CYTOGENETIC STUDIES ON SOME SYNTHETIC STEROIDS IN RELATION TO HUMAN LYMPHOCYTE CHROMOSOMES

ABSTRACT

THESIS

SUBMITTED FOR THE DEGREE OF

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IN

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BY

MD. EQUEBAL AHMAD

SECTION OF GENETICS
DEPARTMENT OF ZOOLOGY
ALIGARH MUSLIM UNIVERSITY
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ABSTRACT

Synthetic steroids have potential application in pharmacology. These steroids possess antitubercular, antitumour, anti-inflammatory, antineoplastic, immunosuppressive and antimicrobial activities. In the present study six synthetic steroidal hormones were taken to evaluate their cytogenetic effects on human lymphocyte chromosomes in vitro. All the synthetic steroids used in the present study were sex hormones two each from estrogens, progestins and androgens.

The sex hormones are used widely in human medicine for a large variety of conditions, apart from their uses as oral contraceptive agents. They are used in the treatment of dysmenorrhoea, endometriosis, dysfunctional uterine bleeding, as replacement therapy in patients with various hormonal disbalances, in gonadal dysgenesis and in women with symptoms of climacteric. They are also used in the prevention of postmenopausal osteoporosis and in the treatment of advanced breast and endometrial carcinoma. In obstetric practice progestins are used in the management of threatened abortion and to prevent premature labour. And local estrogens are used successfully to 'prime' the unripe cervix to induction of labour. Estrogens are also used to develop secondary female sex characteristics in trans-sexual males and androgens to suppress these characteristics in trans-sexual females. Androgens are used in the treatment of male hypogonadism, in the treatment of symptoms of climacteric and in advanced mammary tumours in women.
Certain synthetic androgens are also used to induce ovulation in anovulatory women and also in oligospermic males. The use of androgens to improve athletic performance is well known. Apart from the above described applications, these synthetic steroids are used in large number of other conditions.

Cytogenetic studies were carried out to evaluate the genotoxic effects of these synthetic steroids on human system. The genotoxic effects of steroids cannot be assessed on bacterial system because specific receptor molecules needed by the steroids to perform their action are absent in bacteria. Therefore, cytogenetic tests are more appropriate parameters for evaluating the genotoxic effects of steroids. Human peripheral blood lymphocytes were used for this purpose because of the ease of obtaining blood samples, in contrast to other human cell populations (e.g. bone marrow cells), and because these cells are extremely sensitive indicators of the in vivo and in vitro induced chromosomal changes (structural and numerical). It is a single system that provides us with a large number of dividing cells. These are easily cultured to yield large number of mitotic figures for chromosome analysis.

The parameters used in the present study included chromosomal aberrations, sister chromatid exchange analysis and cell growth kinetics assay in the absence as well as in the presence of exogenous metabolic activation system (S9 mix). The students 't' test (paired) was applied for chromosomal aberrations and sister chromatid exchanges. The 2x3 chi-square test was used
for cell growth kinetics studies. The level of significance was compared at
P<0.05 for all parameters. Lymphocytes were exposed to all drugs for three
different durations (i.e. 24, 48 and 72 h.) and for three different dose
concentrations. The synthetic steroids were used with a view to study the
following objectives:

(1) To study the *in vitro* effects of the steroids on human lymphocyte
culturing system.
(2) To study the toxic effects of the steroids on human lymphocyte
chromosomes.
(3) Evaluation of the chromosome damage in parallel in the presence
of metabolic activation (S₉ mix).
(4) To study the effects of the steroids on the frequency of sister
chromatid exchange (SCE).
(5) To find the frequency of SCEs when compared in the presence of
S₉ mix, and finally,
(6) To study the effects of the steroids on cell growth kinetics in the
presence as well as in the absence of S₉ mix.

Out of the six synthetic steroids studied, three were found to have
harmful effects on human peripheral lymphocyte chromosomes. Both
the estrogens i.e. mestranol and quinestrol were found to be genotoxic.
Among progestins, norgestrel was found to be genotoxic whereas,
norethindrone was not having any harmful effect on the chromosomes.
Both the androgens i.e. danazol and oxymetholone also did not show any
harmful effect on the chromosomes.

Mestranol was found to be associated with increase in the incidence of chromosomal aberration. At 10 \( \mu g/ml \) dose concentration the drug did not show any significant result, but at 25 \( \mu g/ml \) concentration highly significant increase in chromosomal aberration was noted at all exposure durations (i.e. 18.00\%, 22.00\% and 27.00\% respectively at 24, 48 and 72 h of incubations). At the highest dose of concentration i.e. 50 \( \mu g/ml \), an interesting result was obtained. A steep decline in chromosomal aberrations and aberrant metaphases were observed. This can be attributed to the toxic effect of the drug on the mitotic index at this concentration. In the presence of metabolic activation the drug induced significant level of chromosomal aberration at 25 \( \mu g/ml \) concentration only (i.e. 29.50\%). Similar trend was noted for this drug in the sister chromatid exchange analysis in the absence as well as in the presence of metabolic activation i.e. highly significant increase in sister chromatid exchange frequency occurred in both cases at 25 \( \mu g/ml \) of dose concentration only (5.04 ± 0.224 and 5.38±0.180 respectively). The result of cell growth kinetics study was again similar for mestranol. The replication indices were found significant only at 25 \( \mu g/ml \) of dose concentration in the absence as well as in the presence of metabolic activation (1.43 and 1.46 respectively).

Another estrogen quinestrol was also found to be genotoxic. Quinestrol did not increase chromosomal aberration frequency at 10
μg/ml of dose concentration. But at 25 μg/ml concentration the drug was found to increase aberrant metaphases as well as chromosomal aberration frequency at all treatment durations (i.e. 18.66%, 22.67% and 39.66% respectively at 24, 48 and 72 h). At 50 μg/ml concentration the aberration frequency first increased i.e. at 24 h duration (33.67%) but decreased later on with the increase in exposure duration (6.00% and 4.33% respectively at 48 and 72). This interesting result may be attributed to cell death at the highest concentration and for longer exposure duration. In the presence of metabolic activation experiment, a dose time relationship was observed and the aberration frequency increased with increase in dose concentration. All the values obtained were significant. Quinestrol increased the SCE frequency, significantly both in the absence as well as in the presence of metabolic activation, only at 25 and 50 μg/ml of dose concentration. A dose time relationship was observed and maximum value of SCE frequency was observed at 50 μg/ml concentration in both cases (4.28±0.090 and 4.68±0.100 respectively). Cell growth kinetics study for quinestrol also reveals a similar trend. The replication indices at 25 and 50 μg/ml concentration were found significant both in the absence (1.46 and 1.40 respectively) as well as in the presence (1.46 and 1.43 respectively) of metabolic activation.

Norethindrone, a progestin, was not found to cause harmful effects on human lymphocyte chromosomes. The drug did not induce significant
level of chromosomal aberration in human lymphocytes at any dose concentration (20, 40 and 75 µg/ml) and any treatment duration in the absence as well as in the presence of metabolic activation system.

Another progestin, norgestrel, was found to be effective in increasing chromosomal aberration frequency as well as aberrant metaphases. A dose time relationship was observed. The drug was not effective at 10 µg/ml concentration at any treatment duration. At 25 and 50 µg/ml of dose concentration the drug enhanced significant level of chromosomal aberration frequency at all durations. The values obtained were 19.00%, 26.33% and 50.33% respectively at 25 µg/ml of concentration, and 22.00%, 28.00% and 54.66% respectively at 50 µg/ml of concentration. In the presence of metabolic activation the values obtained for this drug were significantly high at all concentrations (9.50%, 22.50% and 41.00% respectively). Norgestrel enhanced SCE frequency significantly both in the absence as well as in the presence of S9 mix at 25 and 50 µg/ml of concentrations only. The value observed in the absence of S9 mix were 2.98±0.104 and 4.44±0.086 respectively. In the presence of S9 mix the values were 3.34±0.120 and 5.22±0.136 respectively. Similar results were observed for cell growth kinetics study also. Significant values were obtained at 25 and 50 µg/ml of dose concentration only. Replication indices observed were 1.52 and 1.47 respectively in the absence of S9 mix. In the presence of S9 mix the replication indices were 1.44 and 1.46 respectively.
Both the synthetic androgens, i.e. danazol and oxymetholone, evaluated for their cytogenetic effects in the present study were found to be non-genotoxic. The drugs neither induce significant level of chromosomal aberrations, aberrant metaphases and sister chromatid exchanges nor they affect the mitotic index, either in the absence or in the presence of metabolic activation system.

The overall conclusion that emerges from the results of present investigation reveals that both the estrogens (i.e. mestranol and quinestrol) and one progestin (i.e. norgestrel) seem to be potent mutagens and they are well associated with clastogenic effects in the presence as well as in the absence of metabolic activation system. Various structural and numerical chromosomal changes were observed when the lymphocytes were treated with estrogens and the progestin 'norgestrel'. It was concluded that the use of synthetic steroidal drugs particularly those drugs which are used as oral contraceptives is associated with a significant increase in the incidence of chromosome abnormalities per cell, besides an increase in the number of abnormal cells, and gene mutation which is exhibited as SCE. It is established that there is an apparent relationship of chromosome abnormalities, sister chromatid exchanges and cell proliferation inhibiting effect with mutagenesis, carcinogenesis and teratogenesis.

The phenotypic expression of genetic damage in the drug user, either as visible chromosomal aberrations or the point mutations, is dependent
on the developmental time when the damage occurs (i.e. pre- or postnatal life) and the type of cell in which the damage occurs (i.e. somatic or germinal). Such damage in somatic cells of the developing embryo can lead to malformations and abortions in extreme cases. Any mutational effect on adult somatic cells could lead to cancer, and genetic damage in germ cells of the person taking these drugs would only be manifested in his or her offspring leading to teratogenesis or carcinogenesis.

It is suggested that such type of genetic damage might have occurred due to the ability of these steroids to generate free oxygen radicals in the lymphocytes.

It is, therefore, advisable to be careful of the potential hazards of these drugs to which we are continuously exposed day in and day out in modern life. 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 directly in their unmetabolized or the metabolized state.
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CERTIFICATE

I feel great pleasure in forwarding the thesis entitled "Cytogenetic studies on some synthetic steroids in relation to human lymphocyte chromosomes" submitted by Mr. Md. Equebal Ahmad of the Department of Zoology for the award of the degree of Doctor of Philosophy (Zoology, Genetics) of the Aligarh Muslim University, Aligarh. This is to certify that the subject matter of the thesis as presented by Mr. Ahmad is the actual record of work done by him under my supervision and guidance and the same does not form the part of any other course or degree presented to or taken by him.

Dr. Mohammad Afzal
Reader
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(MD. EQUEBAL AHMAD)
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Introduction
INTRODUCTION

In many ways chemicals are a basic factor in the well-being and effective functioning of the present day societies. The quality of life at both the social and individual levels would be severely restricted if we had to live without petroleum products, plastics, synthetic drugs, synthetic textiles and man-made mineral fibres or without the synthetic materials needed to produce modern electronic devices.

As in the case of drugs, the use of other chemicals implies not only the desired benefits but also adverse effects. We are compelled to compromise between costs and benefits. When the desired and the adverse effects of the use of chemicals are weighed, it is pertinent to ask whether we really need all the two thousand new chemicals synthesized annually and whether all the 60,000 to 70,000 chemicals used daily in industrialized societies are truly essential? It is wondering how long our ecosystem can survive and how long the biosystem, man included, can tolerate the doubling of the consumption of chemicals that occurs every seven years (IARC, 1984).

In fact, humanity is faced today with growing problems of 'chemicalization', and questions of weighing risks and benefits have become more and more relevant. This is because all societies have become increasingly dependent on chemicals. Severe and system-wide damage to the ecosystem has been recognized, such as damage due to acid rain, the pollution of soil and water and increasing rates of certain types of cancer and allergies.

Appropriate toxicity testing has been done only for about 10% of the chemicals used daily and in the work environment only 1% of the chemicals used daily have exposure limits. And these are the chemical carcinogens which are most long-acting chemicals to which human population will be exposed for long time in the future (IARC 1984).

The extent to which human somatic and germinal mutation
frequencies may be increased by exposure to ionizing radiation and to
the variety of chemicals that characterize modern societies has been a
matter of concern in recent years. Somatic mutations, either genetic or
chromosomal, are not transmitted to the offspring of an exposed
individual. However, increase in the frequency of these mutations may
contribute to an increase in the frequency of acquired disorders, for
example, cancer. Increase in the frequency of germinal mutations, genetic
or chromosomal, are likely to contribute to inherited defects in the
offspring of individuals exposed to mutagenic agents (WHO 1985).

The history of chemical mutagenesis goes back to 1962. The first
chemical mutagen discovered was mustard gas or sulfur mustard [D-
(2-chloroethyl) sulfide], when C.Auerbach and her associates first
discovered the mutagenic effects of mustard gas and related compounds
used during world war II. Hundreds of chemicals are now known to have
from slight to very large mutagenic effects.

Increasing evidence indicates that most neoplastic conditions arise
from interaction between external factors and a predisposing genotypic/
phenotypic susceptibility, that may be attributed to, e.g., increased
susceptibility of target cells to transformation, inability to repair certain
kinds of DNA damage or reduced capacity to destroy malignant cells.
Much progress has been made in our understanding of the relationship
to cancer development of such predisposing host factors and
environmental carcinogens. However, one basic mechanism that remains
to be elucidated is the role played by chromosomal aberrations in the
causation and/or evolution of neoplasia, especially the influence of
external factors on the karyotypic pattern of tumour cells.

**MECHANISM OF ACTION OF THE CARCINOGENIC/
MUTAGENIC CHEMICAL AGENTS:**

Chemical mutagens are divided into two classes:

(1) The first class comprises those mutagens which are mutagenic to
both replicating and non-replicating DNA. For example, nitrous acid and alkylating agents (a large class of chemical mutagens that transfer alkyl groups i.e. CH\textsubscript{3}\textsuperscript{-},\textsubscript{3}CH\textsubscript{2}-, etc. to the bases in DNA).

(2) The another class contains those mutagens which are mutagenic only to replicating DNA. For example, includes the acridine dyes, which bind to DNA and increase the probability of mistakes during replication. And base analogs which are purines and pyrimidines with structures similar to the normal bases of DNA. The base analogs must be incorporated into DNA chains in the place of normal bases during replication to exert their mutagenic effects.

Mechanism of action of different well known highly potent mutagens are as follows:

1. **Base Analogs**: The base analogs are mutagens which have structures sufficiently similar to the normal bases so that they are metabolized and incorporated into DNA during replication, but sufficiently different such that they increase the frequency of mispairing of bases and thus mutation. Examples are 5-bromouracil and 2-aminopurine.

   (a) **5-bromouracil**: The pyrimidine 5-bromouracil is a thymidine analog, which exists in two forms, keto and enol forms respectively. If 5-bromouracil is present in its less frequent enol form as a nucleoside triphosphate at the time of its incorporation into a nascent strand of DNA, it will be incorporated opposite guanine in the template strand and cause a GC→AT transition. However, on the other hand, 5-bromouracil may also be incorporated in its more frequent keto form opposite adenine (in place of thymine) and undergoes a tautomeric shift to its enol form during a subsequent replication, it will cause an AT→GC transition. Thus 5-bromouracil induces transitions in both directions, AT↔GC.

   (b) **2-aminopurine**: This purine base analog induces mutations in a
similar manner as described above.

2. **Nitrous Acid**: Nitrous acid \( \text{HNO}_2 \) is a very potent mutagen that acts directly on either replicating or nonreplicating DNA by oxidative deamination of the bases that contain amino groups—adenine, guanine and cytosine. Conversion of the amino groups to keto groups changes the hydrogen-bonding potential of the bases. And thus by nitrous acid transitions may be induced in both directions AT\(\rightarrow\)GC.

3. **Acridines**: The acridine dyes such as proflavin, acridine orange, and a whole series of compounds called ICR-170, ICR-190 etc., are very powerful mutagens that induce frameshift mutations. The ICR compounds have acridine moieties with various side chains, often alkylating agents. The positively charged acridines intercalate or "sandwich themselves" between the stacked base pairs in DNA and cause small additions and deletions usually of a single base-pair, result in reading frameshifts for the portion of the gene distal to the mutation.

4. **Alkylating and Hydroxylating Agents**: Alkylating agents such as nitrogen and sulfur mustards, methyl and ethyl methane-sulfonate (MMS and EMS), nitrosoguanidine (NTG), and many others have several effects on DNA. One major mechanism of mutagenesis by alkylating agents involves the transfer of methyl or ethyl groups to the bases such that their base-pairing potentials are altered and transitions result. For example, ethylation at the 7-N position and at the 6-O position are believed to be the two effects of EMS. 7-Ethylguanine is then believed to base-pair with thymine. Other base alkylation products are believed to somehow "activate" repair processes in much the same way as thymine dimers do. The occasional errors occurring in these repair processes may lead to transversions and frameshift mutations in addition to transitions. Finally some alkylating agents, particularly difunctional alkylating agents (those with two reactive alkyl groups), cross-link DNA strands and/or molecules and induce chromosome breaks and the various kinds of chromosomal aberrations found correlated with breaks.
Nitrosoguanidine (NTG), one of the most potent chemical mutagens known, has been found to induce clusters of closely linked mutations in the segment of the chromosome which is replicating during the mutagenic treatment. Mutants isolated after NTG treatment often carry multiple, closely linked mutations.

The hydroxylating agent hydroxylamine, $\text{NH}_2\text{OH}$, in contrast to many of the alkylating agents, has a very specific mutagenic effect. It induces only $\text{GC} \rightarrow \text{AT}$ transitions. Although the exact mechanism of hydroxylamine mutagenesis is still somewhat uncertain, it apparently acts by hydroxylating the amino group of cytosine. The resulting hydroxylaminocytosine can base-pair with adenine to produce the observed $\text{GC} \rightarrow \text{AT}$ transitions.

It is also assumed that some DNA damaging agents are carcinogenic because they induce mutations. These damages lead to heritable changes in the methylation of cytosine in DNA. Considerable evidence exists to show that gene expression in mammalian cells is in part controlled by methylation of specific DNA sequences (Robin 1987).

Most chemical carcinogens and alkylating or acetylating agents (electrophilic reagents) produce such compounds following metabolism in the body. This means that they have electron deficient centre and will combine with electron rich centres within the cell. Reaction with DNA is the most important process in the initiation of mutagenesis and carcinogenesis, although reaction with certain amino acids in proteins will also occur (Farmer 1982).

**CORRELATION OF MUTAGENESIS AND CARCINOGENESIS:**

For many years it has been recognized that most of the strong mutagenic agents, such as ionizing radiations, ultraviolet light, and chemicals are also carcinogenic, i.e. induce cancers. In recent years, sensitive techniques have been developed to test chemicals and other agents for mutagenicity and carcinogenicity. There are several techniques
for testing a chemical for its mutagenicity and carcinogenicity. But the most sensitive technique is the Ames' *Salmonella* mutagenicity test (Ames et al., 1975). Over a period of several years, hundreds of different chemicals were tested by B.N. Ames and his colleagues. They observed greater than 90% correlation between the mutagenicity and carcinogenicity of the substances tested. Initially several potent carcinogens were found to be non-mutagenic to the tester strains. Subsequently, many of these carcinogens, although non-mutagenic per se, were found to be metabolized to strong mutagenic derivatives in eukaryotic cells. Further, rat liver microsomal fraction was added to the Ames' microbial mutagenicity tests to detect the mutagenicity of metabolic derivatives of substances being tested. Several chemicals which were not mutagenic and carcinogenic by themselves were found to be mutagenic and carcinogenic after being metabolized. For example, nitrates are not mutagenic or carcinogenic by themselves, but they are converted by a series of enzyme catalyzed reactions to nitrosamines, which are highly mutagenic or carcinogenic. Activation was not always required for a chemical to be a mutagen and subsequently carcinogen.

Geneticists generally concur on the idea that there is a strong correlation between mutagenicity and carcinogenicity which was later on proved by Ames and his colleagues. Now there is a general consideration that cancer is caused by somatic mutations. This theory has received strong support from the discovery of cellular oncogenes (cancer-causing genes) and the demonstration that oncogene responsible for human bladder carcinoma resulted from a single base-pair change in its normal cellular counterpart (Reddy et al., 1984).

All cancers exhibit a loss of the normal control of cell division that result in the tumour formation. Cell division is undoubtedly, at least in part under genetic control. Thus, mutation in a gene involved in the control of cell division, like a mutation in any other gene, can cause a loss of function and thus a loss of the normal control of cell division. Thus, nowadays the key question is not that whether there is relation
between mutagenicity and carcinogenicity, but the question is that what proportion of human cancers are caused by somatic mutations (Barbacid, 1986).

It has been found that the products of various protooncogenes play central role in the control of cell division. Oncogenes from human cancer cells have been cloned and characterized, they are frequently found to be derivatives of \textit{c-ras} protooncogenes. The genomes of all vertebrates contain three distinct, but closely related protooncogenes called as \textit{ras} protooncogenes. These are \textit{C-H-ras}, \textit{C-K-ras} and \textit{N-ras} (Barbacid, 1987). The first cellular oncogene to be characterized in detail was derived from a human bladder carcinoma called EJ. When the cellular oncogene present in these EJ bladder tumour cells was cloned and sequenced, it was found to be a derivative of the \textit{C-H-ras} protooncogene. Surprisingly, the oncogenicity of the EJ \textit{C-H-ras} mutant gene was found to result from a single base-pair substitution, that is GC$\rightarrow$TA transversion. Subsequent to the characterization of the \textit{C-H-ras} oncogene present in the EJ human bladder carcinoma line, oncogenic variants of the three \textit{c-ras} protooncogenes have been detected and characterized in a large number of different mammalian cancer cell lines (Bishop, 1982). All the mutations that conferred oncogenicity on the \textit{ras} genes involved one or more of three codons: codons number 12, 59 and 61 (Barbacid, 1987).

**CORRELATION OF MUTAGENESIS AND CARCINOGENESIS WITH CHROMOSOMAL ABERRATIONS:**

Chromosomal damage indicates a biological effect on the genome. It is generally believed that increased rates of chromosomal aberration in a population may indicate an increased cancer risk for the group. The suggestion that increased rates of chromosomal aberrations might, somehow, favour the occurrence of cancer is supported by the observation that among subjects with congenital diseases like Fanconi's anaemia and Blooms' syndrome, both characterized by the presence of abnormally high rates of chromosomal breakage and rearrangement, there is an
increased incidence of leukemia and neoplasm (German, 1972). Cigarette smoke, which is a potent carcinogen, has clastogenic effects in lymphocyte cultures (Vijayalaxmi and Evans, 1982). Ionizing radiations and benzene, which are both leukaemogenic, induces chromosome damage in rat bone marrow cells (Forni et al., 1971a,b). A correlation between the prevalence of chromosomal aberration in lymphocytes and cytological abnormalities in the specific target tissue (i.e., bronchial cells) has been demonstrated in uranium miners, a population at high risk for lung cancer (Brandom et al., 1978). Increased proportions of chromosomal aberrations have been found in the survivors of Hiroshima and Nagasaki (Archer et al., 1981). Chromosomal aberrations are generally accepted as indicators of a mutagenic and, therefore, a potentially malignant event and have been described in more than 5000 cases of various malignant disorders (Mitelman, 1983a). The aberrations are strictly non-random within each group of tumours. This has been demonstrated for all types of cancer and leukaemia, in experimental animals and in humans, where the number of subjects studied has been sufficient to draw conclusions (Mitelman and Levan 1981). Consistent specific aberrations have been discovered in an increasing number of tumour types, the most characteristic aberrations are summarized in Table 1. Cancer-associated nonrandom and specific aberrations are convincing evidence for the fundamental role of chromosomal change in the initiation of the malignant process (Yunis, 1983; Mitelman, 1994).

Table 1. Specific chromosome aberrations in human neoplasia.

<table>
<thead>
<tr>
<th>Tumour type</th>
<th>Chromosome</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Myeloproliferative disorders</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic myeloid leukaemia</td>
<td>t(9;22)(q34;q11)</td>
<td>Nowell and Hungerford (1960)</td>
</tr>
<tr>
<td>Acute myeloid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Condition</td>
<td>Chromosome Abnormality</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------------------------------</td>
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<td>-------------------------</td>
</tr>
<tr>
<td>Leukaemia</td>
<td>t(8;21)(q22;q22)</td>
<td>Rowley (1973)</td>
</tr>
<tr>
<td>Acute promyelocytic leukaemia</td>
<td>t(15;17)(q22;q21)</td>
<td>Rowley et al. (1977)</td>
</tr>
<tr>
<td>Acute monocytic leukaemia</td>
<td>t(9;11)(p22;q23)</td>
<td>Berger et al. (1982),</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hagemeijer et al. (1982)</td>
</tr>
<tr>
<td>Refractory anaemia</td>
<td>del(5)(q12q31)</td>
<td>Sokal et al. (1975)</td>
</tr>
<tr>
<td>Polycythemia vera</td>
<td>del(20)(q11)</td>
<td>Reeves et al. (1972)</td>
</tr>
<tr>
<td>Lymphoproliferative disorders</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Burkitt's lymphoma</td>
<td>t(8;14)(q24;q32)</td>
<td>Manolov and Manolova</td>
</tr>
<tr>
<td>(1972)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute lymphocytic leukaemia</td>
<td>t(9;22)(q34;q11)</td>
<td>Zech et al. (1976)</td>
</tr>
<tr>
<td>Workshop</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leukaemia</td>
<td>t(4;11)((q21;q23)</td>
<td>Third International</td>
</tr>
<tr>
<td>Leukaemia</td>
<td>t(8;14)(q24;q32)</td>
<td>on Chromosomes in</td>
</tr>
<tr>
<td>Leukaemia</td>
<td></td>
<td>(1981)</td>
</tr>
<tr>
<td>Chronic Lymphocytic +12 leukaemia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solid Tumours</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meningioma</td>
<td>-22</td>
<td>Mark et al. (1972), Zang</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1972)</td>
</tr>
<tr>
<td>Cervical carcinoma</td>
<td>?1q</td>
<td>Atkin and Baker (1979)</td>
</tr>
<tr>
<td>Ovarian carcinoma</td>
<td>t(6;14)(q21;q24)</td>
<td>Wake et al. (1980)</td>
</tr>
<tr>
<td>Neuroblastoma</td>
<td>del(1)(p32)</td>
<td>Brodeur et al. (1981),</td>
</tr>
<tr>
<td>Gilbert</td>
<td></td>
<td>et al. (1982)</td>
</tr>
<tr>
<td>Pleomorphic adenoma</td>
<td>t(3;8)(p21;q12)</td>
<td>Mark et al. (1982)</td>
</tr>
<tr>
<td>Seminoma</td>
<td>i(12p)</td>
<td>Atkin and Baker (1982)</td>
</tr>
</tbody>
</table>
Ewing's sarcoma  $t(11;22)(q24;q12)$  Aurias et al. (1983), Turc-Carel et al. (1983)

Malignant melanoma  der$(6)(q21q23)$  Trent et al. (1983), Becher et al. (1983)

Nasopharyngeal carcinoma  der$(3)(q25q27)$  Mitelman (1983)

A survey of the distribution of cancer-associated aberrations shows that only a few of the specific marker chromosomes are pathognomonic for a particular malignant disorder (Mitelman 1981, 1983b). Two characteristic observations deserve special mention here: the $14q^+$ marker chromosome produced by the $t(8;14)(q24;q32)$ and the reciprocal translocation $t(15;17)(q22;q21)$, each being limited to just one cell type, the B-cell lymphocyte and the promyelocyte, respectively. Although all the other types of specific marker chromosomes are usually much more frequent in one or even two disorders they may characteristically, be found in several other tumour types also. Thus, it was concluded from the above study that there is little or no target-cell specificity with the specific chromosomal aberrations in neoplastic disorders.

The aberrations that are involved, preferentially, in different tumour types tend to cluster to certain chromosome types. Thus, some chromosomes, e.g. Nos. 1, 8 and 14 seem to be particularly prone to aberrations during malignant development, whereas others, such as chromosomes 2, 4, 10, 18, 19, X and Y, show no selective involvement (Mitelman and Levan, 1981; Mitelman, 1983b). The studies also indicated that certain specific karyotypic aberrations, in particular monosomy 5 (-5), deletions of the long arm of chromosome 5(5q'), monosomy 7(-7) and deletions of the long arm of chromosome 7(7q'), were more frequent in patients with previous occupational exposure to chemical solvents, insecticides and/or petroleum products or their combustion residues.
One interpretation of this is that the chromosomes most often affected carry genetic material which is important to the regulation of cell proliferation and/or differentiation, and which needs to be manipulated in the process of malignant transformation. It is, certainly, striking that the locations of all oncogenes that have been mapped to specific chromosomal regions so far, agree with the break points of characteristic cancer associated chromosomal aberrations (Rowley, 1983). These studies were further confirmed by Mitelman et al. (1981) and Golomb et al. (1982).

Translocations and deletions or deficiencies involving specific chromosomes and, more importantly, often break points at the same positions on these chromosomes has been observed in certain types of cancer cells. Philadelphia chromosome, an altered chromosome 22 that has lost a large segment of its long arm has been found in various studies in upto 90% of the patients suffering from a specific type of cancer called chronic myelogenous leukemia (Klein, 1982).

Now that the chromosomal locations of several human cellular protooncogenes have been determined, a striking correlation is evident between their locations and chromosomal break points of translocations and deficiencies observed in specific types of cancer cells (Yunis, 1983; Rowley, 1983). These results have led to speculations that the observed chromosome breaks and rearrangements may have caused altered expression of protooncogenes or other important regulatory genes present in the vicinity of the break points.

The Philadelphia chromosome was shown to have been produced by a reciprocal translocation involving the ends of the long arms of chromosomes 9 and 22. The break points on chromosome 9 that give rise to these translocations occur very close to the c-abl protooncogene, and the exchange transfers the c-abl gene to chromosome 22 (Klein, 1982).

Another type of cancer that is consistently (>80%) associated with
specific kind of translocation is Burkitt's lymphoma, a cancer of the antibody producing B-lymphocytes. The translocations observed in B cells of patients with Burkitt's lymphoma invariably involve chromosome 8 and one of the three chromosomes (2, 14 and 22) that carry genes encoding antibody chains. The most common sites of the chromosome breaks that give rise to the translocations between chromosome 8 and 14 in Burkitt's lymphoma cells are in bands q24 and q32, respectively (Kaiser-McCaw et al., 1977; Dalla-Favera, 1982). The c-myc protooncogene is located in band q24 on chromosome 8, and c-myc is transferred adjacent to the heavy chain antibody genes on chromosome 14 by the translocation events. Other chromosomal translocation events have been detected with high frequencies in other types of cancer cells as well, although none with as high an incidence as the translocations associated with chronic myelogenous leukemia and Burkitt's lymphoma (Le Beau and Rowley, 1986; Kaiser-McCaw et al., 1977; Dalla-Favera, 1982; Rabbitts, 1985).

Other types of chromosomal changes present in cancerous cells are "double minutes" (DMs) and "homogenously staining regions" (HSRs). The double-minute chromosomes are super numerary chromosomes that contain the amplified gene and adjacent chromosomal DNA on extrachromosomal circular molecules of DNA. They contain the circular molecules of DNA packaged in nucleosomes and chromatin fibres just like normal chromosomes. The same unit of gene that is present in DMs is often present as the tandemly repeated one within the HSR regions of chromosomes containing amplified genes.

**DNA Damage**: Damage to DNA is likely to be a major cause of cancer and other diseases (Haitt et al., 1977; Ames, 1971). There is an evidence which supports that carcinogens and radiations are likely to induce majority of human cancers and other genetic defects by way of damage to DNA (McCann et al., 1975). Defined precisely, DNA damage is an alteration that constitute a stumbling block for the replication machinery and hence hampers the replication of DNA, endangering the survival of
the cell (Davoret, 1979).

The correlation between radiation-induced damage and cancer has long been apparent to the radiation biologists but through molecular evidence, it has been found that DNA damage is a direct cause of cancer. The evidence comes from patients suffering from *Xeroderma pigmentosum* (Morgan *et al.*, 1996). With certain eukaryotic cells, the consequences of DNA damage can also be assessed by cytogenetic analysis. The human disorders *Ataxia telangiectasia*, Bloom's syndrome and Fanconi's anaemia are genetic diseases characterized by an increased susceptibility to cancer (Walker *et al.*, 1985).

Most chemical carcinogens become electrophilic through an enzymatic activation process, or are electrophilic reagents which react with DNA, RNA and proteins (Miller and Miller 1974). They cause DNA damage and induce various mechanisms of repair. Therefore, determination of DNA repair has been suggested to predict carcinogenicity. Combes (1983) studied the efficacy of DNA repair assays in predicting genotoxicity in bacteria; a review of the literature showed good correlation between DNA repair and mutagenicity in bacterial systems.

**DNA Repair**: DNA is the primary carrier of genetic information and the structural integrity of DNA is a prerequisite for gene expression. It is a known fact that primary structure of DNA is of a dynamic nature and subject to a constant change. All ionizing and UV-radiations as well as multitudes of other chemical agents upset the genetic and metabolic machinery of the living system. The living systems have, therefore, evolved repair processes to maintain structural and functional fidelity of DNA against a large range of insults (Friedberg 1985). The molecular mechanism involved in repair of damaged portions of DNA and their restriction into functionally intact informational units is fundamental to the maintenance of the functional integrity. Defective DNA repair could cause an accumulation of lesions or mutations which might either
be lethal or lead to an altered phenotype, or cause neoplastic
transformation. Repair at the cellular and macromolecular level is
multiple in its form and varies as a function of species, tissues and stage
of the cell cycle (Hart et al., 1979).

An indication of the importance of keeping mutation, both somatic
and germline, at a tolerable level is the multiplicity of repair mechanisms
in organisms ranging from bacteria to humans. Mechanisms for the repair
of damaged DNA are probably universal. *Escherichia coli*, for example,
possesses at least four different mechanisms for the repair of DNA
containing thymine dimers: (1) photoreactivation, (2) excision repair,
(3) post replication recombination repair, and (4) Inducible error-prone
"SOS" repair.

DNA repair in *in vitro* system can follow at least two major pathways.
Various mechanisms are implicated in the repair process which either
brings about reversion of lesions or excision of lesions. The reversion
of lesions may be carried out by photoreactivating enzymes which can
remove pyrimidine dimers generated by UV light (Sutherland *et al*.,
1975) or by certain enzymes like insertases and alkyltransferases (Livnen
*et al*., 1979; Deutsch and Linn, 1979; Newbold *et al*., 1980; Pegg *et al*.,
1982; Harris *et al*., 1983; Yarosh *et al*., 1983). The excision repair has
been described in variety of mammalian cells (Hart and Setlow, 1974).
This may be carried out either by base-excision repair (Shaper and
Grossman, 1980) or Nucleotide excision repair mechanism (Lindahl,
1982). However, even after replication the DNA lesions can be repaired
by post replication repair mechanism as was demonstrated by Rupp and
Howard-Flandar (1968) in *E. coli*.

**MUTAGENICITY/CARCINOGENICITY TEST SYSTEMS**

In recent years, sensitive techniques have been developed to test
chemicals and other agents for mutagenicity and carcinogenicity.
Carcinogenicity tests are usually done with rodents, and more frequently
with newborn mice. These studies involve feeding or injecting the
substance being tested and subsequent examination of the animal for
tumour formation. The mutagenicity tests have often been done in similar
fashion. However, because mutation is a low frequency event and because
maintaining large populations of animals such as mice is an expensive
undertaking, such animal tests have usually been quite insensitive, that
is, low levels of mutagenicity would have seldom been detected.

Long term tests are expensive and time consuming. There is another
problem of predicting whether humans will respond to the carcinogen in
the same way as do the animals (Farmer 1982). There are several instances
in which only one species like mouse was found to respond to the test
and the rats did not. Then, how can we extrapolate the risk from rodents
to humans, a very dissimilar long-lived species. Opposite was also found
in which a chemical was found to be carcinogenic by epidemiological
studies but was non-carcinogenic in rodents (Ames et al., 1987).

Short term tests (STTs) for genotoxic chemicals were originally
developed to study mechanisms of chemically induced DNA damage and
to assess the potential genetic hazard of chemicals to humans. In the
following, better known short-term tests are listed. (Table 2). The number
of tests has increased, considerably, because of accumulating evidence
in support of the somatic mutation theory of carcinogens (Strauss, 1981;
Crawford, 1985; Ames et al., 1987) and because of reports that say many
rodent carcinogens \textit{in vitro} are genotoxic in short term tests (Ames
1979). The \textit{in vitro} short-term tests have the advantage that they can be
conducted relatively quickly and inexpensively compared to long term
carcinogenicity assays with rodents and do not involve testing in animals.
Early studies on the concordance between results from \textit{in vitro} short
term tests and rodent carcinogenicity tests were highly encouraging
Table 2: Methods for the assessment of mutagenicity and carcinogenicity (From annexures V and VIII of Council Directives 79/831/EEC).

Tests for gene mutations:
- *Salmonella typhimurium* reverse mutation assays
- *E. coli* reverse mutation assay
- Gene mutation in *Saccharomyces cerevisiae*
- *In vitro* mammalian cell gene mutation
- Sex-linked recessive lethal test in *Drosophila melanogaster*
- Mouse spot test

Test for chromosome mutations:
- Mitotic aneuploidy, *Saccharomyces cerevisiae*
- *In vitro* mammalian cytogenetic test
- *In vivo* mammalian bone marrow cytogenetic test
- Micronucleus test
- Mammalian germ cell cytogenetics
- Mouse heritable translocation
- Rodent dominant lethal tests

Indicator tests for DNA effects:
- Mitotic recombination, *Saccharomyces cerevisiae*
- DNA damage and repair, unscheduled DNA synthesis
- Sister chromatid exchange *in vitro*

Other indicator tests for carcinogenic potential:
- *In vitro* mammalian cell transformation test

Wide use of short term tests (STTs) for detecting mutagen (Ames, 1979) and a number of animal cancer tests of plant substances have contributed to the identification of many natural mutagens and carcinogens in the human diet (Kapadia, 1982). There is a range of application in which STTs have been used successively from the identification of mutagenic fractions in complex mixtures such as cooked meat (Hatch et al., 1984; Sagimura, 1985) or air pollutants to the early identification of genotoxicity in the development of new chemical products (Tassignon, 1985).
AMES TESTING SYSTEM:

B. Ames and his associates (1975) have developed very sensitive techniques by which large number of chemicals and other agents can be screened for mutagenicity. Their procedures are rapid and inexpensive. Ames' procedures involve the use of specially constructed strains of *Salmonella typhimurium* (a bacterium) that carry auxotrophic mutations of various types: transitions, transversions, and frame shifts. The reversion of these auxotrophic mutants to prototrophy, either spontaneous or induced with various agents, can easily be monitored by placing a known number of mutant cells in a petriplate containing medium supplemented with a trace of the growth factor required by the auxotroph (enough to support a few cell divisions, but not enough to allow the formation of visible colonies) and counting the number of colonies produced by prototrophic revertants after an appropriate period of incubation. The frequency of reversion induced by a particular substance can be compared directly with the spontaneous reversion frequency to obtain an estimate of its mutagenicity. Its ability to induce different types of mutations can be assessed by using a set of test strains that carry different types of mutations - one strain with a transition, one with a frame shift mutation, and so forth.

The genotoxic effects of steroids can not be assessed on bacterial systems because specific receptor molecules needed by the steroids to perform their action are absent in bacteria. Therefore, cytogenetic tests are more appropriate parameters for evaluating the genotoxic effects of steroids.

CYTOGENETIC STUDIES FOR THE ASSESSMENT OF MUTAGENICITY:

The birth of Human Cytogenetics as a discipline in its own right can be said to have taken place around 40 years ago. It emerged directly as a consequence of the development of techniques that enabled us to clearly visualise chromosomes in human somatic cells and the
demonstrations, using these techniques, that a number of congenital abnormalities in man, e.g. Down's syndrome, Kleinefelter's syndrome, etc., were a consequence of inherited constitutional chromosomal anomalies. The emergence of the discipline was very much facilitated by these facts: (i) that human peripheral blood T lymphocytes could be readily stimulated to undergo mitosis in short term culture; (ii) that these cells were easily available in large quantity from a few drops of blood obtainable from virtually any individual; and (iii) that stimulated lymphocytes were ideal cells from the point of view of producing well spread metaphase chromosomes. Modern cytogenetics, of course, is not confined to the study of human chromosomes in lymphocytes and a wide variety of cells have been subjected to analysis and particularly so in the case of studies on human carcinogenesis.

The techniques of cell culture enable us to expose normal human cells in vitro to mutagens and carcinogens and allow us to study the consequences of such exposure on chromosome structure and behaviour in these cells.

Chromosomal aberrations:

The most well-developed method for biomonitoring of genotoxic exposure is the measurement of chromosomal aberrations in human lymphocytes (Lohman et al., 1984). This method was used in studies of the biological effects in humans of exposure to a known mutagen, such as ionizing radiations (Bender and Gooch, 1962; Buckton et al., 1962). The experience gained in radiation cytogenetics was subsequently applied to assess possible genetic damage from exposure to chemicals known or suspected to be carcinogenic in man (Forni, 1979). The evaluation of the cytogenetic effects of chemicals is more complex than that of ionizing radiations, due to the different mechanisms by which different chemicals induce chromosomal aberrations (Wolff, 1982). Induced chromosome aberrations in human lymphocytes were well-developed by the mid-1970s (Evans and O'Riordan, 1975). Recommended
methodology, classification and scoring of structural aberrations and statistical methods have been described (Evans, 1982; Forni, 1979). Chromosome aberrations consist of breakage and rearrangement of chromosomes visualized in the metaphase plate. While some types of aberrations are easily scored (acentric fragments, dicentrics, rings), other types, like reciprocal translocations and pericentric inversions, are evident in conventional preparations only when they give rise to grossly abnormal chromosomes, with peculiar arm ratios (Forni, 1984). Chromosome aberrations are most sensitive to agents that can directly break DNA duplex. However, since most chemical mutagens which cause chromatid type damage are 'S' phase dependent, the highest yield of aberration is likely to be seen in the first division of the metaphases. Most chemicals undergo metabolic conversion in vivo into the ultimate reactant, the in vivo and in vitro results of chromosomal aberration tests are directly not comparable (Evans, 1982). Therefore, rat liver metabolic activation system (S9 mix) is used in in vitro culture to compare the in vivo observations.

Sister Chromatid Exchanges:

In comparison to chromosomal aberration method, sister chromatid exchange (SCE) analysis is more sensitive and nonspecific for the chemical in question. SCEs involve breakage of double stranded DNA in both chromatids followed by an exchange of whole DNA duplexes. The SCEs, like chromosome aberrations, induced by S phase-dependent agents, are formed by unpaired lesions that are present when the cell passes through S-phase and the chromosome replicates. This is the most sensitive method to detect the mutagenic property of the chemical and to determine which of the metabolites of a premutagenic and precarcinogenic agent might be the most likely ones to interact with DNA and cause its effects (Perry and Evans, 1975; Latt et al. 1981). SCE represent symmetrical reciprocal exchanges of segments between sister chromatids; generally, they do not result in alteration of the chromosome morphology or the genetic information. This detection
method is exquisitely sensitive as an \textit{in vitro} screening test. For many
mutagenic chemicals very low concentration is required to induce a
significant response in SCEs than to produce effective chromosomal
aberration (Perry and Evans, 1975; Stetka and Wolff, 1976). However,
the DNA lesions that induce SCE are not necessarily the same as those
that induce point mutations or chromosome breakage (Carrano \textit{et al.},
1978).

\textbf{Micronucleus Test:}

Micronucleus test can be applied successfully to diverse human
tissues and is not unusually difficult or time-consuming (Heddle \textit{et al.},
1982; Stich \textit{et al.}, 1983), but it has not been validated for biomonitoring
purposes. Some preliminary results, reported by Stich \textit{et al.} (1983), show
a relationship between betel chewing and the induction of micronuclei
in buccal mucosa. Micronuclei are thought to originate from aberrant
cell division and unequal chromosome distribution, although Heddle \textit{et al.}
(1982) reported that damaged chromosomes in micronuclei of colon
cells may separate without cell division. The main advantages of this
method are that it may be specific for clastogenic effects and that it
measures effects directly in the target tissues of exposed persons.
Micronuclei are acentric chromosome fragments or whole chromosomes
lagging behind in the normal karyokinesis (Sorsa, 1984). These
fragments or chromosomes are excluded from the post-mitotic daughter
cells and form additional small nuclei or micro nuclei. Thus, the
induction of micronucleated cells represents either chromosomal
breakage (clastogenicity) or a failure in the spindle fibre mechanism
(turbagenicity). The conventional micronucleus test in rodents is
designed to detect the damage in the polychromatic erythrocytes of bone
marrow. Extensive chemical evidence obtained from this test shows that
micronucleus formation is qualitatively correlated closely with
chromosomal breakage or loss. In humans micronucleated cells have been
visualized, likewise, in dividing tissues, e.g., in bone marrow \textit{in vivo}
(Krogh Jensen and Nyfors, 1979) and in cultured lymphocytes \textit{in vitro}
(Heddle et al., 1978). Hogstedt et al. (1983) was the first to show that human \textit{in vivo} exposure to cigarette smoke can be detected as an increased frequency of micronuclei in the cultured lymphocytes of smokers.

\textbf{Cell Cycle Kinetics:}

An additional check on the potential of the mutagen to produce an effect on the cells can be made by analyzing the proportion of mitotic cells in their first, second or subsequent metaphases for each treatment group and calculating a proliferation index (PI) or mitotic index (MI).

\textbf{STEROIDS: USES AND APPLICATIONS:}

The steroids, a group of structurally related compounds which are widely distributed in animals and plants, include sterols (from which the name steroid is derived), vitamin D, the bile acids, a number of sex hormones, the adrenal cortex hormones, some carcinogenic hydrocarbons, certain sapogenins, etc. Steroids are group of lipids based on a skeleton of four fused rings, the 1-2, cyclopentanoperhydrophenantherene system. They are derivatives of cholesterol.

Present study is concerned with steroidal hormones. In mammals five important groups of hormonal steroids occur: progestins, glucocorticoids and mineralocorticoids with 21 carbon atoms, androgens with 19 and estrogens with 18 carbon atoms.

Sex hormones produced by the gonads and adrenals are necessary for conception, embryogenic maturation, and development of primary and secondary sexual characteristics at puberty. The gonadal hormones are used therapeutically in replacement therapy and, in the case of estrogen, for contraception and osteoporosis. The adrenal cortex produces two major classes of steroid hormones. The adrenocorticosxteroids (glucocorticoids and mineralocorticoids) and the adrenal androgens. Their synthesis is stimulated by corticotropin
(ACTH). Hormones of the adrenal cortex are used in replacement therapy, in the treatment and management of inflammatory diseases such as rheumatoid arthritis, in the treatment of severe allergic reactions, and in the treatment of some cancers. In the present study six hormonal steroids were evaluated for their cytogenetic effects on human lymphocytes, two each from estrogens, progestins and androgens.

1. ESTROGENS: Estrogenic hormones are secreted by the ovarian follicles. They either stimulate or regulate the growth and development of the uterus, the vaginal mucous membrane and also other structures such as mammary glands, subcutaneous fat, axillary and pubic hair and certain elements in the skin. The later includes the secondary female sex characteristics. Therefore, the estrogens are also called as female sex hormones. Estrogens are secreted throughout the period of activity of the ovaries, though at varying rates at different times of the menstrual cycle (Reynolds and Kathleen, 1996; Hardman and Limbird, 1996).

The naturally occurring estrogens can be prepared synthetically. Improvement of natural estrogens can also be done by synthetic modification. For example the potency 17 b-estradiol, the most potent of natural estrogens, can be further improved by the addition of a side chain forming ethinyl estradiol. Both the estrogens used in the present study are synthetic i.e. Mestranol and Quinestrol.

Estrogens are used as substitution therapy after the appearance of menopausal symptoms. Estrogens are also used in young women in whom there is failure of steroidogenesis. Applied locally, the estrogens are useful in the treatment of atrophic or senile vaginitis, vulvovaginitis or cervicitis resulting from hypoestrogenesis. A number of menstrual irregularities may be treated with these molecules like amenorrhoea, secondary amenorrhoea etc. They are of value in decreasing electrolyte imbalance, headache, tension, breast engorgement and nipple tenderness in premenstrual tension, however they are of dubious value in relieving neurological and mood changes in that disorder. In endometriosis,
estrogens are effective for only a short time, in eventually resulting endometrial hyperplasia. In dysfunctional uterine bleeding, combined treatment with estrogens and progestogens is used (Herfindal and Gourley, 1996; Melmon and Morelli, 1992).

Since estrogens suppress gonadotropin (FSH) release and also inhibit blastocyst implantation, they are used as contraceptives. A contrasting use is to assist reproduction as an adjunct in the prevention of habitual abortion. They may be used in the induction of parturition and in the postpartum period to reduce breast engorgement.

Estrogens decrease plasma cholesterol and hence have been used as hypocholesteremic drugs, but side effects are usually unacceptable to the male recipient. Estrogenic also are used to treat acne vulgaris and hirsutism. They are also used to inhibit the growth of prostatic cancer of men and carcinoma of the breast or carcinoma elsewhere in the reproductive tract of women (Becker 1995).

2. PROGESTINS: Progestins or progestogens are second type of hormones produced in the ovaries. Although it originates in the cells which may also produce estrogens and that it has a molecular structure very similar to that of the estrogens, progesterone has a unique physiological action. Under its influence the numerous minute glands which line the uterine cavity are transformed into secreting glands. This alteration is part of the change which is essential to provide the implantation of a fertilized ovum and for the continuing development of placenta. This endometrial alteration requires the cooperation of an estrogen. In the absence of an estrogen, a progestin that is devoid of estrogenic activity, will exert an atrophic effect on the endometrium (Katzung, 1995; Brody, 1994).

Progestins also cause a change in the cervical secretions to suppress "ferning" a dendritic crystallization of cervical muco polysaccharides when the cervical mucous in not dendritic, when it forms a tight net of fibers, through which sperms can not pass. The antifertility effect of
some progestins possibly may be due in part to the suppression formation of ferning. Progestins in high doses suppress the pituitary release of lutenizing hormone and the hypothalamic release of the LH-releasing factor (LRF), thus preventing ovulation. Progestins also decrease uterine mobility, which may contribute to a contraceptive effect. In addition they antagonize the endometrial actions of estrogens, especially the natural estrogens. Progestins have the ability to stimulate development of the glandular portions of the mammae. They also exert some effect upon the capacity of tissues to retain water in the intercellular spaces. They also have a thermogenic action (Becker, 1995).

The synthetic progestins used in this study are Norethindrone and Norgestrel. These progestins used cyclically in the treatment of infertility in which the uterus is not receptive to implantation. The progestins sustain the secretory endometrium during the third and fourth weeks of the menstrual cycle. They are used cyclically with estrogens in the treatment of secondary amenorrhoea and dysfunctional uterine bleeding. They also may be used to lessen premenstrual tension, although they cause salt and water retention, which is a factor in this disorder. The effect to suppress the release of LH and LRF is used to prevent ovulation, not only with some oral contraceptives but also in the treatment of primary dysmenorrhoea and endometriosis. In sexual infantilism in the female, progestins may be combined with estrogens to bring about genital development and maturation. Progestins may decrease breast size in mastodynia (Kuhl, 1996; Brody, 1994).

In pre-eclampsia and toxemia of pregnancy due to hormonal imbalance, progestins plus estrogens may improve the condition, even though both types of hormones can cause salt and water retention and estrogens can cause hypertension. They may be used in huge doses as adjunctive treatment in endometrial carcinoma. They have been used in the past to prevent habitual abortion or in the treatment of threatened abortion. An intriguing use of progestins is to stimulate respiration in the Pickwickian syndrome (Hardman and Limbird, 1996).
3. ANDROGENS: Natural androgens or male hormones (i.e. testosterone) are synthesized and secreted from interstitial cells or Leydig cells of testes. However, it is mainly the metabolite, dihydrotestosterone, which stimulates and maintains the secondary sex organs; these are the penis, prostate gland, seminal vesicles, vas deferens and scrotum. It also exerts sustaining effects on the spermatogenic cells, and it stimulates the development of bones, muscles, nerves, skin and hair growth, and emotional responses to produce the characteristic adult masculine traits. Testosterone itself regulates the hypophyseal release of LH. This group of combined actions of this hormone is termed androgenic actions. Testosterone also antagonizes a number of the effects of estrogens, and sometimes is employed clinically for this purpose. This is specially important in the suppression of metastatic carcinoma of the breast. Since it promotes development of the clitoris, which is an anatomic homolog of the penis, androgens may increase the libido of women (Herfindal and Gourley, 1996).

The naturally occurring androgens (androsterone, testosterone) are derivatives of androstane. Testosterone and its esters (testosterone propionate) and derivatives (methyl testosterone) are the most commonly used androgenic steroids. In addition to their androgenic properties, however, these compounds exert widespread anabolic effects and promote the retention of calcium. In attempts to dissociate the virilizing and anabolic properties (for use in women) a number of compounds with high anabolic and androgenic ratios have been prepared. However, it has not been possible yet to abolish completely the androgenic effects.

The two synthetic androgens used in the present study are Danazol and Oxymetholone. These and other synthetic androgens are used for varieties of purposes, e.g. among men or youths with hypogonadism (enuchism, klinefelter's syndrome). They have been employed to facilitate development of adult masculine characteristics when the adolescent process has been delayed. In cryptorchidism they may be used
adjunctively with gonadotropins. They also are very useful in therapy of patients with hypopituitarism and with Addison’s disease. They are of significant value in the treatment of frigidity and occasionally in impotence. The use of androgens for relief of impotence not associated with evidence of testicular under activity (psychic causes) is known to be futile in most cases. Low doses of androgens have been used in pituitary dwarfism to accelerate growth, but care must be exercised not to arrest growth by epiphyseal closure. They also are used to promote hematopoiesis. In large supratherapeutic doses, anabolic steroids increase athletic performance and aggressiveness. Female performance is improved but at the expense of virilization and acne vulgaris (Melmon and Morelli, 1992).

With estrogens, androgen therapy may be efficacious in the treatment of the menopause. The anabolic effects are possibly of some benefit in the post climacteric persons, and they may retard osteoporosis. They also help relieve vasomotor instability in post menopausal women in whom estrogens alone do not relieve symptoms. In functional dysmenorrhoea androgens may give relief through an antiestrogenic action. They may be used to treat endometriosis. They also may be used in the treatment of postpartum breast engorgement and for suppression of lactation.

**METABOLISM OF THE STEROID HORMONE:**

Naturally occurring estrogens and their esterified or conjugated derivatives are readily absorbed through the gastrointestinal tract, skin and mucous membranes. Estrogens are also quickly absorbed when administered intramuscularly. Administered orally, estradiol is rapidly metabolized and partially inactivated by the microsomal enzymes of the liver. The synthetic estrogen analogs e.g. ethinyl estradiol and mestranol, are well absorbed after oral administration, and through the skin or mucous membranes. Mestranol is quickly oxidized to ethinyl estradiol, which is metabolized more slowly than the naturally occurring estrogens
by the liver and peripheral tissues. Being fat soluble they are stored in adipose tissue from which they are slowly released. Therefore, the synthetic estrogen analogs have a prolonged action and high potency as compared to natural estrogens. Estrogens are transported in the blood bound to serum albumin and sex-hormone binding globulin. They are hydroxylated in the liver to derivatives that are subsequently glucoordinated or sulfated. The physiological inactive products are excreted in the urine. Oral effectiveness of synthetic estrogens is more because these are metabolized more slowly, whereas, naturally occurring estrogens are destroyed almost totally in a single pass through the liver. The half-life of estradiol is 40-50 minutes, whereas other estrogens persist much longer. Drugs that induce the hepatic microsomal mixed oxygenase system (e.g. phenobarbital, phenytoin, rifampin etc.) will accelerate estrogen metabolism.

Progesterone is rapidly absorbed after its administration by any route. It has a short-half-life in the plasma, since it is almost completely metabolized in one passage through the liver. The glucuronidated metabolite (pregnanediol glucuronide) is excreted by the kidney. Whereas, synthetic progestins are less rapidly metabolized. Synthetic progestins used in contraception are more stable to first pass metabolism, allowing for lower doses when administered orally. Norethindrone and norgestrel have the same characteristics. These two are also called as norestosterone progestins because of their structural similarity to the androgen. They also possess some androgenic activity.

Like the estrogens and progestins, androgens bind to a specific nuclear receptor in a target cell. Although testosterone itself is the active ligand in muscle and liver, in other tissues it must be metabolized to derivatives such as 5-a-dihydrotestosterone (DHT). Testosterone after diffusing into the cells of the prostate, seminal vesicles, epididymis and skin, is converted to DHT, which binds to the receptor. In the brain testosterone is biotransformed to estradiol. The hormone/receptor complex binds to DNA and stimulates the synthesis of specific RNAs
and proteins. Testosterone analogs that cannot be converted to DHT have less effect on the reproductive system than they do on the skeletal musculature. Testosterone is rapidly absorbed by the liver and other tissues, and is metabolized to relatively or completely inactive compounds that are excreted primarily in the urine but also in the feces. Danazol has a longer half life in the body than does the naturally occurring androgens.

Synthetic steroids may be metabolized in human by the same mechanisms as are the natural compounds.

**MECHANISM OF STEROID HORMONE ACTION**

Steroid action entails all of the sequential and simultaneous biochemical steps from the binding of hormone to a cellular receptor to the final biochemical or physiological adjustment that is the outcome of the presence of the particular steroidal hormone in sufficient amount. Steroid hormone action is typical of those hormones that primarily affect the cell nucleus. After steroid binding and activation of the cytoplasmic receptor, the hormone receptor complex is translocated into the nucleus by a characteristically temperature dependent process. Once the complex is in the nucleus, it binds to chromatin and activates RNA polymerase. This is followed by a copying of a specific DNA region that contains the code for the sequence of amino acids comprising a specific protein. The newly synthesized mRNA is transported out of the nucleus to the rough endoplasmic reticulum where it participates in protein synthesis. The newly synthesized protein is either incorporated into cellular components, used to activate an intracellular regulatory protein or it is secreted by the cell. The hormone-receptor complex may remain in the nucleus and exert its effects for several hours before it dissociates and re-enters the cytoplasm. The steroid is metabolized to an inactive form and subsequently diffuses out of the cell.
STEROID HORMONES AND CARCINOGENESIS:

Several sex steroids have been studied for their carcinogenic potentials (Lang and Redmann, 1979). Estrogens induce the formation of endogenous DNA adducts both in human and animals and can ultimately lead to cancer development (Liehr et al., 1986; Liehr, 1990). Contraceptive steroids cyproterone acetate can have a tumour inducing potential in rat liver in addition to its tumour promoting activity (Wolff, 1993). Estradiol is carcinogenic in experimental animals. Administration of estrogens leads to an increase in endometrial cancer in women (IARC, 1979). Some other possible complications of contraceptive use include breast cancer, and cancer of the uterus, cervix and vagina.

There is a high risk of development of cancer with excessive use of steroids. Several steroids have been reported to possess tumourogenic and carcinogenic activities (Kay, 1981). Several steroids have been reported to be mutagenic and carcinogenic on the basis of short term tests (Dunkel et al., 1985). Yet various steroids have been shown to play an active role in human carcinogenesis. Specially the steroidal hormones and their derivatives have been demonstrated to be mutagenic and carcinogenic in the bacterial as well as in the animal testing system (Kay, 1981; Metzler, 1984).

Estrogenic hormones have been shown to possess the tumourogenic activity and are assumed to serve as regulators of tumourous growth. These hormones probably maintain the neoplastic state of the cells in a variety of well characterized experimental animal tumour systems (Katzene llenbogen, 1986). Growth promoting effect on hepatocarcinoma has also been demonstrated to be mediated by estrogen receptors in the mated rats (Kohigashi et al., 1986). Steroids have been found to play a role in regulating aromatase activities in breast and endometrial cancer (James et al., 1986). Contraceptive steroids have been assessed for toxicological and carcinogenic hazards (Heywood, 1986). Moreover, the stereochemical complementarity of DNA and reproductive steroid
hormones have been found to correlate with biological activity (Lawrence et al., 1986).

The following points of possible mechanisms is speculative for neoplastic growth:

1. Steroid hormones may increase the binding of chemical carcinogens to cellular constituents e.g. by influencing metabolic activation system.

2. Steroid hormones may activate oncogenic virus production e.g. mammary tumour virus in mice.

3. Exposure to hormones may result in lesions (preneoplastic) which provide an environment for the survival of cells with abnormal growth potentials.

4. These hormones may be immunosuppressive and could thus influence tumour occurrence and growth.

5. They may influence the rate of progression of preneoplastic cells to neoplastic cells.

6. They may preferentially stimulate proliferation of abnormal cell populations.

7. They may stimulate the DNA synthesis and mitosis essential for fixation of the transformed state.

8. Steroid hormones, by stimulating the proliferation of normal cells with a definite number of cell divisions, may exhaust the normal cell population and thus eliminate their inhibitory influence over the proliferation of abnormal cells (Nandi, 1978).

STEROID HORMONES: EMBRYOTOXICITY AND TERATOGENICITY:

Many steroidal sex hormones cause antifertility, embryotoxicity and foetotoxicity in several species, and such effects are usually dose
related. Some estrogens also produce teratogenic effects and impaired fertility in exposed offspring. Certain progestins, testosterone and testosterone derivatives have a virilizing effect on the foetus.

In humans, birth defects have been observed in foetuses after maternal ingestion of various drugs. However, only in limited number of cases it is possible to ascribe a particular defect to specific drug. Masculinization of external genitalia in female foetuses have been observed after their exposure in utero to large doses of progesterone. Advancement of skeletal maturation has been noted (Breibart et al., 1983). It has been reported that more congenital abnormalities occur in infants born to women who became pregnant while taking oral contraceptives (Nora et al. 1978). Significant embryolethality was observed after the administration of steroid (ethinylestradiol) in rats (Joshi et al., 1983). Injection of sex steroids lead to incomplete development of genital tracts in mice and rats (Bern, 1979; Kohrman, 1978). A VACTERL syndrome was seen in a child of a mother given hormone therapy at the beginning of pregnancy (Kaufman, 1973). Chromosome abnormalities (principally triploidy and tetraploidy) in foetuses have been reported to be more common following the use of oral contraceptives by their mothers (Alberman et al., 1976; Carr, 1970; Harlap et al., 1979).

The mutagenic potential of a few steroidal hormones used as oral contraceptives (OCs) has been studied in human with both positive and negative results. Carr (1967) found chromosomal abnormalities in 6 out of 8 spontaneous abortions in women who became pregnant after stopping the intake of oral contraceptive pills. This observation highlighted the possible mutagenic effect of oral contraceptives in female germ cells. Estradiol was reported to induce structural chromosomal aberrations in human embryonic fibroblasts (Serova and Kerkis, 1974). Increased incidence of vaginal and cervical adenocarcinomas have been reported in female offspring of mothers who have had synthetic estrogens during the first trimester of pregnancy (Greenwald et al., 1971; Herbst, 1971).
Cytogenetic study of peripheral blood lymphocyte culture was done on 50 babies born to mothers after oral contraceptive use, 23 women using oral contraceptives and 20 women who had never used oral gestagens. The chromosome analysis on the progeny revealed one baby to have D/G translocation type Mongolism. Cytogenetic changes of increased chromosome breakage and satellite association were observed in the 23 patients using oral contraceptives over the 20 patients on no gestagen therapy. (McQuarrie et al., 1970).

STEROID HORMONES: MUTAGENICITY/GENOTOXICITY/CLASTOGENICITY:

Steroid hormones play a major role in the growth, development and metabolism. They diffuse through the cell membrane and after conjugating with specific receptor proteins in the cytoplasm, 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 (Thomas and Thomas, 1993). Several sex steroids have been studied for their mutagenic and clastogenic potentials (Wheeler et al., 1986). Estrogens have been identified as mitotic inhibitors. It was suggested that structural specificity is required for mitotic inhibition (Rao and Engelberg, 1967). Estrogens are mitotic poisons, which at high concentration cause metaphase arrest, abnormal cell division and chromosomal aberrations (Wheeler et al., 1986). Contraceptive steroids induce DNA repair synthesis in rat hepatocyte cultures, indicating DNA damage (Neuman et al., 1992). The most potent of naturally occurring estrogens, estradiol, binds to isolated DNA and induce fragmentation (Yamafugi et al., 1971; P.O.P Tso Lu, 1964; Blackburn et al., 1974). It was found to induce very low frequencies of hyperploidies in human synovial cells in vitro (Lycette et al., 1970) and various numerical aberrations in pig oocytes in vitro (McGaughhey, 1977). In human embryonic fibroblasts and kidney epithelial cells, estradiol was reported to induce structural chromosomal aberrations (Serova and Kerkis, 1974). Simultaneous addition of
estradiol, progesterone and human chorionic gonadotropin (HCG) increased the SCE frequency in human lymphocyte culture, but their separate administration did not induce SCEs (Sharma and Das, 1986). Estradiol was found to be negative in Ames' test (Lang and Redmann, 1979) and does not induce point mutations in mammalian cells in vitro (Drevon et al., 1981). Estradiol has been found to induce chromosomal aberrations and sister chromatid exchanges in human lymphocyte cultures in vitro (Ahmad et al., 2000a). Mestranol, the synthetic estrogen, has been found to induce chromosomal aberrations, sister chromatid exchanges and micronuclei formation in mouse bone marrow cells though it was non-mutagenic in Ames' test both with or without S9 mix (Dhillon et al., 1994). Badr and Badr (1974) found lyndiol (lymstranol and mestranol) not to affect the frequency of dominant lethal mutations in mice. Similarly Dutokowski et al. (1979) reported non-genotoxic effects for mestranol and norethindrone on micronuclei formation and induction of SCEs in lymphocytes cultured from six women. Neumann et al. (1992) found cyproterone acetate (CPA), a contraceptive steroid, to induce DNA repair synthesis in rat hepatocyte cultures, indicating DNA damage. Many other hormonal drugs have also been reported to be mutagenic and clastogenic (McQuarrie et al., 1970; Wheeler et al., 1986).

Some other sex steroidal hormones used as oral contraceptives (OCs) have been found to be mutagenic in plant chromosomes (Hakeem and Amer, 1965; Kabarity and Khodary, 1967; Goswami, 1978; Kabarity and Mazrooei, 1984). Ovral 50, a contraceptive steroid was found to be genotoxic in mammals like mice and dogs (Williams et al., 1968; Devi and Reddy, 1986). Badr and Badr (1974) demonstrated a high incidence of dominant lethal mutations in female mice given a large dose of OCs. OCs were not mutagenic in Drosophila melanogaster (Paradi, 1981).

Goh (1967) reported an increased number of chromosomal breaks and rearrangements in lymphocyte cultures of women taking OCs. This finding was confirmed by McQuarrie et al. (1970), Littlefield et al.
(1971) and Badr et al. (1972). But Shapiro et al. (1972) did not find such effects. Husum et al. (1982) found no significant increase in the frequency of sister chromatid exchanges in the peripheral lymphocytes of women using OCs, whereas Murthy and Prema (1979, 1983) observed a significantly increased frequency of SCEs in OC users. In vitro studies of human lymphocyte cultures treated with OCs failed to induce a genotoxic effect (Stenchever, 1969; Timson, 1969; Devi and Reddy, 1986). Anovlar 21, a combination drug and an OC containing the estrogen ethinyl estradiol and the progestin norethisterone acetate, was found to induce chromosomal aberrations in mouse bone marrow cells in a dose-time related manner, whereas the drug was unable to induce micronuclei (Shyama et al., 1991).

Reports on the mutagenicity of non-sex steroids is controversial. Some of these molecules do not exhibit mutagenicity in Ames/Salmonella test system but are clastogenic in mammals (Bali et al., 1990; Singh et al., 1994). A few of such drugs inhibit DNA synthesis in rapidly dividing liver cells in vitro at low dose concentrations (Loeb et al., 1973). Dexamethasone, a non-sex steroid, has been found to induce chromosomal aberrations and sister chromatid exchanges in human lymphocytes in vivo but not in vitro (Ahmad et al., 2000 b) whereas, other corticosteroid hydrocortisone induces chromosomal aberrations and sister chromatid exchanges in human lymphocytes in vitro (Shadab et al., 1999).

Estradiol was found to induce C-mitoses, polyploidies and micronuclei in human peripheral lymphocytes in vitro but was unable to induce structural chromosomal aberrations and SCEs (Banduhn and Obe, 1985). Wheeler et al. (1986) found that only estrogens have the ability to induce mitotic inhibition and aneuploidy in Chinese hamster cells in vitro. Estrogens studied were diethylstilbestrol (non-steroidal), dienestrol, hexestrol, b-estradiol, ethinyl estradiol and estriol. Most potent was diethylstilbestrol and least potent was estriol. In the same study progesterone, estrone and testosterone were also evaluated, but
none were found to cause mitotic disturbances. The steroidal hormones estradiol-17b, estriol, estrone and ethinyl estradiol were found to induce and enhance sister chromatid exchange in Chinese hamster ovary cells \textit{in vitro} (Kochhar, 1988). In a study in 44 women by Pinto (1986) it was observed that significant increase in chromosome aberrations was associated with the use of hormonal contraceptives. Earlier, in a similar study it had been found that oral contraceptives have no effect on SCE (Husum \textit{et al.}, 1981). It was observed by Littlefield \textit{et al.} (1975) that synthetic hormones do not directly damage lymphocyte chromosomes Kabarity and Mazrooei (1984) found that Anovlar can produce major cytological abnormalities. It induced a mitodepressive effect, produced abnormal prophases and considerable number of micronuclei. Women using an estrogen-progestogen combination contraceptive exhibited an increased frequency of sister chromatid exchange. These elevated SCE frequencies tended to decline 3 months after the discontinuation of the pill. Women using a progestogen injectable contraceptive had unaltered SCE rates (Murthy and Prema, 1983).

Very little study has been done to evaluate the genotoxic effects of androgens.

**STEROID HORMONES AND APOPTOSIS:**

Apoptosis is the scattered, random deaths of cells in healthy tissues. During apoptosis cells undergo shrinkage and separation from their neighbours, membrane blebbing, a characteristic form of nuclear chromatin condensation, nuclear membrane breakdown, and cytolysis into condensed apoptotic bodies. This process evokes little gross inflammation, though the shrivelled cells and apoptotic bodies are phagocytosed by surrounding cells and macrophages (Kerr, 1971; Kerr \textit{et al.} 1972; Wyllie, 1981).

It has been observed that glucocorticoids evoke apoptosis in rodent thymocytes \textit{in vivo} and \textit{in vitro}, and also in lymphocytes \textit{in vitro} (Dougherty and White, 1945; Munck and Wira, 1970). DNA lysis occurs
in glucocorticoid evoked apoptosis in thymocytes.

Sex steroids are also associated with apoptosis (Huggins and Hodges, 1941; Huggins et al., 1941). In the prostate, the glandular epithelium shows the morphological changes of apoptosis beginning about 3 days after castration (Martikainen and Isaacs, 1990). Antiandrogen or androgen withdrawal induce apoptosis in the prostate, in prostate organ cultures (Martikainen and Isaacs, 1990b), androgen-dependent prostatic carcinomas, and in rat and human prostate derived cell lines in vitro (Tenniswood et al., 1992; Kyprianon, et al., 1990). Replacement of androgens can restore the prostatic epithelium. Glucocorticoids repress apoptosis in the prostate. Several specific genes and gene products have been associated with the apoptosis that follows androgen removal.

The steroid-dependent cyclical regression of the uterine epithelium is another example of apoptosis occurring subsequently to hormone removal (Hopwood and Levison, 1976). Estrogen stimulates uterine epithelial growth; progesterone stimulates its differentiation. Removal of progesterone or use of antiprogestins results in morphological apoptosis of the epithelial cells without gross change in the stroma (Rotello et al., 1992). Interestingly, prolonged antiprogestin exposure in rats resulted in continued apoptosis of epithelial cells accompanied by invasion by granulocytes: a hallmark of inflammation not usually associated with the apoptotic process (Rumpell et al., 1993).

Thus there is a difference that addition of glucocorticoids and removal of sex steroids evoke apoptosis and prolonged exposure to sex steroids can also lead to apoptosis.

DEFINITION OF THE PROBLEM AND OBJECTIVES OF THE PRESENT WORK

In many ways chemicals are a basic factor in the well-being and effective functioning of present day societies, which were not
encountered by human ancestors, and to which he has not been adapted specifically. Among these substances are food additives, synthetic drugs, narcotics, antibiotics, cosmetics, contraceptives, pesticides, alcohol, tobacco in various forms, various air and water pollutants, petroleum products, plastics, synthetic textiles, man-made mineral fibres and different other synthetic materials. The quality of life at both the social and individual levels would be severely restricted without the use of most of the above described substances. Of all these chemicals, synthetic drugs and contraceptives can be far more dangerous to the population in terms of genetic defects than radiation because the use of these drugs is indispensable for survival. As a result, it is important to determine their mutation frequency in human cells.

Synthetic steroidal hormones have potential application in pharmacology (Loeb et al., 1973; Kochhar 1988). These steroids possess antitubercular, antitumour, anti-inflammatory, antineoplastic, immunosuppressive, antibacterial, antimicrobial and antiviral activities (Killie, 1971; Haynes, 1974). The sex hormones are used widely in human medicine for a large variety of conditions, apart from their uses as oral contraceptive agents. They are used in the treatment of dysmenorrhoea, endometriosis, dysfunctional uterine bleeding, as replacement therapy in patients with gonadal dysgenesis and in women with symptoms of climacteric. They are also used in the prevention of postmenopausal osteoporosis, treatment of advanced breast and endometrial carcinoma. In obstetric practice, progestins are used in the management of threatened abortion and to prevent premature labour. And local estrogens are used successfully to 'prime' the unripe cervix to induction of labour. Estrogens are also used to develop secondary female sex characteristics in trans-sexual males and androgens to suppress these characteristics in trans-sexual females. Androgens are used in the treatment of male hypogonadism, in the treatment of symptoms of climacteric and in advanced mammary tumours in women. Clomiphene citrate is used to induce ovulation in anovulatory women and also in
oligospermic males. The use of androgens to improve athletic performance is well known. Sex hormones are also used in veterinary medicine and as supplements to feed stuffs to promote growth of food animals.

The cytogenetic studies conducted on the possible chromosome damaging effect of steroidal hormones so far is inconclusive and controversial. A large number of steroids have been studied for their teratogenic (Joshi et al., 1983), carcinogenic (Rosenfield et al., 1983), mutagenic and clastogenic (Wheeler et al., 1986) potentials. Although numerous literatures have been produced on the genotoxicity of estrogens and progestins, very little attention has been paid to the androgens which are being used extensively.

Keeping in view the practical application of these compounds, genotoxicity study through a battery of in vitro assays have been attempted. Three groups of sex steroidal hormones have been chosen viz. (a) estrogens (b) progestins and (c) androgens. Two widely used and potent synthetic hormones from each group were evaluated for their harmful cytogenetic effects on humans.

These steroids have been used with a view to study the following objectives:

1. To study the in vitro effects of the steroids on human lymphocyte culturing system.
2. To study the toxic effects of the steroids on human lymphocyte chromosomes.
3. Evaluation of the chromosome damage in parallel in the presence of metabolic activation (S$_9$ mix).
4. To study the effects of the steroids on the frequency of sister chromatid exchange (SCE).
5. To find the frequency of SCEs when compared in the presence of S$_9$ mix, and finally,
6. To study the effects of the steroids on cell growth kinetics in the presence as well as in the absence of S$_9$ mix.
Materials & Methods
MATERIALS AND METHODS

The in vitro evaluation of the mutagenic and clastogenic potentials of six synthetic sex steroidal hormones were undertaken with human peripheral blood lymphocytes. Blood was obtained from healthy voluntary donors who were aged between 20-30 years, belonging to both sexes and having similarity in socioeconomic status. All the donors were not occupationally exposed to any sort of possible mutagens viz. drugs, alcohol and irradiation. Each of the six synthetic steroids were tested on human lymphocytes. The genetic end points used were chromosomal aberrations, sister chromatid exchanges and cell cycle kinetics assays in the absence as well as in the presence of exogenous metabolic activation system (S9 mix).

DESCRIPTION OF TEST CHEMICALS

The test chemicals evaluated in the present investigation were six synthetic steroids, which were all sex steroidal hormones. Two estrogens namely mestranol and quinestrol, two progestins namely norethindrone and norgestrel, and two androgens namely danazol and oxymetholone were investigated. The chemical and physical properties of the test chemicals are as following:

1. ESTROGENS

(a) MESTRANOL \((C_{21}H_{26}O_2\) mol. wt. 310.44)
Description: White to creamy white, odorless, crystalline powder; melts within a range of 4° between 146° and 154°C.

Solubility: Freely soluble in chloroform; sparingly soluble in ether; slightly soluble in alcohol; insoluble in water.

Uses: This drug is widely used as oral contraceptive in combination with norethynedrel and several other progestins. It suppresses the pituitary release of gonadotrophins rather than having antiovulatory effect. It is an effective estrogen but it is not marketed as a single entity.

(b) QUINESTROL ($C_{25}H_{32}$ Mol.wt. 364.53)

Description: White powder; melts at about 108°C.

Solubility: Practically insoluble in water; soluble in alcohol, chloroform or ether.

Uses: For estrogen replacement therapy, primarily for relief of vasomotor symptoms. This cyclopentyl ether of ethinyl estradiol is stored in adipose tissue after oral absorption. It is released slowly and metabolized to the parental compound. This drug is applied orally.

2. PROGESTINS

(a) NORETHINDRONE ($C_{29}H_{26}O_2$ mol.wt. 298.42)
**Description**: White to creamy white, odorless, crystalline powder; melts at about 205°C; stable in air.

**Solubility**: Practically insoluble in water; sparingly soluble in alcohol; soluble in chloroform or dioxane; slightly soluble in ether.

**Uses**: Among the progestational drugs, it ranks high in its ability to postpone menstruation. In addition to its progestational actions it has weak estrogenic actions owing to biotransformation to an estrogenic metabolite. This steroid is an important oral contraceptive used alone or combined with an estrogen especially mestranol and ethinyl estradiol. But it is more potent when used in combination.

**(b) NORGESTREL** \((\text{C}_{21}\text{H}_{28}\text{O}_2, \text{mol. wt. 312.45})\)

![Norgestrel molecule](image)

**Description**: White or nearly white, practically odorless, crystalline powder; melts within a range of 4°, between 205° and 212°C.

**Solubility**: Insoluble in water; sparingly soluble in alcohol; freely soluble in chloroform.

**Uses**: A progestin with potentially all the progestational actions, is marketed as an oral contraceptive, both as a single entity product and in combination with ethinyl estradiol. It is free from estrogenic activity. It has androgenic effects in animals but not in humans.
3. ANDROGENS

(a) **DANAZOL** \((C_{22}H_{27}NO_{2}, \text{mol. wt. 337.46})\)

![Chemical structure of danazol](image)

**Description**: Pale yellow crystalline powder; melts at about 225°C.

**Solubility**: Practically insoluble in water; sparingly soluble in alcohol.

**Uses**: This drug is an impeded androgen. It binds to androgen, glucocorticoid and progesterone receptors, but it evokes no glucocorticoid, gestational or estrogenic effects except that it suppresses the release of LH and FSH in women. It suppresses ovarian steroidogenesis, induces the hepatic metabolism of progesterone and binds to α-macroglobulin causing partial displacement of other steroids. It is used in the treatment of endometriosis, in patients who do not or cannot tolerate other drug therapy and in the management of fibrocystic breast disease, periareolar abscesses and several other diseases.

(b) **OXYMETHOLONE** \((C_{21}H_{32}O_3, \text{mol. wt. 332.48})\)

![Chemical structure of oxymetholone](image)

**Description**: White to creamy white crystals or crystalline powder; odorless and stable in air; tautomeric in nature and can exist either as tautomer or as a mixture of both and melts at about 175°C.

**Solubility**: 1 gm in > 10,000 ml water, 40 ml alcohol, 5 ml chloroform, 82 ml ether or 14 ml dioxane.
Uses: An androgenic steroid with relatively greater anabolic activity than androgenic activity. Consequently, it is employed mainly to promote nitrogen metabolism and weight gain in cachexia and debilitating diseases and after serious infections, burns, trauma or surgery. It is also used in treating osteoporosis, hypoplastic and aplastic anaemias. This drug is applied orally.

SELECTION OF CONTROL CHEMICALS

(a) NEGATIVE CONTROL (DIMETHYL SULPHOXIDE; DMSO)

The test chemicals are organic compounds, hence mostly insoluble in water. Therefore, the organic compound, dimethyl sulfoxide (DMSO), which is being successively employed as a solvent for most of the organic chemicals, was used as the solvent for the test chemicals. It has been reported to be non-toxic to animals and humans (Prestcott et al., 1987). This solvent control or negative control provides the baseline for the comparison of the data to see the significant effects.

\[
\begin{align*}
\text{H}_3\text{C} & \quad \text{S} = \text{O} \\
\text{H}_3\text{C} & \\
\end{align*}
\]

Dimethyl Sulphoxide

(b) POSITIVE CONTROL (CYCLOPHOSPHAMIDE; CP)

Cyclophosphamide (endoxan) is the most versatile therapeutic drug which possesses the broadest spectrum and antitumour activity of all alkylating agents (Brock, 1967). It is known to have strong mutagenic effects which have been proved many a time on different systems. Cyclophosphamide acts by forming DNA-crosslinks by the reaction of chloroethyl moieties of CP with adjacent nucleotide bases. It is widely used as positive control in \textit{in vitro} assays due to its potent mutagenic and clastogenic nature,
ASSAY SYSTEMS USED

Most of the cytogenetic studies being carried out involve the examination of metaphase chromosomes, mainly because at metaphase, chromosomes are thick, short, and chromatids are separate and easily visible. Thus, the evaluation of chromosomal damage at metaphase helps to study a greater number of dividing figures and gives more precise and detailed picture of the clastogenic agents than the anaphase and telophase analysis.

Chromosomal changes can be studied in any cycling cell population, or in any non-cycling cell population that can be stimulated by a mitogenic agent to enter the cell cycle. In animals there are several cell types that fit for these criteria, however for human studies, to all intents and purposes, there are only two cell types that are practically suitable. These are bone marrow cells, which are a cycling population, and the peripheral blood lymphocytes, which are normally non-cycling, but can be stimulated to divide and to enter cell cycle by in vitro culturing with a mitogen such as phytohaemagglutinin (PHA). However, because of the ease of obtaining blood samples, in contrast to bone marrow samples, and because these cells are extremely sensitive indicators of the in vivo and in vitro induced chromosomal changes (structural and numerical); human peripheral blood lymphocytes were utilized for investigating the genotoxicity of six selected steroids. The parameters studied included chromosomal aberrations, sister chromatid exchange analysis and cell growth kinetics assay in the absence as well as in the presence of exogenous metabolic activation system.
PREPARATION OF S9 LIVER /MICROSOME FRACTION

The S9 liver microsomal fraction was the exogenous metabolic activation system utilized for the present investigation. It was prepared as follows:

1. INDUCTION OF RAT LIVER ENZYMES:

For preparing S9 fraction, the standard procedures as recommended by Ames et al (1975) and Maron and Ames (1983) were followed. Swiss albino healthy rats (Wister strain, obtained from Disease Free Small Animal House, Haryana Agriculture University, Hissar, India), each weighing about 200 gm was given 0.1% (1 mg/ml) of phenobarbitone in drinking water for one week for the induction of liver enzymatic activities.

2. REMOVAL OF LIVERS FROM THE RATS:

The rats were sacrificed through cervical decapitation. In order to ensure the sterility of S9 preparation, the liver lobes were removed aseptically using sterile surgical tools in laminar flow cabinet. Proper care was taken not to cut into the oesophagus or the intestine which could otherwise cause the contamination of liver homogenate. The removed liver lobes were immediately placed in chilled 0.15 M KCl.

3. PREPARATION OF LIVER HOMOGENATE FRACTION (S9 FRACTION):

The whole procedure was carried out at 0-4°C using sterilized solutions and glassware. The livers were washed in fresh chilled KCl several times so as to remove the traces of haemoglobin which inhibits enzyme activity. The washed livers were transferred to a beaker containing three volumes of 0.15 M KCl (3 ml/gm wet liver) and after mincing with sterile scissors these were homogenized within a tissue homogenizer. The homogenate was centrifuged in refrigerated centrifuge for 10 minutes at 9000 rotation per minute (rpm). The supernatant (S9
fraction) was decanted and saved in 1 ml aliquots in polypropylene storage vials, and stored in liquid nitrogen till further use.

4. PREPARATION OF S₉ MIX:

The S₉ mix from S₉ fraction was prepared fresh every time for use in the cultures. It contained 0.04 ml/ml S₉ fraction, 8 µM MgCl₂, 33 µM KCl, 5 µM glucose-6-phosphate (G-6-P), 4 µM NADP, 100 µM Na₂HPO₄ and NaH₂PO₄ buffer with 7.4 pH. 0.8 ml of S₉ mix was added every time along with the test chemicals in the culture.

IN VITRO TESTING OF CHROMOSOMAL ABERRATION

PREPARATION OF CULTURE MEDIA:

Culture media from RPMI-1640 medium (GIBCO BRL, Life Technologies) with L-glutamine and Hepes buffer without NaHCO₃ was always prepared in advance and stored at 4°C but the storage never lasted longer than a week. 10.4 gm of medium was dissolved in 100 ml of sterilized distilled water by gentle shaking. Antibiotic - Antimycotic [100 X lyophilized (10,000 units/ml pencillin G sodium, 10,000 µg/ml streptomycin sulfate, 25 µg/ml amphotericin B) GIBCO BRL, Life Technologies] was also added and the pH was adjusted between 6.8-7.2 with N/10 NaHCO₃ and HCl. The media was then filter sterilized in the negative pressure millipore filtration assembly by using 0.22 µM millipore filters. This 10X filtered stock medium was then stored in sterilized, and tightly capped glass bottles at 4°C in dark.

PREPARATION OF WORKING MEDIA:

The above prepared stock medium was used for making the working media as follows: 10 ml of this stock medium was dissolved in 90 ml of sterilized triple distilled water. 20 ml of fetal calf serum (Gibco BRL, Life Technologies) was also added. 2 ml of phytohaemagglutinin-M (PHA-M; Gibco BRL, Life Technologies) and 1 ml of heparin (500 IU/ml; Micro Lab) was added to the total volume of working media as a mitogen and anticoagulant respectively.
Preparation of culture media and working media was done under sterile condition in the laminar flow cabinet.

**COLLECTION OF BLOOD SAMPLES:**

Peripheral blood was taken from male and female healthy donors for each culture. The donors were occupationally not exposed to any sort of mutagen. The blood was taken fresh every time with needles (21 gauge) and disposable syringes (steriware). Heparin (500 IU/ml; Micro Lab) was used as anticoagulant. The blood was collected in glass vials which were tightly capped, gently mixed and stored at 4°C for half an hour to separate red blood cells (RBCs) from plasma.

**SETTING OF THE CULTURE:**

Lymphocyte cultures were carried out as per Moorehead *et al.* (1960) with slight modifications by adding 0.8 ml of plasma containing white blood cells (WBCs) in 6 ml of working culture media. The culture vials were then tightly capped to avoid CO₂ loss. This was done under laminar flow cabinet and spirit lamp was also used to avoid contamination. The culture vials were gently shaken to mix their contents, and were incubated at 37°C in dark for 72 hours. Colchicine (0.2 µg/ml; Micro Lab) was added to the culture 2½ hours prior to harvesting to arrest the cells at metaphase stage.

**SELECTION OF DOSES:**

Preliminary screening of the steroids with various concentrations was undertaken for determining their influence on the mitotic index (MI). Only those doses, which did not drastically reduced the MI but yielded sufficient number of scorable metaphases, were considered for the final experimental protocol. On the basis of these, the lower and the medium doses were selected and the highest dose selected was normally the maximum tolerated dose (MTD) beyond which the MI showed a steep decline. Following these norms the three doses selected for the various steroids were: 10, 25 and 50 µg/ml for Mestranol, Quinestrol and
Norgestrel and 25, 50 and 100 µg/ml for Danazol and Oxymetholone. The doses selected for Norethindrone were 20, 40 and 75 µg/ml

STUDIES ON THE STEROIDS WITHOUT $S_o$ Mix:

In the experiments where no exogenous metabolic activation was incorporated, the cultures were exposed to three different concentrations of each steroid for 24, 48 and 72 hours. For this purpose the steroids were added to the cultures at 0, 24 and 48 hours after the initiation of cultures for 72, 48 and 24 hours of exposure respectively. Positive (Cyclophosphamide or CP, $1 \times 10^{-7}$ M) and negative (Dimethyl sulphoxide or DMSO, 5 µl/ml) control cultures were also simultaneously set for all treatment durations.

STUDIES ON THE STEROIDS WITH $S_o$ Mix:

In the metabolic activation experiments, 30 hours old cultures were given six hours treatment with different concentrations of each steroid in the presence of $S_o$ mix.

The cells were collected after centrifugation and the pellet was washed twice in the pre-warmed medium to remove the chemical and the $S_o$ mix. Parallel cultures receiving the same dose of the steroids for similar treatment duration but without $S_o$ mix were also simultaneously set for comparison. DMSO (5 µl/ml; SRL, Mumbai) and CP ($1 \times 10^{-7}$ M; Khandelwal Labs, Mumbai) were used as negative and positive controls respectively in these metabolic activation experiments.

HARVESTING OF THE CULTURES:

After the 72 hours of cultures being completed, the culture vials were taken out from the incubator. These were gently shaken to mix their contents and then transferred to different centrifuge tubes. The cells were spundown by centrifugation (10 minutes; 1200 rpm) and the supernatant discarded. Pre-warmed hypotonic solution (0.075 M KCl) was added to the centrifuge tubes, while shaking continuously with the help of cyclomixer.
Hypotonic treatment (0.075 M KCl) was carried out for 10-12 minutes at 37°C and the cells recollected by centrifugation. The cell pellet was suspended in 7 ml freshly prepared chilled fixative (3:1 : Methanol: Acetic acid) which was added drop by drop with a pasteur pipette while continuously shaking the pellet so as to avoid formation of clots. In order to ensure proper fixation, the cells were kept suspended in the fixative for a minimum period of one hour but preferably overnight. The cells were washed thrice with fresh fixative before preparing the slides.

PREPARATION OF SLIDES AND STAINING:

After giving the final washing in the fixative, the cells were resuspended in 0.2 ml of fresh fixative. Two to three drops of this cell suspension were dropped on clean, grease free, pre-chilled and wet microscope slides which were then air dried.

One-day old slides were stained in 5% Giemsa (Loba Chem, India) for 3 minutes. After that the slides were rinsed in triple distilled water and then air dried. The slides were dipped in xylene for 5 minutes before mounting in DPX (BDH).

ANALYSIS OF THE CELLS:

In order to avoid bias in scoring of the chromosomal anomalies, all slides were coded prior to scoring. A total of 300 well spread metaphases were analysed for each concentration of the steroid and for each time duration, to analyze various chromosome and chromatid type aberrations. For scoring various chromosome and chromatid type aberrations, the following method as described by Savage (1975) and Bloom (1981) was followed.

A. CHROMOSOME TYPE ABERRATIONS:

Analysis of chromosomal aberrations included the following parameters:

1. Terminal and interstitial deletions
2. Asymmetrical interchanges (usually dicentrics)
3. Asymmetrical interchange (centric ring)
4. Symmetrical interchanges (reciprocal translocations)
5. Symmetrical interchanges (peri-and paracentric inversions)

B. CHROMATID TYPE ABERRATIONS:

Chromatid-type aberrations are generally classified in the same way as chromosome-type aberrations. The apparent unit of involvement in a chromatid-type aberration is, in most cases, the single chromatid, and not the whole chromosome as seen for chromosome type aberrations. The examination of the chromatid type aberrations considered the following parameters:

1. Terminal deletions:
2. Interstitial deletions
3. Achromatic lesions ("gaps")
4. Isochromatid deletions
5. Asymmetrical interchanges (interarm interchanges, asymmetrical chromatid exchanges)
6. Symmetrical interchanges (symmetrical chromatid exchanges 7. Asymmetrical and symmetrical intrachanges (interarm intrachanges)
7. Triradials

C. MULTIPLE ANOMALIES:

1. Polyploidy: An increase in the chromosome number in excess of diploid set in multiple of the haploid (N) number.
2. Endoreduplications: The chromosome reduplication occurring in the absence of sister chromatid separation.
3. Cells with multiple anomalies: The cells with 10 or >10 aberrations.
4. Pulverization: The extreme fragmentation of the chromatin material.

SISTER CHROMATID EXCHANGE (SCE) ANALYSIS:

Mechanism of the sister chromatid exchange (SCE) detection: Sister chromatid exchange is a sensitive, rapid and objective method of observing reciprocal exchanges between sister chromatids. This method depends upon the phenomenon of 5-bromo-2-deoxyuridine (BrdU), an analogue of thymidine, incorporation into DNA in place of thymidine. After two rounds of cell division the chromatids are labelled with BrdU and consequently get differentially stained with Hoechst stain. The BrdU incorporation quenches the fluorescence of the fluorochrome 33258 Hoechst. Therefore, the light energy is absorbed but not emitted by such dyes which results in the reduced staining of chromatid with Giemsa (Latt and Wolleb, 1975; Latt, 1976).

Labelling of chromosomes with BrdU: Sister chromatid exchange analysis was carried out following the standard procedure of Latt et al. (1981) with suitable modifications. The cells in the cultures were exposed to nucleoside, BrdU (Sigma) after 24 hours of initiation of cultures, at the final concentration of 2 μg/ml. The culture vials were tightly capped and covered with tin foil to avoid light exposure and incubated at 37°C for another 48 hours in the dark.

Studies on the steroids without S, mix: After 48 hours of the initiation of cultures, three different log doses of each of six steroids were added into these cultures which did not contain any S, mix. The culturing was further continued for 24 hours. Positive (CP, 1x10^{-7}M), negative (DMSO, 5 μl/ml) and untreated (normal) controls were also simultaneously studied. The detailed information regarding donors, doses, durations etc. has already been described.

STUDIES ON THE STEROIDS WITH S, MIX: The steroids along with S, mix was added into the cultures after 48 hours of its initiation and were reincubated at 37°C. After 90 minutes of pulse treatment, the cells
were spun down and supernatant discarded. The cells were then washed with pre-warmed medium twice so as to remove the traces of the steroids and liver metabolites. Finally, the cell pellets were resuspended in fresh and warm working media supplemented with fetal calf serum, PHA-P, antibiotics- antimycotic and BrdU, and cultured for another 24 hours in the dark at 37°C. In order to compare the effects of the steroids with and without metabolic activation, parallel cultures with same concentrations of the steroids and same exposure durations but without S<sub>9</sub> mix were also simultaneously set. Positive (CP, 1x10⁻⁷M), negative (DMSO, 5μl/ml) and untreated (normal) controls in the presence of S<sub>9</sub> mix were also simultaneously studied.

**Slide preparation and staining:** After 2½ hours of colchicine treatment, the cultures were harvested and processed following the same procedure as described for the chromosomal aberration analysis.

The methods of Perry and Wolff (1974) and Latt et al. (1981) with slight modifications were followed for the differential staining of SCEs. One day old slides were dipped in 0.5 μg/ml of 33258 Hoechst stain (Sigma) dissolved in double distilled water in horizontal couplin jar. The slides were rinsed twice in distilled water. The air-dried slides were then put in flat glass dish with the layer of cells facing upwards. These were covered by thick layer (2-3 cm) of phosphate buffer (pH 6.8) and exposed to UV lamp (15W, 254 nm; Philips) from a distance of 10-15 cm for 30-45 minutes. The slides were taken out from the buffer, washed twice in double distilled water and air dried. These were then incubated in 2XSSC (0.3M sodium chloride, 0.3M sodium citrate, pH 7.0) at 65°C in water bath for 90 minutes using vertical couplin jars. The slides were taken out and rinsed in distilled water. The air dried slides were then stained in giemsa for 3 minutes and again rinsed in distilled water. The dried slides were dipped in xylene for 5 minutes and mounted in DPX.

**Analysis of the cells:** All slides were coded prior to scanning so as to avoid any confusion. 50 second-division metaphases with differentially
stained chromatids were scored for each steroid treatment in the absence as well as in the presence of S₉ mix. The interstitial exchanges between two sister chromatids were scored as two exchanges and the terminal exchanges were scored as a single exchange. Students 't' test was applied for calculating the significance of difference between the treated and the controls.

**CELL CYCLE KINETICS:**

The cells undergoing first (M₁), second (M₂) and third (M₃) division were detected by studying the BrdU labelled differentially stained chromosomes following the methodology of Tice et al. (1976) and Crossen and Morgan (1977). The cells with both the chromatids being darkly stained were scored as M₁ cells while those with one dark and one lightly stained chromatids as M₂ cells, and those having mixture of both the differentially stained and uniformly stained chromatids were scored as M₃ metaphases. 50 well spread metaphases were scored for each concentration and each treatment duration from each donor in the absence as well as in the presence of S₉ mix. The replication index (RI) was calculated according to the formula of Tice et al (1976) as given below. The deviation from the controls was determined by using Chi-Square test.

\[
RI = (M₁ \times 1 )+(M₂ \times 2 )+(M₃ \times 3 )/100
\]

**STATISTICAL ANALYSIS:**

Standard deviation (SD) and standard error (SE) were calculated using the following formula -

Mean \( \bar{X} = \frac{X}{n} \)

Variance \( \delta^2 = X^2 - \frac{\Sigma x^2}{n-1} \)

\( \delta = \sqrt{X^2 - \frac{\Sigma x^2}{n-1}} \)

\( SE = \frac{SD}{\sqrt{n}} \)
where \( x = \) variable

\( n = \) number of observations

\( \sum x^2 = \) sum of square of individual variables

\( \bar{X} = \) Mean of variables

2x3 Chi-Square test (\( x^2 \)) for homogeneity test of variance was used to analyse the cell growth kinetics with the normal control. The level of significance was tested from standard statistical tables of Fisher and Yates (1963).

The endoreduplication, ploidies, pulverizations and cells with multiple anomalies were not included in the statistical analysis.

Student's two tailed 't' test was used for calculating the statistical significance in SCE and chromosomal aberrations by comparing the effects induced by different steroids with the respective controls. Following formula was used for this purpose.

\[
t = \frac{\bar{X}_1 \text{ (control)} - \bar{X}_2 \text{ (treated)}}{\sqrt{(SE \text{ of control})^2+(SE \text{ of treated})^2}}
\]

The statistical significance was calculated from Fisher and Yates table at \( (n_1+n_2-2) \) degree of freedom (df) at 0.05% level of significance (P).
Results
RESULTS

The following drugs were evaluated for their genotoxic effects on human lymphocytes. The students 't' test (paired) was applied for chromosomal aberrations and sister chromatid exchanges. The 2x3 chi-square test was used for cell growth kinetics studies. The level of significance was compared at P < 0.05 for all parameters.

1. ESTROGENS

1. Mestranol

(a) Chromosomal aberrations:

The results of chromosomal aberration analysis are presented in table 3. At 10 mg/ml of dose, the drug was not effective at any exposure duration. At 24, 48 and 72 hours of incubation, the percentage of chromosomal aberrations were 3.67%, 4.67% and 5.00% respectively. These values were not significant as compared to the normal control value (3.00%). The percentage of aberrant metaphases were not significantly high. At 25 mg/ml of dose concentration, the drug affects both the aberrant metaphases as well as chromosomal aberration frequency. At 24, 48 and 72 hours of incubation the percentage of chromosomal aberrations were 18.00%, 22.00% and 27.00% respectively which were highly significant at P < 0.05 compared to the normal control value (Figures A-C in Appendix). However, peculiar results from observations were obtained at the highest concentration (i.e. 50 mg/ml). The frequency of chromosomal aberrations and aberrant metaphases decreased (as compared to the 25 mg/ml dose) and significant difference was noted at all exposure times. The decrease in the chromosomal aberration may be attributed to the toxic effects of the drug at this concentration on the mitotic index. The values observed were only 5.00%, 6.00% and 6.67% respectively for 24, 48 and 72 hours of exposure. The CP gave a significant increase in the frequency of aberrant metaphases and chromosomal aberrations (29.00%, 52.00% and 66.67%) for all exposure times i.e. 24, 48 and 72 hours of incubation.
The results of the frequency of chromosomal aberrations in lymphocytes after mestranol treatment in the presence of metabolic activation is given in table 4. Again at lower concentration (i.e. 10 mg/ml) the aberration frequency was low. The percentage of aberration was 4.50%. This value was insignificant as compared to the normal control value i.e. (4.00%). At 25 mg/ml of concentration the aberration percentage was 29.50%. This value was significantly high statistically (at p < 0.05) (Figures D & E in Appendix). When the dose was increased i.e. at 50 mg/ml concentration, the aberration frequency decreased i.e. it became 10.00%. This value was not significantly high. The aberration percentage for CP was 49.50% which was highly significant statistically.

(b) Sister Chromatid Exchange (SCE):

Data on the mean frequency of sister chromatid exchange per cell in human lymphocytes after mestranol treatment is presented in table 5. At 10 mg/ml of dose concentration the value of mean SCE frequency was 1.90±0.099. This value was not significant. At 25 mg/ml of dose concentration, the mean SCE frequency was 5.04±0.224 which was highly significant as compared to the normal control value (i.e. 1.10±0.099) at p < 0.05. But when the dose was further increased to 50 mg/ml, the value of mean SCE frequency showed a steep decline. It was only 1.78±0.017. The drug was found to be effective in enhancing the SCE frequency only at 25 mg/ml of concentration. The mean SCE frequency for CP was 6.36±0.152, which was highly significant compared to the normal control value.

Table 6 documents the mean SCE value per cell in human lymphocytes after mestranol treatment in the presence of metabolic activation (S⁹ mix). At 10 mg/ml dose concentration the value of mean SCE was 1.74±0.126. This value was insignificant. At 25 mg/ml concentration, there was a significant increase in the SCE frequency i.e. 5.38±0.180 as compared to the value of normal control (1.46±0.090). At the highest concentration (i.e. 50 mg/ml), the drug slightly enhanced
Table 3: Chromosomal aberrations in human lymphocytes after Mestranol treatment.

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Duration (h)</th>
<th>Metaphase</th>
<th>Percent aberration</th>
<th>Types of aberration (%)</th>
<th>Aberration/Cell ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Metaphase</td>
<td>Incl. gap</td>
<td>Excl. gap</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mestranol</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>24</td>
<td>300</td>
<td>4.33</td>
<td>2.67</td>
<td>3.67</td>
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<td></td>
<td>48</td>
<td>300</td>
<td>6.00</td>
<td>3.67</td>
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<td>72</td>
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<td>Normal</td>
<td>72</td>
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<td>2.33</td>
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<tr>
<td>DMSO (5µl/ml) (-ve control)</td>
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<td>300</td>
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<td>2.00</td>
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<tr>
<td>CP (1x10^-7M) (+ve control)</td>
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<td>14.33</td>
<td>13.67</td>
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<td>300</td>
<td>28.67</td>
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<td>39.00</td>
</tr>
</tbody>
</table>

CP : Cyclophosphamide; DMSO : Dimethyl sulphoxide
SE : Standard error
* : Significant at P<0.05
Table 4: Chromosomal aberrations in human lymphocytes after Mestranol treatment with and without metabolic activation (S₉ mix).

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Metabolic Phase</th>
<th>Percent Aberration</th>
<th>Types of Aberration (%)</th>
<th>Aberration/Cell ± SE</th>
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<td>Activation</td>
<td>Metaphase</td>
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<tr>
<td></td>
<td></td>
<td>Percent Incl</td>
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<td></td>
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<td>Metaphase gap</td>
<td>gap</td>
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</tr>
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<td>Mestranol</td>
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<td>-S₉</td>
<td>300</td>
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<td>3.67</td>
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<tr>
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<td>-S₉</td>
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<td>+S₉</td>
<td>200</td>
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<td>-S₉</td>
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<td>4.33</td>
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<tr>
<td></td>
<td>+S₉</td>
<td>200</td>
<td>5.00</td>
<td>3.50</td>
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<td></td>
<td></td>
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<tr>
<td>Normal</td>
<td>-S₉</td>
<td>300</td>
<td>3.67</td>
<td>2.33</td>
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<tr>
<td></td>
<td>+S₉</td>
<td>200</td>
<td>4.00</td>
<td>2.00</td>
</tr>
<tr>
<td>DMSO (5µl/ml)</td>
<td>-S₉</td>
<td>300</td>
<td>4.33</td>
<td>1.67</td>
</tr>
<tr>
<td>(-ve control)</td>
<td>+S₉</td>
<td>200</td>
<td>4.50</td>
<td>2.00</td>
</tr>
<tr>
<td>CP (1x10⁻⁷M)</td>
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<td>16.67</td>
<td>14.67</td>
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<tr>
<td>(+ve control)</td>
<td>+S₉</td>
<td>200</td>
<td>20.00</td>
<td>19.00</td>
</tr>
</tbody>
</table>

CP: Cyclophosphamide, DMSO: Dimethyl sulphoxide
SE: Standard error
*: Significant at P<0.05
Table 5: Sister Chromatid Exchange (SCE) in human lymphocytes after Mestranol exposure in vitro.

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Duration (h)</th>
<th>No of Methaphase</th>
<th>Total SCE</th>
<th>Range</th>
<th>SCEs/Cell ±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mestranol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>48</td>
<td>50</td>
<td>95</td>
<td>0-6</td>
<td>1 90±0 099</td>
</tr>
<tr>
<td>25</td>
<td>48</td>
<td>50</td>
<td>252</td>
<td>2-10</td>
<td>5 04±0 224*</td>
</tr>
<tr>
<td>50</td>
<td>48</td>
<td>50</td>
<td>89</td>
<td>0-5</td>
<td>1 78±0 017</td>
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<td>Control</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>48</td>
<td>50</td>
<td>55</td>
<td>0-4</td>
<td>1 10±0 099</td>
</tr>
<tr>
<td>DMSO (5µl/ml)</td>
<td>48</td>
<td>50</td>
<td>64</td>
<td>0-4</td>
<td>1 28±0 110</td>
</tr>
<tr>
<td>CP (1x10⁻⁷ M)</td>
<td>48</td>
<td>50</td>
<td>318</td>
<td>3-13</td>
<td>6 36±0 152*</td>
</tr>
</tbody>
</table>

Table 6: Sister Chromatid Exchange (SCE) in human lymphocytes after Mestranol exposure with metabolic activation (S9 mix).

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Duration (h)</th>
<th>No of Methaphase</th>
<th>Total SCE</th>
<th>Range</th>
<th>SCEs/Cell ±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mestranol</td>
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<td></td>
</tr>
<tr>
<td>10</td>
<td>48</td>
<td>50</td>
<td>87</td>
<td>1-6</td>
<td>1 74±0 126</td>
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<tr>
<td>25</td>
<td>48</td>
<td>50</td>
<td>269</td>
<td>2-11</td>
<td>5 38±0 180*</td>
</tr>
<tr>
<td>50</td>
<td>48</td>
<td>50</td>
<td>95</td>
<td>1-5</td>
<td>1 90±0 117</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>48</td>
<td>50</td>
<td>73</td>
<td>0-5</td>
<td>1 46±0 090</td>
</tr>
<tr>
<td>DMSO (5µl/ml)</td>
<td>48</td>
<td>50</td>
<td>63</td>
<td>0-5</td>
<td>1 26±0 093</td>
</tr>
<tr>
<td>CP (1x10⁻⁷ M)</td>
<td>48</td>
<td>50</td>
<td>389</td>
<td>4-14</td>
<td>7 78±0 150*</td>
</tr>
</tbody>
</table>

CP Cyclophosphamide, DMSO Dimethyl sulphoxide  
SE Standard error  
* Significant at P<0.05
Table 7: Analysis of cell growth kinetics after Mestranol exposure in vitro.

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Cells Scored</th>
<th>Percent cells in M&lt;sub&gt;1&lt;/sub&gt; M&lt;sub&gt;2&lt;/sub&gt; M&lt;sub&gt;3&lt;/sub&gt;</th>
<th>Replication Index</th>
<th>2x3 Chi-square test</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mestranol</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>200</td>
<td>36 55 09</td>
<td>1.73</td>
<td>Not significant</td>
</tr>
<tr>
<td>25</td>
<td>200</td>
<td>62 33 05</td>
<td>1.43*</td>
<td>Significant</td>
</tr>
<tr>
<td>50</td>
<td>200</td>
<td>33 54 13</td>
<td>1.80</td>
<td>Not significant</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>200</td>
<td>32 55 13</td>
<td>1.81</td>
<td></td>
</tr>
<tr>
<td>DMSO (5µl/ml) (-ve control)</td>
<td>200</td>
<td>34 50 16</td>
<td>1.82</td>
<td></td>
</tr>
<tr>
<td>CP (1x10&lt;sup&gt;-7&lt;/sup&gt; M) (+ve control)</td>
<td>200</td>
<td>65 33 02</td>
<td>1.37*</td>
<td>Significant</td>
</tr>
</tbody>
</table>

Table 8: Analysis of cell growth kinetics after Mestranol exposure with metabolic activation (S<sub>s</sub> mix).

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Cell Scored</th>
<th>Percent cells in M&lt;sub&gt;1&lt;/sub&gt; M&lt;sub&gt;2&lt;/sub&gt; M&lt;sub&gt;3&lt;/sub&gt;</th>
<th>Replication Index</th>
<th>2x3 Chi-square test</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mestranol</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>10</td>
<td>200</td>
<td>36 53 11</td>
<td>1.75</td>
<td>Not significant</td>
</tr>
<tr>
<td>25</td>
<td>200</td>
<td>60 34 06</td>
<td>1.46*</td>
<td>Significant</td>
</tr>
<tr>
<td>50</td>
<td>200</td>
<td>39 50 11</td>
<td>1.72</td>
<td>Not significant</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>200</td>
<td>34 54 12</td>
<td>1.78</td>
<td></td>
</tr>
<tr>
<td>DMSO (5µl/ml) (-ve control)</td>
<td>200</td>
<td>33 53 14</td>
<td>1.81</td>
<td></td>
</tr>
<tr>
<td>CP (1x10&lt;sup&gt;-7&lt;/sup&gt; M) (+ve control)</td>
<td>200</td>
<td>66 29 05</td>
<td>1.38*</td>
<td>Significant</td>
</tr>
</tbody>
</table>

CP : Cyclophosphamide;  DMSO : Dimethyl sulfoxide
SE : Standard error
* : Significant at P<0.05
the frequency of SCE (1.90±0.117) in comparison to normal control value. But this value was not significant statistically. The SCE frequency of CP was 7.78±1.50 which was highly significant.

(c) Cell growth kinetics:

The data of the cell growth kinetics in human lymphocytes after mestranol treatment is given in table 7. The replication index at 25 mg/ml concentration was significant (1.43) when compared to the normal control value (1.81). At lowest and highest dose concentration i.e. 10 and 50 mg/ml, the values of replication indices were not significant (1.73 and 1.80 respectively). The CP was found to be effective and also significant at P < 0.05 (1.37). Table 8 shows the effect of mestranol on cell growth kinetics of human lymphocytes in the presence of metabolic activation. The value of replication index was found to be significant only at 25 mg/ml concentration (1.46) as compared to the normal control value (1.78) at P < 0.05. At 10 and 50 mg/ml concentration the value of replication indices obtained were found to be insignificant (1.75 and 1.72 respectively). The positive control value (CP) was 1.38 which was significant statistically.

2. Quinestrol

(a) Chromosomal aberrations:

Table 9 summarises the data on the chromosomal aberration influenced by the drug quinestrol on human lymphocyte chromosomes. At 10 mg/ml of dose concentration the aberration frequency observed was 4.33%, 3.67% and 9.00% for 24, 48 and 72 hours of exposure durations. The aberration percentage at 10 mg/ml for 72 hours of exposure was significant. When the dose was increased i.e. at 25 mg/ml concentration, the aberration percentage observed, showed an increase with the increase in exposure duration (Figures F-H in Appendix). The actual values observed were 18.66%, 22.67% and 39.66% for 24, 48 and 72 hours of exposure respectively. These values are significantly
higher than the normal control value (i.e. 3.00%) at P < 0.05. At the highest dose concentration i.e. at 50 mg/ml, the aberration frequency first increased but decreased later on with the increase in exposure duration. The aberration percentage for 24 hours of exposure was 33.67% which was highly significant statistically in comparison to the normal control value. But for 48 and 72 hours of exposure durations the percentage of aberrations were 6.00% and 4.33% respectively. This interesting result may be attributed to cell death at the highest concentration and for longer exposure duration. The positive control, CP, being a potent mutagen, influences the rate of aberration at all exposure duration (i.e. 29.00%, 52.00% and 66.67% respectively for 24, 48 and 72 hours of exposure duration).

The effects of quinestrol on human lymphocytes in the presence of metabolic activation (S\text{\textsubscript{g}} mix) has been presented in table 10. The mean percentage value at 10 mg/ml was 9.50% in the presence of S\text{\textsubscript{g}} mix, which was significantly higher than the aberration value observed for normal control (4.00%) at P < 0.05. At 25 mg/ml of dose concentration, the drug enhanced the rate of aberration (22.50%) which was highly significant at P < 0.05. At 50 mg/ml of dose concentration the value of aberration percentage observed was 51.50% which was again highly significant statistically in comparison to the normal control value (Figures I & J in Appendix). Thus a dose time relationship was observed. For positive control the value was again significantly high (49.50%).

\textbf{(b) Sister Chromatid Exchange:}

Table 11 shows the effect of quinestrol on the frequency of SCE in human lymphocytes. At 10 mg/ml concentration the mean SCE frequency observed was 1.72±0.100. Maximum SCE frequency was observed at 25 mg/ml dose concentration (3.50±0.140). At the highest dose concentration the value of mean SCE frequency observed was maximum (2.38±0.090). Thus at all the dose concentrations the mean SCE values observed were significantly higher than the normal control value.
(1.10±0.099) at P < 0.05. The data of the mean SCE frequency value of the positive control was 6.36±0.152. This value was highly significant compared to the normal control value.

The data of SCE frequency in human lymphocytes after quinestrol exposure in the presence of metabolic activation is presented in table 12. The value observed at 10 mg/ml concentration was not significant (1.84±0.086) as compared to the normal control value (1.46±0.090). AT 25 and 50 mg/ml concentration the mean SCE values observed were 3.78±0.099 and 4.68±0.100 respectively. Both these values were highly significant as compared to the normal control value at P < 0.05. The slight increase in the observations in the presence of S9 mix in comparison to the values observed in the absence of S9 mix may be due to the metabolic activation of the drug by liver enzymes. The value for CP was 7.78±0.150 which was highly significant at P < 0.05.

(c) Cell growth kinetics:

The effect of quinestrol on the cell growth kinetics of human lymphocytes is presented in Table 13. No significant effect of the drug was observed at the lowest (10 mg/ml) concentration. The replication index (RI) was 1.74 for 10 mg/ml concentration of the drug. At 25 and 50 mg/ml of dose concentrations, lower replication indices were observed (i.e. 1.46 and 1.40 respectively). These values were significant as compared to the normal control value (1.81) at P < 0.05. CP also showed a decrease in the replication index (1.37) which was significant at P < 0.05 in comparison to the normal control value.

Table 14 represents the data of the effect of quinestrol on the cell growth kinetics of human lymphocytes in the presence of metabolic activation (S9 mix). Here also the RI was higher for 10 mg/ml concentration, the value observed was 1.75. The value was not significant statistically compared to the normal control (1.78). At 25 and 50 mg/ml, the replication indices observed were 1.46 and 1.43 which were highly significant in comparison to the normal control value of 1.78 (at P < 0.05). The observed value for CP was 1.38 which was highly significant.
Table 9: Chromosomal aberrations in human lymphocytes after Quinestrol treatment.

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Duration (h)</th>
<th>Meta-</th>
<th>Percent aberration</th>
<th>Types of aberration (%)</th>
<th>Aberration/ Cell ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>phase</td>
<td>Metaphase Incl. gap</td>
<td>Chromatid</td>
<td>Metaphase Excl. gap</td>
</tr>
<tr>
<td>Quinestrol</td>
<td></td>
<td>Scan-</td>
<td></td>
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<tr>
<td>10</td>
<td>24</td>
<td>300</td>
<td>5.43</td>
<td>3.33</td>
<td>4.33</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>300</td>
<td>7.00</td>
<td>5.33</td>
<td>3.67</td>
</tr>
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<td>25</td>
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<td>12.33</td>
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<td>24.67</td>
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<td>16.00</td>
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<td>38.67</td>
<td>36.00</td>
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<td>50</td>
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<td>300</td>
<td>28.00</td>
<td>26.67</td>
<td>19.00</td>
</tr>
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<td>300</td>
<td>36.33</td>
<td>35.33</td>
<td>4.67</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>300</td>
<td>42.67</td>
<td>42.00</td>
<td>3.00</td>
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<td>Control</td>
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<td></td>
</tr>
<tr>
<td>Normal</td>
<td>72</td>
<td>300</td>
<td>4.33</td>
<td>2.33</td>
<td>2.67</td>
</tr>
<tr>
<td>DMSO (5µl/ml)</td>
<td>72</td>
<td>300</td>
<td>0.03</td>
<td>2.00</td>
<td>2.33</td>
</tr>
<tr>
<td>(-ve control)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP (1x10^-3M)</td>
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<td>300</td>
<td>14.33</td>
<td>13.67</td>
<td>15.33</td>
</tr>
<tr>
<td></td>
<td>0.29±0.048</td>
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<td></td>
</tr>
<tr>
<td>(+ve control)</td>
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<td>300</td>
<td>19.67</td>
<td>18.67</td>
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</tr>
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<td></td>
<td>0.52±0.066</td>
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<td>72</td>
<td>300</td>
<td>28.67</td>
<td>27.33</td>
<td>39.00</td>
</tr>
</tbody>
</table>

CP : Cyclophosphamide;  DMSO : Dimethyl sulphoxide
SE : Standard error
* : Significant at P<0.05
Table 10: Chromosomal aberrations in human lymphocytes after Quinestrol treatment with and without metabolic activation (S₉, mix).

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Metabolic Activation</th>
<th>Percent aberration</th>
<th>Types of aberration (%)</th>
<th>Aberration/Cell ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Metaphase Scanned</td>
<td>Metaphase Incl gap</td>
<td>Chromatid +s.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Metaphase Excl gap</td>
<td>Chromatid +s.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Incl +s.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Excl +s.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total +s.</td>
<td></td>
</tr>
<tr>
<td>Quinestrol 10</td>
<td>-S₉</td>
<td>6 67</td>
<td>4 33</td>
<td>3 33</td>
</tr>
<tr>
<td></td>
<td>+S₉</td>
<td>9 00</td>
<td>5 50</td>
<td>7 00</td>
</tr>
<tr>
<td>Quinestrol 25</td>
<td>-S₉</td>
<td>12 67</td>
<td>7 00</td>
<td>11 00</td>
</tr>
<tr>
<td></td>
<td>+S₉</td>
<td>17 50</td>
<td>15 00</td>
<td>15 50</td>
</tr>
<tr>
<td>Quinestrol 50</td>
<td>-S₉</td>
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<td>26 00</td>
<td>23 33</td>
</tr>
<tr>
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<td>+S₉</td>
<td>31 00</td>
<td>30 00</td>
<td>29 00</td>
</tr>
<tr>
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<td>-S₉</td>
<td>3 67</td>
<td>2 33</td>
<td>3 00</td>
</tr>
<tr>
<td></td>
<td>+S₉</td>
<td>4 00</td>
<td>2 00</td>
<td>3 50</td>
</tr>
<tr>
<td>DMSO (5µl/ml)</td>
<td>-S₉</td>
<td>4 33</td>
<td>1 67</td>
<td>3 00</td>
</tr>
<tr>
<td></td>
<td>(+ve control) +S₉</td>
<td>4 50</td>
<td>2 00</td>
<td>2 50</td>
</tr>
<tr>
<td>CP (1x10⁻⁷M)</td>
<td>-S₉</td>
<td>16 67</td>
<td>14 67</td>
<td>17 00</td>
</tr>
<tr>
<td></td>
<td>(+ve control) +S₉</td>
<td>20 00</td>
<td>19 00</td>
<td>26 50</td>
</tr>
</tbody>
</table>

CP Cyclophosphamide, DMSO Dimethyl sulphoxide
SE Standard error
* Significant at P<0.05
Table 11: Sister Chromatid Exchange (SCE) in human lymphocytes after Quinestrol exposure *in vitro*.

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Duration (h)</th>
<th>No. of Methaphase</th>
<th>Total SCE</th>
<th>Range</th>
<th>SCEs/Cell ±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinestrol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>48</td>
<td>50</td>
<td>86</td>
<td>0-6</td>
<td>1.72±0.100</td>
</tr>
<tr>
<td>25</td>
<td>48</td>
<td>50</td>
<td>175</td>
<td>1-8</td>
<td>3.50±0.140*</td>
</tr>
<tr>
<td>50</td>
<td>48</td>
<td>50</td>
<td>214</td>
<td>1-7</td>
<td>4.28±0.090*</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>48</td>
<td>50</td>
<td>55</td>
<td>0-4</td>
<td>1.10±0.099</td>
</tr>
<tr>
<td>DMSO (5µl/ml)</td>
<td>48</td>
<td>50</td>
<td>64</td>
<td>0-4</td>
<td>1.28±0.110</td>
</tr>
<tr>
<td>CP (1x10⁻⁷ M)</td>
<td>48</td>
<td>50</td>
<td>318</td>
<td>3-13</td>
<td>6.36±0.152*</td>
</tr>
</tbody>
</table>

Table 12: Sister Chromatid Exchange (SCE) in human lymphocytes after Quinestrol exposure with metabolic activation (*S*, mix).

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Duration (h)</th>
<th>No. of Methaphase</th>
<th>Total SCE</th>
<th>Range</th>
<th>SCEs/Cell ±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinestrol</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>10</td>
<td>48</td>
<td>50</td>
<td>92</td>
<td>0-7</td>
<td>1.84±0.086</td>
</tr>
<tr>
<td>25</td>
<td>48</td>
<td>50</td>
<td>189</td>
<td>1-8</td>
<td>3.78±0.099*</td>
</tr>
<tr>
<td>50</td>
<td>48</td>
<td>50</td>
<td>234</td>
<td>1-7</td>
<td>4.68±0.100*</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>48</td>
<td>50</td>
<td>73</td>
<td>0-5</td>
<td>1.46±0.090</td>
</tr>
<tr>
<td>DMSO (5µl/ml)</td>
<td>48</td>
<td>50</td>
<td>63</td>
<td>0-5</td>
<td>1.26±0.093</td>
</tr>
<tr>
<td>CP (1x10⁻⁷ M)</td>
<td>48</td>
<td>50</td>
<td>389</td>
<td>4-14</td>
<td>7.78±0.150*</td>
</tr>
</tbody>
</table>

CP : Cyclophosphamide; DMSO : Dimethyl sulphoxide
SE : Standard error
* : Significant at P<0.05
Table 13: Analysis of cell growth kinetics after Quinestrol exposure *in vitro*.

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Cells Scored</th>
<th>Percent cells in $M_1$</th>
<th>$M_2$</th>
<th>$M_3$</th>
<th>Replication Index</th>
<th>2x3 Chi-square test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinestrol</td>
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<td></td>
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<td>10</td>
<td>200</td>
<td>40</td>
<td>42</td>
<td>18</td>
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<td>Not significant</td>
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<td>25</td>
<td>200</td>
<td>58</td>
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<td>04</td>
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<td>Significant</td>
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<td>50</td>
<td>200</td>
<td>62</td>
<td>36</td>
<td>02</td>
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<td>Significant</td>
</tr>
<tr>
<td>Normal</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>DMSO (5µl/ml)</td>
<td>200</td>
<td>32</td>
<td>55</td>
<td>13</td>
<td>1.81</td>
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</tr>
<tr>
<td>(-ve control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP (1x10$^{-7}$ M)</td>
<td>200</td>
<td>65</td>
<td>33</td>
<td>02</td>
<td>1.37*</td>
<td>Significant</td>
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<tr>
<td>(+ve control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Table 14: Analysis of cell growth kinetics after Quinestrol exposure with metabolic activation (S$_9$ mix).

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Cells Scored</th>
<th>Percent cells in $M_1$</th>
<th>$M_2$</th>
<th>$M_3$</th>
<th>Replication Index</th>
<th>2x3 Chi-square test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinestrol</td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<td>200</td>
<td>37</td>
<td>51</td>
<td>12</td>
<td>1.75</td>
<td>Not significant</td>
</tr>
<tr>
<td>25</td>
<td>200</td>
<td>61</td>
<td>32</td>
<td>07</td>
<td>1.46*</td>
<td>Significant</td>
</tr>
<tr>
<td>50</td>
<td>200</td>
<td>60</td>
<td>37</td>
<td>03</td>
<td>1.43*</td>
<td>Significant</td>
</tr>
<tr>
<td>Normal</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO (5µl/ml)</td>
<td>200</td>
<td>34</td>
<td>54</td>
<td>12</td>
<td>1.78</td>
<td></td>
</tr>
<tr>
<td>(-ve control)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>CP (1x10$^{-7}$ M)</td>
<td>200</td>
<td>66</td>
<td>29</td>
<td>05</td>
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<td>Significant</td>
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<tr>
<td>(+ve control)</td>
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</tr>
</tbody>
</table>

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

1. Norethindrone

(a) Chromosomal aberrations:

Table 15 documents the effects of the drug norethindrone on the chromosomal aberration frequency of human lymphocytes. A wide range of concentrations (i.e. 20, 40 and 75 mg/ml) of the drug were used to see the genotoxic effects. It did not show any significant increase in the frequency of chromosomal aberrations at any of the concentrations nor were they effective at any exposure time. The percentage of aberrations observed at 20 mg/ml were 2.67%, 4.67% and 4.34%. At 40 mg/ml these values were 5.66%, 5.33% and 4.67% and at 75 mg/ml the values observed were 5.00%, 8.00% and 8.34%. Three different values were observed at each dose concentration for three different exposure durations i.e. 24, 48 and 72 hours respectively. All these values were statistically insignificant as compared to the normal control value i.e. 3.00%. The aberration caused by CP were 29.00%, 52.00% and 66.67% respectively for 24, 48 and 72 hours of exposure durations. These values were highly significant in comparison to the normal control value at P < 0.05.

The effects of norethindrone on chromosomal aberration frequency of human lymphocytes in the presence of metabolic activation has been described in table 16. The drug was found to be unable to induce any clastogenic effect in this case. At 20, 40 and 75 mg/ml concentrations, the aberration percentages observed were 4.00%, 5.50% and 6.00% respectively. These values were not significant statistically as compared to the normal control value (4.00%). The CP in the presence of S9 mix induces a significant genotoxic effect on the human lymphocytes. The aberration frequency observed for CP was 9.50%.

(b) Sister Chromatid Exchange:

The results of SCEs induced by norethindrone in human lymphocytes has been summarised in table 17. The mean SCE frequencies
of the drug at 20, 40 and 75 mg/ml of dose concentrations were 1.18±0.055, 1.24±0.060 and 1.30±0.066 respectively. These values were not significant statistically compared to the normal control value (1.10±0.099). The mean SCE frequency was enhanced by the positive control drug (CP) and the value observed was 6.36±0.152 which was highly significant.

Norethindrone did not prove to be effective in enhancing the SCE frequency in human lymphocytes even in the presence of S9 mix at any of the dose concentrations. The results have been presented in table 18. The mean SCE frequency values at 20, 40 and 75 mg/ml were 1.30±0.065, 1.24±0.060 and 1.34±0.067 respectively. All these values were not significant at P < 0.05 as compared to the normal control value i.e. 1.46±0.090. Cyclophosphamide was able to enhance SCE frequency (7.78±0.150) which was highly significant statistically compared to the normal control value.

Thus, the drug norethindrone was not able to induce chromosomal aberrations or to enhance SCE frequency in human lymphocytes.

(c) Cell Growth Kinetics:

Table 19 shows the analysis of cell growth kinetics after norethindrone exposure in human lymphocytes. The 2x3 chi-square test indicated no significant deviations from the normal control culture. The values of replication indices at 20, 40 and 75 mg/ml concentrations were 1.79, 1.81 and 1.75 respectively which were not significant as compared to the normal control value (1.81) at P < 0.05 whereas the replication index for CP was 1.37 which was statistically significant in comparison to the normal control value.

The effects of norethindrone on cell growth kinetics of human lymphocytes have been shown in table 20. Again the values of replication indices obtained at different concentrations were not significant when statistically compared to the normal control value. The actual values
Table 15: Chromosomal aberrations in human lymphocytes after Norethindrone treatment.

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Duration (h)</th>
<th>Metaphase Scanned</th>
<th>Percent aberration</th>
<th>Types of aberration (%)</th>
<th>Aberration/Cell ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Chromatid</td>
<td>Chromosome</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>24</td>
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<td></td>
<td>72</td>
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<td>2.00</td>
<td>4.33</td>
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<td>300</td>
<td>6.67</td>
<td>1.67</td>
<td>4.33</td>
</tr>
<tr>
<td></td>
<td>72</td>
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<td>7.33</td>
<td>3.00</td>
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<td></td>
<td>72</td>
<td>300</td>
<td>8.00</td>
<td>3.67</td>
<td>5.67</td>
</tr>
<tr>
<td>Control</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Normal</td>
<td>72</td>
<td>300</td>
<td>4.33</td>
<td>2.33</td>
<td>2.67</td>
</tr>
<tr>
<td>DMSO (5µl/ml)</td>
<td>72</td>
<td>300</td>
<td>4.00</td>
<td>2.00</td>
<td>2.33</td>
</tr>
<tr>
<td>(-ve control)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP (1x10⁻⁷M)</td>
<td>24</td>
<td>300</td>
<td>14.33</td>
<td>13.67</td>
<td>15.33</td>
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<td>0.29±0.048</td>
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<td>(+ve control)</td>
<td>48</td>
<td>300</td>
<td>19.67</td>
<td>18.67</td>
<td>28.00</td>
</tr>
<tr>
<td>0.52±0.066</td>
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<td></td>
<td>72</td>
<td>300</td>
<td>28.67</td>
<td>27.33</td>
<td>39.00</td>
</tr>
<tr>
<td>CP</td>
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<td></td>
</tr>
<tr>
<td>SE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

CP: Cyclophosphamide, DMSO: Dimethyl sulfoxide
SE: Standard error
* Significant at P<0.05
Table 16: Chromosomal aberrations in human lymphocytes after Norethindrone treatment with and without metabolic activation (S₉ mix).

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Metabolic Activation</th>
<th>Metaphase Scanned</th>
<th>Percent aberration Metaphase</th>
<th>Types of aberration (%)</th>
<th>Aberration/Cell ± SE</th>
</tr>
</thead>
<tbody>
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<td>Incl.</td>
<td>Excl.</td>
<td>Chromatin</td>
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<tr>
<td>Norethindrone</td>
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<td></td>
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</tr>
<tr>
<td>20</td>
<td>-S₉</td>
<td>300</td>
<td>5.33</td>
<td>1.67</td>
<td>2.67</td>
</tr>
<tr>
<td></td>
<td>+S₉</td>
<td>200</td>
<td>7.50</td>
<td>3.50</td>
<td>3.50</td>
</tr>
<tr>
<td>40</td>
<td>-S₉</td>
<td>300</td>
<td>7.67</td>
<td>2.67</td>
<td>2.33</td>
</tr>
<tr>
<td></td>
<td>+S₉</td>
<td>200</td>
<td>5.50</td>
<td>3.50</td>
<td>4.50</td>
</tr>
<tr>
<td>75</td>
<td>-S₉</td>
<td>300</td>
<td>6.00</td>
<td>1.67</td>
<td>3.00</td>
</tr>
<tr>
<td></td>
<td>+S₉</td>
<td>200</td>
<td>8.50</td>
<td>5.00</td>
<td>4.50</td>
</tr>
<tr>
<td>Control</td>
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</tr>
<tr>
<td>Normal</td>
<td>-S₉</td>
<td>300</td>
<td>3.67</td>
<td>2.33</td>
<td>3.00</td>
</tr>
<tr>
<td></td>
<td>+S₉</td>
<td>200</td>
<td>4.00</td>
<td>2.00</td>
<td>3.50</td>
</tr>
<tr>
<td>DMSO (5µl/ml)</td>
<td>-S₉</td>
<td>300</td>
<td>4.33</td>
<td>1.67</td>
<td>3.00</td>
</tr>
<tr>
<td>(-ve control)</td>
<td>+S₉</td>
<td>200</td>
<td>4.50</td>
<td>2.00</td>
<td>2.50</td>
</tr>
<tr>
<td>CP (1x10⁻⁷M)</td>
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<td>14.67</td>
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<td>+S₉</td>
<td>200</td>
<td>20.00</td>
<td>19.00</td>
<td>26.50</td>
</tr>
</tbody>
</table>

CP : Cyclophosphamide; DMSO : Dimethyl sulphoxide
SE : Standard error
* : Significant at P<0.05
Table 17: Sister Chromatid Exchange (SCE) in human lymphocytes after Norethindrone exposure in vitro.

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Duration (h)</th>
<th>No of Methaphase</th>
<th>Total SCE</th>
<th>Range</th>
<th>SCEs/Cell ±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norethindrone</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>20</td>
<td>48</td>
<td>50</td>
<td>59</td>
<td>0-4</td>
<td>1.18±0.055</td>
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<tr>
<td>40</td>
<td>48</td>
<td>50</td>
<td>62</td>
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<td>1.24±0.060</td>
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<tr>
<td>75</td>
<td>48</td>
<td>50</td>
<td>65</td>
<td>0-5</td>
<td>1.30±0.066</td>
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<td>Control</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>48</td>
<td>50</td>
<td>55</td>
<td>0-4</td>
<td>1.10±0.099</td>
</tr>
<tr>
<td>DMSO (5µl/ml)</td>
<td>48</td>
<td>50</td>
<td>64</td>
<td>0-4</td>
<td>1.28±0.110</td>
</tr>
<tr>
<td>CP (1x10⁻⁷ M)</td>
<td>48</td>
<td>50</td>
<td>318</td>
<td>3-13</td>
<td>6.36±0.152*</td>
</tr>
</tbody>
</table>

Table 18: Sister Chromatid Exchange (SCE) in human lymphocytes after Norethindrone exposure with metabolic activation (S₉ mix).

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Duration (h)</th>
<th>No of Methaphase</th>
<th>Total</th>
<th>Range</th>
<th>SCEs/Cell ±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norethindrone</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>48</td>
<td>50</td>
<td>65</td>
<td>0-4</td>
<td>1.30±0.065</td>
</tr>
<tr>
<td>40</td>
<td>48</td>
<td>50</td>
<td>62</td>
<td>0-5</td>
<td>1.24±0.060</td>
</tr>
<tr>
<td>75</td>
<td>48</td>
<td>50</td>
<td>67</td>
<td>0-4</td>
<td>1.34±0.067</td>
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<tr>
<td>Control</td>
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<td></td>
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<tr>
<td>Normal</td>
<td>48</td>
<td>50</td>
<td>73</td>
<td>0-5</td>
<td>1.46±0.090</td>
</tr>
<tr>
<td>DMSO (5µl/ml)</td>
<td>48</td>
<td>50</td>
<td>63</td>
<td>0-5</td>
<td>1.26±0.093</td>
</tr>
<tr>
<td>CP (1x10⁻⁷ M)</td>
<td>48</td>
<td>50</td>
<td>389</td>
<td>4-14</td>
<td>7.78±0.150*</td>
</tr>
</tbody>
</table>

CP: Cyclophosphamide, DMSO: Dimethyl sulphoxide
SE: Standard error
* Significant at P<0.05
Table 19: Analysis of cell growth kinetics after Norethindrone exposure *in vitro*.

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Cells Scored</th>
<th>Percent cells in $M_1$</th>
<th>$M_2$</th>
<th>$M_3$</th>
<th>Replication Index</th>
<th>2x3 Chi-square test</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Norethindrone</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>200</td>
<td>38</td>
<td>45</td>
<td>17</td>
<td>1.79</td>
<td>Not significant</td>
</tr>
<tr>
<td>40</td>
<td>200</td>
<td>32</td>
<td>55</td>
<td>13</td>
<td>1.81</td>
<td>Not significant</td>
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<tr>
<td>75</td>
<td>200</td>
<td>40</td>
<td>45</td>
<td>15</td>
<td>1.75</td>
<td>Not significant</td>
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<tr>
<td><strong>Control</strong></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Normal</td>
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<td>32</td>
<td>55</td>
<td>13</td>
<td>1.81</td>
<td></td>
</tr>
<tr>
<td>DMSO (5µl/ml)</td>
<td>200</td>
<td>34</td>
<td>50</td>
<td>16</td>
<td>1.82</td>
<td></td>
</tr>
<tr>
<td>CP (1x10⁻⁷ M)</td>
<td>200</td>
<td>65</td>
<td>33</td>
<td>02</td>
<td>1.37*</td>
<td>Significant</td>
</tr>
</tbody>
</table>

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

Table 20: Analysis of cell growth kinetics after Norethindrone exposure with metabolic activation (S₉ mix).

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Cells Scored</th>
<th>Percent cells in $M_1$</th>
<th>$M_2$</th>
<th>$M_3$</th>
<th>Replication Index</th>
<th>2x3 Chi-square test</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Norethindrone</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>200</td>
<td>38</td>
<td>46</td>
<td>16</td>
<td>1.78</td>
<td>Not significant</td>
</tr>
<tr>
<td>40</td>
<td>200</td>
<td>41</td>
<td>45</td>
<td>14</td>
<td>1.73</td>
<td>Not significant</td>
</tr>
<tr>
<td>75</td>
<td>200</td>
<td>36</td>
<td>53</td>
<td>11</td>
<td>1.75</td>
<td>Not significant</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>200</td>
<td>34</td>
<td>54</td>
<td>12</td>
<td>1.78</td>
<td></td>
</tr>
<tr>
<td>DMSO (5µl/ml)</td>
<td>200</td>
<td>33</td>
<td>53</td>
<td>14</td>
<td>1.81</td>
<td></td>
</tr>
<tr>
<td>CP (1x10⁻⁷ M)</td>
<td>200</td>
<td>66</td>
<td>29</td>
<td>05</td>
<td>1.38*</td>
<td>Significant</td>
</tr>
</tbody>
</table>

CP: Cyclophosphamide; DMSO: Dimethyl sulphoxide
SE: Standard error
* Significant at P<0.05
obtained were 1.78, 1.73 and 1.75 respectively at 20, 40 and 75 mg/ml concentrations. The value of replication index for normal control was 1.78. Whereas, the replication index value for CP was 1.38 which was significantly high at $P < 0.05$ when compared to normal control value.

Thus norethindrone was not found to affect the cell turnover rates of human lymphocytes \textit{in vitro}.

2. \textbf{Norgestrel}

\textbf{(a) Chromosomal aberrations:}

Table 21 summarises the results of chromosomal aberrations in human lymphocytes after norgestrel exposure \textit{in vitro}. The percentage of aberrations observed at 10 mg/ml were 3.33\%, 3.00\% and 5.67\% after 24, 48 and 72 hours of exposures. All these values were not significant statistically as compared to the normal control value (i.e. 3.00\%). When the dose of the drug was increased to 25 mg/ml the aberration frequencies also showed an increase for different exposure durations. At 25 mg/ml concentration the observed aberration frequencies were 19.00\%, 26.33\% and 50.33\% for 24, 48 and 72 hours of treatment durations respectively. All these values were highly significant at $P < 0.05$ when compared to the normal control value. At 50 mg/ml dose concentration, the aberration frequencies showed a further increase, and the values obtained were 22.00\%, 28.00\% and 54.66\% respectively for 24, 48 and 72 hours of exposure durations (Figures K-M in Appendix). Again these values were highly significant statistically. For CP, the positive control, the aberration frequency values obtained were 29.00\%, 52.00\% and 66.67\% for 24, 48 and 72 hours of treatment durations. These values were also highly significant. Thus, a time dose relationship was observed for norgestrel treatment. At 25 and 50 mg/ml of dose concentrations a high frequency of chromosome aberrations and aberrant metaphases were observed for all exposure times.

Results obtained after the exposure of lymphocytes to norgestrel
in the presence of metabolic activation are presented in table 22. The drug was found to be effective in all treatments and an increased frequency of aberrant metaphases as well as chromosomal aberrations were observed, when the lymphocytes were exposed to the drug in the presence of S₉ mix. The values of aberration frequency obtained were 9.50%, 22.50% and 41.00% respectively for 10, 25 and 50 mg/ml of dose concentrations. These data were highly significant at P < 0.05 when compared to the normal control value i.e. 4.00%. The percentage of chromosomal aberrations was 49.50% for CP which was highly significant.

(b) Sister Chromatid Exchange (SCE):

Data on the mean frequency of SCE for lymphocytes after the treatment of norgestrel is presented in the table 23. The maximum frequency of SCE per cell was found to be 4.44±0.086 at 50 mg/ml of dose concentration. This value was highly significant than the mean SCE value of normal control (1.10±0.099) at P < 0.05. The increased mean SCE frequency was also observed at 25 mg/ml of dose concentration (2.98±0.104) but with less magnitude in comparison to that of 50 mg/ml concentration. This value was also significant at P < 0.05. The mean SCE frequency at 10 mg/ml was not significant when compared to the normal control value. This value was 1.60±0.141. The mean SCE frequency for CP was observed to be 6.36±0.152 which was highly significant statistically.

The data on the mean SCE frequency of lymphocytes when treated with norgestrel in the presence of metabolic activation are given in table 24. Here again both the doses i.e. 25 and 50 mg/ml enhanced the SCE frequency significantly, but the maximum value was recorded at 50 mg/ml concentration. The actual values obtained at 25 and 50 mg/ml dose concentration were 3.34±0.120 and 5.22±0.136 respectively (Figures N & O in Appendix). Both these values were significantly higher than
the mean SCE values of normal control i.e. $1.46 \pm 0.126$ at $P < 0.05$. At 10 mg/ml concentration the value observed was $1.78 \pm 0.105$ which was not significant statistically. The observed mean SCE frequency was highest for CP i.e. $7.78 \pm 0.150$ which was also highly significant when compared to the normal control value.

(c) Cell Growth Kinetics:

The data for cell growth kinetics of the drug norgestrol is summarised in table 25. The replication indices at 25 and 50 mg/ml of dose concentration was found to be 1.52 and 1.47 respectively. Both these values were statistically significant at $P < 0.05$ when compared to the normal control value i.e. 1.81. The replication index observed at 10 mg/ml was 1.82 which was not significant. The frequencies of $M_1$ cells were high and that of $M_2$ cells were lower at 25 and 50 mg/ml of dose concentrations. The value obtained for CP was 1.37 which was highly significant at $P < 0.05$.

Table 26 summarises the results obtained for cell growth kinetics of human lymphocytes after norgestrel treatment in the presence of metabolic activation. The pattern of results obtained was same as it was in the absence of metabolic activation. But here, slightly decreased values of replication indices were observed at 25 and 50 mg/ml of doses. At 25 mg/ml the value was 1.44 and at 50 mg/ml it was 1.46. Both these values of replication index were significant at $P < 0.05$ when compared to the normal control value i.e. 1.78. At 10 mg/ml of dose again the value observed was insignificant (1.80) at $P < 0.05$. For CP result obtained was highly significant (1.38).

III. ANDROGENS

1. Danazol

(a) Chromosomal aberrations:

The data of chromosomal aberrations in human lymphocytes when
Table 21: Chromosomal aberrations in human lymphocytes after Norgestrel treatment.

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Duration (h)</th>
<th>Metaphase Scanned</th>
<th>Percent aberration Metaphase</th>
<th>Types of aberration (%)</th>
<th>Aberration/Cell ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Incl. gap</td>
<td>Excl. gap</td>
<td>Chromatid</td>
</tr>
<tr>
<td>Norgestrel</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>24</td>
<td>300</td>
<td>6.00</td>
<td>3.67</td>
<td>3.33</td>
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<tr>
<td></td>
<td>48</td>
<td>300</td>
<td>5.67</td>
<td>2.67</td>
<td>3.00</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>300</td>
<td>8.33</td>
<td>4.33</td>
<td>4.00</td>
</tr>
<tr>
<td>25</td>
<td>24</td>
<td>300</td>
<td>12.00</td>
<td>10.33</td>
<td>11.33</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>300</td>
<td>26.67</td>
<td>22.00</td>
<td>18.00</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>300</td>
<td>38.33</td>
<td>36.00</td>
<td>29.00</td>
</tr>
<tr>
<td>50</td>
<td>24</td>
<td>300</td>
<td>18.33</td>
<td>12.00</td>
<td>14.00</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>300</td>
<td>28.67</td>
<td>25.00</td>
<td>15.67</td>
</tr>
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<td></td>
<td>72</td>
<td>300</td>
<td>40.67</td>
<td>38.00</td>
<td>34.33</td>
</tr>
<tr>
<td>Control</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>72</td>
<td>300</td>
<td>4.33</td>
<td>2.33</td>
<td>2.67</td>
</tr>
<tr>
<td>DMSO (5µl/ml)</td>
<td>72</td>
<td>300</td>
<td>4.00</td>
<td>2.00</td>
<td>2.33</td>
</tr>
<tr>
<td>(-ve control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP (1x10^-3M)</td>
<td>24</td>
<td>300</td>
<td>14.33</td>
<td>13.67</td>
<td>15.33</td>
</tr>
<tr>
<td>(+ve control)</td>
<td>48</td>
<td>300</td>
<td>19.67</td>
<td>18.67</td>
<td>28.00</td>
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<td></td>
<td>72</td>
<td>300</td>
<td>28.67</td>
<td>27.33</td>
<td>39.00</td>
</tr>
</tbody>
</table>

CP : Cyclophosphamide;  DMSO : Dimethyl sulfoxide
SE : Standard error
* : Significant at P<0.05
Table 22: Chromosomal aberrations in human lymphocytes after Norgestrel treatment with and without metabolic activation (S<sub>S</sub>, mix).

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Metabolic Activation</th>
<th>Percent aberration</th>
<th>Types of aberration (%)</th>
<th>Aberration/Cell ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Metaphase Scanned</td>
<td>Incl gap</td>
<td>Excl gap</td>
<td>Chromatid</td>
</tr>
<tr>
<td>Norgestrel</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>-S&lt;sub&gt;S&lt;/sub&gt;</td>
<td>300</td>
<td>8 33</td>
<td>6 67</td>
</tr>
<tr>
<td></td>
<td>+S&lt;sub&gt;S&lt;/sub&gt;</td>
<td>200</td>
<td>10 50</td>
<td>8 50</td>
</tr>
<tr>
<td>25</td>
<td>-S&lt;sub&gt;S&lt;/sub&gt;</td>
<td>300</td>
<td>11 67</td>
<td>9 33</td>
</tr>
<tr>
<td></td>
<td>+S&lt;sub&gt;S&lt;/sub&gt;</td>
<td>200</td>
<td>17 50</td>
<td>14 00</td>
</tr>
<tr>
<td>50</td>
<td>-S&lt;sub&gt;S&lt;/sub&gt;</td>
<td>300</td>
<td>18 33</td>
<td>16 67</td>
</tr>
<tr>
<td></td>
<td>+S&lt;sub&gt;S&lt;/sub&gt;</td>
<td>200</td>
<td>26 50</td>
<td>25 00</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>-S&lt;sub&gt;S&lt;/sub&gt;</td>
<td>300</td>
<td>3 67</td>
<td>2 33</td>
</tr>
<tr>
<td></td>
<td>+S&lt;sub&gt;S&lt;/sub&gt;</td>
<td>200</td>
<td>4 00</td>
<td>2 00</td>
</tr>
<tr>
<td>DMSO (5µl/ml)</td>
<td>-S&lt;sub&gt;S&lt;/sub&gt;</td>
<td>300</td>
<td>4 33</td>
<td>1 67</td>
</tr>
<tr>
<td>(-ve control)</td>
<td>+S&lt;sub&gt;S&lt;/sub&gt;</td>
<td>200</td>
<td>4 50</td>
<td>2 00</td>
</tr>
<tr>
<td>CP (1x10&lt;sup&gt;7&lt;/sup&gt;M)</td>
<td>-S&lt;sub&gt;S&lt;/sub&gt;</td>
<td>300</td>
<td>16 67</td>
<td>14 67</td>
</tr>
<tr>
<td>(+ve control)</td>
<td>+S&lt;sub&gt;S&lt;/sub&gt;</td>
<td>200</td>
<td>20 00</td>
<td>19 00</td>
</tr>
</tbody>
</table>

CP = Cyclophosphamide,  DMSO = Dimethyl sulfoxide  
SE = Standard error  
* = Significant at P<0.05
Table 23: Sister Chromatid Exchange (SCE) in human lymphocytes after Norgestrel exposure in vitro.

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Duration (h)</th>
<th>No of Methaphase</th>
<th>Total SCE</th>
<th>Range</th>
<th>SCEs/Cell ±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norgestrel</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>48</td>
<td>50</td>
<td>80</td>
<td>0-6</td>
<td>1 60±0 141</td>
</tr>
<tr>
<td>25</td>
<td>48</td>
<td>50</td>
<td>149</td>
<td>1-8</td>
<td>2 98±0 104*</td>
</tr>
<tr>
<td>50</td>
<td>48</td>
<td>50</td>
<td>222</td>
<td>2-12</td>
<td>4 44±0 086*</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>48</td>
<td>50</td>
<td>55</td>
<td>0-4</td>
<td>1 10±0 099</td>
</tr>
<tr>
<td>DMSO (5µl/ml)</td>
<td>48</td>
<td>50</td>
<td>64</td>
<td>0-4</td>
<td>1 28±0 110</td>
</tr>
<tr>
<td>(-ve control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP (1x10⁻⁷ M)</td>
<td>48</td>
<td>50</td>
<td>318</td>
<td>3-13</td>
<td>6 36±0 152*</td>
</tr>
<tr>
<td>(+ve control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 24: Sister Chromatid Exchange (SCE) in human lymphocytes after Norgestrel exposure with metabolic activation (S₉ mix).

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Duration (h)</th>
<th>No of Methaphase</th>
<th>Total SCE</th>
<th>Range</th>
<th>SCEs/Cell ±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norgestrel</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>48</td>
<td>50</td>
<td>89</td>
<td>0-5</td>
<td>1 78±0 105</td>
</tr>
<tr>
<td>25</td>
<td>48</td>
<td>50</td>
<td>167</td>
<td>1-10</td>
<td>3 34±0 120*</td>
</tr>
<tr>
<td>50</td>
<td>48</td>
<td>50</td>
<td>261</td>
<td>2-13</td>
<td>5 22±0 136*</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>48</td>
<td>50</td>
<td>73</td>
<td>0-5</td>
<td>1 46±0 090</td>
</tr>
<tr>
<td>DMSO (5µl/ml)</td>
<td>48</td>
<td>50</td>
<td>63</td>
<td>0-5</td>
<td>1 26±0 093</td>
</tr>
<tr>
<td>(-ve control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP (1x10⁻⁷ M)</td>
<td>48</td>
<td>50</td>
<td>389</td>
<td>4-14</td>
<td>7 78±0 150*</td>
</tr>
<tr>
<td>(+ve control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CP    Cyclophosphamide,  DMSO  Dimethyl sulfoxide  
SE    Standard error   
*     Significant at P<0.05
Table 25: Analysis of cell growth kinetics after Norgestrel exposure in vitro.

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Cells Scored</th>
<th>Percent cells in M1</th>
<th>M2</th>
<th>M3</th>
<th>Replication Index</th>
<th>2x3 Chi-square test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norgestrel</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>200</td>
<td>37</td>
<td>44</td>
<td>19</td>
<td>1.82</td>
<td>Not significant</td>
</tr>
<tr>
<td>25</td>
<td>200</td>
<td>53</td>
<td>42</td>
<td>05</td>
<td>1.52*</td>
<td>Significant</td>
</tr>
<tr>
<td>50</td>
<td>200</td>
<td>56</td>
<td>41</td>
<td>03</td>
<td>1.47*</td>
<td>Significant</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>200</td>
<td>32</td>
<td>55</td>
<td>13</td>
<td>1.81</td>
<td></td>
</tr>
<tr>
<td>DMSO (5µl/ml)</td>
<td>200</td>
<td>34</td>
<td>50</td>
<td>16</td>
<td>1.82</td>
<td></td>
</tr>
<tr>
<td>CP (1x10^-7 M)</td>
<td>200</td>
<td>65</td>
<td>33</td>
<td>02</td>
<td>1.37*</td>
<td>Significant</td>
</tr>
</tbody>
</table>

Table 26: Analysis of cell growth kinetics after Norgestrel exposure with metabolic activation (S₉ mix).

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Cells Scored</th>
<th>Percent cells in M1</th>
<th>M2</th>
<th>M3</th>
<th>Replication Index</th>
<th>2x3 Chi-square test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norgestrel</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>200</td>
<td>38</td>
<td>44</td>
<td>18</td>
<td>1.80</td>
<td>Not significant</td>
</tr>
<tr>
<td>25</td>
<td>200</td>
<td>61</td>
<td>34</td>
<td>05</td>
<td>1.44*</td>
<td>Significant</td>
</tr>
<tr>
<td>50</td>
<td>200</td>
<td>57</td>
<td>40</td>
<td>03</td>
<td>1.46*</td>
<td>Significant</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>200</td>
<td>34</td>
<td>54</td>
<td>12</td>
<td>1.78</td>
<td></td>
</tr>
<tr>
<td>DMSO (5µl/ml)</td>
<td>200</td>
<td>33</td>
<td>53</td>
<td>14</td>
<td>1.81</td>
<td></td>
</tr>
<tr>
<td>CP (1x10^-7 M)</td>
<td>200</td>
<td>66</td>
<td>29</td>
<td>05</td>
<td>1.38*</td>
<td>Significant</td>
</tr>
</tbody>
</table>

CP : Cyclophosphamide, DMSO : Dimethyl sulphoxide
SE : Standard error
* : Significant at P<0.05
exposed to the drug danazol are presented in table 27. For this drug the
dose concentrations selected for the evaluation of genotoxic potential
were 25, 50 and 100 mg/ml. Here at all concentrations the values obtained
for the percentage of chromosomal aberrations were not significant at
any exposure duration. The values obtained for 25 mg/ml were 4.33%,
4.67% and 6.00%, for 50 mg/ml these were 3.33%, 5.00% and 5.00%,
and for 100 mg/ml of dose concentration the values obtained were
6.00%, 8.00% and 7.00%. All these values were obtained respectively
at 24, 48 and 72 hours of exposure durations. All these values were found
insignificant statistically when compared to the normal control data i.e.
6.00%. The percentage aberrations of CP were 29.00%, 52.00% and
66.67% respectively at 24, 48 and 72 hours of exposure durations. These
values were highly significant when compared to the normal control value
at P<0.05.

When the lymphocytes were exposed to the drug danazol in the
presence of metabolic activation (S<sub>m</sub> mix), the values obtained for the
percentage of chromosomal aberrations were all insignificant, as were
reported in the absence of S<sub>m</sub> mix. All the values are documented in table
28. The percentage aberration observed were 4.50%, 5.50% and 8.00%
respectively at 25, 50 and 100 mg/ml of dose concentrations. When
compared to the normal control value i.e. 4.00%, all the above values
were found to be insignificant at P < 0.05. For CP the value obtained
was 49.50% which was highly significant as compared to the normal
control value at P < 0.05.

(b) Sister Chromatid Exchange (SCE):

The results are presented in table 29. The mean SCE value observed
at 25, 50 and 100 mg/ml of dose concentrations were 1.12±0.019,
1.34±0.012 and 1.44±0.069. These values were not significant when
compared to the normal control value i.e. 1.10±0.099. For positive
control the value for mean SCE frequency was 6.36±0.152. This value
was highly significant as compared to the normal control value at P < 0.05.
Table 30 describes about the results obtained after the exposure of lymphocytes to danazol in the presence of S₉ mix. The pattern of result was same as it was in the absence of metabolic activation. The values of mean SCE frequency at 25, 50 and 100 mg/ml of dose concentrations were 1.34±0.125, 1.18±0.113 and 1.50±0.119. All these values were not significant as compared to the normal control value i.e. 1.46±0.090 at P < 0.05. For CP the value obtained was highly significant (7.78±0.150) statistically when compared to normal control.

(c) Cell Growth Kinetics:

Table 31 shows the analysis of cell growth kinetics after danazol exposure in human lymphocytes. The 2x3 chi-square test indicated no significant deviation from the normal control culture. The replication indices at 25, 50 and 100 mg/ml of dose concentrations were 1.84, 1.88 and 1.91 respectively. These values were not significant when compared to the normal control value i.e. 1.81. The result obtained for CP was 1.37. This value was highly significant when compared to the normal control value at P<0.05.

When the above experiment was repeated in the presence of metabolic activation, the result obtained was quite similar i.e. all the values obtained were found to be insignificant. The result has been documented in table 32. Replication index values for 25, 50 and 100 mg/ml dose concentrations were 1.81, 1.84 and 1.81 respectively. These values were not significant at P < 0.05 when compared to the normal control value (1.78). For CP the result was highly significant statistically i.e. 1.38.

2. Oxymetholone
(a) Chromosomal aberrations:

The results of chromosomal aberrations in human lymphocytes after exposure to the drug oxymetholone have been presented in table 33. The drug was found to be ineffective in inducing chromosomal aberrations in human lymphocytes at any dose concentration and for any exposure
Table 27: Chromosomal aberrations in human lymphocytes after Danazol treatment.

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Duration (h)</th>
<th>Metaphase Scanned</th>
<th>Percent aberration</th>
<th>Types of aberration (%)</th>
<th>Aberration/Cell ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Metaphase Incl gap</td>
<td>Excl gap</td>
<td>Chromatid</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Chromosome</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>Danazol</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>25</td>
<td>24</td>
<td>300</td>
<td>6 33</td>
<td>3 00</td>
<td>4 33</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>300</td>
<td>7 00</td>
<td>4 67</td>
<td>4 67</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>300</td>
<td>7 67</td>
<td>4 67</td>
<td>5 00</td>
</tr>
<tr>
<td>50</td>
<td>24</td>
<td>300</td>
<td>6 33</td>
<td>3 00</td>
<td>3 00</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>300</td>
<td>6 00</td>
<td>3 67</td>
<td>4 33</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>300</td>
<td>7 33</td>
<td>4 00</td>
<td>4 33</td>
</tr>
<tr>
<td>100</td>
<td>24</td>
<td>300</td>
<td>7 00</td>
<td>3 67</td>
<td>4 67</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>300</td>
<td>7 67</td>
<td>3 33</td>
<td>6 00</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>300</td>
<td>7 33</td>
<td>4 33</td>
<td>5 33</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>72</td>
<td>300</td>
<td>4 33</td>
<td>2 33</td>
<td>2 67</td>
</tr>
<tr>
<td>DMSO (5µl/ml) (-ve control)</td>
<td>72</td>
<td>300</td>
<td>4 00</td>
<td>2 00</td>
<td>2 33</td>
</tr>
<tr>
<td>CP (1x10^7M) (+ve control)</td>
<td>24</td>
<td>300</td>
<td>14 33</td>
<td>13 67</td>
<td>15 33</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>300</td>
<td>19 67</td>
<td>18 67</td>
<td>28 00</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>300</td>
<td>28 67</td>
<td>27 33</td>
<td>39 00</td>
</tr>
</tbody>
</table>

CP: Cyclophosphamide, DMSO: Dimethyl sulfoxide
SE: Standard error
* Significant at P<0.05
Table 28: Chromosomal aberrations in human lymphocytes after Danazol treatment with and without metabolic activation (S₉ mix).

| Treat- | Meta- | Meta- | Percent aberration | Types of aberration (%) | Aberration/Cell ± SE |
| ment (µg/ml) | bolic | Metaphase | | Chromatid | Chromosome | Total |
| | Activation | | Metaphase | Incl. | Excl. | gap | gap | | | |
| Danazol | | | | | | | | | | |
| 25 | -S₉ | 300 | 5.67 | 2.67 | 4.33 | 0.33 | 4.66 | 0.05±0.029 |
| | +S₉ | 200 | 6.50 | 3.00 | 3.50 | 1.00 | 4.50 | 0.05±0.035 |
| 50 | -S₉ | 300 | 6.33 | 3.67 | 3.33 | 1.67 | 5.00 | 0.05±0.028 |
| | +S₉ | 200 | 6.00 | 2.50 | 4.50 | 1.00 | 5.50 | 0.06±0.035 |
| 100 | -S₉ | 300 | 7.33 | 3.00 | 5.67 | 2.33 | 8.00 | 0.08±0.023 |
| | +S₉ | 200 | 8.50 | 4.50 | 6.00 | 2.00 | 8.00 | 0.08±0.028 |
| Control | | | | | | | | | |
| Normal | -S₉ | 300 | 3.67 | 2.33 | 3.00 | 0.33 | 3.33 | 0.03±0.027 |
| | +S₉ | 200 | 4.00 | 2.00 | 3.50 | 0.50 | 4.00 | 0.04±0.035 |
| DMSO (5µl/ml) | -S₉ | 300 | 4.33 | 1.67 | 3.00 | 0.67 | 3.67 | 0.04±0.028 |
| (-ve control) | +S₉ | 200 | 4.50 | 2.00 | 2.50 | 1.00 | 3.50 | 0.04±0.034 |
| CP(1x10⁻⁷M) | -S₉ | 300 | 16.67 | 14.67 | 17.00 | 16.33 | 33.33* | 0.33±0.027 |
| (+ve control) | +S₉ | 200 | 20.00 | 19.00 | 26.50 | 23.00 | 49.50* | 0.50±0.028 |

CP: Cyclophosphamide, DMSO: Dimethyl sulfoxide, SE: Standard error, *: Significant at P<0.05
Table 29: Sister Chromatid Exchange (SCE) in human lymphocytes after Danazol exposure in vitro.

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Duration (h)</th>
<th>No. of Methaphase</th>
<th>Total SCE</th>
<th>Range</th>
<th>SCEs/Cell ±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Danazol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>48</td>
<td>50</td>
<td>56</td>
<td>0-6</td>
<td>1.12±0.019</td>
</tr>
<tr>
<td>50</td>
<td>48</td>
<td>50</td>
<td>67</td>
<td>0-7</td>
<td>1.34±0.012</td>
</tr>
<tr>
<td>100</td>
<td>48</td>
<td>50</td>
<td>72</td>
<td>0-7</td>
<td>1.44±0.069</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>48</td>
<td>50</td>
<td>55</td>
<td>0-4</td>
<td>1.10±0.099</td>
</tr>
<tr>
<td>DMSO (5µl/ml)</td>
<td>48</td>
<td>50</td>
<td>64</td>
<td>0-4</td>
<td>1.28±0.110</td>
</tr>
<tr>
<td>CP (1x10^{-7} M)</td>
<td>48</td>
<td>50</td>
<td>318</td>
<td>3-13</td>
<td>6.36±0.152*</td>
</tr>
</tbody>
</table>

Table 30: Sister Chromatid Exchange (SCE) in human lymphocytes after Danazol exposure with metabolic activation (S9 mix).

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Duration (h)</th>
<th>No. of Methaphase</th>
<th>Total SCE</th>
<th>Range</th>
<th>SCEs/Cell ±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Danazol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>48</td>
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<td>67</td>
<td>0-6</td>
<td>1.34±0.125</td>
</tr>
<tr>
<td>50</td>
<td>48</td>
<td>50</td>
<td>59</td>
<td>0-7</td>
<td>1.18±0.113</td>
</tr>
<tr>
<td>100</td>
<td>48</td>
<td>50</td>
<td>75</td>
<td>0-7</td>
<td>1.50±0.119</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>48</td>
<td>50</td>
<td>73</td>
<td>0-5</td>
<td>1.46±0.090</td>
</tr>
<tr>
<td>DMSO (5µl/ml)</td>
<td>48</td>
<td>50</td>
<td>63</td>
<td>0-5</td>
<td>1.26±0.093</td>
</tr>
<tr>
<td>CP (1x10^{-7} M)</td>
<td>48</td>
<td>50</td>
<td>389</td>
<td>4-14</td>
<td>7.78±0.150*</td>
</tr>
</tbody>
</table>

CP = Cyclophosphamide; DMSO = Dimethyl sulphoxide
SE = Standard error
* = Significant at P<0.05
Table 31: Analysis of cell growth kinetics after Danazol exposure in vitro.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cells Scored</th>
<th>Percent cells in</th>
<th>Replication Index</th>
<th>2x3 Chi-square test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$M_1$</td>
<td>$M_2$</td>
<td>$M_3$</td>
</tr>
<tr>
<td>Danazol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>200</td>
<td>37</td>
<td>42</td>
<td>21</td>
</tr>
<tr>
<td>50</td>
<td>200</td>
<td>38</td>
<td>36</td>
<td>26</td>
</tr>
<tr>
<td>100</td>
<td>200</td>
<td>35</td>
<td>39</td>
<td>26</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>200</td>
<td>32</td>
<td>55</td>
<td>13</td>
</tr>
<tr>
<td>DMSO (5μl/ml) (-ve control)</td>
<td>200</td>
<td>34</td>
<td>50</td>
<td>16</td>
</tr>
<tr>
<td>CP (1x10^{-7} M) (+ve control)</td>
<td>200</td>
<td>65</td>
<td>33</td>
<td>02</td>
</tr>
</tbody>
</table>

Table 32: Analysis of cell growth kinetics after Danazol exposure with metabolic activation ($S_s$ mix).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cells Scored</th>
<th>Percent cells in</th>
<th>Replication Index</th>
<th>2x3 Chi-square test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$M_1$</td>
<td>$M_2$</td>
<td>$M_3$</td>
</tr>
<tr>
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<td>200</td>
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<td>21</td>
</tr>
<tr>
<td>50</td>
<td>200</td>
<td>36</td>
<td>44</td>
<td>20</td>
</tr>
<tr>
<td>100</td>
<td>200</td>
<td>37</td>
<td>45</td>
<td>18</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>200</td>
<td>34</td>
<td>54</td>
<td>12</td>
</tr>
<tr>
<td>DMSO (5μl/ml) (-ve control)</td>
<td>200</td>
<td>33</td>
<td>53</td>
<td>14</td>
</tr>
<tr>
<td>CP (1x10^{-7} M) (+ve control)</td>
<td>200</td>
<td>66</td>
<td>29</td>
<td>05</td>
</tr>
</tbody>
</table>

CP : Cyclophosphamide,  DMSO : Dimethyl sulphoxide
SE : Standard error
* : Significant at $P<0.05$
duration. The aberration percentage observed at 25 mg/ml were 3.00%, 3.67% and 4.34%, at 50 mg/ml these values were 4.67%, 4.66% and 6.66%, and at 100 mg/ml of concentration the percentage obtained were 5.34%, 5.00% and 6.66%. The three different observations at all doses were obtained for different exposure durations i.e. 24, 48 and 72 hours respectively. All these values were not significant in comparison to the normal control value (3.00%) at P < 0.05. The aberrations percentages obtained for CP were 29.00%, 52.00% and 66.67% respectively for 24, 48 and 72 hours of exposure durations. These values were highly significant statistically.

Table 34 shows the effects of oxymetholone on the chromosomal aberrations in human lymphocytes in the presence of metabolic activation. The percentage of aberrations observed for 25, 50 and 100 mg/ml of dose concentrations were 4.50%, 3.50% and 7.50% respectively. These values were totally insignificant in comparison to the normal control value (4.00%) at P < 0.05. The percentage of aberrations induced by CP was highly significant statistically. This value was 49.50%.

Thus the drug oxymetholone was not effective in inducing chromosomal aberrations in human lymphocytes either in the presence or absence of metabolic activation.

(b) Sister Chromatid Exchange (SCE):

When the lymphocytes were exposed to the drug oxymetholone to see the increase in mean SCE frequency, it was observed that this drug is not potent enough to enhance the SCE frequency significantly. The results has been presented in the table 35. The mean SCE frequency observed at 25, 50 and 100 mg/ml of dose concentrations were 1.18±0.083, 1.24±0.077 and 1.38±0.080 respectively. All these values were insignificant statistically, compared to the normal control value (1.10±0.099). For CP the value was 6.36±0.152 which was highly
significant at $P < 0.05$, when compared to normal control value.

Table 36 shows the effects of oxymetholone on the mean SCE frequency of human lymphocytes in the presence of metabolic activation. The values of mean aberration frequency obtained for 25, 50 and 100 mg/ml of concentrations were $1.64 \pm 0.103$, $1.78 \pm 0.103$ and $1.66 \pm 0.095$ respectively. These values were found insignificant when compared to the normal control value ($1.46 \pm 0.090$) at $P < 0.05$. CP enhanced the SCE frequency greatly in the presence of $S_9$ mix. The value obtained was $7.78 \pm 0.150$ which was highly significant statistically.

(c) Cell Growth Kinetics:

Table 37 shows the analysis of cell growth kinetics after oxymetholone exposure in human lymphocytes. The 2x3 chi-square test indicated no significant deviations from the control cultures. The replication index values obtained were 1.88, 1.81 and 1.77 respectively at 25, 50 and 100 mg/ml concentrations. These values were not significant in comparison to the normal control value (i.e. 1.81) at $P < 0.05$. Cyclophosphamide was found to have a statistically significant effect when compared to normal control value. The value obtained was 1.37.

In the presence of metabolic activation, human lymphocytes were exposed to oxymetholone to analyse the cell growth kinetics. The results obtained have been summarised in table 38. At 25, 50 and 100 mg/ml concentrations the drug did not show any significant result. The replication indices observed were 1.90, 1.78 and 1.74, which were all insignificant statistically in comparison to the normal control value (1.78). The replication index observed for CP was 1.38 which was highly significant at $P < 0.05$ when compared to the normal control value.

Thus, oxymetholone was not found to affect the cell turnover rates of the lymphocytes either in the presence or in the absence of $S_9$ mix in vitro.
<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Duration (h)</th>
<th>Metaphase Scanned</th>
<th>Percent aberration</th>
<th>Types of aberration (%)</th>
<th>Aberration/Cell ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Metaphase Incl gap</td>
<td>Metaphase Excl gap</td>
<td>Chromatid</td>
<td>Chro-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>mosome</td>
</tr>
<tr>
<td>Oxymetholone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>24</td>
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<tr>
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<td>24</td>
<td>300</td>
<td>6.00</td>
<td>3.00</td>
<td>3.67</td>
</tr>
<tr>
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<td>300</td>
<td>6.00</td>
<td>4.67</td>
<td>4.00</td>
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<td>5.33</td>
</tr>
<tr>
<td>Control</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>72</td>
<td>300</td>
<td>4.33</td>
<td>2.33</td>
<td>2.67</td>
</tr>
<tr>
<td>DMSO (5µl/ml)</td>
<td>72</td>
<td>300</td>
<td>4.00</td>
<td>2.00</td>
<td>2.33</td>
</tr>
<tr>
<td>(-ve control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP (1x10^-7M)</td>
<td>24</td>
<td>300</td>
<td>14.33</td>
<td>13.67</td>
<td>15.33</td>
</tr>
<tr>
<td>(+ve control)</td>
<td>48</td>
<td>300</td>
<td>19.67</td>
<td>18.67</td>
<td>28.00</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>300</td>
<td>28.67</td>
<td>27.33</td>
<td>39.00</td>
</tr>
</tbody>
</table>

CP : Cyclophosphamide, DMSO : Dimethyl sulfoxide
SE : Standard error
* : Significant at P<0.05
Table 34: Chromosomal aberrations in human lymphocytes after Oxymetholone treatment with and without metabolic activation (S₉ mix).

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Metabolic Activation</th>
<th>Percent aberration</th>
<th>Types of aberration (%)</th>
<th>Aberration/Cell ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Metaphase Scanned</td>
<td>Metaphase</td>
<td>Chromatid</td>
<td>Chromosome</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Incl gap</td>
<td>Excl gap</td>
<td></td>
</tr>
<tr>
<td>Oxymetholone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>-S₉ 300</td>
<td>4.67  2.67</td>
<td>2.00  0.33</td>
<td>2.33</td>
</tr>
<tr>
<td></td>
<td>+S₉ 200</td>
<td>3.50  1.50</td>
<td>3.50  1.00</td>
<td>4.50</td>
</tr>
<tr>
<td>50</td>
<td>-S₉ 300</td>
<td>4.33  3.67</td>
<td>2.67  1.33</td>
<td>4.00</td>
</tr>
<tr>
<td></td>
<td>+S₉ 200</td>
<td>5.00  3.00</td>
<td>3.00  0.50</td>
<td>3.50</td>
</tr>
<tr>
<td>100</td>
<td>-S₉ 300</td>
<td>5.00  2.33</td>
<td>4.00  2.33</td>
<td>6.33</td>
</tr>
<tr>
<td></td>
<td>+S₉ 200</td>
<td>5.00  3.50</td>
<td>5.00  2.50</td>
<td>7.50</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>-S₉ 300</td>
<td>3.67  2.33</td>
<td>3.00  0.33</td>
<td>3.33</td>
</tr>
<tr>
<td></td>
<td>+S₉ 200</td>
<td>4.00  2.00</td>
<td>3.50  0.50</td>
<td>4.00</td>
</tr>
<tr>
<td>DMSO (5µl/ml)</td>
<td>-S₉ 300</td>
<td>4.33  1.67</td>
<td>3.00  0.67</td>
<td>3.67</td>
</tr>
<tr>
<td>(-ve control)</td>
<td>+S₉ 200</td>
<td>4.50  2.00</td>
<td>2.50  1.00</td>
<td>3.50</td>
</tr>
<tr>
<td>CP (1x10⁻³M)</td>
<td>-S₉ 300</td>
<td>16.67 14.67</td>
<td>17.00 16.33</td>
<td>33.33*</td>
</tr>
<tr>
<td>(+ve control)</td>
<td>+S₉ 200</td>
<td>20.00 19.00</td>
<td>26.50 23.00</td>
<td>49.50*</td>
</tr>
</tbody>
</table>

CP : Cyclophosphamide, DMSO Dimethyl sulphoxide
SE : Standard error
* : Significant at P<0.05
Table 35:  Sister Chromatid Exchange (SCE) in human lymphocytes after Oxymetholone exposure *in vitro*.

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Duration (h)</th>
<th>No of Methaphase</th>
<th>Total SCE</th>
<th>Range</th>
<th>SCEs/Cell ±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxymetholone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>48</td>
<td>50</td>
<td>59</td>
<td>0-4</td>
<td>1.18±0.083</td>
</tr>
<tr>
<td>50</td>
<td>48</td>
<td>50</td>
<td>62</td>
<td>0-5</td>
<td>1.24±0.077</td>
</tr>
<tr>
<td>100</td>
<td>48</td>
<td>50</td>
<td>59</td>
<td>0-5</td>
<td>1.38±0.080</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>48</td>
<td>50</td>
<td>55</td>
<td>0-4</td>
<td>1.10±0.099</td>
</tr>
<tr>
<td>DMSO (5µl/ml)</td>
<td>48</td>
<td>50</td>
<td>64</td>
<td>0-4</td>
<td>1.28±0.110</td>
</tr>
<tr>
<td>CP (1x10⁻⁷ M)</td>
<td>48</td>
<td>50</td>
<td>318</td>
<td>3-13</td>
<td>6.36±0.152*</td>
</tr>
</tbody>
</table>

CP: Cyclophosphamide, DMSO: Dimethyl sulfoxide
SE: Standard error
* Significant at P<0.05

Table 36: Sister Chromatid Exchange (SCE) in human lymphocytes after Oxymetholone exposure with metabolic activation (S9 mix).

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Duration (h)</th>
<th>No of Methaphase</th>
<th>Total SCE</th>
<th>Range</th>
<th>SCEs/Cell ±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxymetholone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>48</td>
<td>50</td>
<td>82</td>
<td>0-6</td>
<td>1.64±0.103</td>
</tr>
<tr>
<td>50</td>
<td>48</td>
<td>50</td>
<td>89</td>
<td>0-6</td>
<td>1.78±0.103</td>
</tr>
<tr>
<td>100</td>
<td>48</td>
<td>50</td>
<td>83</td>
<td>1-7</td>
<td>1.66±0.095</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>48</td>
<td>50</td>
<td>73</td>
<td>0-5</td>
<td>1.46±0.090</td>
</tr>
<tr>
<td>DMSO (5µl/ml)</td>
<td>48</td>
<td>50</td>
<td>63</td>
<td>0-5</td>
<td>1.26±0.093</td>
</tr>
<tr>
<td>CP (1x10⁻⁷ M)</td>
<td>48</td>
<td>50</td>
<td>389</td>
<td>4-14</td>
<td>7.78±0.150*</td>
</tr>
</tbody>
</table>

CP: Cyclophosphamide, DMSO: Dimethyl sulfoxide
SE: Standard error
* Significant at P<0.05
**Table 37:** Analysis of cell growth kinetics after Oxymetholone exposure **\textit{in vitro}**.

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Cells Scored</th>
<th>Percent cells in (M_1)</th>
<th>(M_2)</th>
<th>(M_3)</th>
<th>Replication Index</th>
<th>2x3 Chi-square test</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oxymetholone</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>200</td>
<td>31</td>
<td>50</td>
<td>19</td>
<td>1.88</td>
<td>Not significant</td>
</tr>
<tr>
<td>50</td>
<td>200</td>
<td>35</td>
<td>53</td>
<td>14</td>
<td>1.81</td>
<td>Not significant</td>
</tr>
<tr>
<td>100</td>
<td>200</td>
<td>39</td>
<td>45</td>
<td>16</td>
<td>1.77</td>
<td>Not significant</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>200</td>
<td>32</td>
<td>55</td>
<td>13</td>
<td>1.81</td>
<td></td>
</tr>
<tr>
<td>DMSO (5µl/ml) (-ve control)</td>
<td>200</td>
<td>34</td>
<td>50</td>
<td>16</td>
<td>1.82</td>
<td></td>
</tr>
<tr>
<td>CP (1x10^{-7} M) (+ve control)</td>
<td>200</td>
<td>65</td>
<td>33</td>
<td>02</td>
<td>1.37*</td>
<td>Significant</td>
</tr>
</tbody>
</table>

**Table 38:** Analysis of cell growth kinetics after Oxymetholone exposure with metabolic activation \((S_9\text{ mix})\).

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Cells Scored</th>
<th>Percent cells in (M_1)</th>
<th>(M_2)</th>
<th>(M_3)</th>
<th>Replication Index</th>
<th>2x3 Chi-square test</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oxymetholone</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>200</td>
<td>29</td>
<td>52</td>
<td>19</td>
<td>1.90</td>
<td>Not significant</td>
</tr>
<tr>
<td>50</td>
<td>200</td>
<td>36</td>
<td>50</td>
<td>14</td>
<td>1.78</td>
<td>Not significant</td>
</tr>
<tr>
<td>100</td>
<td>200</td>
<td>37</td>
<td>52</td>
<td>11</td>
<td>1.74</td>
<td>Not significant</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>200</td>
<td>34</td>
<td>54</td>
<td>12</td>
<td>1.78</td>
<td></td>
</tr>
<tr>
<td>DMSO (5µl/ml) (-ve control)</td>
<td>200</td>
<td>33</td>
<td>53</td>
<td>14</td>
<td>1.81</td>
<td></td>
</tr>
<tr>
<td>CP (1x10^{-7} M) (+ve control)</td>
<td>200</td>
<td>66</td>
<td>29</td>
<td>05</td>
<td>1.38*</td>
<td>Significant</td>
</tr>
</tbody>
</table>

CP : Cyclophosphamide, DMSO : Dimethyl sulphoxide  
SE : Standard error  
* : Significant at \(P<0.05\)
Discussion
DISCUSSION

The cytogenetic effects of sex steroidal hormones (two each from estrogens, androgens and progestins) have been evaluated in vitro in the present study. The advantages and disadvantages of the test systems used, the parameters assayed and the effect of the steroids evaluated on these parameters are discussed below:

A. GENERAL CONSIDERATIONS:

All the results of mutagenicity (genotoxicity) evaluation of the steroids are eventually aimed at human safeguards. It is, therefore, ideal to evaluate them directly on humans who are exposed to these steroids, or test them for genotoxicity on a system that has the same sensitivity of response as the human genome. The human peripheral blood lymphocyte culture system is a single system that provides us with a large number of dividing cells. These can be easily cultured to yield large number of mitotic figures from chromosome analysis. The lymphocytes also contain some metabolizing enzyme systems that are able, to some extent, to activate certain promutagens into a mutagenically active state (Buckton and Evans, 1973).

Studies on exposed individuals, and on cultured cells, have shown that the human peripheral blood lymphocyte is an extremely sensitive indicator of both in vivo and in vitro induced chromosomal structural changes. These changes in chromosome structure offer readily scored morphological evidence of damage to the genetic material. Although problems exist in the extrapolation from in vitro results to the in vivo situation, the lymphocyte offers several advantages as a test system in the following ways (Evans, 1982; Evans et al., 1979; Evans, 1970; Buckton and Evan, 1973):

(i) Easy availability of large number of human cells. A few ml of peripheral blood can be easily and repeatedly obtained from an individual and each 1 ml of blood contains 1-3x10^6 small lymphocytes.
(ii) The lymphocytes are distributed throughout the body, circulate in all tissues and a proportion are long-lived as well.

(iii) Virtually all the peripheral blood lymphocytes are a synchronized cell population in the same $G_0$ or $G_1$ stage of mitotic interphase and, in healthy individuals, these cells are only infrequently involved in mitotic proliferation \textit{in vivo}.

(iv) A proportion of the lymphocytes can be stimulated by mitogens to undergo mitosis in culture. They are easy to culture and thus provide a ready source of dividing cells for the scoring of chromosome aberrations.

(v) There are excellent techniques available for making chromosome preparations from lymphocytes and the cells have a low spontaneous chromosome aberration frequency (Buckton and Evans, 1973).

Some disadvantages include:

(i) Although the lymphocytes are a synchronized population, there are different sub-populations of cells even within the same individual. These can have varying responses to different mitogenic agents and probably differing intermitotic times which may be further influenced by variations in culture conditions.

(ii) As in other cell systems, there is the possibility of non-uniformity of exposure and of preferential growth in culture of less heavily damaged cells.

(iii) It is now recognised that in man only T-lymphocytes, i.e. the thymic-derived cells, can be stimulated in culture by phytohaemagglutinin (PHA) to undergo mitosis. The T-lymphocyte is closely concerned with the immune response, thus, where an analysis of \textit{in vivo} exposure is to be undertaken, any recent previous exposure of an individual to an immunological stimulus
may positively or negatively alter the number of cells with chromosome aberrations, depending on when the cells are withdrawn from the individual.

These disadvantages relate largely to the use of lymphocytes for studying mutagenic effects in vivo and are of less relevance to the use of lymphocytes for in vitro mutagen testing.

**IN VIVO VS IN VITRO EVALUATIONS**

It is ideal to study the effect of chemicals directly on persons exposed to these chemicals or drugs. But ethical reasons preclude the experimental approach on evaluation of genotoxicity on humans. The in vivo study is possible only in specific cases when individuals have been exposed to the agents either as a chemotherapeutic necessity (e.g. medicines) or occupational inevitability (e.g. exposure to styrene, aniline, polyvinyl chloride) or accidental reasons (e.g. exposure to radiation, atom bomb explosions etc.).

The genotoxicity evaluation among patients on therapy is difficult. Patients may be on multiple therapy and the effects observed may either be additive, synergistic or antagonistic to each other. In vivo studies in humans have been found to have significant effect on chromosomal aberrations (Pinto, 1986) and SCE (Murthy and Prema, 1983).

The in vivo cytogenetic evaluation also has its shortcomings. The cytogenetic abnormalities found in exposed persons represent the most drastic and obvious expression of genetic damage while the frequent and subtle genetic changes like mutations are not detected. Another limitation is that the peripheral lymphocytes which are cultured are not in the dividing cell cycle at the time when the chemicals exert their influence in vivo. Drugs which can cause primary genetic lesions in the G1 phase only can manifest their detrimental effects while agents that cause genetic damage in the S-phase may not exhibit cytogenetic damage in lymphocyte cultures of person exposed to these agents (Gebhart, 1982).
These shortcomings of the in vivo evaluations can be overcome by in vitro treatment of peripheral blood lymphocytes in cultures. The experimental approach can be used and the effect of drugs/chemicals can be evaluated at a range of concentrations (including sub-toxic and toxic levels) on lymphocyte cultures of healthy individuals. This also permits the evaluation of effects of different time exposure to the drug and fate of lesion after consecutive cell division and the effect of drug during the G1 as well as S-phase of the cell cycle. In vitro test, however, has its own shortcomings because there is no metabolizing system of the drugs and chemicals in it. But lymphocytes have been known to metabolise at least some compound, e.g. activation of polycyclic hydrocarbon benzo (a) pyrene. The presence of erythrocytes in cultures is also known to aid in metabolising compounds like aniline, HCl (Wilmer et al., 1984), sodium selenite (Ray and Altenburg, 1978) and styrene and its analogues (Norppa et al., 1983; Norppa and Tursi, 1984).

In the present study two estrogens mestranol and quinestrol, two progestins norethindrone and norgestrel, and two androgens danazol and oxymetholone were evaluated for their cytogenetic effects on human lymphocytes in peripheral blood cultures in vitro. All the drugs were also evaluated in the presence of metabolic activation (S9 mix.)

**CHROMOSOME ABERRATIONS:**

Chromosomal aberrations are categorised as structural and numerical chromosomal aberrations.

**I. Structural aberrations:**

The broadest classification of structural chromosomal aberrations is based on whether they are formed before or after semiconservative DNA replication. On this basis the types of structural chromosomal damage which can be cytologically distinguished at metaphase can be divided into two main groups: Chromosome-type aberrations and chromatid-type aberrations (Evans, 1962). The circulating lymphocyte
is in the $G_0$ or $G_1$ phase of mitosis and exposure to ionizing radiations and certain other mutagenic agents (radiomimetic chemicals) during this stage produces chromosome-type damage where the unit of breakage and reunion is the whole chromosome (i.e. both chromatids at the same locus). However, cells exposed to these agents while in the S or $G_2$ stages of the cell cycle, after the chromosome has divided into two sister chromatids, yield chromatid-type aberrations and only the single chromatid is involved in breakage or exchange. Other agents (e.g. some of the alkylating agents) will produce only chromatid-type aberrations although the cells are exposed to the mutagen whilst in $G_0$ or $G_1$ phase. Therefore, chromosome type aberrations are produced by errors in DNA repair in unreplicated DNA, i.e. in $G_1$ or $S$ phase in DNA regions that have not yet been replicated. When observed at metaphase or interphase, chromosome-type aberrations involve a change in both chromatids of the chromosome. Whereas, chromatid-type aberrations are produced by errors in DNA repair in $G_2$ or $S$ phase in DNA regions that have already replicated at the time of exposure or by errors in DNA replication on a damaged DNA template. When observed at metaphase, chromatid type aberrations involve only one of the two sister chromatids, with the exception of isochromatid fragments or deletions that involve both chromatids. Isochromatid deletions are thus difficult to distinguish from chromosome-type terminal deletions. They can be classified, if needed, into chromatid-type or chromosome-type on the basis of the other, clearly distinguishable types observed in a particular study. For the purposes of the present discussion, it is important to note that after cell division and a subsequent round of DNA replication, chromatid type aberrations can be converted into the so-called derived-chromosome type aberrations. The probability of this occurrence varies for different types of aberrations depending on the likelihood of lethality at the intervening cell division resulting from loss of an acentric chromosomal fragment.

(A) Chromosome-type aberrations: Studies on somatic metaphases show that seven classes of chromosome-type aberrations can be
distinguished cytologically. Aberration types 1-5 involve only a single chromosome and are known as intrachanges whereas types 6 and 7 involves the exchange of parts between chromosomes and therefore are classified as interchanges.

1. **Terminal deletions**: These are paired acentric fragments which result from a simple break across the chromosome and they are not associated with an obvious exchange aberration.

2. **Minutes** (interstitial, isodiametric or dot deletions): These are pairs of acentric fragments, smaller in size than terminal deletions, characteristically appear as paired spheres of chromatin. These are intercalary deletions.

3. **Acentric rings**: These are paired segments of chromatids without a centromere and are joined to give a ring.

4. **Centric rings**: Ring structures containing a centromere. The centric ring can be easily distinguished morphologically from the acentric type and is generally accompanied by one acentric fragment.

5. **Inversions**: Chromosome inversions can be classified into two categories: (a) **paracentric inversions** where both points of breakage and reunion lie on the same arm of the chromosome; and (b) **pericentric inversions** where the points of breakage and inversion lie on opposite sides of the centromere.

   In cells where two points of pericentric exchange are of unequal distance from the centromere, the abnormal chromosome can be easily distinguished by the altered position of the centromere. However, where the exchange points are equally distant from the centromere, and in the case of paracentric inversions, there is no change in the relative position of the centromere. The detection of such inversions may, therefore, be possible only through the use of chromosome banding techniques.

6. **Reciprocal translocations** (symmetrical interchange): These are
aberrations which involve breakage of two chromosomes and the reciprocal exchange of broken segments. Conventional staining methods will not allow the detection of reciprocal translocations involving the exchange of two equal-sized segments, and banded chromosome preparations are required. Interchanges may also occur in the centromere regions of two chromosomes giving whole-arm exchanges. If they involve acrocentric chromosomes are referred to as centric fusions or Robertsonian translocations.

(7) **Dicentric or poly-centric aberrations** (asymmetrical interchange): Aberrations which arise from an exchange between two or more chromosomes results in the centric products re-uniting in such a way to form a dicentric or polycentric structure and an associated acentric fragment.

Aberration types (1), (2) and (3) i.e. terminal deletions, minutes and acentric rings, are often loosely grouped together as acentric fragments. These are not associated with any obvious exchange event resulting in a rearrangement. Fragments associated with an exchange event e.g. the fragments observed in association with a dicentric, are scored as part of the exchange and not as separate aberrations in their own right.

**(B) Chromatid-type aberrations**: Chromatid-type aberrations are induced by ionizing radiations when cells are exposed in the S or $G_2$ stages of interphase. Chromosome-type damage are produced in cells irradiated in $G_1$. However, many chemical agents and viruses cause only chromatid-type damage even though the cells are exposed to the agent whilst in the $G_1$ phase and examined in their first subsequent division. These aberrations are a consequence of errors in replication that occur at the DNA synthesis, or S phase following exposure.

In contrast to the chromosome-type aberrations, according to some scientists, chromatid breaks and particularly gaps are unreliable indicators of real damage to the genetic material. Because gaps are also
caused by technical artefacts e.g. "poor" culture conditions, and the use of "drastic" processes during slide preparation. For this reason, if chromatid gaps and breaks are to be scored and included in the aberrations yield, it is particularly important that "control" blood cultures are cultured in a similar way and at the same time as those exposed to the agent being tested (Evans and O'Riordan, 1975). But another group of researchers strongly support the idea that chromatid breaks and gaps are also indicators of genetic damage, because the frequency of chromatid breaks and gaps increases with the increase in the concentration and exposure duration of mutagen in comparison to the normal control culture. In the present study chromatid breaks and gaps have been considered as indicators of genetic damage.

(1) **Chromatid and isochromatid gap**: The gap or achromatic lesion appears as a non-staining and constricted region in the chromatid arm and the apparently "broken" segments of the chromatid area are in alignment. Where the gap involves both chromatid arms at the same position, this is referred to as an isolocus or isochromatid gap.

(2) **Chromatid break**: Where there is a discontinuity with displacement in the chromatid arm so that the broken chromatid ends are not aligned. An apparently "simple" break results in a terminal deletion.

(3) **Chromatid minutes**: Single, or unpaired, intercalary fragments.

(4) **Chromatid acentric rings**: Intercalary fragments joined to give ring structures.

(5) **Centric rings**: Intrachanges resulting in chromatid ring structures in which the centromere is included in the ring.

(6) **Inversions**: Paracentric inversions are not readily scorable, but pericentric inversions may be detected because of the close pairing of chromatids.

(7) **Isochromatid aberrations**: These involve exchanges between sister
chromatids and may be confused with chromosome type terminal deletions. They may be distinguished from chromosome type aberrations, however, since the majority of isochromatid breaks involve a union between sister chromatids either proximal or distal to the point of breakage.

(8) Symmetrical interchanges: This type of aberration involves an exchange (exchanges) between two (or more) chromosomes. Simple exchanges between one chromatid in each of two chromosomes results in a configuration having four arms and referred to as quadri-radial.

(9) Asymmetrical interchanges: An exchange between two or more chromosomes resulting in the formation of one (or more) dicentric chromatids.

Within each major group of chromosome-type and chromatid-type aberrations is a range of different types of aberrations, broadly defined as deletions and exchanges. Large terminal or interstitial chromosome-type deletions observable under the light microscope are almost always lethal to the cells as a result of loss of the acentric fragment at the first or second cell division after formation. Whereas in case of chromatid-type terminal and interstitial deletions one daughter cell is viable (the one receiving the undamaged chromatid). It can be reasonably argued, however, that the mechanism of formation and general shape of the dose-response curve for small deletions that cause transmissible mutations is similar to that for larger (microscopically observable) interstitial deletions. Exchange aberrations can be intrachromosomal or interchromosomal. Each class includes transmissible (stable) and non-transmissible (unstable) types. Intrachromosomal exchanges can produce transmissible inversions involving the centromere (pericentric) or within a chromosome arm (paracentric). Besides intrachromosomal exchange can also produce centric and acentric rings which are most frequently non-transmissible because of loss of acentric chromosomal material at division (i.e. the acentric ring itself or the acentric fragment
accompanying a centric ring). Acentric rings are the same as the interstitial deletions described earlier.

Interchromosomal exchanges can produce reciprocal translocations that are transmissible, and dicentrics (with an accompanying acentric fragment) that are almost always non-transmissible. A reciprocal chromatid-type translocation between homologous chromosomes in mitotic crossing-over leads to the production of homozygosity for heterozygous loci for genes located distal to the translocations. This mechanism is relevant to producing homozygosity of recessive mutant tumour suppressor genes in cells that were originally heterozygous at these loci. Chromosome-type reciprocal translocations are transmitted to both daughter cells and do not cause loss of heterozygosity, whereas chromatid-type reciprocal translocations have a one-quarter probability of being present in a daughter cell. This is an important consideration for predicting potentially adverse outcomes attributable to chromosomal changes. The analysis of reciprocal translocations and inversions has until recently been difficult, relying on one or another version of chromosome banding. The recent development of whole-chromosome painting probes (Tucker et al., 1993), chromosome arm-specific probes (Lucas et al., 1996) and even total karyotypic differentiation (Specicher et al., 1996) have made the analysis much easier. These transmissible aberrations can be analysed in human and mouse cells. An aberration is a rare occurrence in comparison with the amount of induced DNA damage, most of which is efficiently repaired. The kinetics of repair of different types of DNA damage affects the types of structural alterations produced.

Chromosome and chromatid gaps:

One class of chromosomal change that requires separate attention is gaps or achromatic lesions. These changes appear as unstained regions of a chromosome where no obvious break or displacement is observed microscopically. The gaps have variously been attributed to damaged
DNA regions that have unwound or decondensed sites of DNA repair, and have been considered harbingers of cell death. Their frequency can clearly be increased by exposure to radiation and mutagenic chemicals but their further significance is unclear. When gaps are observed at the first metaphase after treatment, they are generally not seen at subsequent metaphases, as would be predicted if gaps are sites of decondensation, or repair, or indicate imminent cell death. The idea that they represent sites of damage is perhaps supported by the observation that the number of chromosomes breaks in treated cells which are induced to undergo premature chromosome condensation is very much higher than what is observed in normal metaphase cells. There is no agreement on how to distinguish deletions from gaps or how to interpret data on gaps, especially when they are the only chromosomal change observed in an assay. The United Kingdom Environmental Mutagen Society subcommittee on guidelines for mutagenicity testing (Scott et al., 1983) provides what is perhaps the most reasonable approach: an observation of gaps alone indicates the potential for true chromosomal aberrations but is insufficient for a firm conclusion to be drawn.

Gaps Vs Breaks:

The distinction between chromatid gap and break is very arbitrary. A gap cytogenetically is distinguished from a break depending on the length of the non-staining region in the chromosome. If the attenuated region is shorter than the width of the chromatid, it is classified as a gap and if longer than the width it is a break. But the problem in this classification is that chromosome at prometaphase are elongated with a small width of chromatid while at late metaphase they are condensed with larger width. This results in classification of more breaks in early metaphase and more gaps in late metaphase.

Various studies have been done to look into the nature of gaps and breaks. A combination of studies with light microscopy, transmission electron microscopy and scanning electron microscopy of x-ray induced
aberrations in *Vicia faba* showed that gaps and breaks are different manifestations of the same event and that gaps may be complete breaks (Brecher, 1977). Breaks, for example, are thought to be a complete loss of material at the non-staining region with displacement of the distal fragment, whereas gaps are thought to have a chromatin bridges. But electron microscopic studies (Op.cit.) demonstrated that few breaks had chromatin material bridging the space while some gaps had no such chromatin bridges. Thus, the distinction between the two is arbitrary at the light microscopy level and both are apparently different manifestations of the same phenomenon.

Comings (1974) offered a hypothesis regarding the nature of gap. According to him gaps and breaks represent the same basic lesions but differ in the time at which the lesions are induced. If a break in chromatin fibre occurs when the chromatin is fully extended (e.g. G₁, S and early G₂ phase), subsequent condensation of chromosome results in a chromatid break. But if the chromosome is already partially condensed (e.g. G₂ phase) and a break occurs, a few fibres still hold the chromosome together, resulting in a gappy appearance.

Silver staining technique of the chromatid core has shown that the chromatid core is not continuous in chromatid gaps (Satya Prakash *et al.*, 1981). Some authors suggest that gaps are produced due to loss of despiralization of both DNA as well as chromosomal proteins (Stoain and Raicu, 1975). An increase in constriction and gaps in human chromosomes following mercapto-ethanol exposure suggests that gaps may occur from damage to the protein moiety of the chromatid causing deficient folding of the chromatin fibre in metaphase chromatid (Brogger, 1982).

However, under the influence of mutagen the frequencies of both gaps and breaks increase. It has been suggested by many authors that gaps are indicative of toxic phenomena from genetic point of view and the aberrations could be useful sensitive indicators of chemically induced

Any damage altering the normal morphology of a chromosome (be it because of damage to DNA and chromatin or to the packing proteins of chromosomes) could interfere with normal functioning of the chromosome and ultimately to that of cell causing genotoxicity. Therefore, gaps were included in the present study for analysis.

Numerical Alterations:

Hyperploidy and hypoploidy i.e. increase and decrease in the number of chromosomes may result from incorrect segregation of chromosomes at anaphase and failure of the chromosomes to leave the metaphase plate or anaphase, so that they are not included in either daughter nucleus. In addition, partial chromosomal aneuploidy can arise from chromosomal deletions and from certain segregants of exchange aberrations. Increasing knowledge about the mechanisms of chromosome movement during mitosis has allowed identification of a number of steps at which induced changes can lead to aneuploidy (Preston, 1996). Traditionally, aneuploidy has been assessed by counting chromosomes at metaphase. More frequently, fluorescence hybridization techniques have been added. For example, the proportion of micronuclei containing whole chromosomes can be assessed by using a probe that can detect the kinetochore (Eastmond and Tucker, 1989). This approach can, of course, detect only aneuploidy that results from lagging chromosomes. Whole chromosome painting probes or preferably artificial bacterial chromosome probes that provide more discrete signals, can be used for the detection of aneuploidy in interphase cells (Kim et al., 1995; Bell et al., 1996).

Changes in ploidy (duplication of whole chromosome complement) are also induced, generally as a result of inhibition of cytokinesis or failure of all chromosomes to segregate. The outcome can either be
polyploidy or endo reduplication. The later is a specific version of polyploidy in which replicated chromosomes maintain a parallel association along their lengths even at C metaphase (Therman et al., 1983). Specific assays are not available for detecting changes in ploidy, but they should be noted when observed in the course of other assays for chromosomal alterations.

ABERRATION YIELD AND TYPE IN RELATION TO TIME IN CULTURE:

Probably the most important \textit{in vitro} factor which influences the chromosome aberrations is the time factor during which the cells are maintained in culture. After two days in culture at 37°C the majority of transformed lymphocytes generally undergo their first mitosis. Preparation made from cells cultured for longer periods will contain increasingly high proportions of cells in their second or subsequent divisions. Consequently, if lymphocytes with chromosome type damage fail to undergo repeated division \textit{in vitro} due to their genetic unbalance or mechanical difficulties at anaphase, examination of cells after 72 h of culture may not reflect the true \textit{in vivo} aberration level. Furthermore, a proportion of the chromosome-type aberrations observed at this later time may be really of a "derived" type resulting from a duplication of aberrations that were initially chromatid type. Thus, for tests which are designed to detect the presence or absence of chromosome damage in lymphocytes following \textit{in vivo} exposure of individuals to a suspected mutagenic agent, the blood samples should only be cultured for up to 50 hours at 37°C. The same restriction applies to \textit{in vitro} studies but consideration must also be given to the fact that the agent and the test may itself inhibit cell development and so it is necessary to prolong the time of culture to obtain mitotic cells.

SISTER CHROMATID EXCHANGE (SCE):

SCE results from the breakage and rejoining of DNA at apparently homologous sites on the two chromatids of a single chromosome. They
were first visualized by Taylor et al. (1957) who used tritiated thymidine to differentially label the DNA of replicating cells and autoradiography to distinguish the silver grain pattern on the two sister chromatids. Latt (1973) and Perry and Wolff (1974) made this technique more simple. In principle, SCEs can be observed in any cells that has completed two, or the first of two, replication cycles in the presence of BrdU. The most common procedure for human lymphocytes is to grow the cells in BrdU for two replication cycles.

SCEs are most efficiently induced by substances that form covalent adducts with the DNA, or interfere with DNA precursor metabolism or repair (Perry and Evans, 1975; Wolff, 1977; Perry, 1980; Carrano and Thompson, 1981; Latt et al., 1981). It is well documented that SCEs are produced during DNA replication (Wolff et al., 1974) and that the polarity of DNA is maintained in the process of exchange (Taylor, 1958; Wolff and Perry, 1975). The molecular mechanism by which the exchange is formed is not known but, since there is an absolute requirement for DNA synthesis, hypotheses have implicated the mechanisms of this process in SCE formation (Bender et al., 1974; Painter, 1980; Cleaver, 1981; Shafer, 1982). Baseline SCE frequencies are increased in some human diseases such as Bloom's syndrome (Chaganti et al., 1974) and multiple sclerosis (Sutherland et al., 1980; Vijayalaxmi et al., 1983). Cells from patients with the inherited disease Xeroderma pigmentosum are hypersensitive to the induction of SCEs by ultraviolet radiation and alkylating agents (Wolff et al., 1975; Wolff, 1977). There is no clear relation, however, between the repair capacity of these cells and their sensitivity to SCE induction (de Weerd-Kastelein et al., 1977).

Relevance of sister-chromatid exchange:

An SCE represents the breakage of 4 strands of DNA (2 double helices), a switch of the strands from one to the other arm of the same chromosome, and the rejoining of the strands in their new locations.
The question is whether the breakage and rejoining events occur without producing any modification in the genetic code. There is evidence suggesting that this exchange process might not always be error-free. In particular, a considerable amount of information has been gathered concerning the relationship between induced SCEs and other genetic effects in vivo and in vitro.

Because the technique for enumerating SCEs is relatively simple, this assay has been used quite extensively in genetic toxicology studies. It has been amply demonstrated that the frequency of SCE is dramatically increased when cells, or animals including human beings, are exposed to known mutagens or carcinogens (Perry and Evans, 1975; Latt et al., 1981; Lambert et al., 1982). Moreover, in earlier experiments using Chinese hamster cells in culture, it was found that the induction of SCEs was linearly related to the increase in single-gene mutations (HPRT locus), when the cells were exposed to several substances each of which differed in the type of lesion produced in DNA (Carrano et al., 1978; Carrano and Thompson, 1982). The ratio of induced SCE to induced mutation differed for each of the agents, suggesting that each type of lesion formed is processed differently by the cell favouring either the formation of SCEs or mutations. It is possible that the same lesion is capable of producing both an SCE and a mutation and/or that the lesions that form mutations are a subset of those that form SCEs. The induction of mutations has been shown to be correlated with the induction of SCEs in mice for one chemical, ethylnitrosourea (Jones et al., 1985). In the same study, the induction of SCEs in mouse splenocytes after intraperitoneal administration of the chemical it was found that a linear relation is existing between SCEs and proportional to the increase in mutations at the HPRT locus. It suggests that the mutation-SCE relation is not unique to in vitro studies.

Other investigations have compared the induction of SCEs to either the induction of transformation in vitro or tumour promotion in vivo. In a series of experiments, the induction of SCEs was shown to be
linearly and positively correlated with the induction of transformation of Syrian hamster embryo cells in vitro (Popescu et al., 1981). This relationship was held for exposure to five chemicals, but not for X-irradiation. The ratio of induced SCEs to induced transformation frequency varied for each chemical. This is analogous with the results found for the SCE-to-mutation ratio described above. Cheng et al. (1981) examined the induction of SCEs in mouse bone marrow, regenerating liver, and alveolar macrophages following intraperitoneal injection of ethylcarbamate and related chemicals. The relative potencies in inducing SCE paralleled the reported activities for the induction of lung adenomas in mice. For ethyl carbamate, the doses for the significant induction of SCEs in alveolar macrophages and lung adenomas were similar. Further, the slopes of the dose responses showed that the two end-points were of comparable sensitivity.

The results of early studies on rabbits demonstrated that it was possible to measure an increase in SCEs in peripheral blood lymphocytes for many months following repeated low-level exposure to mitomycin C in vivo (Stetka et al., 1978). Increased SCEs in human beings have been observed in cigarette smokers (Lambert et al., 1978; Carrano, 1982) and in individuals undergoing chemotherapy (Lambert et al., 1978; Musilova et al., 1979; Gillian Clare et al., 1982). The results of these studies suggest that a subpopulation of lymphocytes with high SCE frequency may be present for long periods after termination of exposure. These cells may represent persistent damage in long-lived lymphocytes or a sensitive subpopulation (Carrano and Moore, 1982). If this is true, they may offer a more appropriate approach to the quantification of persistent damage in vivo.

Factors potentially influencing SCE frequency:

The baseline SCE frequency in human peripheral lymphocytes averages about 7-10 per cell but has been reported to range from about 2 to 45 per cell in unexposed individuals (Alhadeff and Cohen, 1976;
Lambert et al., 1976; Crossen et al., 1977; Galloway, 1977; Carrano et al., 1980). Even within the same laboratory the baseline frequency may vary by a factor of two or more. Several potential sources of variation have been identified and they generally fall into two categories:

(a) Culture factors associated with the *in vitro* growth of the lymphocytes; and (b) biological factors associated with the genotype, life style, or general health of the individual. The biological factors usually cannot be mitigated and must be accounted for in the selection of appropriate control cohorts or ultimately in the statistical analysis.

(a) **Culture-associated factors**: A major source of variation can be attributed to the concentration of BrdU relative to the number of lymphocytes in the culture. The frequency of SCE has been shown to increase by as much as 50%, when the BrdU concentration is raised 10-fold (Carrano et al., 1980). The ability of BrdU to induce SCE has been well documented in human and other cell systems (Wolff and Perry, 1974; Lambert et al., 1976; Latt and Juergens, 1976; Mazrimas and Stetka, 1978) and it has been further shown that the SCE frequency can increase if the BrdU concentration is held constant, but the cell number decrease (Stetka and Carrano, 1977). To avoid this situation, the effect of BrdU is minimized by standardizing both the concentration of BrdU and white blood cells at culture initiation.

Other culture-associated factors influencing SCE frequency are culture time (Carrano *et al.*, 1980; Lindblad and Lambart, 1981; Das and Sharma, 1984), culture temperature (Speit, 1980; Das and Sharma, 1984) and serum used (McFee and Sherill, 1981). To avoid all sorts of bias, standardized procedure is used.

(b) **Biological factors**: Biological factors include sex, age, diet, genotype, medication and smoking. Each of these potentially plays a role in the induction or expression of SCEs. Although some information is available on the influence of these factors, the consequences are not always clear-cut.
Clear documentation of a sex difference for baseline SCE frequencies is absent (deArce, 1981; Waksvik et al., 1981; Carrano and Moore, 1982; Livingston et al., 1983). However, there is evidence for increased SCE frequencies in women taking oral contraceptives (Murthy and Prema, 1979).

The independence of age and baseline SCE frequency in lymphocytes in adults has been observed (Hollander et al., 1978; Livingston et al., 1983). Lower SCE frequencies have been found in infants (Seshadri et al., 1982), in cord blood (Ardito et al., 1980) and in children with a mean age of 1.5 years (Husgafvel-Pursiainen et al., 1980). De Arce (1981) reported that people in the age range of 30-40 years had a higher number of SCEs per cell than those between the ages of 0-10 or 60-70 years. Schmidt and Sanger (1981) found a significant increase in SCE with age when comparing age groups of 1-18 months, 10-13 years, 26-32 years and 63-85 years. However, this might be due to differential, BrdU incorporation between cultures. With the exception of lower SCE frequencies in new born infants, the weight of evidence suggests that baseline SCE frequencies do not change with age.

The role of genetic factor in the baseline SCE frequency is controversial, with majority of reports suggesting that there is no significant difference in the SCE frequency of different families (Cohen et al., 1982; Pederson et al., 1979; Waksvik et al., 1981; Butler, 1981).

Various drugs have been found to increase SCE frequency in human lymphocyte chromosomes in vitro (Ahmad et al., 2000a). Increase in SCE frequency is also associated with nutrition factors in certain cases e.g. in children suffering from severe protein calorie malnutrition (Murthy et al., 1980), which decreased after nutritional rehabilitation. Increased SCEs have also been reported in chronic alcoholics (Butler et al., 1981). Elevated SCE frequency has also been observed in cigarette smokers (Lambert et al., 1978; Ardito et al., 1980; Hopkin and Evans, 1980; Lambert and Lindblad, 1980; Carrano, 1982; Husum et al., 1982)
Livingston et al., 1983). However, this effect has not been observed in all studies (Hollander et al., 1978; Hedner et al., 1983).

Since the frequency of SCE has been found to be greatly increased on exposure to mutagenic agents, therefore, SCE is considered to be sensitive indicator of DNA damage (Latt, 1974; Perry and Evans, 1975; Carrano et al., 1978; Gebhart, 1981, 1982, 1984).

**Chromosomal Aberration Vs Sister Chromatid Exchange:**

It has been found that there is a positive correlation between chromosomal aberration and SCE frequency for most of the mutagenic chemicals (Latt, 1974). But, there are some mutagenic agents which cause increase in chromosomal aberration only and not in SCE frequency e.g. X-rays (Perry and Evans, 1975), certain anti-tubercular drugs in combination in vivo (Madhuri et al., 1983) and some antibiotics in vitro (Manjula et al., 1984). On the other hand there are other mutagenic chemicals that increase SCE frequency only and do not increase chromosomal aberrations e.g. aniline (Abe and Sasaki, 1977; Ishidate and Odashina, 1977). Another group with a small number of mutagenic agents are also there which neither cause chromosome aberrations nor induce SCEs e.g. sodium azide (Arenaz and Nilan, 1981).

Thus SCEs and chromosome aberrations are two different endpoints depicting cytogenetic damage due to mutagenic action. The mechanism of these endpoints is probably different, one system can not replace the other. Therefore, both the endpoints should be scored to assess the genetic damage caused by drugs and chemicals.

**CELL GROWTH KINETICS:**

Cell cycle kinetics analysis is based on the differential staining technique of sister chromatids. It is a sensitive method for detecting delay or stimulation in human lymphocyte cultures. Potential of the mutagenic agent to produce an effect on the cells is tested by analysing the proportion of mitotic cells in their first, second and third replication
cycle ($M_1$, $M_2$ and $M_3$ respectively). (Tice et al., 1976; Crossen and Morgan, 1977; Morimoto and Wolff, 1980).

The rate at which mitosis occurs is measured by the mitotic index or proliferation index or replication index. A statistical difference between the test compound treated and control groups indicates the ability of the chemical to induce cell-cycle delay or cytotoxicity. In some instances this type of information can be important for indicating that metabolic activation in the test compound had occurred even though it was not clastogenic. Thus the index gives an idea about the stimulation of lymphocytes to divide and the mitotic activity in culture (Melarango and Smith, 1984; Sinha et al., 1984). Any change in mitotic index following drug exposure compared to concurrent control, provides information on mitostimulative or mitodepressive activity of the drug.

Treatment resulting in SCE formation has been found to induce cell cycle delay (Morimoto, 1983; Craig-holmes and Shaw, 1976). However, the relationship between the proliferation rate as measured by cell growth kinetics and the induction of SCEs is controversial: It has been found that SCE levels in B-lymphocytes (early proliferating cells) were significantly lower than those in T-lymphocytes from the same individual (Santessen et al., 1979). Again Snope and Rary (1979) and Block et al. (1982) also found a lower SCE frequency in the more rapidly dividing cell populations. Lindblad and Lambert (1981) found that though the percentage of B and T lymphocytes did not correlate with SCE frequency, a significantly higher SCE rate was presented in slowly proliferating cultures. On the other hand, low SCE and longer cell cycle were observed in leukemic cells (Becher et al., 1981) and increased SCE rate and shortened cell cycle time were observed in trisomic cells of a Down syndrome mosaic (Heidemann et al., 1983). Many other workers however failed to find any relationship between SCE rate and cell cycle kinetics for example, in second generation metaphases collected from different culture ties (Beek and Obe 1979). Morimoto and Wolff (1980) and Morgan and Crossen (1981) also found no significant differences
in SCE frequency. Guilotto et al. (1980) also concluded that the incidence of SCE appeared to be independent of the proliferating properties of cultured lymphocytes, such as length of the cell cycle, fast or delayed response to PHA and number of divisions performed in vitro. Lambert et al. (1982) set up repeated cultures in three tissue culture media and found a great variability in proliferation kinetics between tissue culture media and between donors. Speit et al. (1986) also studied the SCE frequency and proliferation of cells in repeated human lymphocyte cultures. They found no systematic relationship between the proliferation of culture and basal SCE values. Also, a great variation in the two parameters was observed in repeated cultures.

B. CYTOGENETIC EFFECTS OF STEROIDAL HORMONES

I. Estrogens:

According to Wheeler et al. (1986) estrogens are mitotic poisons which at high concentrations, cause metaphase arrest, abnormal cell division and chromosomal aberrations.

The overall conclusion that emerge from the results of present investigation reveals that estrogens are potent mutagens and they are well associated with a significant increase in the incidence of chromosomal abnormalities per cell as well as with an increase in the number of abnormal cells in lymphocyte cultures in vitro, when compared to the normal control culture. Both of the estrogens used in the present study i.e. mestranol and quinestrol, were found to be genotoxic. These synthetic steroidal hormones induce chromosomal aberrations in the absence as well as in the presence of metabolic activation (S9 mix.) Although at higher doses of these drugs the frequencies of chromosomal aberrations decreased drastically, probably due to cell death.

The two estrogens, mestranol and quinestrol, taken for the analysis are widely used drugs in the treatment of varieties of diseases. Mestranol
is a widely used oral contraceptive, besides it is being used in hormonal replacement therapy. It is a combination drug which is used with ethynodiol diacetate, norethynodrel or norethindrone. Quinestrol is used in replacement therapy which gets metabolized inside the body to ethinylestradiol. This metabolic product is the most widely used estrogen in oral contraceptive combination and one of the most potent estrogen known.

Reports on the mutagenic activity of sex steroids or oral contraceptives in vitro and in vivo are contradictory. Goh (1967) demonstrated a variety of complex chromosomal rearrangements in the peripheral blood leucocytes of women taking oral contraceptives. Carr (1967) found that there is no evidence of an increase in congenital anomalies in pregnancies which result after discontinuing oral contraceptives, although in another investigation he reported chromosome abnormalities in 6 out of 8 abortuses collected from women who became pregnant after taking oral contraceptives. Two types of anomalies were observed triploidy and X-monosomy. Trisomy was the most common type of chromosomal defect observed at birth (Carr, 1970). Goh and Carr suggested that the imbalance in steroidal hormones in the blood was the causative factor of chromosomal breaks. A somewhat lower frequency of chromosomal breaks was found in cross cultures after the women had stopped taking the pills. However, Stenchever and his associates (1969) did not find obvious chromosomal effects in vitro of various gestagens in lymphocyte cultures with varied concentrations and with controls. Goh (1967) suggested that the oral contraceptives may cause chromosome breaks as do some other drugs and pointed out that the breakage was not observed in peripheral venous sampling after discontinuation of "the pills". In another study by McQuarrie et al. (1970), cytogenetic changes of increased chromosome breakage and satellite association were observed in 23 patients using oral contraceptives. Cytogenetic aberrations in lymphocytes from women taking oral contraceptives have also been observed by Badr et
In contrast, another group of scientists demonstrated that oral contraceptive pills do not have potential to induce chromosome breakage in pill users (Shapiro et al., 1972). It was also found that synthetic hormones may induce cytogenetic abnormalities in germ cells of rats, dogs and hamsters. Littlefield et al. (1975) suggested, after comparing the results of cytogenetic studies of lymphocytes culture from normal women, pregnant women and women taking oral contraceptives, that the increase in breakages in cultures from the oral contraceptive users was not related with length of time on pill or stage of the pill cycle, and thus the synthetic hormones do not directly damage lymphocyte chromosomes. Timson (1969) dealt with the \textit{in vitro} and \textit{in vivo} cytogenetic effects of lyndiol (an oral contraceptive). He observed no chromosome abnormalities in either conditions. The same steroid was again studied by Bishun et al. (1975), who also did not find any significant effect on chromosomal aberration incidence. Another study involving chromosomal examinations of babies whose mothers had taken oral contraceptives was undertaken by Rice-Wray et al. (1970). They did not find significant level of structural or chromosomal abnormalities in the cells examined from the babies. Fitzgerald et al. (1973) compared the results of chromosomal aberrations in lymphocytes of women taking oral contraceptives for over 4.5 years from control women. He also reported no increase in chromosomal aberrations in their cells. DeGutierrez and Lisker (1973) studied 35 patients on mestranol and ethynodiol acetate, and failed to confirm any chromosomal abnormalities in the women after culturing their blood for 65 hours. These results were also confirmed by Mills et al. (1975), and Matton-Van-Leuven et al. (1974). On the other hand Littlefield and Mailhes (1975) observed a statistically significant increase in average breakages in a series of cultures from the group of oral contraceptive users. Rohbom and Hansmann (1974) found a slight but not significant increase of non-disjunction due tonorethisterone acetate treatment. Estradiol, the most potent naturally occurring estrogen, was found to induce very low
frequencies of hyperploidies in human synovial cells in vitro (Lycette et al., 1970), and various numerical aberrations in pig oocytes in vitro (Mc Gaughey, 1977). In human embryonal fibroblasts and kidney epithelial cells, estradiol was reported to induce structural chromosomal aberrations (Serova and Kerkis, 1974). In contrast, Banduhn and Obe (1985) observed no structural chromosomal aberration in human lymphocytes in vitro after estradiol treatment. In another study the use of hormonal contraceptives was found to be associated with a highly significant increase of chromosome aberrations (Pinto, 1986).

Ovral 50, an oral contraceptive, was found to be genotoxic in mice (Devi and Reddy, 1986) whereas, the same drug was unable to induce genotoxic effects in human lymphocyte cultures in vitro. Anovlar 21, another oral contraceptive containing estrogen, ethinyl estradiol, and progestin, norethisterone acetate, causes a statistically significant increase in chromosomal aberration in cultured mouse bone marrow (Shyama et al., 1991). Dhillon et al. (1994) found that mestranol was capable of causing chromosomal aberrations in mouse bone-marrow cells in vitro. Ethinyl estradiol, a potent estrogen, was found to induce chromosomal aberrations in male Syrian hamster kidney cells in vivo and in mouse bone-marrow cells in vivo. Estradiol-17b another potent estrogen was found to induce various structural chromosomal aberrations in human lymphocyte chromosomes in vitro (Ahmad et al., 2000a).

In the present study of the various chromosomal aberrations induced by mestranol and quinestrol, chromatid, and chromosome fragments and breaks were more frequent. In certain cases, at higher doses, multiple anomalies were also observed. Similar observations have been made earlier by McQuarrie et al. (1970) in human lymphocytes, and in anovlar treated plant chromosomes by Kabarity and Mazrooei (1984). Breaks and structural aberrations observed in the present study were not restricted to any particular chromosome or segment and were random. Few metaphases were also observed with 45 chromosomes and few others with 47 chromosomes. A few metaphases were observed with
endoreduplication in quinestrol treated cultures at highest dose and time duration. This effect might have resulted due to inhibition of microtubule formation during cell division. In most of the metaphases, more than one chromosomes were found affected. Because the breaks were random, no any particular fragile site was observed for any particular chromosome. It has been observed in earlier studies that the common effects of steroid hormones on chromosomes are stickiness in diakinesis and metaphase, and bridges and sometimes lagging chromosomes in anaphase (Hakeem and Amer, 1965). Anovlar has been found to cause chain of bivalents in addition to stickiness, which may be due to the sticking of the ends of the different bivalents with each other (Kabarity and Mazrooei, 1984). The trend of abnormalities caused by mestranol and quinestrol were similar. Quinestrol might have been metabolized into ethinyl estradiol, its parent compound, by the human lymphocytes as has been reported by Iskandar and Vijayalaxmi (1981). The potency of ethinyl estradiol as a mutagen is controversial (Stenchever et al., 1969).

In the observation of different chromosomal abnormalities in lymphocyte cultures in vitro, caused by estrogen mestranol and quinestrol, it was found that chromatid breaks were more frequent than chromosome breaks. This indicates that the drug introduces replication errors or breaks replicated chromatids (Ahmad et al., 2000a). Shyama et al. (1991) suggested that frequent chromatid breaks were due to the effects of the drug on the DNA strand at its late S-phase or after the DNA has replicated or duplicated. The peripheral lymphocytes are cells which remain in G0-stage of their cell cycle and do not divide inside the body. But the majority of chemical mutagens affect the chromosomes only when the cell is dividing and passing through the S-phase. When the DNA is uncoiled during S-phase for replication of the DNA molecule, the mutagens make action on it. Therefore, it is necessary for the cells to go through S-phase at least ones between treatment with the chemical mutagen and observations. So, the mitogen (PHA) stimulates
the lymphocytes to enter cell cycle in vitro. It can be said that the frequency of chromatid type aberrations observed, following exposure of $G_0$ cells to the drug, are produced by errors in DNA repair in cells in $G_2$ or S-phase. The chromatid type aberrations may be due to errors in DNA regions that have already replicated at the time of exposure or by errors in DNA replication on a damaged DNA template. Aberration is a rare occurrence in comparison to the amount of induced DNA damage, most of which is efficiently repaired. The kinetics of repair of different types of DNA damage affects the types of structural alterations produced. Most clastogenic chemicals produce aberrations almost exclusively by errors of DNA replication on an adducted template. However, given the relatively slow overall rate of repair of DNA damage induced by most chemicals, there is least probability to produce aberrations by repair error. The probability of obtaining such coincident repair is greatly enhanced by using a DNA repair inhibitor, such as cytosine arabinoside.

Chromosome-type aberrations are formed in cells in $G_1$ treated with chemicals that are commonly described as S-phase-dependent (Preston and Gooch, 1981). Thus, it is essential in any cytogenetic assay for so-called S-phase dependent chemicals to ensure that the cells observed at metaphase have passed through the S-phase between treatment and sampling. If the induced frequency of all aberration classes is required, cells should be sampled for metaphase analysis at their first mitosis after a single treatment. Since several types of aberration produce cell lethality and/or loss of an acentric fragment at division, observation at the second or subsequent mitosis after treatment leads to a lower aberration frequency than that induced (Preston, 1999).

The frequency of chromatid aberrations produced by DNA replication errors is influenced most strongly by the dose to the cell, the phase of the cell cycle, DNA repair kinetics, DNA replication fidelity and maintenance of cell cycle checkpoints. Thus, different cell types can have different sensitivity to chromosomal aberration induction as a consequence of differences in these factors.
Numerical changes which were also observed in the case of mestranol and quinestrol treatment (i.e. monosomics, trisomics and a rare event of diploidy) can arise from errors in chromosomal segregation. The apparent complexity of the control and the mechanisms of the mitotic process means that alterations of a number of components can result in failure to segregate the sister chromatids to separate daughter cells or in a failure to segregate a chromosome to either pole (Bickel and Orr-Weaver, 1996). The mechanisms underlying chromosomal loss are pertinent to those involved in the formation of centromere-containing micronuclei.

A limited set of chemicals has been demonstrated to cause aneuploidy through interaction with components of chromosome movement. These include benomyl, griseofulvin, nocodazole, colchicine, colcemid, vinblastine and taxol. These chemicals affect tubulin polymerization or spindle microtubule stability. Till date, other mechanisms of aneuploidy induction have not been clearly identified.

Three different doses i.e. 10, 25 and 50 mg/ml concentrations and three different time durations i.e. 24, 48 and 72 h were taken for this study. It was observed that at 10 mg/ml concentration mestranol is unable to induce significant level of chromosomal aberrations. Whereas quinestrol induces significant increase in chromosomal aberration only for 72 h of incubation. Both the drugs show maximum activity at 25 mg/ml of dose concentration, and the highest increase for 72 h of treatment. Whereas, at the highest concentration (50 mg/ml) mestranol did not induce significant increase in chromosomal aberration, but the other drug quinestrol affects the chromosome only for 24 h of treatment duration. This decline in the frequency of aberration with increase in the dose concentration for both the drugs and with increase in time duration at 50 mg/ml concentration for quinestrol, may be due to some or all of the following reasons, namely (i) repair of damaged genetic material; (ii) elimination of the drug and its metabolites; (iii) elimination of cells/chromosomes with damaged genetic material; (iv) inactivation of the
drug and its metabolites; (v) and due to cell cycle delay or cell killing effect.

Both the synthetic steroidal estrogens i.e. mestranol and quinestrol, were also evaluated for their cytogenetic effects in the presence of metabolic activation. Both the drugs were found to be ineffective at 10 mg/ml dose concentration whereas, highly effective at 25 mg/ml concentration. And also found effective at 50 mg/ml concentration in contrast to the results obtained in the absence of S9 mix. This increase in the chromosomal abnormalities may be due to the action of the metabolites of the drugs. Drugs are metabolized by the microsomal activation system. In this case the metabolites of the drug might have been more potent cytogenetically than the drug themselves and consequently have caused more chromosomal abnormalities. But these metabolites were less potent than the drugs in terms of their abilities to induce probable cell billing effect. The effect of mestranol on human lymphocyte chromosomes in the presence of S9 mix has not been studied earlier. But it has not been found potent enough to cause reverse mutation in *Salmonella typhimurium* in the presence of S9 mix. (Dhillon et al., 1994). However, in various other studies, in combination of different drugs in the presence of S9 mix, mestranol has been found to cause reverse mutation in *Salmonella typhimurium*. Quinestrol has not been studied earlier for its genotoxic effects either in the presence or in the absence of metabolic activation. But, ethinylestradiol, the parent molecule of quinestrol (to which it is metabolized to), has been found to be ineffective in causing mutation in *Salmonella typhimurium* and in Chinese hamster lung V79 cells in the presence of metabolic activation.

It was observed in the present investigation that both the estrogens mestranol and quinestrol affected the SCE frequency in a similar fashion that had been observed in the case of chromosomal aberrations. Maximum value of SCE was observed at 48 h of treatment durations for all dose concentrations. But statistically significant value of SCE was observed only for 25 mg/ml dose concentration in the case of mestranol
both in the presence and absence of $S_9$ mix. Quinestrol increased the frequency of SCE in human lymphocyte chromosomes in the absence of $S_9$ mix only for 25 mg/ml concentration. Whereas in the presence of $S_9$ mix the drug was effective at two concentrations i.e. 25 mg/ml and 50 mg/ml, but the frequency of SCE was maximum at 50 mg/ml concentration. The decrease in the frequency of SCE at the highest concentration might have been due to the cell killing effect of the drug. Similar type of observations have been made earlier also by different groups of scientists. Murthy and Prema (1979) reported an increased SCE frequency in peripheral lymphocytes of oral contraceptive users. It was again observed that estrogen progestogen combination contraceptive exhibited an increased frequency of SCE but the progestogen injectable contraceptive alone did not (Murthy and Prema, 1983). Similar results for estradiol were observed by Banduhn and Obe (1985). Dose response effect was observed by Kochhar (1988) for four different estrogens in cultured Chinese hamster ovary cells. The drug mestranol was found to induce SCE in mouse bone-marrow cells (Dhillon et al., 1994). Another group of researchers observed that the use of oral contraceptives had no influence on SCE (Husum et al., 1982). Hill and Wolff (1983) reported that estradiol and estriol were unable to induce SCE in human lymphocytes. Estradiol-17b was found to be effective in contrast to the above reports in inducing SCE in human lymphocytes in vitro in the presence as well as in the absence of $S_9$ mix (Ahmad et al., 2000). The combination of drugs mestranol and norethisterone were found to be ineffective in inducing SCE.

The mechanism of sister chromatid exchange is that it is produced during DNA synthesis and is presumed to be a consequence of errors in the replication process itself, perhaps at the site of stalled replication complexes (Painter, 1980; Heartlein et al., 1983). The creation of intracellular condition that slows down replication, for example, could also induce sister chromatid exchange (Preston, 1999).

The overall results of the present investigations on the cell growth
kinetics show that both the drugs inhibit *in vitro* proliferation of lymphocytes in the presence as well as in the absence of metabolic activation. It has been reported earlier that the estrogen, estradiol, strongly inhibits proliferation of human lymphocytes, but another estrogen, estriol, was a weak inhibitor (Hill and Wolff, 1983). Estradiol also inhibits DNA and protein synthesis in mouse fibroblasts which may result in the inhibition of cell proliferation, whereas the estriol did not inhibit DNA and protein synthesis (Kuchler and Grauer, 1962). Both in the presence and absence of metabolic activation estradiol was found to inhibit cell proliferation (Ahmad *et al.*, 2000a). Mestranol and quinestrol has not been evaluated for their cell growth inhibiting potential earlier, but the present finding is supported by earlier studies on most of the estrogens. Proliferation of lymphocytes in culture is dependent on the time of phytohaemagglutinin (PHA) mediated transformation of small resting (G<sub>0</sub>) lymphocytes into blast cells and the length of the cell cycle after the proliferation has taken place (Morimoto and Wolff, 1980). It has also been observed that T-lymphocytes respond to PHA first (Greaves *et al.*, 1974). As activated T-cells are necessary to produce a later B-cell response, so it is possible that these estrogens slow down the T-cell proliferation, thus B-cell transformation is delayed in treated cultures. On the other hand it has also been reported that the estrogens accelerate the mitotic activity immediately after administration of the first dose (Gopala Krishna and Inamdar, 1971). However, accelerated level never exceeds normal level that is established in the target tissues in due course. Cell growth is inhibited after estrogen exposure due to an extended G<sub>2</sub> phase. The average length of time for G<sub>2</sub> in actively dividing lymphocytes is 3.5 h (Hill and Wolff, 1983). The cells may be arrested in G<sub>2</sub> or may stop division due to damage to DNA and cell division may be blocked. Such G<sub>2</sub> blocks are well known after cells are X-rayed. Chinese hamster ovary cells has been reported to be blocked in G<sub>3</sub> phase when treated with nitrosourea (Rao and Rao, 1976). In the same study by using the premature chromosome condensation technique,
it was observed that a linear relationship exists between the number of extensively damaged $G_2$ chromosomes and the accumulation of $G_2$ cells in treated cultures, and it was suggested that chromosome damage is a contributing factor to the $G_2$ arrest (Rao and Rao, 1976).

**Progestins:**

The two important and widely used progestins i.e. norethindrone and norgestrel, were evaluated for their genotoxic potential. The results reveal the fact that norethindrone was not potent enough to cause genotoxic damage in human lymphocytes *in vitro*. Whereas the other progestin norgestrel was highly effective in inducing chromosomal aberrations at higher doses with a dose time relationship.

Norethindrone is an important oral contraceptive which is used either alone or in combination with mestranol or ethinylestradiol (both estrogens). Norgestrel which is a synthetic progestin with potentially all the actions and uses of progestins, it is marketed only as an oral contraceptive both as a single entity product and in combination with ethinyl estradiol. Genotoxic effects of either of two drugs have not been studied earlier in any test system. Whereas, genotoxic effects of various other progestins have been evaluated, Goh (1967) and Carr (1967) have reported chromosomal abnormalities in the peripheral blood lymphocytes of women taking oral contraceptives. Goh (1967) suggested that oral contraceptives may cause chromosome breaks and pointed out that the breakage was not observed in peripheral venous sampling after discontinuation of the pills and suggested that the individuals are capable of removing "mutant cells". Although it might be possible to remove the somatic mutant cells by discontinuation of the steroidal contraceptive, the germ cells may not be removed so easily. Ovulation can accomplish this slowly. The chances of pregnancy incorporating the mutant cell are possible if these cells are affected (McQuarrie *et al.*, 1970). Therefore, the greatest danger of oral contraceptives lies in the potential damage to the gametes. Since contraceptive hormones are in circulation, they
presumably have free access to germ cells. The existence of chromosome damage in these cells can lead to a hazardous situation.

Badr et al. (1972) have also reported about the cytogenetic aberration causing potential of progestogens in lymphocytes from women taking oral contraceptives. Whereas other studies have not demonstrated increased chromosome breakages in women taking progestins as oral contraceptive (Shapiro et al., 1972). Littlefield et al. (1975) have observed chromosome damage in lymphocytes from women exposed to progestins. They concluded that the effect of synthetic hormones on chromatin is not directly related to concentration of circulating hormone molecules or length of time of exposure. It has been reported that norethindrone does not have potential to induce cytogenetic damage in female swiss albino mouse bone-marrow cells in vitro. Norethindrone acetate, a synthetic derivative of norethindrone, was also studied by different groups of scientists and the result found was again contradictory (Stenchever et al., 1969; Shyama et al., 1991). Norgestrel has been reported to be studied in combination with ethinylestradiol. It induces cell transformation in primary baby rat kidney. The embryolethality induced by combination drug norethindrone and ethinylestradiol might have been due to mutation caused by these drugs.

In the presence of metabolic activation, similar types of results were observed. Norethindrone was not able to induce any genotoxic damage whereas norgestrel induced chromosomal aberrations with increased intensity, and a time and dose relationship observed may be due to the more potent metabolites in the case of norgestrel.

Norgestrel was found to induce aberrations such as breaks, gaps, terminal deletions and pulverization. A dose dependent increase in aberrations was also noted. Among the various aberrations observed, gaps, breaks and terminal deletions were very frequent and they increased significantly at higher doses. Gaps were found in significant frequencies even at 25 mg/ml, whereas breaks were observed in significant
frequencies only at 50 mg/ml dose concentration. CP also induced significant frequencies of aberrations. Metaphases with fragmentation and multiple aberrations were observed with CP-treated cultures. Aberration frequencies increased with increase in treatment duration of the various aberrations observed, breaks and gaps were seen at all time intervals, but pulverization was noted in few metaphases at maximum treatment duration and highest dose concentration only. In the case of norgestrel it was observed that chromatid breaks were more common than the chromosome breaks, as has been observed in the case of the estrogens in the present study. This indicates that the drug affects the DNA strand either at its late S-phase or after the DNA has replicated or duplicated.

The present result on SCE analysis shows that norethindrone did not induce significant increase in SCE frequency at any treatment duration and for any dose concentration. But the drug norgestrel was found to enhance the SCE frequency at significant level at 25 and 50 ug/ml concentration both in the presence as well as in the absence of $S_9$ mix. However, here also, higher incidence of SCE was observed in the presence of $S_9$ mix.

In some earlier studies contradictory results have been found regarding the SCE inducing potential of progestins. Husum et al. (1982) observed that oral contraceptives containing progestins do not enhance SCE frequency in the cultured lymphocytes of user women, though Murthy and Prema (1979) observed significant increase in SCE frequency in a similar study. In another study Murthy and Prema (1983) found that women using progestogen contraceptives have normal SCE rates. This may mean that the increased SCE rates observed in the combination type contraceptive users could be due to mutagenic activity of the estrogen or its metabolites. Norethindrone acetate has been found to increase significant level of SCE frequency in human male lymphocytes in vitro. There exists a relationship between increased SCE rates and altered mutation frequency (Carrano et al., 1979), the long term persistence of
increased SCEs in hormonal contraceptive users may have some ill effects on the health of users. It may be important to find out whether the changes in SCEs associated with the use of contraceptives could be reversible. Prema and Murthy (1983) reported that the frequency of SCE in eight women during the use of combinational contraceptives increased significantly and markedly decreased later on, in those women who discontinued the contraceptive. This may mean that cells with increased SCEs are being replaced with cells with 'normal' SCEs in these women. It may, therefore, be argued that due to persistent use of contraceptives the increased SCEs might have resulted into cell death because of excessive and intense point mutations.

The overall results of the present investigations on the cell growth kinetics showed that Norethindrone did not inhibit the \textit{in vitro} proliferation of lymphocytes both with and without metabolic activation. However, the other progestin studied i.e. norgestrel, strongly inhibited the proliferation of lymphocytes and arrested the growth both in the presence as well as in the absence of metabolic activation. The effect was more prominent in the presence of metabolic activation. Earlier studies show negative result about the cell proliferation inhibiting potential of progestins. Norethindrone acetate in combination with ethinylestradiol do not affect the mitotic index significantly (Shyama \textit{et al.}, 1991). Similar type of result was obtained for norgestrel in combination with ethinyl estradiol by Kabarity and Mazrooei, (1984). It may be suggested that inhibited growth of cells after norgestrel treatment was observed due to an extended $G_2$ phase.

The reduction in mitotic index (MI) with increasing dose concentration of norgestrel may be attributed to inhibition of DNA synthesis, preventive effect of the drug on protein binding in the cells and induction of genetic damage. Widemayer and Shaver (1972) studied the chromosome complement of blastocysts recovered from rabbits injected with progesterone or estradiol during the time of ovum maturation or the first cleavage division. They found that the spindle
fibre apparatus was disturbed due to the treatment. A lower mitotic index than normal was found in whole mounts of those blastocysts.

Androgens:

In the present study the results of the androgens i.e. danazol and oxymethalone show that both the drugs are not potent enough to induce statistically significant increase in chromosomal aberrations in human lymphocyte chromosomes in vitro either in the presence or in the absence of metabolic activation (S₉ mix). Very little work has been done on androgens in the past time. Testosterone did not induce gene mutation in Salmonella typhimurium and shown negative result in micronucleus test in vivo (Ingerowski et al., 1981). SCE analysis reveals the fact that both the drugs did not enhance the SCE frequency either in the presence or in the absence of metabolic activation. The values obtained were not signification statistically at P < 0.05. Similar types of results were obtained for cell growth kinetics assay. Both the drugs i.e. danazol and oxymethalone were found unable to inhibit cell proliferation in the presence as well as in the absence of metabolic activation.

Thus it was concluded that androgens in general, and danazol and oxymethalone in particular are not genotoxic and do not have potential to induce significant level of structural or numerical chromosomal aberrations, sister chromatid exchanges and cell proliferation inhibiting effect at any concentration and for any exposure duration in human lymphocyte chromosomes. It was observed that metabolites of both the drugs (i.e. drugs in the presence of S₉ mix) were also ineffective in producing any type of genotoxic effect.

Thus, the overall conclusion may be derived from the present investigation that the use of synthetic steroidal drugs, particularly those drugs which are used as oral contraceptives, is associated with a significant increase in the incidence of chromosome abnormalities per cell as well as with an increase in the number of abnormal cells, and also with gene mutation which is exhibited as SCE. It is established that
there is an apparent relationship of chromosome abnormalities, sister chromatid exchanges and cell growth kinetics with mutagenesis, carcinogenesis (Mitelman and Pero, 1979; Sandberg, 1980) and teratogenesis (Purtilo et al., 1978; Joshi et al., 1983). Therefore, in vitro screening for chromosome aberrations and other cytogenetic damage has become an established tool in the evaluation of potential hazards to the genetic material due to environmental agents. Synthetic steroidal drugs particularly those drugs which are used as contraceptives have been more widely studied than any other drug in history (Pinto, 1986). However, the great limitation of this morphological approach is that only gross genetic damage is visible in chromosomal aberration and negative findings do not exclude other types of genetic damage such as those resulting in point mutations. Although point mutations are visible in the case of SCEs, but when the value of SCE is insignificant the point mutations are not considered hazardous, although a single base pair change sometimes lead to death of the cell. Therefore, there must be a system to detect single gene mutations also. The present observation also suggest that for a complete evaluation of mutagenic effects, a battery of test systems should be used, since no single test can be expected to detect all mutagens and mutations.

The phenotypic expression of genetic damage, either as visible chromosome aberrations or single point mutations, is dependent on the developmental time when the damage occurs, namely pre-or post-natal life and the type of cell, somatic or germinal, in which the damage occurs (German, 1979). If such damage occurs in somatic cells of the developing embryo it can lead to malformations and abortions in extreme cases. The malformations could be the result of (a) chromosome breakage with consequent extensive cell death; (b) unbalanced stable chromosome abnormalities; (c) single gene mutations. The teratogenic effects of synthetic steroidal drugs might have been the result of genetic damage reported in several studies (Nora and Nora, 1973, 1975; Janerich et al., 1974, 1980; Royal College of General Practitioners, 1976; Janerich and
Piper, 1978; Bracken et al., 1978; Rothman and Louik, 1978; Kasan and Andrews, 1980; Harlap et al., 1979; Joshi et al., 1983). The reasons could be explained by one or more of the above described mechanisms. An excess of chromosomally abnormal spontaneous abortions have been reported in mothers who use synthetic steroids as oral contraceptives when compared with non-users (Alberman et al., 1976). Any genetic damage occurring in germ cells of a person taking these drugs would only be manifested in his or her offspring either in the form of single gene mutations or chromosome abnormalities leading to teratogenesis or carcinogenesis. Even if a recessive mutation or balanced chromosome abnormality is not expressed in the first generation, it could possibly manifest several generations later, due to fetal germ or somatic cell mutations.

The theoretical possibility of genetic damage during post-natal life will apply directly to the health of the drug user. Any mutational effect on adult somatic cells could lead to cancer and although data are accumulating on the possible association between the use of these drugs and different types of cancers, yet these are not conclusive and still more study need to be done (Nissen et al., 1978; Royal College of General Practitioners, 1981; Swan and Petitti, 1982; Weiss et al., 1976).

Therefore, care should be taken of the potential hazards of such drugs, especially if they are in use for longer periods of time. The basic chemical structure of all synthetic steroids used in this study is same i.e. they are composed of 1,2, cyclopentanoperhydrophenanthrene ring. Then what is the factor which is responsible for a drug to become genotoxic? May be the molecular structure of a drug is the deciding factor to determine whether the drug is harmful or not. Another mechanism involved is the generation of free oxygen radicals in the growth medium which results in an increase of chromosome breakage and SCE rates of human lymphocyte cultures. This can be prevented in the presence of the enzyme superoxide dismutase (Emerit et al., 1981).
Free oxygen radical species are supposed to be generated by electrophilic groups on the synthetic steroids (Islam et al., 1991). The synthetic steroids initiate the SOS response and thus brings about the mutation in DNA. The DNA damage and/or mutation with the concomitant SOS-like response brought about by mutagenic steroids might also play an important role in the neoplastic transformation. Oxygen radical formation in the living system increases the risk of chemical carcinogenesis and certain steroids might be instrumental in the causation of cancer owing to their typical ring structure which could intertact with the DNA bases on the one hand and might also provide a favourable micro environment for the generation of toxic radicals on the other. The mutagenicity appears to involve the formation of hydrogen peroxide as well as superoxide and hydroxy radicals (Islam et al., 1991).

Therefore, it can be concluded that androgens (danazol and oxymetholone) and the progestin, norethindrone, do not produce free oxygen radicals in the lymphocytes. Their molecular structure differed from the estrogen (mestranol and quinestrol) and the progestin, norgestrel, in such a way that they were not able to produce free oxygen radicals and thus were not able to induce significant level of cytogenetic damage in the lymphocytes. Whereas, both the estrogens (mestranol and quinestrol) and the progestin, norgestrel, were having such a molecular structure that they generated free oxygen radicals in the human lymphocytes and induced mutagenesis.

It is, therefore, advisable to be careful of the potential hazards of these drugs to which we are continuously exposed day in and day out in modern life. 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 directly in their unmetabolized as well as metabolized state.
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Appendix
Aberrations induced by Mestranol

Fig.A. Cell with chromatid gap and chromatid break.

Fig.B. Cell showing diploidy

Fig.C. Cell with quadri-radial chromosome
Aberrations induced by Mestranol

Fig. D. Cell with chromatid gap, chromatid break and displaced fragment.

Fig. E. Cell with chromatid break, secondary constriction and dicentric chromosome
Aberrations when treated with Quinestrol

Fig.F.  Cell with chromatid break

Fig.G.  Cell with extreme chromosome shortening

Fig.H.  Cell showing chromatid gap and chromatid break
Aberrations when treated with Quinestrol

Fig. I. Cell with endoreduplication

Fig. J. Cell with chromatid gap and chromatid separation
Some of the anomalies when treated with Norgestrel

Fig.K. Cell showing chromatid deletion and chromatid break

Fig.L. Cell showing chromosome deletion, chromatid deletion, displaced fragment and double minute
Some of the anomalies when treated with Norgestrel

Fig. M. Cell showing pulverization

Fig. N. Cell with sister chromatid exchanges

Fig. O. Cell with sister chromatid exchanges
Publications
Genotoxic effects of estradiol-17β on human lymphocyte chromosomes

Md. Equebal Ahmad, G.G.H.A. Shadab, Afsahul Hoda, Mohammad Afzal

Section of Genetics, Department of Zoology, Aligarh Muslim University, Aligarh 202002, India

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Abstract

The cytogenetic effect of a hormonal steroid, estradiol-17β, was assessed in peripheral blood human lymphocyte culture. Sister chromatid exchanges (SCE) and chromosome aberrations (CA) were scored as genetic end points. Significant induction of CA was observed at 25 μg/ml and 50 μg/ml concentrations of estradiol-17β in the absence of microsomal activation. The drug was effective in all treatments in the presence of rat liver S9 microsomal fraction (S9 mix) and exhibited increased frequency of chromosomal aberrations. The drug was effective in increasing the SCE frequency which was found to be maximum at the dose of 50 μg/ml concentration (i.e., 4.34 ± 1.22) both with and without metabolic activation. It was found that estradiol-17β itself and possibly its metabolites are potent mutagens beyond a particular dose in human lymphocytes. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Estradiol 17β, Chromosomal aberration, Sister chromatid exchange, Lymphocyte

1. Introduction

Sex hormonal steroids are used as medicine for human for a large variety of conditions, apart from their uses as oral contraceptive agents, viz., in the treatment of dysmenorrhea, endometriosis, dysfunctional uterine bleeding, climacterics and in the prevention of postmenopausal osteoporosis. They diffuse through the cell membrane and after conjugating with specific receptor proteins in the cytoplasm, pass through the nuclear pore to form steroid–DNA complexes. This complex alters the gene expression of many cellular processes including enzyme synthesis [1]. Estradiol also binds to isolated DNA [2,3] and induces fragmentation [4]. Several sex steroids have been studied for their teratogenic [5], carcinogenic [6], mutagenic and clastogenic [7] potentialities. Estrogens have been identified as mitotic inhibitors. It was suggested that the structural specificity is required for mitotic inhibition [8]. Estrogens are mitotic poisons, which at high concentration, cause metaphase arrest, abnormal cell division and CA [7]. Estrogens also induce the formation of endogenous DNA adducts both in human and animals and can ultimately lead to cancer development [9,10]. Contraceptive steroids induce DNA repair synthesis in rat hepatocytes cultures, indicating DNA damage [11]. These drugs can also have a tumor inducing potential in rat liver in addition to its tumor...
promoting activity [12]. Estradiol is carcinogenic in experimental animals. Administration of estrogens leads to an increase in endometrial cancer in women, and estradiol can be expected to have a similar effect [13]. Estradiol-17β is one of the sex hormones, and occurs in two forms alpha and beta. Beta estradiol has the greatest physiological activity of naturally occurring estrogens. The alpha form is relatively inactive. Estradiol was found to induce very low frequencies of hyperploidies in human synovial cells in vitro [14] and various numerical aberrations in pig oocytes in vitro [15]. In human embryonic fibroblasts and kidney epithelial cells, estradiol was reported to induce structural chromosomal aberration [16]. Simultaneous addition of estradiol, progesterone and human chorionic gonadotrophin (HCG) increased the SCE frequency in human lymphocyte culture, but their separate administration did not induce SCEs [17]. The hormone is negative in the Ames test [6] and does not induce point mutations in mammalian cells in vitro [18]. In the present study, we investigated the genotoxic effect of estradiol-17β on human chromosomes in lymphocytes cultures in vitro.

2. Materials and methods

2.1. Chromosomal aberration analysis

Metaphase chromosome analysis for the detection of CAs was performed according to conventional technique [19]. Lymphocyte cultures were set up by adding 0.5 ml of heparinized whole blood, from two adult and healthy male 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 (1 mM; Gibco). Lymphocytes were stimulated to divide by adding 0.1 ml of phytohaemagglutinin-M (PHA-M; Microlab). The cultures were incubated at 37°C and 5% CO₂, for 72 h in the dark. Estradiol-17β (Wyeth Lab, India), at a final concentration of 10, 25 and 50 μg/ml was added for 24, 48 and 72 h of duration by adding the steroid after 0, 24 and 48 h in reverse order after the initiation of cultures. Dimethylsulphoxide (DMSO 5 μg/ml; SRL Bombay, India) was added to the simultaneously kept cultures for 72 h of incubation as negative control. And for positive control, cyclophosphamide (CP 1 × 10⁻⁷ M) was added for 24, 48 and 72 h of incubation.

In the metabolic activation experiments, 6 to 30 h old cultures were given treatment with 10, 25 and 50 μg/ml concentrations of the drug in the presence of rat liver S₉ microsomal fraction. Liver S₉ fraction was prepared from healthy albino rats induced with phenobarbital and it was stored in liquid nitrogen until use. S₉ mix was freshly prepared for use. The cells were collected after centrifugation and the pellets were washed twice in the pre-warmed medium to remove the chemical and the S₉ mix. The parallel cultures receiving the same doses of the drug for similar treatment duration but without S₉ mix were also simultaneously set for comparison. Colchicine (0.20 μg/ml; Microlab) was added to the cultures prior to harvesting. The cells were collected by centrifugation (10 min; 1200 rpm), hypotonitric treatment (0.075 M KCl) was given for 10–12 min at 37°C 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.

2.2. Sister chromatid exchange analysis

Analysis of SCEs was carried out following the fluorescent plus Giemsa techniques [20,21]. The cells in the cultures were exposed to 5-bromo-2-deoxyuridine (BrdU 2 μg/ml; Sigma) after 24 h of the initiation of cultures. The test compounds were added together with the BrdU. To minimize photolysis of BrdU another 48 h of cultures were maintained in the dark.

For SCE analysis in the presence of metabolic activation system, the drug along with S₉ mix was added into the culture after 48 h of its initiation and re-incubated at 37°C. After 90 min of this pulse treatment the cells were spun down and the supernatant discarded. The cells were washed twice to remove the traces of the steroid and the liver metabolites. Finally, the cells pellets were re-suspended in
fresh medium supplemented with fetal calf serum, PHA-M and BrdU, and put to culture for another 24 h in the dark at 37°C. For comparison, parallel cultures without S$_{0}$ mix were also set simultaneously. Hoechst 33258 stain (0.5 µg/ml, Sigma) was used along with 10% Giemsa stain for differential staining of the sister chromatids. The slides were coded prior to scoring, and 50 well-spread metaphase cells were scanned per concentration and the number of exchanges scored.

3. Results

3.1 Chromosomal aberrations

The chromosomal aberration frequencies in human lymphocytes induced by 10 to 50 µg/ml estradiol-17β are presented in Table 1. At 10 µg/ml of dose, the drug was not effective at any exposure time. At 25 µg/ml and 50 µg/ml of concentration, the drug affects both the aberrant metaphases as well as chromosomal aberration frequency. At 25 µg/ml, the values for 24, 48 and 72 h of incubations were 18.20%, 18.80% and 48.60%, respectively, while at 50 µg/ml the values were 19.33%, 21.00% and 54.34% at 24, 48 and 72 h of incubations, respectively, which were highly significant at $p < 0.05$ compared to the normal control value.

The drug was effective in all treatments and an increased frequency of aberrant metaphases as well as chromosomal aberrations were observed when treated with S$_{0}$ mix (Table 2). These values were 9.60%, 20.00% and 18.00% at 10, 25 and 50 µg/ml of concentrations, respectively. These data were highly significant at $p < 0.05$ when compared to normal control (3.5%). The percentage of chromosomal aberration was 61.00% for CP, which was highly significant.

3.2 Sister chromatid exchanges

Data on the mean frequency of SCEs for cells after the treatment of estradiol-17β are presented in Table 3. The enhanced SCE rate was observed and at

### Table 1

<table>
<thead>
<tr>
<th>Treatment ($µg/ml$)</th>
<th>Duration (h)</th>
<th>Metaphase scanned</th>
<th>Percent aberration metaphase</th>
<th>Types of aberration (%)</th>
<th>Aberration/cell + SE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Estradiol 17β</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>24</td>
<td>300</td>
<td>7.33</td>
<td>3.70</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>300</td>
<td>5.00</td>
<td>2.70</td>
<td>0.03 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>300</td>
<td>8.30</td>
<td>1.50</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>25</td>
<td>24</td>
<td>300</td>
<td>11.34</td>
<td>12.90</td>
<td>0.18 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>300</td>
<td>26.67</td>
<td>11.50</td>
<td>0.19 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>300</td>
<td>39.70</td>
<td>21.30</td>
<td>0.48 ± 0.04</td>
</tr>
<tr>
<td>50</td>
<td>24</td>
<td>300</td>
<td>16.00*</td>
<td>13.33</td>
<td>0.19 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>300</td>
<td>23.00*</td>
<td>12.67</td>
<td>0.21 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>300</td>
<td>39.67</td>
<td>23.67</td>
<td>0.54 ± 0.05</td>
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<td><strong>Control</strong></td>
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<td>Normal</td>
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<td>300</td>
<td>2.30</td>
<td>1.30</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>300</td>
<td>3.30</td>
<td>2.00</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>DMSO (5 µg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP (−ve control)</td>
<td>24</td>
<td>300</td>
<td>23.00</td>
<td>24.00</td>
<td>0.30 ± 0.03</td>
</tr>
<tr>
<td>(1 × 10 M)</td>
<td>48</td>
<td>300</td>
<td>39.00</td>
<td>44.00</td>
<td>0.68 ± 0.09</td>
</tr>
<tr>
<td>(+ve control)</td>
<td>72</td>
<td>300</td>
<td>40.00</td>
<td>45.00</td>
<td>0.66 ± 0.09</td>
</tr>
</tbody>
</table>

CP: Cyclophosphamide, DMSO: dimethyl sulfoxide, SE: standard error
Significantly at $p < 0.05$
Table 2
Chromosomal aberrations in human lymphocytes after estradiol-17β treatment with and without metabolic activation

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Metabolic activation</th>
<th>Metaphase scanned</th>
<th>Percent aberration metaphase</th>
<th>Types of aberration (%)</th>
<th>Aberration/cell ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Including gap</td>
<td>Excluding gap</td>
<td>Chromatid</td>
</tr>
<tr>
<td>Estradiol-17β</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>S₀</td>
<td>300</td>
<td>8.70*</td>
<td>7.90*</td>
<td>6.60</td>
</tr>
<tr>
<td></td>
<td>+ S₀</td>
<td>200</td>
<td>10.50*</td>
<td>9.00*</td>
<td>8.60</td>
</tr>
<tr>
<td>25</td>
<td>S₀</td>
<td>300</td>
<td>11.30*</td>
<td>11.30*</td>
<td>12.50</td>
</tr>
<tr>
<td></td>
<td>+ S₀</td>
<td>200</td>
<td>16.00</td>
<td>15.00*</td>
<td>15.00</td>
</tr>
<tr>
<td>50</td>
<td>S₀</td>
<td>300</td>
<td>10.00*</td>
<td>9.30*</td>
<td>8.30</td>
</tr>
<tr>
<td></td>
<td>+ S₀</td>
<td>200</td>
<td>14.00</td>
<td>13.00*</td>
<td>13.50</td>
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<td>Control</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>− S₀</td>
<td>300</td>
<td>2.30</td>
<td>1.70</td>
<td>1.30</td>
</tr>
<tr>
<td></td>
<td>+ S₀</td>
<td>200</td>
<td>4.50</td>
<td>3.00</td>
<td>3.50</td>
</tr>
<tr>
<td>DMSO (5 µg/ml)</td>
<td>− S₀</td>
<td>300</td>
<td>3.30</td>
<td>1.70</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>+ S₀</td>
<td>200</td>
<td>4.50</td>
<td>3.50</td>
<td>3.50</td>
</tr>
<tr>
<td>CP (1 × 10⁻⁷ M)</td>
<td>− S₀</td>
<td>300</td>
<td>6.30*</td>
<td>4.67*</td>
<td>10.00</td>
</tr>
<tr>
<td></td>
<td>+ S₀</td>
<td>200</td>
<td>40.50*</td>
<td>39.50*</td>
<td>37.50</td>
</tr>
</tbody>
</table>

CP: Cyclophosphamide, DMSO: dimethyl sulphoxide, SE: standard error

50 µg/ml of concentration the maximum frequency of SCE per cell was found to be 4.34 ± 1.22. This value was highly significant compared to the mean SCE value of normal control, i.e., 1.74 ± 0.14.

In the presence of S₀ mix, both doses (25 and 50 µg/ml) enhanced the SCE frequency significantly, i.e., 4.58 ± 0.41 and 6.08 ± 1.31, respectively (Table 4). Both these values were significantly higher than

Table 3
Sister chromatid exchange (SCE) in human lymphocytes after estradiol-17β exposure in vitro

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Duration (h)</th>
<th>No of metaphases</th>
<th>Total SCE</th>
<th>Range</th>
<th>SCEs/cell ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol 17β</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>48</td>
<td>50</td>
<td>76</td>
<td>0-6</td>
<td>1.52 ± 0.14</td>
</tr>
<tr>
<td>25</td>
<td>48</td>
<td>50</td>
<td>171</td>
<td>1-9</td>
<td>3.42 ± 0.52²</td>
</tr>
<tr>
<td>50</td>
<td>48</td>
<td>50</td>
<td>217</td>
<td>2-11</td>
<td>4.34 ± 1.22²</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>48</td>
<td>50</td>
<td>87</td>
<td>0-5</td>
<td>1.74 ± 0.14</td>
</tr>
<tr>
<td>DMSO (5 µg/ml)</td>
<td>48</td>
<td>50</td>
<td>96</td>
<td>0-6</td>
<td>1.92 ± 0.12²</td>
</tr>
<tr>
<td>(− ve control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP (1 × 10⁻⁷ M)</td>
<td>48</td>
<td>50</td>
<td>340</td>
<td>2-11</td>
<td>6.80 ± 1.29*</td>
</tr>
<tr>
<td>(+ ve control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CP: Cyclophosphamide, DMSO: dimethyl sulphoxide, SE: standard error

Significant at p < 0.05
Table 4
Sister chromatid exchange (SCE) in human lymphocytes after estradiol-17β with metabolic activation

<table>
<thead>
<tr>
<th>Treatment (μg/ml)</th>
<th>Duration (h)</th>
<th>No of metaphases</th>
<th>Total SCE</th>
<th>Range</th>
<th>SCEs/cell ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol 17β</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>48</td>
<td>50</td>
<td>97</td>
<td>0-5</td>
<td>1.94 ± 0.50</td>
</tr>
<tr>
<td>25</td>
<td>48</td>
<td>50</td>
<td>229</td>
<td>1-11</td>
<td>4.58 ± 0.41*</td>
</tr>
<tr>
<td>50</td>
<td>48</td>
<td>50</td>
<td>304</td>
<td>2-12</td>
<td>6.08 ± 1.31*</td>
</tr>
<tr>
<td>Controls</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>48</td>
<td>50</td>
<td>78</td>
<td>0-5</td>
<td>1.56 ± 0.12</td>
</tr>
<tr>
<td>DMSO (5 μg/ml)</td>
<td>48</td>
<td>50</td>
<td>89</td>
<td>0-6</td>
<td>1.78 ± 0.11</td>
</tr>
<tr>
<td>CP (1 × 10⁻³M)</td>
<td>48</td>
<td>50</td>
<td>380</td>
<td>4-12</td>
<td>7.60 ± 1.34*</td>
</tr>
<tr>
<td>(+ ve control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CP: Cyclophosphamide, DMSO: dimethylsulphoxide, SE: standard error
*Significant at p < 0.05

the mean SCE value of 1.56 ± 0.12 observed in normal controls (p < 0.05)

4. Discussion

The results of the present investigation demonstrate that the use of estradiol-17β is associated with clastogenicity. A significant increase in the incidence of chromosomal abnormalities per cell as well as an increase in the number of abnormal cells were observed when compared with normal control. At 10 μg/ml of concentration, the results were insignificant for all treatment duration (24, 48 and 72 h). However, at 25 μg/ml concentration a significant increase in aberration frequency was observed. But the maximal effect was observed at the highest concentration, an observation in line with the majority of clastogens. Of the various chromosomal aberrations induced by estradiol-17β, chromatid and chromosome fragments and breaks were more frequent than other types of aberrations, such as ring and bridge formation, acentric fragments, etc.

The increased clastogenicity by estradiol-17β at the longest treatment duration as compared to the shorter treatment duration could be attributable to the inherent ability of the human lymphocytes to metabolize certain compounds per se [22]. The common effects of steroid hormone on chromosomes are stickiness in diakinesis and metaphase, and bridges and sometimes lagging chromosomes in the anaphase [23]. Similar observations were made in Anovlar (a steroid) treated plant chromosomes [24]. Chromatid breaks were seen more frequently than chromosome breaks in the metaphase plates analyzed. This indicates that the drug introduces replication errors or breaks replicated chromatids. Estradiol-17β induced reversible mitotic delay in HeLa cells, which were localized at the beginning of mitosis. The duration of mitotic lag was dose dependent [25]. The present data on the time–response analysis revealed a significant increase in chromosomal aberration at 72 h of treatment [26].

In the presence of S, a microsomal activation system, the clastogenic effects of estradiol-17β were more prominent. In the absence of S, mix, the chromosomal aberrations observed were also significant but slightly less. The time–response analysis in the presence of S, revealed significant increase at 48 h of incubation and decreased later on [27]. The maximum frequency of chromosomal aberration was found at 48 h long treatment on mouse bone marrow cells, while it decreased later on and gave no significant result. This suggests that the drug and/or its metabolites were active during this (48th h) period. The decline in the frequency of aberrations observed at the later time intervals may be due to some or all of the following reasons: (i) repairs of damaged genetic material, (ii) elimination of the drug and its metabolites, (iii) elimination of cells/chromosomes with damaged genetic material, (iv) or due to cell
cycle delay and cell killing effect, and (v) inactivation of drug or its metabolites. The results obtained from the SCE analyses indicated the drug has strong SCE inducing potential. The frequency of SCE further increases if the drug is treated in the presence of S9 mix. There exists a relationship between increased SCE rates and altered mutation frequency [28]. Therefore, it can be concluded that estradiol-17β is capable of attacking the genetic material directly in its unmetabolized as well as metabolized state.

Because of the apparent relationship among chromosome abnormalities, mutagenesis and carcinogenesis [29], in vivo and in vitro screening for chromosone aberrations is an established tool in the evaluation of the potential hazards to the genetic material due to environmental agents. The theoretical possibility of genetic damage during post-natal life will apply directly to the health of the drug user. Any mutational effect on adult somatic cells could lead to cancer [30]. People should, therefore, be careful and aware of the potential hazards of such drugs, especially if they are continuously exposed to for such long periods.

Acknowledgements

Thanks are due to Dr Md Asim Azfer, Lecturer, Department of Zoology, for his valuable suggestions and to the Chairman of the Department of Zoology for his kind help and providing necessary facilities to complete this work.

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Cytogenetic effects of steroidal drug dexamethasone on human lymphocytes.

Md. Equebal Ahmad, G.G.H.A. Shadab and Mohammad Afzal
Section of Genetics, Department of Zoology, Aligarh Muslim University, Aligarh - 202 002 (India)

[Received 11 November 1998; Accepted 19 April 1999]

ABSTRACT: Dexamethasone, a steroidal hormone has potential application in pharmacology. This drug possesses anti-inflammatory, immunosuppressive, antibacterial, antimicrobial and antiviral activity. The cytogenetic effects of this drug was studied on human chromosomes in lymphocyte cultures in vitro and in vivo. There was no increase in the chromosomal aberration and sister chromatid exchange (SCE) frequency with dexamethasone at any concentration which was equivalent to plasma level and slightly higher than the plasma level in vitro. However, a significant increase in the frequency of chromosome aberration as well as SCE was seen in patients given therapeutic doses of dexamethasone when compared to the control of two types—(a) Arthritis patients before starting the drug therapy, and (b) the normal healthy individuals from the general population. The most frequently observed aberrations were chromatid gaps and breaks. Therefore, our study indicates that dexamethasone is clastogenic in vivo but not in vitro.

Key words: Dexamethasone - Chromosome aberration - Sister chromatid exchanges - Human lymphocytes - Arthritis patients

Introduction

Hormonal steroids are being widely used therapeutically. They form steroid-DNA complex after entering the cells, alter the gene expression and ultimately alter the expression of many cellular processes including enzyme synthesis. Quite a large number of hormonal steroids have been studied for their teratogenic (Joshi et al., 1983), carcinogenic (Lang and Redman, 1979) mutagenic and clastogenic (Wheeler et al., 1986) potentialities. However, most of the work has been concentrated on the sex hormones, while very little attention has been paid to the other members being extensively used as steroidal drug. Dexamethasone is one of the corticosteroidal drugs which is primarily used for curing severe inflammations, allergic reactions, shock, neoplasia and adrenal insufficiencies.

Reports on the mutagenic effects of corticosteroids are controversial. It could not exhibit mutagenicity in the Ames/Salmonella test system (Bali et al., 1990).

It has been reported that the administration of low dose of dexamethasone to rapidly dividing cells results in an inhibition on DNA synthesis in the liver cells in vitro (Loeb et al., 1973). The clastogenic effect of corticosteroid hormones on human lymphocytes is evident (Azfer and Afzal, 1996, Ilinskikh, 1979). The frequencies of sister chromatid exchanges (SCEs) and micronuclei also increase with dose, and thereby indicates the dose related clastogenicity of the drug in the in vivo system (Bali et al., 1990; Singh et al., 1994). A negative result was obtained by Dhillon (1990) that corticosteroids could not induce clastogenicity in human chromosomes. In the present study we investigated the effect of dexamethasone on human chromosomes in lymphocyte cultures in vitro and in vivo in the Indian population.

Materials and Methods

Chromosomal aberration analysis: Peripheral blood samples were obtained from healthy adults who were not exposed to mutagen. 0.5 ml of blood (lymphocyte) was cultured into 4.5
ml of TC-199 medium supplemented with 20% foetal calf serum, phytohaemagglutinin and antibiotics Dexamethasone (Wyeth Lab India), at a final concentration of 20, 40 and 60 µg/ml, was added at the 24, 48 and 72 h old cultures. Cultures without the drug served as controls.

In vivo studies were conducted in Arthritis patients from Jawaharlal Nehru Medical College, AMU, Aligarh. Patients who were on therapeutic doses of dexamethasone for over 4 weeks were examined. Two types of controls were taken: (i) Arthritis patients before taking any treatment and (ii) normal healthy individuals from the general population.

Whole blood was cultured by the method of Moorehead et al., (1960) and were prepared by flame drying method and stained with giemsa (10%) and screened for the presence of chromosomal aberrations.

Sister chromatid exchange (SCE) analysis: SCE analysis was performed using the fluorescent plus giemsa techniques of Perry and Wolff (1974). Bromodeoxyuridine (5' BrdU) was added to a concentration of 3 µg/ml. Simultaneously, 20, 40 and 60 µg/ml doses of dexamethasone were added in respective cultures at the 24 h. The cultures were prepared by air drying. One day old slides were stained with 50 µg/ml Hoechst 33258 in the dark for 30 min. They were further washed with distilled water, mounted in 2x SSC with cover slip, and exposed to sunlight for 2 h. They were again washed and stained in 5% giemsa. SCE were scored blindly in 50 M.I. cells per concentration.

Statistical analysis: The differences between the data of control and the experimental groups were statistically evaluated with Student’s ‘t’ test.

Results and Discussion

The concentrations tested were not cytotoxic to the lymphocytes. Very high and cytotoxic concentrations were not tested. No significant increase in the chromosomal aberration frequency could be observed in any of the treated cultures as compared to the normal cultures (Table 1). Time-dose relationship could not prove the genotoxicity of dexamethasone. The aberrations commonly observed were breaks, gaps, exchange and fragments at all concentration and duration of treatment.

Results obtained in the in vivo study of the effect of dexamethasone are summarised in Table 2. The difference between the values of general control and patient control in regard to chromosomal aberrations (3.00 ± 0.60 and 3.38 ± 0.62) was not significant. This indicates that Arthritis disorder per se does not exert any influence on the chromosomal aberration frequency. The frequency of chromosomal aberrations was significantly increased in patients under dexamethasone therapy as compared to the two types of controls (P < 0.05). The frequency of SCE analysis in human lymphocyte after dexamethasone exposure in vitro is presented in Table 3. The mean SCE frequency of the drug at 20, 40 and 60 µg/ml of doses were not significant as compared to the normal controls.

The results of in vivo SCE frequencies in human lymphocytes is described in Table 4. The difference between the mean SCE of the general control and patient control was statistically not significant. The mean SCE frequency of the patient exposed to dexamethasone on the other hand was found to be significantly higher than the mean SCE value observed in both the controls.

The results of the present investigation showed that dexamethasone did not induce chromosomal aberration in vitro but significantly increased the aberration frequency in vivo. The differences in the in vivo and in vitro studies can be explained on the basis of the fact that some chemicals and drugs are not mutagenic and clastogenic by themselves but require metabolic activation (Wright, 1980). It is probable that the products of dexamethasone metabolism could be clastogenic (Iskandar and Vijayalaxmi, 1981). The possibility that the increase in the aberrations in the cells of patients on dexamethasone therapy could be due to their Arthritis disorder is ruled out by the fact that there was no significant difference in the chromosomal aberration frequency between the untreated Arthritis patients and the normal healthy individuals from the general population. A predominance of chromatid gaps and breaks was observed suggesting, that the drug acts mostly in the late S or G2 phase of cell-cycle. The chromosomal aberration observed mainly in groups of A, B and C chromosomes are probably due to their larger size and more DNA contents. Similar observations were also made by Madhuri et al. (1981) in the case of combined chemotherapy with isoniazid and para-aminosalicylic acid in vivo, while Manjula et
Dexamethasone in human lymphocytes

113

Fig. 1

Fig. 2

Fig 3

Fig 4

Figs 1-4. 1. Analysis of human lymphocyte chromosomes treated with dexamethasone in vitro. 2. Chromosomal aberration analysis of patients exposed to dexamethasone. 3. Sister chromatid exchange in human lymphocyte after dexamethasone exposure in vitro. 4. Sister chromatid exchange in human lymphocyte of the patients exposed to dexamethasone. (CD-Control dexamethasone; GC-General control; PC-Patient control; PED-Patient exposed to dexamethasone).


It has been reported that glucocorticoids induce clastogenicity in human lymphocytes (Bali et al., 1990). The higher doses of the drug exhibit clastogenicity but with a lesser magnitude. The reduction could be attributed to their toxic effect at the highest concentration and the cells with increased aberrations could have been eliminated as a result of apoptosis (Thompson, 1994) and got replaced with normal cells perhaps due to compensatory division in the remaining healthy cells. Our findings are further corroborated by the reports of Loeb et al., (1973) and Rubin (1982)
indicating that the continuous presence of the
steroidal drug inhibits DNA synthesis, prevention
of protein binding of the drug causes reduction in
mitotic cell population and produce induction of
genetic damage following the treatment of the
glucocorticoid drugs in both animals and humans.

The non-mutagenicity of dexamethasone in the
in vitro system has been reported by Singh et al.,
(1994).

The increased frequency of chromosomal aberrations in vivo in patients continuously exposed to dexamethasone may be attributed to
Dexamethasone in human lymphocytes

the inherent ability of the human lymphocytes to metabolize certain compounds. Moreover, as dexamethasone has a longer biological half-life (36-54 h) than other corticosteroids, patients using dexamethasone had continuous presence of the drug which affected DNA and spindle forming microtubules (Banduhn and Obe, 1985). In the case of in vivo study it is clear that in arthritis patients adverse effect of dexamethasone in the lymphocytes did not give sufficient time for the possible repair of induced lesions in the DNA.

The frequency of SCE were enhanced in the patient who were under the regime of therapy SCE arise from the DNA breaks and the reunion of broken and exchanged fragments at the homologous loci (Latt et al., 1981). The observed rise in the frequency of SCE could be attributed to the dexamethasone’s influence on increasing the number of DNA lesions. The present study is in line with Naumova (1977) and Ilinskikh (1979) who observed DNA suppression and bone marrow deterioration effects due to the glucocorticoids. From the present findings it appears that dexamethasone is a genotoxic drug. The theoretical possibility of genetic damage during post-natal life will apply directly to the health of the drug user. Any mutational effect on adult somatic cells could lead to cancer (Weiss, 1976). One should, therefore, be mindful of the potential hazards of such drugs, especially if they are continuously used for such longer periods of time.

Acknowledgements

Thanks are due to Dr. M Asim Azfer, Lecturer, Department of Zoology for his valuable suggestions and to the Chairman, Department of Zoology for his help and providing necessary facilities to compile this work.

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Correspondence to
Dr. Mohammad Afzal
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EFFECT OF HYDROCORTISONE ON HUMAN LYMPHOCYTE CHROMOSOMES IN VITRO

G. G. H. A. Shadab, Md. Equebal Ahmed and Mohammad Afzal

Section of Genetics, Deptt. of Zoology, Aligarh Muslim University
Aligarh-202 002, U.P. India

Abstract
Hydrocortisone is a potent steroidal drug used therapeutically for a number of reasons. Hormonal steroids have been reported to cause damage to human chromosomes. To test genotoxicity, human lymphocytes were cultured in TC-199 medium supplemented with 20% fetal calf serum, phytohaemagglutinin and antibiotics. Hydrocortisone (Wyeth Lab India) at a final concentration of 10.25 and 50 µg/ml, was added at the 24, 48 & 72 hrs old culture. Moorehead et al. (1960) flame drying method were used for in vitro culture & staining was done with Giemsa (10%). Chromosome aberration analysis, Sister chromatid exchange (SCE) analysis and Statistical analysis was done. Present study showed that hydrocortisone affects the lymphocytes in vitro and induce chromosomal aberrations with increased frequency in the presence of metabolic activation, maximum aberration was recorded at 25 µg/ml concentration SCE also increased with higher dose but was less mutagenic at the highest concentration. Metabolic activation did not alter result in this case. It is concluded that this drug could inhibit the proliferation of human lymphocytes.

Introduction
Hormones are informational molecules secreted by endocrine glands in response to changes in the environment inside or outside the body. Hormones may stimulate or inhibit specific biological processes in the target organs to modify their activities. They play vital role in metabolic regulation, growth and control of several body functions. Natural Steroidal hormones (e.g. gonadal and adrenocortical hormones) or their synthetic derivatives perform their functions by forming steroid-DNA complexes that influence the gene expression (Rubin et al., Makin et al.). Several naturally occurring as well as synthetic steroids have since long been widely used therapeutically (Azfar 96). Several steroids have been reported to possess anti inflammatory (Boitralik et al.), anesthetic (Evelynne et al.), anabolic (Fennessey et al.), angiostatic (Larrian et al.), antiangiogenic (Judah et al.), cardioactive (Karel & Thomas) and antidiabetic (Ryooji et al.), activities.

Address for correspondence: *Dr. Mohammad Afzal, Reader, Section of Genetics, Deptt. of Zoology, A M U Aligarh-202 002, U. P. India.
Certain steroids have also been reported to control energy metabolism throughout pregnancy (Bird et al.). Steroid alkaloid formulations have been used for skin disorder treatment (Chain et al.). Also there are some steroidal compounds which increase resistance against drugs & toxic agents (Kourounakis). Sex hormonal steroids are used in human medicine for a large variety of conditions, apart from their uses as oral contraceptive agents viz in the treatment of dysmenorrhoea, endometriosis and dysfunctional utrine bleedings and climacterics. They are also used in the prevention of postmenopausal osteoporosis. Progestines have been used in the management of threatened abortion and to prevent premature labour.

Inspite of their therapeutic use there are many reports indicating the teratogenic (Joshi et al), mutagenic (McQuarrie et al, Wheeler et al) and carcinogenic (Lang and Redmann, Rosenfield et al) potentialities of these hormones. Most of the above reports are concerned with oral contraceptives or sex hormones being widely used and very little work has been done to evaluate the effects of other hormones being extensively used as drugs. Keeping this in mind the present experiment was done to observe the genotoxicity of some widely used steroidal drug. We evaluated the genotoxicity of several steroids in human invitro. Present work is about hydrocortisone which is a potent glucocorticoid and used primarily for curing severe inflammations, allergic reactions, shock, neoplasias and adrenal insufficiency (Labus & Lennon).

Materials and Methods

(a) In vitro testing of chromosomal aberrations—

(i) Preparation of culture media—

Tissue culture medium (TC 199, Flow Lab) with L-glutamine and Hepar buffer without NaHCO₃ was always prepared in advance and stored at 4°C but the storage never lasted longer than a week. 1.574 gm of medium was dissolved in 100 ml of triple distilled water by gentle shaking. Penicillin (100 IU/ml) & streptomycin (100 IU/ml Hoechst) were also added and the pH was adjusted from 6.8-7.2 with N/10 NaHCO₃ and HCl. The Medium was then filtered and sterilized in the negative pressure millipore filtration assembly by using 0.45 um millipore filters. This filtered medium was used for culture of lymphocytes.

(ii) Collection of blood samples—

Peripheral blood was collected from the healthy adult donors (20-25 years old). Blood was taken fresh everytime through vein puncture under septic conditions using disposable needles (21 gauge) and disposable syringes (Steriware). Heparin (500 IU/ml; Micro Lab) was used as anticoagulant. The tightly capped glass vials filled with blood were gently mixed and stored at 4°C for half an hour to separate red blood cells (RBCs) from plasma.

(iii) Setting of the cultures—

Lymphocyte culturing was done by adding 0.8 ml of plasma containing white blood cells (WBC) in 4.5 ml of culture medium supplemented with 0.1 ml phytohaemagglutinin-P (PHA-P, Microlab) and 15% foetal calf serum (Gibco). The
tightly capped culture vials were gently mixed and incubated at 37°C in dark for 72 hours. Colchicine (0.20 µg/ml Microlab) was added 2½ hours prior to harvesting to arrest the cells at metaphase stage.

(iv) Selection of doses

Only those doses, which do not reduce the MI but yield sufficient number of scoreable metaphases, were chosen for the experiment. On this basis the lower and the medium doses were selected and the highest dose selected was normally the maximum tolerated dose (MTD) beyond which the MI showed a steep decline. Following these norms the different doses selected for hydrocostisone were 10, 25 and 50 µg/ml.

(v) Studies with and without 59-mix.

Without 59-mix the cultures were exposed to three different concentrations for 24, 48 and 72 hours. Positive (CP, 1x10⁻⁷ m) and negative (DMSO, 5 µl/ml) control cultures were also simultaneously set for all treatment durations. For studying result with metabolic activation, 30 hours old cultures were given 5 hours treatment with different concentration of hydrocostisone in the presence of 59-mix. The parallel culture without 59-mix were done for comparison simultaneously. The DMSO (5 µl/ml; SRL, Bombay) were used as negative and positive controls respectively.

(vi) Harvesting of the cultures

After 72 hours the cultures were centrifuged (10 min, 1200 rpm) & the buttons of cells were saved by discarding the supernatant. Hypotonic treatment (0.075 M KC1) was carried out for 10-12 minutes at 37°C and the cells were recollected in 5 ml freshly prepared chilled fixative before preparing the slides.

(vii) Slide preparation and staining

After final washing in the fixative, the cells were resuspended in 0.2 ml of fresh fixative. Two to three drops of this cells suspension were dropped on clean, grease free, prechilled wet microscope slides which were then air dried. One day old slides were stained in carbol fuschin (Sigma) for 15 minutes and rinsed in 0.95% alcohol and finally in absolute alcohol for proper differentiation. After air drying, these slides were dipped in xylene for 5 minutes before mounting in depex (BDH).

(viii) Analysis of the Cells

To avoid mistakes in scoring of the chromosomal anomalies, all slides were coded prior to scoring. A total of 300 well spread metaphases were analysed for each concentration of the steroid testing and for each time duration to analyse various chromosome and chromatid-type aberrations. Evans' method of analysis was followed.

b) Analysis of Sister Chromatid Exchanges (SCE)

(i) SCE detection mechanism

Sister chromatid exchange is a sensitive rapid and objective method of observing reciprocal exchanges between sister chromatids. In this 5-bromo-2-deoxyuridine (BrdU) incorporate with DNA in place of thymidine (Azfer and Afzal). After two rounds of cell division the chromatids are labelled with BrdU and consequently these differentiate with Hoechst stain. Due to BrdU incorporation
the light energy is absorbed but not emitted by dyes which results in the reduced staining of Chromatid with Giemsa (Latt and Wolleb\textsuperscript{34}, Latt\textsuperscript{31,23}).

(ii) Labelling of chromosome with BrdU—

SCE analysis was carried out following the standard procedure of Latt et al.\textsuperscript{23} with suitable modifications. The cells in the cultures were exposed to nucleoside, BrdU (Sigma) after 24 hours of culture initiation at the final concentration of 2 \( \mu g/ml \). The culture vials were tightly capped and covered with tin foil to avoid light exposure and incubated at 37\(^\circ\)C for another 48 hours in dark. Doses of hydrocortisone were 10, 25 and 50 (\( \mu g/ml \)) with negative control (DMSO 5 \( \mu g/ml \)) & positive control (CP 1 \( \times 10^{-7} \) m).

(iii) Studies on the steroids without and with S9—

After 48 hours of the culture initiation, three different log doses of each steroids were added into the cultures without S9 mix. The culturing was further continued for 24 hours. Positive, negative and untreated controls were also simultaneously studied. For studying metabolic activation, steroids along with S9-mix was added into the cultures after 48 hours of its initiation and reincubated at 37\(^\circ\)C. After centrifugation the cell pellets were suspended in fresh, warm medium supplemented by foetal calf serum, PHA-P and BrdU and cultured for another 24 hours in dark at 37\(^\circ\)C. Positive, negative and control in the presence of S9-mix were also simultaneously studied for comparison.

(iv) Slide preparation and staining—

After 2½ hours of colchicine treatment, the cultures were harvested and processed following the same procedure as for the chromosomal aberration analysis. For the differential staining of SCEs, the methods of Perry and Wolft\textsuperscript{31} and Latt et al.\textsuperscript{23} with slight modifications were followed—

(v) Analysis of the cells—

100 second division metaphases (50 metaphases/donor) with differentially stained chromatids were scored for each steroid doses of steroid treatment in the absence of S9 and 50 metaphases (25 metaphases/donor) were scored for each treatment in the presence of S9 mix. The interstitial exchanges between two sister chromatids were scored as two exchanges and the terminal exchanges were scored as a single exchange. Students 't' test was applied for calculating the significance of difference between the treated and the controls.

c) Statistical Analysis—

All the dates were analysed statistically. Standard deviation (SD) and standard error (SE) were calculated by using the following formula—

\[
\text{mean } = \frac{X}{n} \\
\text{variance } = \frac{x^2 - n \times X^2}{n - 1} \\
\text{SE} = \frac{SD}{\sqrt{n}}
\]

Where \( X \) = variable
\( n \) = number of observations
\( x^2 \) = sum of square of individual variables
\( X \) = mean of variable.
Table 1
Chromosomal aberrations in human lymphocytes after Hydrocortisone treatment

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Duration (hr)</th>
<th>Metaphase scanned</th>
<th>Percent aberration Metaphase Including gap</th>
<th>Types of aberration (%) Chromatid</th>
<th>Chromosome</th>
<th>Total</th>
<th>Aberration/Cell±SE</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>24</td>
<td>300</td>
<td>7.3</td>
<td>3.7</td>
<td>0.0</td>
<td>3.7</td>
<td>0.04±0.01</td>
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<td>48</td>
<td>300</td>
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<td>2.7</td>
<td>0.03±0.00</td>
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<td>5.3</td>
<td>3.7</td>
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<td>3.3</td>
<td>0.07±0.01</td>
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<tr>
<td>25</td>
<td>24</td>
<td>300</td>
<td>11.3</td>
<td>11.3</td>
<td>12.3</td>
<td>5.6</td>
<td>18.0*±0.02</td>
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<tr>
<td></td>
<td>48</td>
<td>300</td>
<td>22.7</td>
<td>20.0</td>
<td>11.3</td>
<td>7.3</td>
<td>18.6*±0.02</td>
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<td>21.3</td>
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<tr>
<td>DMSO (5 µg/ml)</td>
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<tr>
<td>(−ve control)</td>
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<td></td>
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<td></td>
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<tr>
<td>CP (1x10⁻⁷ M)</td>
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<td>300</td>
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<td>24.0</td>
<td>6.0</td>
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<tr>
<td>(−ve control)</td>
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<td>300</td>
<td>48.0</td>
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<td>9.5</td>
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<td>35.0</td>
<td>48.0</td>
<td>20.0</td>
<td>68.0*±0.07</td>
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CP: Cyclophosphamide; DMSO: dimethyl sulfoxide; SE=standard error *significant at P<0.05.
Table 2
Chromosomal aberrations in human lymphocytes after Hydrocortisone treatment with and without metabolic activation

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<th>Types of aberration (%)</th>
<th>Aberration/Cell ±SE</th>
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</tr>
<tr>
<td></td>
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<td>200</td>
<td>40.5</td>
<td>39.5</td>
<td>42.5</td>
</tr>
</tbody>
</table>

CP: Cyclophosphamide; DMSO: dimethyl sulphoxide; SE = standard error; *significant at P < 0.05.
Students two tailed 't' test was used for calculating the statistical significance in SCE and chromosomal aberrations by comparing the effects induced by different doses of steroids with the respective control. The following formula was used for this purpose:

$$t = \frac{X_1 \text{ (control)} - X_2 \text{ (treated)}}{(\text{SE of control})^2 + (\text{SE of treated})^2}$$

Results

**a) Chromosomal aberrations**—Table-1 summarises the data on the influence of chromosomal aberrations. At 10 μg/ml of dose significant increase in the percentage of chromosomal aberrations was found when the drug was continued for 72 hours of incubation (7.3%). This value is higher than the normal control value (2.3%). At 24 and 48 hours of incubation, this dose did not influence the chromosomal aberrations (3.7% in 2.7%).

At 25 μg/ml of concentration the drug influenced the chromosomal aberration in all exposure times. The values were 18.0%, 18.6% and 48.7% at 24.48 72 hrs. of incubation. Increased frequency at this dose showed the significant result compared to the normal control at P < 0.05. However at 50 μg/ml of concentration no significant value were observed (4.0% & 3.5%). The decreased value may be attributed to the toxic effect at higher concentrations.

**b) Sister chromatid Exchange (SCE)**—

Table-3 shows the effect of hydrocortisone on the frequency of SCE in human lymphocytes. The mean SCE frequency at 10 μg/ml did not enhance it significantly (2.46 ± 0.19) when this is compared with normal control value (2.32 ± 1.7). The concentration with 25 μg/ml had a mean SCE frequency of 3.66 ± 0.28 which was significantly higher than the mean SCE value observed in normal control (P < 0.05). No significant value was observed at higher concentration i.e. 50 μg/ml. The mean SCE value was 2.5 ± 0.18, not found significant at P < 0.05. The data of the mean SCE frequency value of the positive control drug (CP) was 4.60 ± 0.68. The value was highly significant compared to normal control.

The effect of hydrocortisone on human lymphocytes in the presence of S9 mix has been summarised in Table-2. The mean percentage value at 10 μg/ml was 9.0 in the presence of S9 mix which was significantly higher than the mean percent of chromosomal aberration value of 2.3% observed in normal control (P < 0.05). At 25 μg/ml of concentration, the drug enhanced the rate of aberrations (20.0%), which gave a statistically significant value at P < 0.05. At 50 μg/ml of concentration no significant value were observed (4.0% & 3.5%). The decreased value may be attributed to the toxic effect at higher concentrations.
Table 3
Sister Chromatid Exchange (SCE) in human lymphocytes after Hydrocortisone exposure in vitro.

<table>
<thead>
<tr>
<th>Treatment (ug/ml)</th>
<th>Duration (hr)</th>
<th>No. of metaphases</th>
<th>Total SCE</th>
<th>Range</th>
<th>SCEs/Cell ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrocortisone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>48</td>
<td>50</td>
<td>123</td>
<td>0–6</td>
<td>2.46 ± 0.19</td>
</tr>
<tr>
<td>25</td>
<td>48</td>
<td>50</td>
<td>183</td>
<td>1–8</td>
<td>3.66 ± 0.36*</td>
</tr>
<tr>
<td>50</td>
<td>48</td>
<td>50</td>
<td>125</td>
<td>0–6</td>
<td>2.5 ± 0.28</td>
</tr>
<tr>
<td>Control Normal</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO (5 ug/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(−ve control)</td>
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<td></td>
</tr>
<tr>
<td>CP (1X10⁻⁷ M)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(+: ve control)</td>
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</tr>
</tbody>
</table>

CP : Cyclophosphamide; DMSO : dimethyl sulphoxide; SE : standard error
* significant at P < 0.05.

Table 4
Sister Chromatid Exchange (SCE) in human lymphocytes after Hydrocortisone exposure with metabolic activation (S₉ mix).

<table>
<thead>
<tr>
<th>Treatment (ug/ml)</th>
<th>Duration (hr)</th>
<th>No. of metaphases</th>
<th>Total SCE</th>
<th>Range</th>
<th>SCEs/Cell ± SE</th>
</tr>
</thead>
<tbody>
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<td>48</td>
<td>50</td>
<td>89</td>
<td>0–4</td>
<td>1.78 ± 0.16</td>
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<tr>
<td>25</td>
<td>48</td>
<td>50</td>
<td>197</td>
<td>0–8</td>
<td>3.94 ± 0.37*</td>
</tr>
<tr>
<td>50</td>
<td>48</td>
<td>50</td>
<td>144</td>
<td>0–6</td>
<td>2.88 ± 0.24*</td>
</tr>
<tr>
<td>Control Normal</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO (5 ug/ml)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>(−ve control)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>GP (1X10⁻⁷ M)</td>
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<tr>
<td>(+: ve control)</td>
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</tr>
</tbody>
</table>

CP : Cyclophosphamide; DMSO : dimethyl sulphoxide; SE : standard error
* significant at P < 0.05.
μg/ml of concentration in the presence of S9 mix enhanced the frequency of SCE. At 25 μg/ml, the mean SCE frequency was 3.94 ± 0.37 which was significant at P < 0.05 when compared with that of the normal control SCE (1.56 ± 0.18). A slightly decreased SCE frequency was observed and found significant with 50 μg/ml compared to normal control. The enhanced SCE at higher level of concentration of the drug may be due to the metabolic activation by liver enzyme (S9 mix). An increased mean SCE frequency was observed by CP (6.50 ± 1.30).

Discussions

Chromosome aberration assessment serves as a good indicator of the clastogenic effects of any genotoxic agent (Gebhart\textsuperscript{19}, Buckton & Evans\textsuperscript{7}, Hsu\textsuperscript{12}). Present study very evidently established the clastogenic potential of hydrocortisone in human lymphocyte cultures. Its effect was maximum at 25 μg/ml concentration. The highest concentration (50 μg/ml) also caused clastogenicity but with a lesser magnitude. This may be due to cell cycle delay and cell killing effect caused by the drug at the highest concentration (Ball \textit{et al.}\textsuperscript{25}). The present findings are further supported by the reports of Loeb \textit{et al.}\textsuperscript{35}, Naumova\textsuperscript{36} and Rubin\textsuperscript{33} indicating the suppression of DNA synthesis, prevention of protein binding of the drug at its higher doses, reduction in mitotic cell population and induction of genetic damage after the hydrocortisone and other glucocorticoid treatment in man.

Hydrocortisone also enhanced the frequency of chromosomal aberration in the presence of metabolic activation. SCE arise from DNA breaks and the reunion of broken exchanged fragments at almost homologous loci (Lett \textit{et al.}\textsuperscript{32}). We observed rise in the frequency of SCE with higher dose of hydrocortisone, but with lesser magnitude at the highest concentration. The effect was same even in the presence of metabolic activation. The rise may be due to the hydrocortisone's influence in increasing the number of DNA lesions.

Because of the apparent relationship between chromosome abnormalities, mutagenesis and carcinogenesis (Sandberg\textsuperscript{35}), \textit{in vivo} and \textit{in vitro}, screening for chromosome aberrations is an established tool in the evaluation of potential hazards to the genetic material due to environmental agents. The theoretical possibility of genetic damage during post-natal life will apply directly to the health of the drug user. Any mutational effect on adult Somatic cells could lead to cancer (Weiss \textit{et al.}\textsuperscript{36}). People should, therefore be careful and aware of the potential hazards of such drugs, especially if they are continuously exposed to for such a long periods.

Acknowledgements

Authors express their gratitude to Dr. Asim Azfer Lecturer for his expert & valuable suggestions and incessant encouragement and the Chairman, Department of Zoology for providing laboratory facilities.

References

CORRIGENDUM

Due to typographical error, in the result, introduction and discussion section, the dose concentrations of steroids have been written as mg/ml, it should be read as μg/ml.