل المسمي أنو فلار –١ على احداث ضرر أولى للمادة الوراثية فى الإنسان. ولتحقيق هذا الغرض تم تعليم المادة الوراثية بادخال مشابه القاعدة المسمى ٥ – بروموذى أوكسى يوريدين ثم الصبغ بطرق خاصة بعد معاملة الخلايا الليمفاوية بالعقار قيد الدراسة. هذا وقد تم اختيار خمس تركيزات توازي نصف، واحد، اثنان، خمس ، عشرة أضعاف الجرعة اليومية المستخدمة. وتم عزل الخلايا الليمفاوية من عينات دم أصحاء وزرعها في بيئة خاصة تم تحضينها على درجة حرارة ٣٧م لمدة ٧٢ ساعة ثم عوملت بالكولسميد لوقف الانقسام عند دور الوضع المتوسط وجمعت الخلايا وتم صبغها بطريقة الفلورسنت والجيمسا. هذا وأوضحت نتائج التحليل المجهري لقياس متوسطات التبادلات بين الكروماتيدات الشقيقة أن التركيزات المستخدمة ذات قدرة موجبة ومعنوية في احداث زيادات جوهرية في التبادل بين الكروماتيدات الشقيقة. وتم الحصول على علاقة خطية بين التركيز وبين متوسط التبادلات للخلية. وتم تعضيد نتائج الفحص المجهري بالتحليل الاحصائي وتم الحصول علي قرابة ست أضعاف المجموعة الضابطة من تبادلات بين الكروماتيدات الشقيقة. هذا وقد برهن البحث على قدرة عقار منع الحمل أنوفلار –١ على احداث ضرر أولى للمادة الوراثية للإنسان.