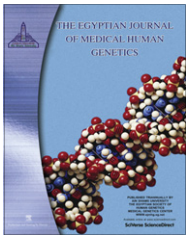
The Egyptian Journal of Medical Human Genetics (2012) 13, 301–305

Ain Shams University

The Egyptian Journal of Medical Human Genetics

www.ejmhg.eg.net
www.sciencedirect.com



ORIGINAL ARTICLE

Genotoxic damage in cultured human peripheral blood lymphocytes of oral contraceptive users

Falaq Naz [a], Smita Jyoti [a], Nishat Akhtar [b], Mohammad Afzal [a], Yasir Hasan Siddique [a,*]

[a] *Human Genetics and Toxicology Laboratory, Section of Genetics, Department of Zoology, Aligarh Muslim University, Aligarh 202002, UP, India*
[b] *Department of Obstetrics and Gynecology, J.N. Medical College, Aligarh Muslim University, Aligarh 202002, UP, India*

Received 18 May 2012; accepted 2 June 2012
Available online 29 June 2012

KEYWORDS

Oral contraceptives;
Chromosomal aberrations;
DNA damage;
Sister chromatid exchanges;
Human lymphocytes

Abstract Synthetic progestins and estrogens have been reported to be toxic in various experimental models. Their prolonged use has been reported to induce cancer in humans. In the present study the effects of oral contraceptives were studied among users using chromosomal aberrations, sister chromatid exchanges and DNA damage as a parameter, in cultured human peripheral blood lymphocytes. The study was performed on 25 women (users) and 25 age match controls. No significant difference was observed in chromosomal aberrations and DNA damage. A significant increase was observed in sister chromatid exchanges (SCEs) Cell among users. The results obtained and the risk of oral contraceptives (OCs) genotoxicity have been discussed.

1. Introduction

Oral contraceptives are the combination of estrogen and progesterone. They are used to treat various hormonal disturbances, premenstrual syndrome and ovarian cysts [1]. The composition of pills varies from country to country due to the responsiveness variation from individual to individual or one population to another [2]. Earlier reports suggest the genotoxic effects of synthetic progestins in cultured human peripheral blood lymphocytes [3–7]. In these studies the genotoxic effects were observed at very high doses and, the reactive oxygen species (ROS) was suggested as a causative agent for the genotoxic damage [8,9]. The conversion of estrogen into catechol estrogens and quinines, via redox reactions causes oxidative damage to DNA [5,10]. There are both positive as well as negative reports regarding the genotoxic effects of estrogens and synthetic progestins [11–13]. In the present study an attempt has been made to investigate the possible genotoxic effects among oral contraceptives (OCs) users (females) by using chromosomal aberrations, sister chromatid exchanges and DNA damage as a parameter.

* Corresponding author.
E-mail address: yasir_hasansiddique@rediffmail.com (Y.H. Siddique).
Peer review under responsibility of Ain Shams University.

Production and hosting by Elsevier

1110-8630
http://dx.doi.org/10.1016/j.ejmhg.2012.06.002

Table 1 Chromosomal aberrations in cultured human peripheral blood lymphocytes of oral contraceptive users and non-users (control).

Subjects	Total abnormal metaphases excluding gapsgroup	Mean ± SE	Total metaphases with aberrations including gaps	Mean ± SE	Chromatid breaks	Mean ± SE	Chromosomal breaks	Mean ± SE	Chromatid Gaps	Mean ± SE	Chromosome Gaps	Mean ± SE
Control	29	1.16 ± 0.213	41	1.64 ± 0.421	18	0.72 ± 0.197	4	0.16 ± 0.124	16	0.64 ± 0.163	3	0.12 ± 0.103
OC users	31	1.24 ± 0.221	41	1.64 ± 0.321	20	0.68 ± 0.102	3	0.12 ± 0.113	14	0.56 ± 0.142	4	0.16 ± 0.118

$P > 0.005$ NS (Non significant).

2. Subjects and methods

2.1. Chemicals

RPMI 1640, fetal calf serum, phytohaemagglutinin-M, antibiotic-antimycotic mixture (Invitrogen); 5-bromo-2-deoxyuridine, Giemsa stain (Merck, India), Tris·HCl, EDTA, Triton-X, Trichloro acetic acid and diphenylamine, colchicine (SRL, India).

2.2. Human lymphocyte culture

A sample of heparinized venous blood was obtained from 25 women taking an OCs preparation containing levonorgestrel and ethinylestradiol as a content for 12 months as a means of contraception following their first pregnancy. Age matched healthy 25 controls were evaluated along with the above subjects. A written informed consent was obtained from all subjects. The mean age of 25 women using OCs was 28.5 years and of the 25 controls was 27.5 years. Individuals answered a questionnaire relating to lifestyle factors. The OC users were not having any other addictions such as smoking, alcohol drinking etc. nor have any history of disease. Briefly, heparinized blood sample (0.5 ml), was obtained from each female and was placed in a sterile cultured tube containing 7 ml of RPMI 1640 medium, supplemented with fetal calf serum (1.0 ml), antibiotic–antimycotic mixture (1.0 ml) and phytohaemagglutinin-M (0.1 ml). The culture tubes were placed in the incubator at 37 °C for 48 h [14].

2.3. Chromosomal aberration analysis

After 47 h, an amount of 0.2 ml of colchicine (0.2 μg/ml) was added to the culture tubes. Cells were centrifuged at 800 g for 10 min. The supernatant was removed and 8 ml of pre warmed (37 °C) 0.075 M KCl (hypotonic solution) was added. Cells were re-suspended and incubated at 37 °C for 15 min. The supernatant was removed by centrifugation, at 800 g for 10 min, and subsequently 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 cells were stained in 3% Giemsa solution in phosphate buffer (pH 6.8) for 15 min. About, 300 metaphases were examined for the occurrence of different types of abnormality. Criteria to classify different types of aberration were in accordance with the recommendation of Environment Health Criteria 48 for Environment Monitoring of Human Population [15].

2.4. Sister chromatid exchanges

For sister chromatid exchange analysis, bromo deoxyuridine (10 μg/ml) was added at the beginning of the culture and the mitotic arrest was attempted, 1 h prior to harvesting by adding 0.2 ml of colchicine (0.2 μg/ml). Hypotonic treatment and fixation were performed as described for chromosomal aberration analysis. The sister chromatid average was taken from an analysis of metaphase during second cycle of division. A total of 25 well spread metaphases were scored per individual [16].

2.5. Quantitative assay for DNA fragmentation

Quantitative assay for DNA fragmentation was performed according to the protocol of Burton [17]. After incubation of 48 h, the cell suspension containing $1–10 \times 10^6$ cells in a 1 ml volume was prepared for each donor. About, 0.8 ml of cell suspension was transferred to a micro centrifuge tube and 0.7 ml of ice cold lysis buffer (5 mM Tris Cl, pH 8.0/20 mM EDTA/0.5% (v/v Triton X-100) was added. The tubes were vortexed and allowed to lyse for 30 min at 4 °C. The tubes were centrifuged for 15 min at 15,000 g (4 °C) and the supernatant was transferred to a labeled conical glass tube. About 0.65 ml of 5% trichloroacetic acid (TCA) was added to the pellet in a micro centrifuge tube and 1.5 ml of 10% TCA to the sample in a labeled glass tube. The sample was precipitated overnight at 4 °C. The tubes were centrifuged for 10 min at 2500 g at room temperature and the supernatant was removed and about 0.65 ml of 5% TCA was added to the pellet. The tubes were boiled for 15 min at 100 °C in a water bath. After cooling down to room temperature the sample was centrifuged at 2500 g for 5 min. About 0.5 ml of each supernatant (from both glass and micro centrifuge tubes) was added to the labeled glass tube. About, 1 ml of diphenylamine reagent was added to each tube and was incubated for 4 h at 37 °C [18]. Finally the absorbances were noted at 600 nm in a spectrophotometer and the results were expressed in the percentage of DNA fragmented as follows:

$$\%\text{Fragmented DNA} = \frac{\text{absorbance of supernatant}}{\text{absorbance of supernatant} + \text{pellet}}$$

2.6. Statistical analysis

Student's *t*-test was used for the analysis of data obtained for chromosomal aberrations and sister chromatid exchanges and χ^2 (Chi-square) was applied for the analysis of DNA damage.

3. Results and discussion

The frequency of cells with aberration in OC users was almost similar to their age matched controls. Gaps and breaks of chromosome as well as chromatid were observed, but dicentric, exchanges and rearrangements were not observed. The mean values obtained were not statistically significant (Table 1). A significant difference ($P > 0.005$) in the number of SCEs/cell was observed between OC users (3.9 ± 0.076) and their age matched control (2.62 ± 0.068) (Table 2). The mean value for the DNA damage was (0.1172 ± 0.0020) in OC users and (0.1148 ± 0.0025) in controls. The values were not statistically significant (Table 3). The results of the present study suggest that the OC users have significantly higher frequencies of SCEs/cell, but the CAs and DNA damage were not significant. An increase in the frequencies of SCEs and CAs has been reported during pregnancy [19,20]. The concentration of the estrogens is increased during pregnancy, which may be the possible reason of an increase in SCEs [21]. Earlier studies performed on the genotoxic potential of steroids have shown to cause chromosomal damage, induction of SCEs and formation of endogenous adducts [7,22–24]. The results of the present study show that the OCs are not potent in inducing DNA

Table 2 Distribution of Sister chromatid exchanges (SCEs) among women using oral contraceptives (OCs).

Subjects	SCE per cell	
	OC users	Control
1	4.2	2.3
2	4.1	2.5
3	4.3	2.9
4	3.9	2.7
5	3.5	2.4
6	3.6	2.3
7	3.7	2.6
8	3.3	3.1
9	3.7	2.2
10	3.8	2.3
11	4.1	2.4
12	4.0	2.5
13	3.2	3.2
14	3.2	2.9
15	3.3	2.8
16	4.2	2.7
17	4.1	3.2
18	4.3	2.2
19	4.4	2.8
20	4.0	2.7
21	4.3	2.4
22	4.2	2.3
23	4.1	2.8
24	3.9	2.1
25	4.4	3.3
Mean ± SE	3.91 ± 0.076*	2.62 ± 0.068

* $P > 0.005$ (Significant with respect to control).

Table 3 Estimation of DNA damage among oral contraceptives (OCs) users and non-users.

Subjects	OC users	Control
1	0.11	0.10
2	0.12	0.11
3	0.12	0.12
4	0.13	0.11
5	0.12	0.12
6	0.10	0.13
7	0.11	0.13
8	0.13	0.12
9	0.14	0.11
10	0.12	0.09
11	0.11	0.08
12	0.10	0.11
13	0.11	0.12
14	0.12	0.11
15	0.11	0.12
16	0.13	0.13
17	0.13	0.11
18	0.12	0.12
19	0.12	0.12
20	0.12	0.13
21	0.11	0.12
22	0.12	0.11
23	0.11	0.10
24	0.10	0.12
25	0.12	0.13
Mean ± SE	0.1172 ± 0.0020	0.1148 ± 0.00252

$P > 0.005$ NS (Non significant).

damage/chromosomal aberrations, but are able to increase the number of SCEs/cell. The study performed by Biri et al. [25] also showed the increase in SCEs/Cell in OC users. The SCEs are the cytological manifestation of interchanges between DNA replication products at apparently homologous loci. It is more sensitive indicator of genotoxic effects than structural aberrations [26,27]. The induction of SCE is also correlated with the induction of cancer [28]. Besides various reports on the genotoxicity of steroids some of the progestogens have been studied in short and long term toxicology studies in rodents and dogs or monkeys for their carcinogenic activity, no significant or unusual toxicities were reported [29]. In some cases the oral contraceptives have also been suggested to be having a role in preventing ovarian cancer by both suppressing ovulation and altering the tumor promoting milieu [30]. The results obtained in the present study are contrary to the studies performed by other workers [13,31,32]. This may be due to the new hormonal formulations and preparations developed in an attempt to reduce the adverse effects of OCs, such as the reduction in the estrogen content. Estrogen such as ethinylestradiol, in the liver undergoes aromatic hydroxylation and the product, 4-hydroxyestrone, 3,4-dihydroxy 1,3,5 (10)–oestratien-17-one (4-OHE) is carcinogenic in male Syrian golden hamster kidney tumor model [33,34]. The genotoxic potential of steroids is determined by the metabolic conditions in the test system and the human body. The extrapolation from the experimental data to humans is not only difficult but also complex. The most of the studies conducted for genotoxic potential of steroids involve the concentration in microgram per ml range whereas the therapeutic plasma concentration ranges from nanogram or picogram per ml [7]. The present study was conducted on the women taking OCs for one year and the increase in SCEs may be due to the variable hormonal profile among them. Further, the occupation and work place, polymorphic metabolizing genes and efficiency of DNA repair can also affect the response against OCs.

Acknowledgements

The authors are thankful to the University Grant Commission (UGC), New Delhi for the award of project entitled: ''Biochemical and Cytogenetic effects of Oral Contraceptives among women of different reproductive histories'' {F.No. 39-582/2010(SR)} to Dr. Yasir Hasan Siddique, Department of Zoology, Aligarh Muslim University, Aligarh.

References

[1] Speroff L, Glass RH, Kase NG, editors. Oral contraception. In: Clinical gynecologic endocrinology and infertility. Lippincott Williams & Wilkins; 1999. p. 867–944.
[2] Goldzieher JW. Selected aspects of the pharmacokinetics and metabolism of ethinyl estrogens and their clinical implications. Am J Obst Gynecol 1990;163:318–22.
[3] Kochhar TS. Steroid hormone enhanced sister chromatid exchange in cultured CHO cells. Experientia 1988;44:62–3.
[4] Shyama SK, Rahiman MA. Genotoxicity of lynoral (ethynilestradiol, an oestrogen) in mouse bone marrow cells, in vivo. Mutat Res 1996;370:175–80.
[5] Siddique YH, Afzal M. Genotoxic potential of cyproterone acetate: a possible role of reactive oxygen species. Toxicol In vitro 2005;19:63–8.
[6] Siddique YH, Ara G, Beg T, Afzal M. Genotoxic potential of medroxyprogesterone acetate in cultured human peripheral blood lymphocytes. Life Sci 2006;80:212–8.
[7] Joosten HFP, van Acker FAA, van den Dobbelsteen DJ, Horbach GJMJ, Krajnc EI. Genotoxicity of hormonal steroids. Toxicol Lett 2004;151:113–34.
[8] Siddique YH, Afzal M. Evaluation of genotoxic potential of synthetic progestin chlormadinone acetate. Toxicol Lett 2004;153:221–5.
[9] Siddique YH, Beg T, Afzal M. Genotoxic potential of ethylestradiol in cultured mammalian cells. Chem Biol Interact 2005;151:141–4.
[10] Siddique YH, Beg T, Afzal M. Antigenotoxic effects of ascorbic acid against megestrol acetate induced genotoxicity in mice. Hum Exp Toxicol 2005;24:121–7.
[11] Littlefild LG, Mailhes JB. Comparison of chromosome breakage in lymphocytes and fibroblasts from control women taking oral contraceptives. Fertil Steril 1975;26:828–32.
[12] Hundal BS, Dhillon VS, Sidhu IS. Genotoxic potential of estrogens. Mutat Res 1997;389:173–81.
[13] Dhillon VS, Singh JR, Singh H, Kler RS. In vitro and in vivo genotoxicity evaluation of hormonal drugs v. mestranol. Mutat Res 1994;322:173–83.
[14] Carballo MA, Alvarez S, Boveris A. Cellular stress by light and Rose Bengal in human lymphocytes. Mutat Res 1993;288:215–22.
[15] IPCS. International programme in chemical system, Environmental health criteria 46, guidelines for the study of genetics effects in human populations, vol. 46. Geneva: WHO; 1985. p. 25–54.
[16] Perry P, Wolff S. New Geimsa method for the differential staining of sister chromatids. Nature 1974;251:156–8.
[17] Burton KA. Study of the condition and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem J 1956;62:315–23.
[18] Siddique YH, Ara G, Beg T, Afzal M. Assessment of cell viability, lipid peroxidation and quantification of DNA fragmentation after the treatment of anticancerous drug Mitomycin C and curcumin in cultured human blood lymphocytes. Exp Toxicol Pathol 2010;62:503–8.
[19] Murthy PBK, Prema K. Further studies on sister chromatid exchange frequency in users of hormonal contraceptives. Mutat Res 1983;119:351–4.
[20] Ghosh R, Ghosh PK. Sister chromatid exchanges in the lymphocytes of control women, pregnant women and women taking oral contraceptives: effects of cell culture temperature. Environ Mol Mutagen 1988;12:179–83.
[21] Sharma T, Das BC. Higher incidence of spontaneous sister chromatid exchanges (SCEs) and X-ray induced chromosome aberrations in peripheral blood lymphocytes during pregnancy. Mutat Res 1983;174:27–33.
[22] Blakey DC, White INH. Unscheduled DNA synthesis caused by norethindrone and related contraceptive steroids in short-term male rat hepatocyte culture. Carcinogenesis 1985;6:1201–5.
[23] Shimomura M, Higashi S, Mizomoto R. 32P-postlabeling analysis of DNA adducts in rats during estrogen-induced hepatocarcinogenesis and effect of tamoxifen on DNA adduct level. Japanese J Can Res 1992;83:438–44.
[24] Yager JD, Fifield DS. Lack of hepatogenotoxicity of oral contraceptive steroids. Carcinogenesis 1982;3:625–8.
[25] Biri A, Civelek E, Karahalil B, Sardas S. Assessment of DNA damage in women using oral contraceptives. Mutat Res 2002;521:113–9.
[26] Tucker JD, Preston RJ. Chromosome aberrations, micronuclei, aneuploidy, sister chromatid exchanges, and cancer risk assessment. Mutat Res 1996;365:147–59.
[27] Tucker JD, Aulleta A, Cimino MC, Dearfield KL, Jacobson Kram D, Tice RR, et al. Sister chromatid exchanges: second

report of the gene tox programme. Mutat Res 1993;297: 101–80.
[28] Gebhart E. Sister chromatid exchange (SCE) and structural chromosome aberrations in mutagenicity testing. Hum Genet 1981;58:235–54.
[29] Jordan A. Toxicology of progestogens of implantable contraceptives for women. Contraception 2002;65:3–8.
[30] Siskind V, Green A, Bain C, Purdie D. Siskind beyond ovulation: oral contraceptives and epithelial ovarian cancer. Epidemiology 2000;11:106–10.
[31] Fridrichova I. Cytogenetic study of blood in women who had used oral contraceptives. Neoplasm 1990;37:545–53.
[32] Pinto MR. Possible effects of hormonal contraceptives on human mitotic chromosomes. Mutat Res 1986;169:149–57.
[33] Liehr JG, Fen WR, Sirbasku DA, Ari-Ulubelen A. Carcinogenicity of catechol estrogens in Syrian hamsters. J Steroid Biochem 1986;24:353–6.
[34] Li JJ, Li SA. Estrogen carcinogenesis in Syrian hamster tissues: role of metabolism. Fed Proc 1987;46:1858–63.