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Original Research Article

EFFECT OF SYNTHETIC PROGESTERONE (NORETHISTERONE) ON HUMAN CHROMOSOMES: A CYTOGENETIC STUDY FROM INDIA

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ABSTRACT

Birth restriction methods dates back to prehistoric times, half a million years ago. Modern contraceptive methods constitute most contraceptive use. Nearly 800 million married or in-union women are projected to be using contraception in 2030. Norethisterone (synthetic progesterone) is used for many therapeutic purposes, and is being used by millions of women in India. The present study was carried out during August 2009 to July 2011 on sixty fertile females within reproductive age group. Chromosomal analysis was carried out to find the effects of synthetic progesterone (Norethisterone) on human chromosomes in lymphocyte culture *in vitro* in three groups at 0, 75 µg, 100 µg of drug per ml respectively and observed for chromosomal aberrations like break, gap, dicentric chromosome and chromosomal association. Chromosomal aberrations were significantly increased at higher concentrations. Mean chromosomal gaps at 0µg/ml, 75µg/ml and 100 µg/ml concentration were 6.90, 7.62 and 10.58 respectively and mean chromosomal breaks in that same concentration were 6.63, 7.28 and 10.08 respectively. 30 samples of the 60 showed chromosomal associations and 5 showed dicentric chromosomes. There is a direct correlation between increase in concentration of Norethisterone and structural chromosomal aberrations, which may be carried to next generation, and lead to anomalies in progeny of woman taking such high doses of synthetic progesterone.

KEY WORDS: Norethisterone, synthetic progesterone, cytogenetics, chromosomal aberrations, Oral contraceptive Pills.

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INTRODUCTION

Birth restriction dates back to prehistoric times, half a million years ago [1]. Around 1850 BC the Ancient Egyptians believed that crocodile dung mixed with honey and milk had contraceptive properties. In 1923 a pure sample of estrogen was obtained, which was an important step in the development of oral contraception. In the 1950s, Pincus, Garcia, and Rock found that progesterone and 19-nor progestins prevented ovulation in women. Clinical studies in the 1950s in Puerto Rico and Haiti established the virtually complete contraceptive success of the norethynodrel-mestranol combination. In 1962 ORTHO-NOVUM (norethindrone plus mestranol) was approved by Food and Drug Administration (FDA) for use as a contraceptive agent [2].

Modern contraceptive methods constitute most contraceptive use. Nearly 800 million married or in-union women are projected to be using contraception in 2030, and growth in the number of contraceptive users will be uneven

across regions [3]. Carr *et al.* explained possible mutagenic effect of combined oral contraceptives (OC) (estrogen plus progesterone), on female germ cells, but was not able to give evidences of adverse effects of individual components of OC [4]. Singh O S *et al.* observed increase in triploidy in abortuses from women conceiving within six months of discontinuing oral contraceptives and it was confirmed that consumption of these hormones was resulting in numerical chromosomal aberrations [5].

Norethisterone (synthetic progesterone) is used for many therapeutic purposes, and is being used by millions of women in India. When used as an oral contracep-tive, norethisterone usually is given at a dose of 0.5 to 2.0 mg daily in combination with mestranol or ethinylestradiol (synthetic estrogen hormones). In the contraceptive "mini-pill," it is used continuously at a daily dose of 0.35 mg. Norethisterone acetate is also administered in several hormone replacement therapies. For other medicinal uses, daily doses of norethisterone range from 10 to 30 mg [6]. This study will provide the basis for evaluating effects of synthetic progesterone (Norethisterone) on chromosomes of human lymphocytes *in vitro*. It is an attempt to find out safety levels of drug used and which will help us to know effects on babies born to females using this drug in OC pills or for other gynecological treatment. Thus, present study involving replicating human cells may be ideal study for finding out the safety profile of oral contraceptives in general and Norethisterone in particular with reference to different types of chromosomal aberrations in woman who are using them, on short term as well as long term basis.

Aim: The present study was undertaken with the aim to study cytogenetic effects of different concentrations of synthetic progesterone (Norethisterone) on human chromosomes, and to find safety levels of this commonly used drug.

MATERIALS AND METHODS

The present Experimental Controlled Study was carried out in collaboration with the department of Anatomy, Jawaharlal Nehru Medical College, Sawangi, Wardha and Kamineni Hospital, Hyderabad. The ethical clearance was taken from institutional ethics committee for the present study work. 60 fertile females within reproductive age group were selected for present study which was carried out during August 2009 to July 2011. After taking detailed history, written consent was taken from each female and 3 ml of venous blood was collected in vacutainer. Healthy Female of reproductive age group (18-45 years) not using any form of hormonal contraceptives, or Hormone replacement therapy (HRT) were included in this study. Women with bad obstetric history, Genetic syndromes, H/O exposure to radiation and chemicals, H/O hypertension, diabetes mellitus or Malignancy were excluded. Chromosomal analysis was carried out to find the effects of synthetic progesterone (Norethisterone) on human chromosomes in lymphocyte culture *in vitro* by the method routinely used in Genetic Laboratory as described by Anjankar SD *et al* [7].

All the 60 samples so collected were divided into 3 parts and group were named as A, B, & C. Group A with no progesterone exposure; Group B with 48 hours progesterone exposure at concentration of (75micro gm of Norethisterone/ ml of medium) and Group C with 48hours progesterone exposure at concentration of (100micro gm of Norethisterone /ml of medium). All materials (blood samples) were brought in laminar flow chamber, presterilised by ultraviolet light for 15 minutes and asepsis maintained thereafter.

In Group A: No drug was added. In Group B: At the end of 24 hours, 300 ìL of stock solution (5 mg Primolut –N tablet in 4 ml of distilled water) was added to each centrifuge tube to obtain final strengths of 75 µg of drug per ml of culture medium and kept for re-incubation at 37^0C till the completion of 70 hours and 40 minutes. In Group C: at the end of 24 hours, 400 ìL of stock solution was added to each centrifuge tube to obtain final strengths of 100 µg of drug per ml of culture medium. Colchicine solution was added (0.25 µL/ml) to arrest cell division in mitosis, to each culture tube (A, B and C groups) and the tubes were further incubated for one hour and twenty minutes at 37^0C. All the culture tubes were centrifuged at 1000 rpm for 10 minutes. Then, the supernatant from each culture tube was discarded. 10 ml of freshly prepared 0.075 M hypotonic KCl solution followed

by pre-fixing and centrifugation, leaving behind ½ to 1 ml deposit in test tube. The deposit was similarly washed twice in chilled fixative at 10 minutes interval to obtain a clear cell suspension. Then slides were prepared by air drying method followed by staining with Conventional Giemsa staining. This all slides were screened first under low power objective of microscope and then under oil-immersion objective. Selected metaphases were photographed using camera and observed for break, gap, dicentric chromosome and chromosomal association.

Gaps are the aligned discontinuity in one or both the chromatids of chromosome, smaller than the width of a chromatid. Break is the aligned discontinuity present in chromosome or chromatid of chromosome, which is equal or larger than the width of chromatid or any nonaligned discontinuity affecting both or single chromatid of chromosome. Dicentric chromosome is a chromosome with two centromeres resulting from the malunion of centric broken ends of two chromosomes (Figure 1).

Statistical analysis: Continuous data are reported as mean ± standard deviation and categorical data as the number (percentage). ANOVA was used for statistical analysis between the groups.

RESULTS

The volunteers were between 20 to 40 years of age. Majority 27 (45%) were between 20 to 25 years of age, and minimum i.e. 4(6.66%) patients were lying in the between 36 to 40 years of age. In 60 volunteers, average total chromosomal aberrations in controls i.e. at 0µg/ml was 6.90 and at 75µg/ml it was 7.62, at concentration of 100µg/ml it significantly high being 10.58. Frequency of total chromosomal aberrations is significantly higher at 100µg/ml concentration as compared to 75µg/ml and 0 µg/ml concentration (Table 1).

The increase in frequency of total chromosomal aberrations between control (0µg/ml) and 75µg/ml concentration was not statistically significant (p value-0.186), there was 38.95 % change of total chromosomal aberrations at 75µg/ml concentration with respect to 0 µg/ml concentration (controls), whereas percentage change over of total chromosomal aberrations at 100µg/ml concentration with respect to 75µg/ml concentration was 53.38% (Table 2). Gaps: Mean chromosomal gaps in controls is 6.90, whereas it was 7.62 at 75µg/ml and 10.58 at 100 µg/ml concentration. Also, specifically group C chromosomes- as mentioned in Denver's Conference [8] were found to be affected maximally (Table 3). Breaks: The mean chromosomal breaks in controls was 6.63, whereas it was 7.28 at 75µg/ml and 10.08 at 100 µg/ml concentration (Table 4). Chromosomal associations: Out of 60 volunteers in this study, 30 showed chromosomal associations. 8 controls (at 0 µg/ml) showed chromosomal associations, 12 samples were observed to have associations at 75µg/ml whereas 10 showed associations at 100µg/ml concentration (Table 5). Dicentric chromosomes: Out of 60 volunteers in this study, 5 showed dicentric chromosomes. Controls showed no dicentric chromosomes, 2 samples were observed to have dicentric chromosomes at 75µg/ml whereas 3 showed dicentric chromosomes at 100µg/ml concentration (Table 6).

Fig. 1: Microscopic photographs of chromosomal aberrations like break, gap, dicentric chromosome and chromosomal association.

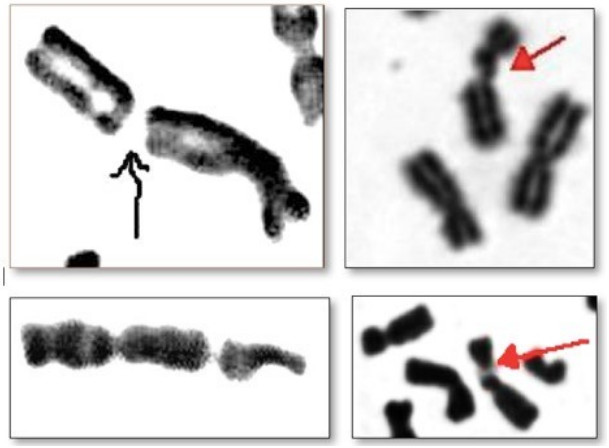

Table 1: Age distribution of volunteers

Age groups	No. of patients	Percentage (%)
20 to 25	27	45%
26 to 30	17	28.33%
31 to 35	12	20%
36 to 40	4	6.66%

Table 2: Total structural chromosomal abnormalities in present study.

ANOVA Test					
Total Aberration	Sum of Squares	Degree of freedom	Mean Square	F	Significance
Between Groups	457.633	2	228.817	26.228	P-0.001
Within Groups	1544.167	177	8.724		
Total	2001.8	179			

Table 3: Comparison of total aberrations between different concentrations of Norethisterone.

Concentration 1	Concentration 2	Mean Difference (I-J)	Std. Error	Sig.(p value)	95% Confidence Interval	
					Lower Bound	Upper Bound
75	0	0.71667	0.53926	0.186	-0.3475	1.7809
100	0	3.68333	0.53926	0.001	2.6191	4.7475
100	75	2.96667	0.53926	0.001	1.9025	4.0309

Table 4: Mean Chromosomal Gaps observed at different concentrations of norethisterone.

Concentrations	Mean Gaps	Std. Deviation
No drug	6.9	2.41
75.00µg/ml	7.62	2.77
100.00µg/ml	10.58	3.56
Total	8.37	3.34

Table 5: Mean Chromosomal breaks observed at different concentrations of norethisterone.

Concentrations	Mean Breaks	Std. Deviation
No drug	6.63	2.19
75µg/ml	7.28	2.64
100µg/ml	10.08	3.14

Table 6: Number of patients showing chromosomal associations at different concentrations of Norethisterone.

Concentrations	No. of Patients	Percentage (%)
No Drug	8	26.66%
75µg/ml	12	40.00%
100µg/ml	10	33.33%
Total	30	100.00%

Table 7: Number of patients showing Dicentric Chromosomes at different concentrations of Norethisterone.

Concentrations	No. of Patients	Percentage (%)
No Drug	0	0
75µg/ml	2	3.33%
100µg/ml	3	5.00%
Total	5	8.33%

DISCUSSION

In spite of special interest of scientists in investigation of contraceptives steroids, their effects on chromosomes are scantily documented and controversial [9]. The aim of this study is therefore to try to elucidate the possibly harmful effects of widely used synthetic progesterone Norethisterone (content of combination oral contraceptive pill) on genetic material, using chromosomal aberrations. In the present study, out of various synthetic progesterones used in OC pills, Norethisterone commonly used by Indian women was selected for study, and large number of females involved in the study were belonging to rural area, which makes the study valuable.

The observations in present study suggested that Norethisterone (synthetic progesterone) induced chromosomal aberrations, which were highest at concentration of 100µg/ml. Chromosomal breakages was the most common type of chromosomal aberrations was observed. Due to environmental, genetic and lifestyle factors, in vivo studies gave variable results. Taking into consideration this fact, in the present study, the effects are observed in *vitro*. This is considered first step in evaluating genotoxic effect on multiplying human lymphocytes.

Ahmed M E *et al.* while working on genotoxicity of norethindrone (norethisterone) used chromosomal aberrations (CA), sister chromatid exchanges (SCE) as parameters [10]. P. Bale Krishna Murthy *et al.* studied effect of the use of an oral contraceptive using frequency of sister-chromatid exchanges (SCEs) as a parameter [11]. Later during his further studies on OC pills same parameter for detecting genetic damage was used by him [12]. Dhillon V S *et al.* evaluated genotoxicity of norethisterone acetate, using chromosomal aberrations and sister chromatid exchanges and frequency of micronuclei formation his in vitro and in vivo assays [13].

Sister chromatid exchange induction in human lymphocytes was negative in study by Ahmad et al, whereas it was positive in study by Dhillon and Dhillon et al. Falaq Naz *et al.* observed no significant difference in chromosomal aberrations and DNA damage, whereas significant increase was observed in sister chromatid exchanges (SCEs) Cell among OCP users [14].

In a recent study by Falaq Naz *et al.* showed hormonal contraceptives seem to exert DNA damage and also have significant effects on blood serum enzymes [15]. Very few authors in the literature have observed these types of chromosomal aberrations, also they were unable to give statistical analysis of these types as observed in present study.

Ahmed M E *et al.* studied genotoxicity of norethindrone(norethisterone) and norgestrel, lymphocytes were exposed to three different concentrations of the drug 20, 40 and 75 ìg /ml for norethindrone [16]. Norgestrel was studied at 10, 25, 50 ìg /ml concentrations. In present study, the drug (Norethisterone) is added at concentration of 75 and 100 µg/ml, more aberrations were seen at high concentration treatment, 100µg/ml. These findings correlate with above author which particularly show more aberrations at high concentration treatment. Out of 30 volunteers showing associations between chromosomes, 8 were controls, 12 volunteers showed associations at concentration of 75µg/ml, and 10 patients showed associations at concentration of 100 µg/ml. This finding is not following dose dependent manner which may be because of variance in genetic and environmental factors. No other author has observed chromosomal associations after progesterone exposure, which is additional finding in the present study. Regarding chromosomal gaps, it was found that, Group C chromosomes are more frequently affected by them, specially at concentrations of 100µg/ml. This may be because of presence of more number of Group C chromosomes in normal karyotype. Siddique *et al.* observed the genotoxic effect of steroid at higher doses [9,16,17]. The authors also concluded that therapeutic doses are safe, but they may be even genotoxic in long term usage [18,19].

Limitation: While studying females in reproductive age group, we found that methodological, biological and lifestyle factors accounted for lot of variations in results, and large part of total variation still remains undetermined and should be studied further in hope of accounting for remaining variance by taking into account the genetic makeup of volunteers, also other endpoints for genetic damage like sister chromatid exchanges, micronuclei formation, DNA damage should be applied for further research in this subject. In vivo studies and molecular studies are required to be conducted in the same subject to correlate their findings with this in vitro study.

CONCLUSION

There is a direct correlation between increase in concentration of Norethisterone and structural chromosomal aberrations, which may be carried to next generation, and lead to anomalies in progeny of woman taking high doses of synthetic progesterone. The findings made from the present study may help physicians to choose progesterone with least adverse effects for their patients. The potential benefits and side effects of the drug, must be weighed up against the deleterious effects. Whole new attitude about rampant use of hormonal steroids these days is required to be changed.

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Conflicts of Interests: None

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