ANALYSIS OF GENOTOXIC EFFECTS OF CERTAIN CLINICALLY USED VASODILATOR DRUGS ON HUMAN GENOME

SUMMARY
OF THE
THESIS
SUBMITTED FOR THE AWARD OF THE DEGREE OF
Doctor of Philosophy
IN
ZOLOGY

BY
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SECTION OF GENETICS
DEPARTMENT OF ZOOLOGY
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The science of genotoxicity mainly concerns with the fact that chemicals, which induce mutations in various experimental models, may conceivably affect the incidence of heritable mutations in man. Chemicals can be tested for their ability to induce chromosomal damage either in microbes, plants, animals, insects, mammals, and cultured mammalian cells. Mammalian cell cultures provide a convenient test system and either established cell lines or human blood lymphocytes can be used in practice. Genotoxicity tests can be defined as in vitro or in vivo tests designed to detect drugs, which can induce genetic damage directly or indirectly by various mechanism of action. These tests enable hazard identification with respect to DNA damage and its fixation in the form of genetic mutations, large-scale chromosomal damage, recombination and numerical chromosome changes. Various chemicals and drugs are tested positive for genotoxicity applying these tests.

Cardiovascular diseases (CVD) are considered as one of the most dreaded challenges the world faces today. These diseases account for nearly a third of all deaths worldwide. These are increasing in developing countries at an alarming rate and it has been estimated that CVD will be the major cause of morbidity and mortality in these countries by the year 2020. The main factor leading to this widespread and chronic problem is hypertension. Hypertension, being chronic, requires long-term drug therapy and thus interaction of antihypertensive drugs and their metabolites is direct and continual.
with the human body. The duration of pharmacological treatment of hypertension may last for decades and, therefore, patients are exposed to prolonged contact with the drugs. Long-term treatments require documentation of long-term safety and efficacy trials including sensitive indices of genotoxic damage.

Antihypertensive vasodilators are drugs that are recommended for the treatment of peripheral vascular diseases, high blood pressure problems, cardiac problems like stroke and other cerebral vascular diseases etc, depending on the function and class of that particular antihypertensive drug. Prolonged drug therapy employing such drugs may produce adverse side-effects, and may even affect the heritable material.

Several studies had earlier been done to evaluate the genotoxicity of commonly recommended cardiovascular drugs and reported in literature. Hydrochlorothiazide, used as a diuretic and antihypertensive agent, was studied to produce micronuclei (MN) in cultured human lymphocytes. A data from 164 marketed antihypertensive drugs showed that out of them, 32 drugs were tested positive in at least one genotoxicity assay, 26 in at least one carcinogenicity assay, and 10 in at least both, one genotoxicity assay and one carcinogenicity assay. Antihypertensive drug nimodipine was studied to induce micronuclei in vitro. Nitrovasodilators, used in nitrate therapy were observed to cause possible genotoxic activity. Likewise some other widely used antihypertensive vasodilators, like, Atenolol, Hydralazine and Dihydralazine, Methyldopa were also
reported to induce some genotoxic effects in cultured human lymphocytes in vitro as well as in vivo in patients.

The cardiac problems are, nowadays, commonly encountered and the treatment is prescribed for decades via drug therapy. Thus, in the present investigation, keeping in mind the widespread, long-term and constant use of vasodilator antihypertensives, four such drugs are tested for their cytogenetic effects in vitro in human peripheral blood lymphocytes. These drugs are Pentoxifylline (Trental®), Isoxsuprine hydrochloride (Duvadilan®), Xanthinol nicotinate (Complamina®) and Metoprolol tartrate (Metolar®). No such type of study has been done previously in three of these drugs, namely, Duvadilan®, Complamina® and Metolar®, whereas, some study has earlier been done over the genotoxicity of the third drug Trental®.

Pentoxifylline (PTX), a derivative of methylxanthine is an oxohexyl-substituted analogue of theobromine. Its chemical formula is \(\text{(3, 7-dimethyl-1-[5-oxo-hexyl] xanthine)}\). Isoxsuprine, or Isoxsuprine hydrochloride, is a beta agonist drug used as a vasodilator in humans and equines. Its chemical formula is 2-hydroxy-2-(4-methoxyphenyl)-1-methyl-N-(1-phenoxy-2-propyl) ethanaminium chloride. Xanthinol nicotinate is a peripheral vasodilator (nictonic acid prodrug). It is the most potent form of niacin (active part of vitamin B3). Its chemical formula is 7-[2-Hydroxy-3-(2-hydroxyethyl-methyl-amino) propyl]-1,3-dimethyl-purine-2,6-dione pyridine-3-carboxylic acid. Metoprolol tartrate is an antihypertensive drug. It is a selective beta1-adrenoreceptor blocking agent. It's chemical formula
is 1-Isopropylamino-3-[4- (2-methoxyethyl) phenoxy]-2-propanol L-(+)-tartrate.

Two highly reliable short-term genotoxicity assays, namely chromosomal aberration (CA) assay and cytochalasin-blocked micronucleus (CBMN) assay, were employed to test drug genotoxicity and cytotoxicity. Different concentrations of the drugs were used for the study and assay parameters like chromosomal aberrations, micronucleus frequencies, frequency of binucleated micronucleated cells, nucleoplasmic bridge and nuclear bud frequencies, ratios of apoptotic and necrotic cells and cell cycle kinetics, were employed to assess and evaluate the results. Values were compared with the concurrent controls and the statistical significance of the results was checked using Student’s $t$-test and $2 \times 3$ Chi-Square ($\chi^2$) test.

Our studies show that all these four test drugs significantly induce changes in the applied parameters, at different concentrations. Significantly high chromosomal aberration frequencies were obtained at 48 hour of incubation in chromosomal aberration assays. The highest response was observed in Isoxsuprine hydrochloride. Rest of the three drugs (Pentoxifylline, Xanthinol nicotinate and Metoprolol tartrate) also gave positive results. Structural chromosomal changes were mainly chromatid-type suggesting the clastogenic nature of the test drugs. Prevalence of chromatid-type structural changes suggested the S-phase dependent nature of these drugs.
Increased drug induced frequencies of micronuclei were analyzed in CBMN assay. The highest values were obtained for drugs Xanthinol nicotinate and Pentoxifylline. This indicated that the test drugs are potent inducers of chromosomal breakage and loss in cultured cells. Frequencies of nucleoplasmic bridges and nuclear buds were also increased with increased drug doses at 24 hour of incubation, suggesting chromosome rearrangement and gene amplification potency of these drugs. Xanthinol nicotinate was highly responsive for these parameters. The other three drugs also gave positive results.

Data for the result of cell division kinetics showed reduced values for parameters like nuclear division index (NDI) and nuclear division cytotoxicity (NDCI) index, for the four test drugs. Reduction in the NDI and NDCI values indicated that the selected drugs disturbed cell-proliferation kinetics and induced cellular toxicity.

As regards the comparative scale of toxicity induced in human lymphocytes, structural basis of mechanism of action could not be deduced, as the scope of the study is primarily the toxicity assay rather than the mechanism of toxicity induced. This can be taken up in future studies with wider experimental designs. Summing up, the four selected vasodilator antihypertensive drugs showed some genotoxic potentiality at applied concentrations in vitro in human cultured lymphocytes and thus required extensive evaluation for in vivo studies also. Their prolonged use may cause some serious side effects in the patients.
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CERTIFICATE

I feel great pleasure to forward the thesis entitled "Analysis of genotoxic effects of certain clinically used vasodilator drugs on human genome" for the degree of Doctor of Philosophy in Zoology (Genetics) of the Aligarh Muslim University, Aligarh. This thesis, as presented by Ms. Asma Frahat Sherwani, consists of subject matter which is the original record of work done by her under my supervision, and the same is not a part of any other course or degree presented to or taken by her.

Mohammad Afzal
(Professor)
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INTRODUCTION
INTRODUCTION

The growing industrialization provide basic comforts and amenities to the human society, however it also exposes humans to the hazards of a variety of chemical substances, pollutants and harmful radiations. Interaction with the chemicals like medicinal products, food stuffs, environmental contaminants, pesticides, clothing apparels, daily-use commodities, cosmetic ranges, plastic and leather wares, and petrochemical products etc present in the environment, is frequent in different forms.

It has now become evident that some of these chemicals can cause cancer in man. More recently, there has been a growing awareness of the possibility that some chemicals may also produce mutations in human germ cells thus influencing the frequency of genetic or heritable diseases. There are many compounds that occur naturally, which are known to be mutagenic and/or carcinogenic (e.g., mycotoxins in foods). The extent to which human population is exposed to different types of environmental agents, chemicals and drugs is alarming. It is important, therefore, that chemicals to which people are exposed, either intentionally (e.g., therapeutically), routinely in the course of their daily life (e.g., in domestic products, cosmetics, etc.), or inadvertently (e.g., in pesticides) are tested for their potential to produce cancer or genetic damage, *i.e.*, mutations (IPSC. WHO, 1985).

Mutagenic chemicals interact with DNA causing changes in its structure. This may result in the loss, addition, or replacement of bases, thus altering their sequence in the DNA and affecting the fidelity of the genetic message. The effect of these mutational changes may be to prevent the synthesis of functional proteins completely (*i.e.*, inhibition of gene
expression) or may lead to the synthesis of proteins with modified structure and enzymes with altered activity and specificity. Where mutations lead to changes in the genetic information carried by male or female germ cells, the progeny of such affected parents may express the mutation as some form of heritable abnormality or disease. When mutations occur in the somatic cells of a complex organism, they may produce irreversible changes in the cell that may ultimately be involved in producing a cancerous growth (IPSC. WHO, 1985).

Damage to DNA may be expressed as mutations at the chromosome level (i.e., chromosomal aberrations or chromosomal mutations) or at the level of the gene (i.e., gene or point mutations). Chromosomal mutations may be observed as changes in the structure of the chromosome (structural aberrations) or in the number of chromosomes in a cell (numerical aberrations or aneuploidy). Structural aberrations are a consequence of DNA damage while numerical changes are usually caused by defects in the accurate distribution of chromosomes during cell division, i.e., DNA damage may not be involved (IPSC. WHO, 1985). Mutations contribute significantly to human diseases and congenital malformations, though the extent of this contribution is not precisely known. For many years, the interaction of chemicals with the genetic material has been studied intensively (Singer and Kusmier, 1982) with a view that the preservation of integrity of DNA as a genetic blueprint is of prime importance for any living organism.

**DNA DAMAGE**

DNA damage, due to environmental factors and normal metabolic processes inside the cell, occurs at a rate of 1,000 to 1,000,000 molecular
lesions per cell per day (Lodish, et al., 2004). The vast majority of DNA damage affects the primary structure of the double helix; that is, the bases themselves are chemically modified.

**Sources of damage**

The source of DNA damage can be subdivided into two main types:

- **Endogenous** damage such as attack by reactive oxygen species produced from normal metabolic byproducts (spontaneous mutation), especially the process of oxidative deamination.

- **Exogenous** damage caused by external agents such as ultraviolet radiation from the sun, other radiation frequencies (X-rays and gamma rays etc.), hydrolysis, certain plant toxins, mutagenic chemicals and cancer chemotherapy and radiotherapy.

**Types of damage**

There are four main types of damage to DNA due to endogenous cellular processes:

- **Oxidation** of bases and generation of DNA strand interruptions from reactive oxygen species.

- **Alkylation** of bases (usually methylation).

- **Hydrolysis** of bases, such as deamination, depurination and depyrimidination.

- **Mismatch** of bases, due to errors in DNA replication.

Damage caused by exogenous agents occurs in many forms, such as:
• **UV-B light** causes crosslinking between adjacent cytosine and thymine bases creating pyrimidine dimers. This is called direct DNA damage.

• **UV-A light** creates mostly free radicals. The damage caused by free radicals is called indirect DNA damage.

• **Ionizing radiation** causes breaks in DNA strands.

• **Thermal disruption** at elevated temperature increases the rate of depurination and single strand breaks.

• **Industrial chemicals and environmental chemicals** create a huge diversity of DNA adducts- ethenobases, oxidized bases, alkylated phosphotriesters and crosslinking of DNA etc.

**DNA DAMAGE AND MUTATION**

DNA damage and mutation are two major types of error in DNA which are fundamentally different from each other. Damages are physical abnormalities in the DNA, such as single and double strand breaks and polycyclic aromatic hydrocarbon adducts. These damages can be recognized by enzymes, and thus they can be correctly repaired if redundant information, such as the undamaged sequence in the complementary DNA strand or in a homologous chromosome, is available for copying. If a cell retains DNA damage, transcription of a gene can be prevented and thus translation into a protein will also be blocked. Replication may also be blocked and/or the cell may die.
In contrast to DNA damage, a mutation is a change in the base sequence of the DNA. A mutation cannot be recognized by enzymes once the base change is present in both DNA strands, and thus a mutation cannot be repaired. At the cellular level, mutations can cause alterations in protein function and regulation. Mutations are copied when the cell replicates.

Although distinctly different from each other, DNA damages and mutations are related because DNA damages often cause errors of DNA synthesis during replication or repair and these errors are a major source of mutation. DNA damages in frequently dividing cells are a prominent cause of cancer. In contrast, DNA damages in the infrequently dividing cells are likely to be a prominent cause of aging.

DNA REPAIR MECHANISMS

Cells cannot function if DNA damage corrupts the integrity and accessibility of essential information in the genome. Depending on the type of damage inflicted on the DNA's double helical structure, a variety of repair strategies have evolved to restore lost information.

Direct reversal

Cells are known to eliminate three types of damage to their DNA by chemically reversing it. These mechanisms do not require a template, since the types of damage they counteract can only occur in one of the four bases. Such direct reversal mechanisms are specific to the type of damage incurred and do not involve breakage of the phosphodiester backbone.

Single strand damage
When only one of the two strands of a double helix has a defect, the other strand can be used as a template to guide the correction of the damaged strand. In order to repair damage to one of the two paired molecules of DNA, there exist a number of excision repair mechanisms that remove the damaged nucleotide and replace it with an undamaged nucleotide complementary to that found in the undamaged DNA strand (Watson, et al. 2004).

- **Base excision repair (BER)**, which repairs damage to a single nucleotide caused by oxidation, alkylation, hydrolysis, or deamination.

- **Nucleotide excision repair (NER)**, which repairs damage affecting longer strands of 2–30 bases.

- **Mismatch repair (MMR)**, which corrects errors of DNA replication and recombination that result in mispaired nucleotides following DNA replication.

**Double-strand breaks**

Double-strand breaks (DSBs), in which both strands in the double helix are severed, are particularly hazardous to the cell because they can lead to genome rearrangements. Two mechanisms exist to repair DSBs (Watson, et al. 2004):

(i) **Non-homologous end joining (NHEJ)** and

(ii) **Recombinational repair** (also known as template-assisted repair or homologous recombination repair).
In NHEJ, DNA ligase IV, a specialized DNA ligase, directly joins the two ends (Wilson, 1997). NHEJ can also introduce mutations during repair. Loss of damaged nucleotides at the break site can lead to deletions, and joining of nonmatching termini to form translocations. NHEJ is important especially before the cell has replicated its DNA, since there is no template available for repair by homologous recombination.

Recombinational repair requires the presence of an identical or nearly identical sequence to be used as a template for repair of the break. The enzymatic machinery responsible for this repair process is nearly identical to the machinery responsible for chromosomal crossover during meiosis. This pathway allows a damaged chromosome to be repaired using a sister chromatid (available in G2 after DNA replication) or a homologous chromosome as a template.

**Translesion synthesis**

Translesion synthesis is a DNA damage tolerance process that allows the DNA replication machinery to replicate past DNA lesions such as thymine dimers or AP sites. It involves the switching out of regular DNA polymerases for specialized translesion polymerases, often with larger active sites that can facilitate the insertion of bases opposite damaged nucleotides.

**DNA DAMAGE CHECKPOINTS**

After DNA damage, cell cycle checkpoints are activated. Checkpoint activation pauses the cell cycle and gives the cell time to repair the damage before continuing to divide. DNA damage checkpoints occur at the G1/S and G2/M boundaries. An intra-S checkpoint also exists.
THE PROKARYOTIC SOS RESPONSE

The SOS response is the term used to describe changes in gene expression in *Escherichia coli* and other bacteria in response to extensive DNA damage. The prokaryotic SOS system is regulated by two key proteins: LexA and RecA. The LexA homodimer is a transcriptional repressor that binds to operator sequences commonly referred to as SOS boxes. The most common cellular signals activating the SOS response are regions of single stranded DNA (ssDNA), arising from stalled replication forks or double strand breaks, which are processed by DNA helicase to separate the two DNA strands (Friedberg, *et al.*, 1995).

EUKARYOTIC TRANSCRIPTIONAL RESPONSES TO DNA DAMAGE

Eukaryotic cells exposed to DNA damaging agents also activate important defensive pathways by inducing multiple proteins involved in DNA repair, cell cycle checkpoint control, protein trafficking and degradation. Such genome wide transcriptional response is very complex and tightly regulated, thus allowing coordinated global response to damage. In an animal different types of cells are distributed amongst different organs which have evolved different sensitivities to DNA damage (Fry, *et al.*, 2004).

DNA REPAIR AND CANCER

Inherited mutations that affect DNA repair genes are strongly associated with high cancer risks in humans. BRCA1 and BRCA2, two famous mutations conferring a hugely increased risk of breast cancer on
carriers, are both associated with a large number of DNA repair pathways, especially NHEJ and homologous recombination.

Cancer therapy procedures such as chemotherapy and radiotherapy work by overwhelming the capacity of the cell to repair DNA damage, resulting in cell death. Cells that are most rapidly dividing, typically cancer cells, are preferentially affected. The side effect is that other non-cancerous but rapidly dividing cells such as stem cells in the bone marrow are also affected. Modern cancer treatments attempt to localize the DNA damage to cells and tissues only associated with cancer, either by physical means (concentrating the therapeutic agent in the region of the tumor) or by biochemical means (exploiting a feature unique to cancer cells in the body).

CHROMOSOMAL ABERRATIONS IN RELATION TO GENOTOXICITY

There are various classes of chromosomal changes that can be induced by exposure to chemical substances. These changes, also known as chromosomal aberrations, are either structural changes in chromosomal shapes or numerical changes in chromosomal counts.

A. Structural aberrations

Following changes are involved in chromosome structure:

Gaps: Unstained regions that are smaller than the diameter or width of the chromatid. The chromatids do not show any misalignment.

Breaks: Damage to the chromatid or isochromatid involving a discontinuity of the chromosomes greater than the width of the chromatid. It may be accompanied by a misalignment of the chromatids or chromosomes.
**Dicentric Chromosomes**: These are chromosomes with two centromeres with or without associated fragments.

**Ring Chromosomes**: aberrant circular chromosomes resulting from the joining of two breaks on separate arms of the same chromosome (generally accompanied by an acentric fragment). They are also called centric rings.

**B. Numerical aberrations**

Changes in the chromosome count is referred to as *Polyploidy*, a condition in which cells display more than double the chromosome number (2n) exact multiples of the haploid chromosome numbers.

- polyploidy (4n)
- hypodiploidy (< 46chr)
- hyperdiploidy (> 46chr)

Visible changes to chromosome structure and morphology have played a very important part as indicators of genetic damage in both clinical and cancer studies. Most of the changes encountered in clinical studies are "secondary" or "derived" aberrations (Savage, 1999)

Primary aberrations are those seen at the first post-induction division, when all the parts are present and there has been no selection by passage through mitosis, nor any modification by subsequent chromosome duplication (Savage, 1976)

**CLASSIFICATION OF PRIMARY CHANGES**
Since the chromosome at metaphase has two (sister-) chromatids, it is convenient (and conventional) to divide all aberrations into two broad types (Savage, 1999):

**Chromosome-type** where the breaks and re-joins always affect both sister-chromatids of a chromosome at any one locus. They are also referred to as an *isochromatid aberrations* (Figure A).

**Chromatid-type** where the breaks and re-joins affect only one of the two sister-chromatids of a chromosome at any one locus (Figure B).

Following an "acute" treatment with any clastogen, surviving cells in later generations carry only *chromosome-type* changes. The presence of *chromatid-type* aberrations in such cells is, therefore, an indicator of an ongoing production of primary structural changes, i.e. of some form of chromosome instability (Savage, 1999)

Nearly all the aberrations appear to result from the interaction (re-joining) of two breaks, so they can be further classified on the basis of where these breaks are situated in relation to the chromosome arms (Savage, 1976)

If the breaks are situated in the arms of different (non-homologous or homologous) chromosomes, they come under the category of *interchanges*.

- If the breaks are in the opposite arms of the same chromosome, they came under the category of *inter-arm intrachanges*.
- If the two breaks are both in the same arm of a chromosome, they came under the category of *intra-arm intrachanges*.

These three categories are often referred to collectively as *Exchanges*.
Figure A. Chromosome – type aberrations (Savage, 1999) (A-asymmetric, S-symmetric).

### Table: Chromosome – Type Aberrations

<table>
<thead>
<tr>
<th>INTERCHANGE</th>
<th>INTER-ARM INTRACHANGE</th>
<th>INTRA-ARM INTRACHANGE</th>
<th>&quot;BREAK&quot; DISCONTINUITY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
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<tr>
<td>A</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>dicentric</td>
<td>centric-ring</td>
<td>interstitial deletion</td>
</tr>
<tr>
<td>S</td>
<td>reciprocal translocation</td>
<td>pericentric inversion</td>
<td>paracentric inversion</td>
</tr>
</tbody>
</table>

Figure B. Chromatid – type aberrations (Savage, 1999) (A-asymmetric, S-symmetric).
Finally, some aberrations appear to arise from a single, open break in just one arm. This category is termed as breaks or discontinuities. Many of them are, in reality, intra-arm intrachanges where one end has failed to join up properly.

Many chromosome-type aberrations which are scored as "simple" two-break interactions actually involve more than two breaks, and often more than two chromosomes, i.e. they are complex exchanges (Savage, and Harvey, 1994).

During the process of re-joining of breaks, interaction between the four ends of two breaks takes place in three ways:

a. Join back to re-form the original chromosomes (restitution) so that no aberration is produced.

b. Re-join in such a way that an acentric fragment is always formed (asymmetrical re-joining). Asymmetrical aberrations are almost always cell-lethal, and so rapidly disappear from a population of continuously dividing cells (Lea, 1955; Savage, 1989).

c. Re-join in a way that never leads to an acentric fragment unless one of the re-joins is incomplete (symmetrical re-joining). Because there is no loss of genetic material, and no mechanical problems at mitosis, most symmetrical forms are transmissible to future cell generations (Savage, 1976, 1995).

As mentioned, re-joining can sometimes be (apparently) incomplete. This is much more frequent for chromatid-type aberrations (typically 30-50% of interchanges) as compared to chromosome-type aberrations (difficult
to measure accurately, but probably around 3-5%). Incompleteness leads to
genetic loss, and so to increased cell lethality.

The four basic categories discussed above are seen in their simplest
forms for chromosome-type aberrations, as shown in Figure B.

Because sister-chromatids tend to adhere, strongly, along their
lengths, many chromatid-type symmetrical forms remain visible without
recourse to special staining methods, as Figure 2 shows. Moreover, the
presence of sister-chromatids allows additional lesion interactions (inter-
chromatid, intra-arm intrachanges) not possible with chromosome-type
changes. As a consequence, there is a much higher frequency of chromatid-
type changes, and a much greater variety of forms, compared with chromosome-types, following a given treatment. Moreover, the interactions
within chromosome arms which are now possible make chromatid-type
aberrations a much more likely source of the complicated
duplications/deletions etc. encountered in clinical and cancer studies.

Combinations of the various categories are frequent, especially for
chromatid-types (interchange/intra-arm intrachange) giving rise to
configurations like triradials (Savage and Harvey, 1994), but these, of
course, constitute a type of complex exchange. Surviving remnants of such
events are responsible for some of the curious anomalies recorded in clinical
and cancer cell studies.

Relationship to the cell cycle

Conventionally, the period between successive mitoses (interphase) is
sub-divided into three phases G₁, S and G₂. For experimental work, further
sub-division of S is possible (Savage, et al., 1984) G1 is the pre-duplication period, when the cell begins to prepare for DNA synthesis and the next mitosis. S-phase is a discrete period of interphase of a few hours duration during which the chromosomal DNA and protein is duplicated, and the new chromatin segregated into the sister-chromatids. If the cell is not going to divide again, it passes out of cycle during this phase into another phase termed G0. From this phase it may, or may not, be possible to call it back into a division cycle. Usually, however, cells pass on to irreversible differentiation with their chromosomes unduplicated.

Most aberration-inducing agents can introduce lesions into the chromatin at all stages of the cell cycle, but relatively few of them can produce actual structural changes in G1, (and therefore give rise to primary chromosome-type changes) or in S and G2 (producing primary chromatid-types). Ionising radiation, restriction endonucleases, and a few chemicals like bleomycin and some antibiotics are amongst those that can produce such changes. Almost all remaining aberration producing agents are S-dependent. Surviving unrepaired lesions from G1 or G2 have to pass through a scheduled S-phase to convert them into exclusively chromatid-type aberrations.

SECONDARY OR DERIVED ABERRATIONS

Although there is an enormous range of primary aberration forms, very few of them are transmissible to future cell generations long term, so only some secondary (or derived) forms are recovered (Savage, 1976; Savage, 1995). Various types of secondary changes are categorized as:

i. Reciprocal translocation:
These changes are derived directly from chromosome-type reciprocal translocations or from one segregation sequence of symmetrical chromatid-type interchanges. They do not involve any mechanical separation problems at anaphase, and usually no genetic loss or imbalance.

ii. Pericentric Inversion:

These changes are derived directly from chromosome-type or chromatid-type pericentric inversions. They have properties very similar to those for reciprocal translocations. Large inversions lead to meiotic bridges, sterility and cell death.

iii. Paracentric inversion:

These changes are derived directly from chromosome-type paracentric inversions, or from one form of chromatid-type intra-chromatid intra-arm intrachange. They are very difficult to detect at the chromosome level unless they are very large (many megabases of DNA). Again the re-joining points can disrupt important genetic sequences, and reverse segments of the reading frame. Large inversions will give problems at meiosis.

iv. Interstitial deletion:

These changes are derived directly from chromosome-type interstitial deletions (double minutes) and from the alternative form of chromatid-type intra-chromatid intra-arm intrachange to that which produces paracentric inversions. Segregation products from some complex chromatid-type interchanges can also carry deletions.
The loss of small segments of a chromosome (usually only in one homologue) is not uncommon. Very occasionally, the loss of quite large segments appears to be compatible with cell survival.

v. **Terminal deletion:**

Derivation of terminal deletion, if genuine, is from various forms of incomplete *chromosome-type* or *chromatid-type* intrachanges and interchanges, followed by telomerase activity to achieve capping. It is not known whether true stable terminal deletions actually exist.

vi. **Interstitial duplication:**

These changes are most likely derived from one form *chromatid-type* inter-chromatid intra-arm intrachange. Some forms of complex *chromatid-type* interchanges can segregate to give surviving chromosomes with duplicated segments. Segments of a chromosome repeat in tandem, sometimes in reverse sequence.

vii. **Interstitial insertion:**

All insertions are derived from complex exchanges, since, by definition, their production requires the interaction of a minimum of 3 lesions. In the formation of these changes, either *chromosome-type* or *chromatid-type*, complex interchanges may be involved, the range of inter-intrachanges in the latter is particularly productive of insertions.

**CYTOGENETIC EFFECTS AND CELL DEATH**

Genotoxic behavior, of some potent DNA-damaging agents causes genotoxic stress-induced cell destruction. Cell death occurs by one of two
mechanisms – necrosis or apoptosis. These are two physiologically different processes. Necrosis is death by injury and apoptosis is death by suicide. Both of these mechanisms have different characteristics.

**Necrosis**

Necrosis is cell death by accident. It is associated with nonphysiological circumstances that disrupt cellular homeostasis (e.g., ischemia, hypoxia and poisoning). Necrosis is caused by membrane dissolution (osmotic lysis, shear stress, pore-forming proteins, loss of ATP). This phenomenon is harmful for cell viability because cellular material (including degradative enzymes) is released into surrounding tissue resulting into inflammatory reactions. This affects contiguous groups of cells. Necrotic cell death is characterized by nuclear swelling, cell swelling, disruption of organelles, and rupture of cell and release of cellular contents, which results into inflammatory response.

**Apoptosis**

Apoptosis is genetically programmed self – destruction of autoreactive or damaged cells (Kerr, *et al.*, 1972; Wyllie, *et al.*, 1980). Cell destruction is induced by new gene synthesis, primarily in response to developmental cues. It requires new RNA and protein synthesis. Inhibitors of transcription or translation prevent apoptosis. It is important for development, homeostasis and elimination of pathogens and tumor cells. It causes deletion of individual cells in the midst of others. But it can be involved in deletion of entire structures also. Apoptosis is followed by fast phagocytosis, and it is anti-inflammatory (housekeeping). Activation of
apoptosis in cells is initiated by various types of DNA damages (Kerr, et al., 1972; Wyllie, et al., 1980).

The symptoms of apoptosis, or programmed cell death, are viability loss accompanied by chromatin condensation, membrane blebbing, cell shrinkage, and packaging of nuclear fragments into small apoptotic bodies (Kerr, et al., 1972; Wyllie, et al., 1980).

Regulation of cell death is essential for normal development and elimination of cells that have suffered serious damage and it is an important defense against the emergence of cancer and other diseases. Perturbations of apoptosis contribute to the pathogenesis of several human diseases including cancer, acquired immunodeficiency syndrome and neurodegenerative disorders (Thompson, 1995).

AN INTRODUCTION TO MUTAGENICITY, CARCINOGENICITY AND GENOTOXICITY

Mutations are permanent hereditary changes in the cell lines, either horizontally in the somatic cells or vertically in the germinal cells of the body. That is, mutations may affect the organism itself through changes in body cells, or they may be passed on to other generations through alteration of the sex cells. Genotoxicity thus precedes mutagenicity although most of genotoxicity is repaired and is never expressed as mutations. Somatic mutations are induced at the cellular level and in the event that they lead to cell death or malignancies, may become manifest as various disorders of tissues or of the organism itself. Somatic mutations are thought to be related to ageing effects or to the induction of atherosclerotic plaques.
Although there is no definitive evidence that exposure to chemicals is responsible for any of the known human genetic disorders, experimental evidence from other mammals have shown that chemicals can produce both chromosomal and gene mutations of the type that are associated with human genetic diseases. There is little direct evidence to suggest that man is any less susceptible than other mammals to the effects of exposure to mutagenic chemicals. Alterations in the structure and function of DNA are believed to play a crucial role in the production of cancer by chemicals. Carcinogenesis is a multistage process that may take years to evolve and a number of different factors influence the progression from a normally functioning cell to an invasive neoplastic tumour. The animal bioassay for detecting carcinogenic chemicals is a prolonged and very expensive scientific study using some hundreds of rodents to which the suspect chemical is administered for most of their life span. Similarly, the specific locus test in mice (Searle, 1975), which is one of the few currently available assays for detecting heritable gene mutations in mammals, requires the examination of many thousands of offspring and is equally expensive and time-consuming. Thus, of the multitude of chemicals introduced into the environment in this century, and the hundreds of new compounds being synthesized each year, only a small fraction can be tested in conventional animal studies. For this reason, the last decade has seen the introduction of a number of relatively rapid tests for detecting mutagenic and carcinogenic chemicals. Such tests are economical in resources and produce results in a matter of weeks. Almost all of these short-term procedures are based on the demonstration of chromosomal damage, gene mutations, or DNA damage, and many of them are in vitro assays. The test organisms range from bacteria and yeasts to
insects, plants, and cultured animal cells, there are also short-term tests in which laboratory animals are exposed to test chemicals for periods of a few hours to, at most, a few weeks. Mammalian cell cultures provide a convenient test system and either established cell lines or human blood lymphocytes can be used in practice, a suspect chemical is first tested using in vitro procedures, to study its ability to react with DNA and thus induce mutations. It may then be necessary to determine its genotoxicity for intact animals by testing in short-term mammalian (in vivo) assays.

**BIOCHEMICAL MECHANISMS OF MUTAGENESIS**

Chemical carcinogens and mutagens act by chemically modifying the bases in DNA, particularly guanine. There are two sites on the guanine molecule to which groups can easily be covalently attached in the presence of reactive metabolites of chemical carcinogens, namely the O₆ and N₇ positions. Substitution at the O₆ position of guanine is most sensitive to produce a permanent mutagenic effect which is perpetuated when the DNA is replicated, whereas N₇ substitutions are usually quickly repaired by the normal DNA repair mechanisms involving excision and replacement of altered base.

The accessibility of DNA bases to chemical attack is more when the DNA is in the process of replication. The likelihood of genetic damage by many mutagens is related to the frequency of cell division. This means that the developing focus is particularly susceptible, and thus many mutagens and carcinogens are also teratogenic. Some mutagens, particularly ionizing radiations, can affect DNA whether or not it is replicating, so that even the female germ cells are sensitive throughout their reproductive life.
GENOTOXICITY TESTING

Genotoxicity is defined as an organism-specific and quantitative measure of the potential of a specific chemical or of electromagnetic environment to cause damage to the DNA of a cell (Corbisier, and Barcelo. 2000). The Mutagenic Index (MI) is binary value of 1 or 0 that is assigned to a compound, where 1 indicates a mutagen (positive) and 0 indicates a non-mutagen (negative). Mutagenicity is an effect that occurs when cellular DNA is altered as a result of its interaction with a chemical entity. DNA may be altered by interaction with a drug in several ways, such as the formation of a covalent bond between the drug and the DNA. The result of the formation of such a covalent bond is a specific adduct or physical alteration of DNA which may lead to such complications as nucleotide strand breakage or the disruption replication or translation. The three principal classes of drug interaction with DNA are intercalators, alkylators, and DNA strand breakers. Most of these effects are the result of irreversible binding of the drug to that DNA and all three modes of interaction have mutagenic consequences. Genotoxic chemicals are generally electrophilic in nature either directly, or through microsomal activation (S9), which results in the ability of the chemical to react with DNA through alkylation. Further, genotoxic changes can also occur indirectly with non-electrophilic entities, which interfere with molecules or enzymes associated with DNA synthesis or chromosomal segregation. Therefore, genotoxic compounds may act directly or indirectly on DNA to produce non-lethal, but harmful heritable changes in the structure or arrangement of genes (Corbisier, and Barcelo 2000).
Genotoxicity testing of New Chemical Entities (NCE) is generally used for hazard identification with respect to DNA damage and its fixation (Madle, et al., 1987). These damages can be manifested in the form of gene mutation, structural chromosomal aberration, recombination, and numerical changes. These changes are responsible for heritable effects on germ cells and impose risk to future generations (Wassom, 1992). In addition it has been well documented that somatic mutations can also play an important role in malignancy (Tennant, et al., 1987). These tests have been used mainly for the prediction of carcinogenicity and genotoxicity because compounds, which are positive in these tests, have the potential to be human carcinogens and/or mutagens (Jena, et al., 2002).

The outcome of genotoxicity tests are needed for the design and conduct of long term carcinogenicity study and interpretation of the test results (Figure C) (Alden, 2000; Kim and Margolin, 1999).

**Principle of genotoxicity testing**

Chemicals that exert their adverse effects through interaction with the genetic material (DNA) of cells are called genotoxic (Brusick, 1987; Galloway, 1994). Most human carcinogens are genotoxic in nature (Jena, et al., 2002). The science of genotoxicity mainly concerns that, chemicals which induce mutations in various experimental models, may conceivably affect the incidence of heritable mutations in man (Davidson, et al., 1986; Douglass, et al., 1988).

Genotoxicity tests can be defined as in vitro or in vivo tests designed to detect drugs, which can induce genetic damage directly or indirectly by various mechanism of action. These tests enable hazard identification with
respect to DNA damage and its fixation in the form of genetic mutations, large-scale chromosomal damage, recombination and numerical chromosome changes. Drugs that are positive in these tests that detect such kind of damage have the potential to be human carcinogens and/or mutagens i.e., may induce cancer and/or heritable defects (Jena, et al., 2002). Genotoxic studies along with data on pharmacokinetic (PK) and toxicokinetic (TK) represent a more rational approach for the assessment of mutagens and carcinogens (Frantz, et al., 1994).

A large number of genotoxicity tests are presently available for use in hazard evaluation. These tests detect the two main categories of mutations, gene mutation and chromosomal aberration, as well as indications of DNA damage. Tests to assess these endpoints can be carried out both in vitro and in vivo, with in vivo tests being conducted in germ cells, as well as in somatic cells. In order to assess adequately any expression of genotoxicity, a simplified systematic approach to the selection of these tests is required. Therefore, there must be an ordered approach using a limited number of well-defined tests that complement each other in terms of endpoints, and that permits a systematic assessment of genotoxicity.

The standard test battery for genotoxicity

For setting up of a battery of tests in genotoxicity assay, it has always been observed that, in vitro tests play a major role due to their high sensitivity and rapidity. Preliminary tests are designed in such a way that it can detect majority of the genotoxic carcinogens (MacGregor, et al., 1995). However, as no single assay has proved capable of detecting mammalian mutagens and carcinogens with an acceptable level of precision and
reproducibility, it is usual scientific practice to apply these assays in batteries. Initial information on the mutagenicity of a substance can be obtained using assays that measure gene mutations and chromosomal damage. Because separate procedures are required to investigate these end points, a battery of assays is needed.

The general features of a standard test battery can be outlined as follows (Hutchinson, 2004):

- It is appropriate to assess genotoxicity in a bacterial reverse mutation test. This test has been shown to detect relevant genetic changes and the majority of the genotoxic rodent carcinogens.

- DNA damage considered to be relevant for mammalian cells and not adequately measured in bacteria should be evaluated in mammalian cells. Several mammalian cell systems are in use. These include systems that detect gross chromosomal damage (in vitro tests for structural and numerical chromosomal aberrations), systems that detect primarily gene mutations, and a system that detects gene mutations and clastogenic effects (mouse lymphoma tk assay).

- An in vivo test for genetic damage should usually be a part of the test battery to provide a test model in which additional relevant factors (absorption, distribution metabolism, excretion) that may influence the genotoxic activity of a compound are included. As a result, in vivo tests permit the detection of some additional genotoxic agents. An in vivo test for chromosomal damage in rodent hematopoietic cells fulfills this need. This test could be either an analysis of chromosomal
aberrations in bone marrow cells or an analysis of micronuclei in bone marrow cells or peripheral blood erythrocytes.

MUTAGENICITY TESTING STRATEGY

A three level approach to genotoxicity testing based on the Level of Concern strategy in the report of the Environmental Contaminants Advisory Committee on Mutagenesis has been adopted by the HPB. That Committee recommended the definition and use of “Levels of Concern” (LOC) to rationalize a systematic approach to testing (Environmental Health Directorate, Canada, 1993).

Chemicals for which human exposure is high or widespread merit the greatest initial concern (LOC III), while those to which few people are exposed at very low dose levels would be of least concern (LOC I). An intermediate level (LOC II) is also defined. Data from at least two different short-term in vitro tests would be standard for the toxicological evaluation of all chemicals for which human exposure is involved. Accordingly, substances assigned initially to LOC I should be subject to two short-term tests, one for gene mutations and the other for chromosomal aberrations in vitro. Chemicals initially assigned to the higher levels of concern (LOC II and III) should be subject to similar scrutiny, but additional data should be obtained initially for chromosomal aberrations in mammalian cells in vivo (Environmental Health Directorate, Canada, 1993)
SHORT TERM IN VITRO GENOTOXICITY ASSAYS USED TO DETERMINE GENOTOXICITY OF TEST CHEMICALS

A. Chromosomal aberration assay

Chemical causing chromosomal aberrations may be identified with an in vitro cytogenetic assay generally referred to as Chromosomal Aberration Test. The chromosome aberration test using cultured mammalian cells is one of the sensitive methods to predict environmental mutagens and/or carcinogens, and is a complementary test to the Salmonella/microsome assay (Ames test) (Ishidate, et al., 1998). The techniques used in the study of chromosomal aberration have been described in detail by Evans (1976) and in the Gene-Tox reports of Latt, et al. (1981) and Preston, et al. (1981). Human peripheral lymphocytes do not divide spontaneously in culture, but can be stimulated to divide by treatment with a mitogen such as phytohaemagglutinin (PHA). Cell cultures are treated with the test chemical and then mitosis is arrested in metaphase with an inhibitor, such as colchicine. The metaphase spreads are examined by light microscopy to detect chromosome or chromatid aberrations, or polyploid cells. A biologically significant increase in the frequency of cells with structural or numerical aberration compared with that of the concurrent control group indicates the chemical is clastogenic or aneugenic, respectively. Aberration types are usually classified into chromatid (involving only one chromatid) and chromosomal or isochromatid (affecting both chromatids) and are clearly described by Evans and O'Riordan (1975), Savage (1976), Basel and Karger (1978) and Scott, et al. (1983). Other classification systems have been devised, such as that of Buckton, et al. (1962), in which chromosome aberrations are classified as stable ($C_s$) such as translocations, or unstable
(C<sub><var>n</var></sub>), such as dicentrics, depending on whether or not they can be maintained through successive cell cycles. Banding techniques (Evans, 1976), commonly used for detailed chromosome analysis, are rarely used for routine screening.

B. Micronucleus assay

The micronucleus assays are considered to be one of the preferred methods for assessing chromosome damage because they enable both chromosome loss and chromosome breakage to be measured reliably. This assay can provide, using simple morphological criteria, different measures of genotoxicity and cytotoxicity including chromosome breakage and loss (micronuclei), chromosome rearrangement (nucleoplasmic bridges), gene amplification (nuclear buds), cell division inhibition, necrosis and apoptosis. This assay also has the added advantage that mitogenic response, a biomarker of immune responsiveness (Chandra, 2002) can be measured by the proportion of cytokinesis-blocked BN and multinucleated cells or the estimated nuclear division index (Fenech, 2000).

Micronuclei (MNI) are expressed in dividing cells that either contain chromosome breaks lacking centromeres (acentric fragments) and/or whole chromosomes that are unable to travel to the spindle poles during mitosis. At telophase, a nuclear envelope forms around the lagging chromosomes and fragments, which then uncoil and gradually assume the morphology of an interphase nucleus with the exception that they are smaller than the main nuclei in the cell, hence, they are called micronucleus (Fenech, 2000). Micronuclei are expressed in cells that have completed nuclear division, and
thus, are ideally scored in the binucleated stage of cell cycle (Fenech and Morley, 1985).

Occasionally nucleoplasmic bridges between nuclei in a binucleated cell are observed. These are probably dicentric chromosomes in which the two centromeres are pulled to opposite poles of the cell and the DNA in the resulting bridge is covered by nuclear membrane. Thus, nucleoplasmic bridges in binucleated cells provide additional and complementary measure of chromosome rearrangement (Fenech, 2000). Sometimes binucleated cells also contain nuclear blebs or buds observed as outgrowths of main nucleus. These are the extruded amplified genes.

The cytokinesis-block MN (CBMN) assay is the preferred method for measuring MNi in cultured human lymphocytes because scoring is specifically restricted to once-divided cells (Fenech, 2002; Fenech and Morley, 1986). In the CBMN assay, cells that have completed one nuclear division are blocked from performing cytokinesis using Cytochalasin -B (cyt -B). Cyt -B is an inhibitor of actin polymerization required for the formation of microfilament ring that constricts the cytoplasm between the daughter nuclei during cytokinesis (Carter, 1967). The CBMN assay is currently the most widely used method for measuring micronucleus frequency in human lymphocytes. The methods for performing the CBMN assay have been described recently (Fenech, 2000).

C. Sister Chromatid Exchange assay

Sister chromatid exchange (SCE) analysis in human peripheral blood lymphocytes has often been applied as a cytogenetic assay for biomonitoring and genotoxicity testing of potentially mutagenic and carcinogenic chemicals
(Natarajan, 2002). This assay is also termed as indicator test. Indicator tests are those that do not directly measure mutations, but rely on other markers. The mechanism of SCE formation was first proposed by Painter in 1980 (Painter, 1980).

The sister chromatid exchange (SCE) assay is a short-term test for the detection of reciprocal exchanges of DNA between two sister chromatids of a duplicating chromosome. SCEs represent the interchange of DNA replication products at apparently homologous loci. The exchange process presumably involves DNA breakage and reunion, although little is known about its molecular basis. Detection of SCEs requires some means of differentially labelling sister chromatids (Latt, 1974; Kato, 1974; and Korenberg and Freedlender, 1974) and this can be achieved by incorporation of bromodeoxyuridine (BrdU) into chromosomal DNA for two cell cycles. SCEs can also be measured in mammals and in non-mammalian systems. Mammalian cells *in vitro* are exposed to the test chemical with and without a mammalian exogenous metabolic activation system, if appropriate, and cultured for two rounds of replication in BrdU containing medium. After treatment with a spindle inhibitor (e.g. colchicine) to accumulate cells in a metaphase-like stage of mitosis (C-metaphase), cells are harvested and chromosome preparations are made (Oecd, 1998).

Because of the ease of preparation and scoring, it is a very widely-used test in the study of mutagens. SCE induction alone is not generally accepted as sufficient evidence to classify an agent as mutagenic. The mechanism of SCE induction is not fully understood, though a number of models have been proposed (Wolff, 1982). Some clastogens induce only a small increase in SCEs, X-irradiation being a particularly striking example.
There is a high background level of SCE compared with chromosome aberrations; it is rare to find many cells without SCEs in untreated samples. This may be partly due to the 5-bromodeoxyuridine (BrdUrd) that is added to the culture medium in order to visualise SCEs, since BrdUrd itself is known to induce SCEs (Latt, et al., 1981). This basal level also implies that cells with SCEs are capable of subsequent growth. SCE evaluation may thus be a more valid indicator than chromosome breakage of events compatible with cell survival, hence its widespread use in mutagenicity screening programmes (IPCS, 1985).

EVALUATION OF GENOTOXICITY IN PHARMACEUTICALS

Pharmaceutical products are one of the fastest growing industries. Human ailments need the use of different drugs for the treatment of one or the other type of disease. Some diseases are chronic and require long-term treatment, thus exposing the human body to drugs and their byproducts for a longer duration of time. Many of these drugs cure health related problems on one hand, yet, on the other hand cause adverse side effects in human body, even affecting the human hereditary material, i.e. DNA at higher levels.

HYPERTENSION

Cardiovascular diseases (CVD) account for nearly a third of all deaths worldwide (WHO, 2002). CVD are increasing day by day in developing countries (Chockalingam and Balaguer-Vinto, 1999; Reddy and Yusuf, 1998) and it has been estimated that CVD will be the major cause of morbidity and mortality in these countries by the year 2020 (Murray and Lopez, 1996). Consequently, the prevention of risk factors for CVD is a public health priority worldwide (WHO, 2002).
Hypertension is an extremely prevalent condition in industrialized countries, accounting for more than 5% of total deaths worldwide (Schork, et al., 1999; Pardell, et al., 2000). About a quarter of the world’s population have been estimated to have hypertension at the turn of the millennium (Kearney, et al., 2005). It has remained an important public health challenge in the developing and the developed world alike (Howson, 1998; Murray and Lopez 1996).

A recent meta-analysis of the prevalence of HTN in India suggested that the prevalence of HTN is increasing steeply in urban areas (Gupta, 1997). Hypertension is a silent killer. Indians are racially predisposed to cardiovascular disease and the increasing burden of hypertension has only added to the problem. Economic constraints and the allure of additional benefits without adverse effects have made lifestyle modifications an attractive proposition in developing and developed countries alike. Blood pressure is a continuum and any increase above optimal level confers additional independent risk of vascular disease, even in ranges previously considered normal (Surya, et al., 2007).

Hypertension (HTN), SBP (systolic blood pressure) >140 mmHg or DBP (diastolic blood pressure) >90 mmHg, is a premier risk factor for CVD, which is easily recognized if sought, and can be treated effectively. Treatment of high blood pressure (BP) has been consistently reported to reduce the risk of CVD (Staessen, et al., 2001). Control of HTN was defined as antihypertensive treatment associated with SBP and DBP less than 140 mmHg and 90 mmHg, respectively (Guidelines Subcommittee. WHO. 1999).
The duration of pharmacological treatment of hypertension may last for decades and, therefore, patients are exposed to prolonged contact with the drugs. Long-term treatments require documentation of long-term safety and efficacy including sensitive indices of genotoxic damage (Furberg, et al., 1996).

In prescribing a drug the knowledge of benefit / risk ratio is of fundamental importance. Among the various adverse reactions that a drug may cause, the possible occurrence of a carcinogenic effect can not be excluded. The development of a tumor may take place after more than 10 and even 20 years of exposure (Giovanni and Antonietta, 2006). In the 2002 edition of Martindale – The Complete Drug Reference there are 164 drugs used in the therapy of hypertension and the large majority of them are marketed in several countries. Along with many other drugs several antihypertensive agents belonging to different groups of this class of drugs were examined for the genotoxicity and carcinogenicity data and some of them were found to have all the genotoxicity and carcinogenicity data required by the ICH – harmonized guidance on genotoxicity testing of pharmaceuticals (Muller, et al., 1999; Snyder and Green, 2001; IARC. 2004).

**CYTOGENOTOXIC EFFECTS OF CARDIOVASCULAR DRUGS – AN INSIGHT**

Vasodilator and antihypertensive drugs are generally used for chronic long term therapy. Because of this long term drug exposure, scientific community has conducted several studies on many of such drugs. Genotoxic, cytotoxic and mutagenic potential of these drugs were evaluated
by measures like sister chromatid exchange (SCE) assay, chromosome aberration (CA) assay, cytokinesis-block micronucleus (CBMN) assay, bacterial mutation assay, comet assay etc.

Hydrochlorothiazide, used as a diuretic and antihypertensive agent, was studied for its genotoxicity in cultured human lymphocytes. This drug produces micronuclei (MN) in cultured human lymphocytes by a mechanism that involves chromosome delay and to a lesser extent through chromosome breakage (Andrianopoulos, et al., 2006).

Brambilla and Martelli (2006) collected data from 164 marketed antihypertensive drugs. Of these 48 drugs were found to have at least one positive finding. 32 drugs were tested positive in at least one genotoxicity assay, 26 in at least one carcinogenicity assay, and 10 in both at least one genotoxicity assay and at least one carcinogenicity assay.

Genotoxic potential of chronic long-term therapy with the antihypertensive drug nimodipine was studied by measures of SCE and MN. A statistically significant increase in the frequency of micronuclei was detected in vitro (Telez, et al., 2001)

Cytogenotoxic effects of the vasodilator drug pentoxifylline, used for the treatment of peripheral vascular diseases and other related diseases, were investigated using cultured Chinese hamster V79 cells and human blood lymphocytes in vitro. The dose response clastogenicity was observed by measures of CA, SCE and MN formation (Bozsakyova, et al., 2001)

Nitrovasodilators, represent a widely-used class of extremely effective antianginal medications capable of causing systemic and coronary
vasodilation whether or not the endothelium is intact. These agents lead to the formation of the reactive free radical nitric oxide (NO). *In vitro* treatment indicated a dose-dependent increase in the frequency of micronucleated lymphocytes with increasing SNP concentrations. Cytotoxicity and cell cycle delay, with a statistically significant difference with respect to control culture, were also observed suggesting a possible genotoxic activity of nitrate therapy (Brambilla, and Martelli, 2006).

A variety of cytogenetic effects of a β- blocker antihypertensive drug Atenolol were observed in vitro. Chronic exposure to this drug led to a statistically significant enhancement of two variables of MN assay i.e. micronuclei and binucleated micronucleated cells (Telez, *et al.*, 2000).

Antihypertensive drugs hydralazine and dihydralazine can induce micronuclei in vitro, the effect depending on time exposure and concentration of the drugs (Chlopkiewicz, 1996).

Methyldopa, a widely used antihypertensive drug, although has no clastogenic action, but causes a significant increase in SCE frequency in human lymphocytes in culture (Cesar and Catarina, 1991).

Regarding the extensive use of various beta-adrenergic blockers for the treatment of a number of cardiovascular problems including cardiac arrhythmias, angina pectoris, and hypertension, eighteen beta-adrenergic blockers have been reviewed and the available literature pertaining to their potential carcinogenicity, mutagenicity, and teratogenicity has been summarized and compared (Jackson and Fishbein, 1986).
Besides cardiovascular drugs, metabolites of some vasodilator drugs like SIN -1 (3 - morpholinosydnonimine), the active metabolite of the vasodilator drug molsidomine, were found to produce a higher level of DNA double -stranded breaks in Tk6 cells and also induces mutagenesis and apoptosis in human lymphoblastoid cell lines (Li, et al., 2002)

Although extensive work has yet not been done in the field related to cytogenotoxicity of cardiovascular drugs, in particular vasodilators and antihypertensives, and thus the above literature survey concerning cytogenotoxicity of these drugs is not very extensive, it indicates that many long- term therapy cardiovascular drugs have genotoxic potential that can not be neglected altogether.

OBJECTIVES AND LIMITATIONS OF THE PRESENT WORK

It is being increasingly recognized that high BP is an important public health problem in developing countries (Fuentes, et al., 2000). New research published in *Lancet* finds that India will bear 60% of the world's heart disease burden in the next two years (Xavier, et al., 2008; Eagle, 2008) In addition, researchers have determined that compared to people in other developed countries, the average age of patients with heart disease is lower among Indian people and Indians are more likely to have types of heart disease that lead to worse outcomes.

Due to this threatening magnitude hypertension has received special attention during last decades. Treatment of hypertension along with other cardiovascular diseases requires long-term therapy involving the use of antihypertensive drugs. These drugs include diuretics, adrenergic beta-antagonists, adrenergic alpha-antagonists, angiotensin-converting enzyme
inhibitors, calcium channel blockers, ganglionic blockers, and vasodilator agents. Vasodilator antihypertensives are generally used for cases of resistant or severe hypertension.

The duration of pharmacological treatment of hypertension may last for decades and, therefore, patients are exposed to prolonged contact with these drugs. Long-term treatments require documentation of long-term safety and efficacy including sensitive indices of genotoxic damage (Furberg, et al., 1996).

In order to assess whether chronic long-term cardiovascular drug therapy may produce chronic toxicological effects, four vasodilator antihypertensive drugs have been selected in the present study.

The work has been conducted with a view to study the following objectives:

1. To study the in vitro effects of the vasodilator antihypertensives through peripheral human lymphocyte culturing system.
2. To study the genotoxic effects of the vasodilator antihypertensives on human chromosomes.
3. The study of DNA damage caused by the vasodilator antihypertensives, at the chromosome level using cytokinesis-block micronucleus (CBMN) assay.
4. To study the effects of vasodilator antihypertensives on cell/ nuclear division kinetics via cell cycle delay.
5. To study the cytotoxic effects (cellular cytotoxicity) of vasodilator antihypertensives on human peripheral lymphocytes.
MATERIALS & METHODS
MATERIALS AND METHODS

Chemicals can be tested for their ability to induce chromosomal damage either in mammals, cultured mammalian cells, insects or in plants. Mammalian cell cultures provide a convenient test system and either established cell lines or human blood lymphocytes can be used. Analysis of metaphase chromosomes in cells from the bone marrow of rats, mice, or hamsters is a well-established technique for studying chromosome damage in vivo. Alternatively, chromosome fragments can be identified as micronuclei in certain bone marrow cells and in other tissues and the "micronucleus test" has proved to be a relatively simple assay for detecting chemicals capable of damaging chromosomes. Cultures of mammalian cells can also be used to investigate the induction of gene mutations by chemicals in vitro.

In the present investigation, four cardiovascular drugs were studied for the evaluation of genotoxicity in human peripheral blood lymphocytes in vitro. Blood donors were 20 – 35 years old healthy males and females. The genetic end points employed were widely used and readily available for evaluating somatic mutations in human beings. These end points were chromosomal aberrations (CA), micronuclei (MNi), binucleated micronucleated (BNMN) cells, nucleoplasmic bridges (NPBR), nuclear buds (NBD) etc. and cell division kinetics. Statistical analysis was performed to ensure and find out significant values.
DESCRIPTION OF TEST DRUGS

Four cardiovascular drugs were selected to assess their cytogenotoxic potential in human peripheral blood lymphocytes in vitro. Three of these drugs namely Pentoxifylline (Trental\textsuperscript{®}), Isoxsuprine hydrochloride (Duvadilan\textsuperscript{®}) and Xanthinol nicotinate (Complamina\textsuperscript{®}) are vasodilators and one drug namely Metoprolol tartrate (Metolar\textsuperscript{®}) is an antihypertensive. Together all of them are grouped as vasodilator antihypertensives. All these drugs were available in injections forms.

1. PENTOXIFYLLINE / TRENTAL\textsuperscript{®}

Pentoxifylline (PTX), a derivative of methylxanthine is an oxohexyl-substituted analogue of theobromine. It was introduced originally for the treatment of peripheral vascular diseases, cerebrovascular disease and for a number of other disorders with defective micro- or macro-circulation

Chemical nomenclature:

Chemical formula of Pentoxifylline is (3, 7-dimethyl-1-[5-oxo-hexyl] xanthine)

Chemical structure:

Structural formula of Pentoxifylline is:

![Structural formula of Pentoxifylline](image)
Indications:

Nonhaemorrhagic stroke, chronic cerebrovascular insufficiency, transient ischaemic attack, intermittent claudication, trophic leg ulcers and gangrene, occlusive circulatory disturbances of the retina and cochlea, improves sperm motility.

Contraindications:

Hypersensitivity, acute myocardial infarction, retinal haemorrhage.

2. ISOXSUPRINE HYDROCHLORIDE / DUVADILAN *

Isoxsuprine, or Isoxsuprine hydrochloride, is a beta agonist drug used as a vasodilator in humans and equines. It is a sympathomimetic agent with p-receptor stimulating effects, dilates blood vessels in skeletal muscle and stimulates the heart. Blood pressure is reported to be little affected but cardiac output increases; peripheral resistance falls while muscle blood flow increases. There is a relaxation of the uterine muscles (Goodman and Gilman, 1965).

Chemical nomenclature:

Chemical formula of Isoxsuprine hydrochloride is 2-hydroxy-2-(4-methoxyphenyl)-1-methyl-N-(1-phenox)-2-propyl) ethanaminium chloride.
Chemical Structure:

Structural formula of Isoxsuprine hydrochloride is:

![Chemical Structure Diagram]

Indications:

Peripheral vascular disorder, intermittent claudication, Buerger's disease, night leg cramps, premature labour, habitual abortion, threatened abortion, cerebral vascular disease.

Contraindications:

Recent haemorrhage, severe anemia, premature detachment of placenta, immediate postpartum, parenteral use in patient with known heart disease.

3. XANTHINOL NICOTINATE / COMPLAMINA *

Xanthinol nicotinate is a peripheral vasodilator (nictonic acid prodrug). It is the most potent form of niacin (active part of vitamin B3), stimulates memory and concentration by improving the brain’s blood flow. It passes easily through cell membranes.

Xanthinol Nicotinate has been shown to increase brain glucose metabolism, improve brain ATP levels and improve brain blood flow (it acts as a vasodilator). As such, Xanthinol Nicotinate has been used to treat short-
term memory disorders, mental flagging (i.e., lack of brain energy that compromises vigilance, concentration and attention) and insufficient blood flow to the arteries and the extremities. Furthermore, Xanthinol Nicotinate has been clinically shown to improve the reaction speed of the elderly.

**Chemical nomenclature:**

Chemical formula of xanthinol nicotinate is 7-[2-Hydroxy-3-(2-hydroxyethyl-methyl-amino) propyl]-1, 3-dimethyl-purine-2, 6-dione pyridine-3-carboxylic acid

**Chemical Structure:**

Structural formula of xanthinol nicotinate \((C_{13}H_{21}N_{5}O_{4} \cdot C_{6}H_{5}NO_{2})\) is:

![Chemical Structure Image]

**Indications:**

Peripheral vascular disease, disordered cerebral function, hyperlipidaemia.

**Contraindications:**

Hypersensitivity towards the product, cardiac decompensation, acute hemorrhaging, cerebral infarction, acute myocardial infarction (this product is not recommended in the case of coronary insufficiency due to the danger
of myocardial infarction), vascular collapse, association with MAO inhibitor or ganglioplegic treatment, gastroduodenal ulcer in its evolutionary phase.

4. **METOPROLOL TARTRATE / METOLAR**

Metoprolol tartrate is an antihypertensive drug. It is a selective beta-l-adrenoreceptor blocking agent. The *in vitro* and *in vivo* animal studies have shown that it has a preferential effect on beta1 adrenoreceptors, chiefly located in cardiac muscle. This preferential effect is not absolute, however, and at higher doses, metoprolol also inhibits beta2 adrenoreceptors, chiefly located in the bronchial and vascular musculature. The tartrate is used in both oral and intravenous formulations.

**Chemical nomenclature:**

Chemical formula of Metoprolol tartrate is 1-Isopropylamino-3-[4- (2-methoxyethyl) phenoxy]-2-propanol L-(+)-tartrate

**Chemical Structure:**

Structural formula of Metoprolol tartrate is:

\[
\begin{align*}
\text{CH}_3\text{OCH}_2\text{CH}_2- & \quad \text{OCH}_2\text{CH}_2\text{NHCH}_2\text{CH}_3: \\
\end{align*}
\]

**Indications:**

Used alone or in combination with other antihypertensive agents, for management of hypertension, long-term management of angina pectoris, myocardial infarction (immediate-release tablets and injection).
Contraindications:

Greater than first-degree heart block, congestive heart failure unless secondary to tachyarrhythmia treatable with beta-blockers, overt or moderate to severe cardiac failure, sinus bradycardia, cardiogenic shock, hypersensitivity to beta-blockers, systolic blood pressure < 100 mm/Hg, MI in patients with heart rate < 45 beats/min.

DESCRIPTION OF CONTROL CHEMICALS

1. Negative control (Dimethyl sulphoxide, DMSO)

In the present investigation the test drugs used are in injection form. So the solvent control used is the organic compound Dimethyl sulphoxide (DMSO), which is being successfully employed as solvent for most of the formulations. It has been reported to be non-toxic to animals and humans (Preston, et al., 1987). This negative control provides the baseline to which the data were compared to see the significant effects.

\[
\text{CH}_3 - S = O - \text{CH}_3
\]

2. Positive control (Cyclophosphamide, CP)

Cyclophosphamide (endoxan) is the most versatile therapeutic drug which possess the broadest spectrum and antitumor activity of all alkylating
agents (Brock, 1967). It has strong mutagenic effects. It acts by forming DNA crosslinks by the reaction of chloroethyl moieties of CP with adjacent nucleotide bases. This drug is widely used as positive control in *in vitro* assays.

\[
\text{\includegraphics[width=0.3\textwidth]{Cyclophosphamide_structure.png}}
\]

**MATERIALS**

1. **Chemicals and biochemicals**
   - Glacial acetic acid
   - Methanol
   - Potassium chloride
   - Potassium dihydrogen phosphate
   - Sodium bicarbonate
   - di-Sodium hydrogen phosphate

   Qualigens (GSK Pharmaceuticals)
   Qualigens (GSK Pharmaceuticals)
   Qualigens (GSK Pharmaceuticals)
   Qualigens (GSK Pharmaceuticals)
   SD Fine (SD Fine Chemicals)
   Qualigens (GSK Pharmaceuticals)

2. **Test drugs and solvent controls**
   - Pentoxifylline
   - Isoxsuprine hydrochloride
   - Xanthinol nicotinate
   - Metprolol tartrate
   - Dimethyl sulphoxide (- control)
   - Cyclophosphamide (+ control)

   Aventis (Aventis Pharma)
   Solvay (Solvay Pharma)
   Cadila Healthcare
   Cipla (Cipla, India)
   Qualigens (GSK Pharmaceuticals)
   Qualigens (GSK Pharmaceuticals)

3. **Solutions and buffers**
Cell culture (CA assay and CBMN assay)

Antibiotic Antimycotic solution  Gibco™ (Invitrogen Corporation)
Colchicine  Loba Chemie
Cytochalasin - B  Gibco™ (Invitrogen Corporation)
Fetal calf serum (FCS)  Sigma - Aldrich
Heparin Sodium (Beparin)  BE (Biological E.)
Phytohaemagglutinin (PHA-M)  Gibco BRL (Life Technologies)
RPMI Medium 1640,  Gibco™ (Invitrogen Corporation)
(with L - glutamine)

Staining
Giemsas stain solution  Merck
Sorensen’s buffer
• KH₂PO₄ (0.1 M)
• Na₂HPO₄ (0.1 M)

4. Software
Microsoft Office 2007
SPSS 11.0 for Windows
Curve Expert 1.3

METHODS

1. In vitro testing of chromosomal aberrations (CA assay)

A. Cell cultures:

a. Preparation of stock culture media

Culture media from RPMI 1640 with L - glutamine was prepared by dissolving the powdered media (x g) in 1 liter (1000 ml) double distilled
autoclaved water by gentle shaking. pH was adjusted at 7.4 with 2 g NaHCO₃. Then 10 ml of antibiotic – antimycotic (10,000 units /ml penicillin G sodium, 10,000 μg /ml streptomycin sulphate and 25 μg /ml amphotericin B) was also added. Contents were mixed gently and thoroughly and the media was then filter sterilized in the negative pressure Millipore filtration assembly by using 0.22 μM millipore filters. This 1X filtered stock medium was then stored in sterilized and tightly capped bottles at 4° C in dark.

b. Preparation of working media

The working medium was prepared from above stored stock medium under the sterile conditions of laminar flow. For the preparation of 100 ml working medium, 79 ml (1X) stock medium, 20 ml fetal calf serum (supplement) and 1ml of heparin (anticoagulant) were mixed respectively and then pH of the solution was checked to 7.4 using pH strips under the range.

c. Collection of blood samples

5 ml peripheral blood from healthy donors was collected by venipuncture under aseptic conditions using sterile disposable syringes (Dispovan®) of 5 ml capacity. Heparin (500 IU / ml; Biological E.) was used as anticoagulant. Blood was stored in sterile collection vial at 4° C for half an hour to separate red blood cells (RBC, s) from plasma. Fresh collection was done every time before each experiment.

d. Setting of the cultures

Culture was set according to the standard techniques of Moorhead, et al. (1960) and Evans, (1976) with few lab modifications under the sterile
conditions of laminar flow hood. 4.5 ml of working medium was taken in 10 - 15 ml capacity sterile culture glass vial and 0.1 ml phytohaemagglutinin - M (PHA - M), a mitogen, was added to it. Lymphocyte culturing was done by adding 0.8 ml of plasma containing white blood cells (WBC,s) in the this medium and the contents were mixed gently. The culture vials were then tightly capped and sealed with parafilm to avoid CO2 loss and culture was incubated at 37° C in dark for 72 hours.

e. Chemical exposure

Human peripheral lymphocytes were incubated for 48 hours at 37°C before the test drugs were added to culture medium. After cultivation for further 21½ hours, i.e. 2½ hours prior to harvesting, Colchicine (0.20 μg/ml) was added to arrest the cells at metaphase stage.

Drug concentrations were selected as per previous work on this class of drugs (Bozsakyova, et al., 2001) on the basis of preliminary cytotoxicity tests. The highest concentration of the drug suppressed the mitotic activity of the cells by nearly 50%.

For control cultures, Dimethyl sulfoxide (negative control) and Cyclophosphamide (positive control) were added to cultures.

f. Harvesting of culture

After 72 hours the cultures were taken out of 37° C incubator temperature and the contents were mixed and transferred to the 15 ml centrifuge tubes for centrifugation. The cells were then spun down by centrifugation (10 minutes, 1000 rpm) and the pellet was saved.
Hypotonic treatment (0.53 % KCl) was done for about 20 minutes at 37° C. Pellet was again saved by centrifugation (10 minutes, 1000 rpm). Saved pellet was then fixed with 5 ml chilled fixative (1:3 acetometanol). Cells were washed 2-3 times with fixative before white pellet was obtained. For proper fixation, cells were kept for 1 hour and preferably overnight at 4° C.

B. Slide preparation and staining

Fixed cells were resuspended in 0.2 ml fresh fixative. Two or three drops of suspended cells were dropped on clean, grease free, pre-chilled wet microscope slides of super deluxe quality. These slides were then air dried and left for over night before staining and storage.

One – day old slides were stained in 5% Giemsa’s stain in Sorensen’s buffer (pH 6.8) for 10 – 15 minutes and then rinsed in distilled water to avoid excess stain before drying.

C. Analysis of the cells

All slides were first marked accordingly. Nearly 100 to 300 metaphases were analyzed for each concentration of test drug. Chromosomal aberrations were scored as chromatid, isochromatid and exchanges as per the classification of Evans (1984) and Savage (1976). Percent aberrant metaphases were also scored.
2. **In vitro Cytokinesis – Blocked Micronucleus (CBMN) assay**

A. **Setting of the culture and harvesting**

CBMN assay was carried out as per standard technique of Michael Fenech (2000) with some lab modifications. In this assay, the initial steps employed in setting the culture were the same as in CA assay, except the test drug exposure time and addition of Cytochalasin – B (a microfilament-assembly inhibitor) to culture medium.

In CBMN assay, human peripheral lymphocytes were incubated for 24 hours before the test drugs were added to culture medium containing 20 % FCS and 0.1 ml PHA – M. Cultures were further incubated at 37°C for 20 hours and Cytochalasin – B (3μg cyt - B / ml) was added to each culture at 44 hour after PHA stimulation.

After 28 hours of adding Cyt – B, cells were harvested by centrifugation. Hypotonic treatment (prewarmed 0.53 % KCl) was given for a very short period of 1 – 2 minutes, followed by cell fixation in fresh and chilled acetomethanol solution (1:3).

B. **Slide preparation and Staining**

Slides were prepared by dropping 2 drops of fixed solution over clean and chilled wet glass slides. They were then air dried and left for one day. One – day old slides were stained in 5% giemsa solution in 50 ml of Sorensen’s buffer (pH 6.8) for 15 – 20 minutes.
C. Analysis of cells

Stained slides were marked accordingly and observed under 100 X lens of light microscope (Olympus-13X50, Japan) with camera attachment (ProgrRes® C3, Jenoptik, Germany) and a monitor to observe and capture cellular details. About 1000 binucleated cells were scored for micronucleus estimation, nucleoplasmic bridges, nuclear buds and binucleated micronucleated cells. About 500 cells were observed for other measures like mono -, bi -, tri – and tetranucleate cells, and apoptotic and necrotic cells (Fenech, 2000)

3. Cell division kinetics

In CBMN assay, the cells with one (M1), two (M2), three (M3) and four (M4) nuclei were scored from a population of 500 viable cells, and apoptotic (nuclear fragmentation with intact cytoplasm) and necrotic cells (vacuolated pale cytoplasm with damaged membrane and intact nucleus or loss of cytoplasm with damaged membrane and partially intact nucleus) along with cells with 1 – 4 nuclei were detected in 500 viable and non- viable cells. Nuclear division index (NDI) was calculated as per the method given by Eastmond and Tucker (1989) using the formula:

\[ NDI = \frac{M1 + 2(M2) + 3(M3) + 4(M4)}{N} \]

Where M1 – M4 represented the number of cells with one to four nuclei and N was the total number of viable cells. The NDI and the proportion of binucleated cells were used as useful parameters for comparing the mitogenic response of lymphocytes and cytostatic effects of drugs examined in this assay.
More accurate assessment of nuclear division status and cell division kinetics was obtained by including necrotic and apoptotic cells in the study. This inclusion resulted in attainment of nuclear division cytotoxicity index (NDCI) using the equation:

\[
NDCI = \frac{(Ap + Nec + M1 + (M2) + 3(M3) + 4(M4))}{N^*}
\]

Where \(Ap\) = number of apoptotic cells, \(Nec\) = number of necrotic cells, \(M1 - M4\) = number of viable cells with 1 -4 nuclei and \(N^*\) = total number of cells scored (viable and non – viable).

The deviation from the control was determined by using Chi – square test for above parameters.

4. Statistical evaluation of the test results

Statistical packages (Microsoft Office excel 2007, SPSS 11.0 for Windows and Minitab 15 English) and statistical calculator were used to analyze results. Mean, Standard Deviation (SD) and Standard Error of Mean (SEM) were calculated first. The standard error for individual observations and for control reading was calculated using the formula

\[
SE = \sqrt{\frac{(1-n) n}{N}}
\]

Where \(n\) indicates individual reading and \(N\) is taken as 100 (for 100 cells of study).

Student’s two tailed ‘t’ test was used for calculating the statistical difference in chromosomal aberrations and MNT (micronucleus test) variables (micronuclei, binucleated micrconucleated cells, nucleoplasmic bridges, nuclear buds etc), by comparing the effects induced by different test
drugs with that of respective controls. The statistical significance was calculated from Fisher and Yates table at \((n_1+n_2-2)\) degree of freedom at 0.005% level of significance \((p)\).

2×3 Chi-Square test (\(\chi^2\)) for homogeneity of variance was used to analyze cell division kinetics of test drugs compared with the control values. The level of significance was tested from standard statistical tables of Fisher and Yates (1963) at \(P < 0.005\%\).

In case of chromosomal alterations conditions like endoreduplication, ploidies, pulverizations and cells with multiple anomalies were not included in the statistical analysis due to their very few numbers.
RESULTS
RESULTS

In the present study, four commonly recommended cardiovascular drugs, i.e., Pentoxifylline (Trental®), Isoxsuprine hydrochloride (Duvadilan®), Xanthinol nicotinate (Complamina®) and Metoprolol tartrate (Metolar®) were assessed for their genotoxic potential in vitro. The genotoxic potentiality was checked at five different concentrations of drugs and the time of exposure was kept constant for each concentration. Two short-term but high potential assays, namely Chromosomal aberration assay and Cytochalasin-block micronucleus assay, were employed to test drug genotoxicity and cytotoxicity. Six parameters were applied to evaluate the results. For statistical analysis, Student’s $t$-test was used for the comparison of chromosomal aberrations, binucleated micronucleated cell frequencies, micronucleus frequencies, nucleoplasmic bridge frequencies and nuclear bud frequencies of test drugs with that of controls. The $2 \times 3$ Chi-Square ($\chi^2$) test was used for nuclear/cell division kinetics and nuclear division cytotoxicity index studies of test drugs and results were compared with controls. The level of significance was studied at $P < 0.005$ for both the parametric tests.

Comparative analysis of the four tested drugs was also done as part of the present study. The drugs were studied for their relative responses to the above used parameters in order to check out the highest response and the lowest one.

1. PENTOXIFYLLINE (TRENTAL®)

(a) Chromosomal aberrations:
The results of chromosomal aberrations analyzed for five different concentrations (0.15 mg/ml, 0.30 mg/ml, 0.60 mg/ml, 1.00 mg/ml and 2.00 mg/ml) Pentoxifylline at 48 hour of incubation are summed up in Table 1. As given in the table, the percentage of aberrant metaphases has increased gradually (40, 53.3, 54.5, 53.3 to 68.7) with the increasing concentrations of the drug except for one value at 0.6 mg/ml concentration, where the percentage of aberrant metaphases has dropped slightly. Aberrations per cell also showed the same pattern of increased values against increasing drug concentrations respectively. Statistically analyzed by applying Student’s t-test, all these values were highly significant at P < 0.005, compared to the negative control value. The results of the positive control were also significant at P < 0.005 at 48 hour of incubation.

The higher frequencies of aberrant metaphases have also been depicted in bar graph where the increasing bar lengths, indicative of the aberrant metaphase frequencies, was observed with increasing concentrations of the drug (Fig. 1).

Regression analysis of aberrations per cell revealed dose-response curve plotted for increasing drug concentration versus increasing aberrations per cell. A positive correlation was found between above two variables (Correlation coefficient R = 0.35). The best fit regression line determined by least squares method along with line equation is shown in Fig. 2.

(b) Micronucleus and Binucleated mirconucleated cell frequencies

The results of the CBMN assay for Micronucleus (MN) frequencies and Binucleated Mirconucleated (BNMN) cell frequencies are depicted in table 2 and table 3 respectively. Table 2 shows that MN induction has
increased with the respective drug concentration. Statistically speaking, the last four values (5.1%, 5.6%, 6.7% and 15.0%, at concentrations of 0.30 mg/ml, 0.60 mg/ml, 1.00 mg/ml and 2.00 mg/ml respectively) were significantly different with respect to control values at (P < 0.005) (Student’s t-test), while at initial drug concentration of 0.15 mg/ml, the difference was insignificant.

The observed frequencies of BNMN cells also increased gradually for the increasing drug concentrations as percent values 2.8, 5.1, 5.6, 6.7 to 10.0. This increase was in line with the increase of MN frequencies, the only difference being that at the highest dose i.e., 2.00 mg/ml, MN frequency is slightly higher (15.0%) as compared to the frequency of BNMN cells (10.0%) of the same concentration. Considering the statistical analysis of BNMN cell frequencies, similar results were obtained as in the case of MN induction. Here the last four values were also found to be highly significant at P < 0.005, as compared to control value for 24 hour of incubation. the first value was insignificant at P < 0.005.

Regression analysis of micronucleus frequencies plotted against drug concentrations reveals dose-response curve. The observed MN frequencies correlated highly and positively (Correlation coefficient R = 0.97) with drug concentrations applied. The best fit regression line (determined by least squares method) and the regression line equation describing it are presented in Fig. 3.

Bar graph analysis of BNMN cell frequencies (minus negative control) drawn against drug concentrations also revealed increased bar
lengths (increased BNMN cell frequencies) with increasing concentrations of the respective drug (Fig. 4).

(c) Nucleoplasmic bridge frequencies:

Table 3 indicates the results of nucleoplasmic bridge (NPB) frequencies induced by the drug pentoxifylline. This table shows relative occurrence of nucleoplasmic bridges in 1000 binucleated cells. Frequencies of NPB, although did not show consistent increase with increasing drug doses, but an over all increase in the effect was seen comparing lowest drug dose (0.15 mg/ml), and highest drug dose (2.00 mg/ml) respectively. At 0.15 mg/ml concentration, the NPB frequency was 12.9 % while at 2.00 mg/ml concentration the NPB frequency was increased to 28.6 %. This result is highly significant at P < 0.005 as compared with the control.

The dose-dependent nucleoplasmic bridge frequencies were also shown in Fig. 4, where increased bar lengths were observed with increasing drug doses (0.15 mg/ml, 0.30 mg/ml, 0.60 mg/ml, 1.00 mg/ml and 2.00 mg/ml respectively) indicating increased NPB frequencies.

(d) Nuclear bud frequencies:

The results of nuclear bud (NB) frequencies are also shown in table 3. These frequencies were low as compared to other parameters shown in the same table i.e. micronuclei and nucleoplasmic bridges. The observed range varied in percentage from 2.6, 3.0, 4.0, 6.6 to 9.0, indicating that the effect got increased with increasing respective drug concentrations (0.15 mg/ml, 0.30 mg/ml, 0.60 mg/ml, 1.00 mg/ml and 2.00 mg/ml). The initial two values, at drug concentrations of 0.15 mg/ml and 0.30 mg/ml respectively,
were not significant at P <0.005 level. The value at 0.60 mg/ml concentration was observed to be significant, while for the last two final concentrations, i.e., 1.00 mg/ml and 2.00 mg/ml respectively, the results were highly significant compared to the control (P < 0.005).

Graphical presentation of NB frequencies drawn against drug doses showed the increased bar length pattern (NB frequencies), with increasing drug concentrations (Fig. 4).

(e) Nuclear Division Index (Cell Division Kinetics):

The extent and progression of nuclear division could be assessed by analyzing the nuclear division index (NDI). The data for nuclear/cell division kinetics of the drug Pentoxifylline is presented in Table 4. This table also contains viable (mononucleated, binucleated, trinucleated and tetrnucleated) cell values. The NDI was 1.25 at lowest drug concentration of 0.15 mg/ml, while at highest concentration of 2.00 mg/ml, the NDI value was 1.06. Other NDI values fell in middle range as 1.10, 1.03 and 1.07 at drug doses of 0.30 mg/ml, 0.60 mg/ml and 1.00 mg/ml respectively. All these values were highly significant at P < 0.005, when compared to control which has an NDI value of 1.24. The calculation of the NDI revealed that there was a significant cell cycle delay detectable after exposing culture cells for 24 h to drug with increasing concentrations (0.15 mg/ml, 0.30 mg/ml, 0.60 mg/ml, 1.00 mg/ml and 2.00 mg/ml).

Regression analysis of the parameter NDI showed statistically significant (P< 0.005) negative correlation between the two variables, i.e. the drug concentrations versus NDI (Correlation coefficient R = 0.54). The best
Table 1: Chromosomal aberrations induced by Pentoxifylline in cultured human peripheral blood lymphocytes.

<table>
<thead>
<tr>
<th>Drug Concentration (mg/ml)</th>
<th>Aberrant metaphase/100 metaphase (±SE)</th>
<th>g b/f other</th>
<th>Aberrations/100 metaphases</th>
<th>Exchange of CA (excluding gaps)</th>
<th>Total no. of CA cell ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15</td>
<td>40±4.8**</td>
<td>20</td>
<td>27</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>0.30</td>
<td>53.3±4.9**</td>
<td>67</td>
<td>21</td>
<td>16</td>
<td>21</td>
</tr>
<tr>
<td>0.60</td>
<td>54.5±4.9**</td>
<td>9</td>
<td>55</td>
<td>45</td>
<td>9</td>
</tr>
<tr>
<td>1.00</td>
<td>53.3±4.9**</td>
<td>7</td>
<td>33</td>
<td>21</td>
<td>13</td>
</tr>
<tr>
<td>2.00</td>
<td>68.7±4.6**</td>
<td>13</td>
<td>50</td>
<td>50</td>
<td>25</td>
</tr>
<tr>
<td>Control</td>
<td>4.33±2.03</td>
<td>3.33</td>
<td>1.33</td>
<td>4.66</td>
<td>0.046±0.02</td>
</tr>
<tr>
<td>Negative</td>
<td>17.33±3.7**</td>
<td>31.33</td>
<td>20.33</td>
<td>51.66</td>
<td>0.52±0.05*</td>
</tr>
</tbody>
</table>


* All values are significant at P < 0.005, ** All values are highly significant at P < 0.005, Student’s t–test.
Fig. 1. Frequencies of aberrant metaphases plotted against different concentrations of Pentoxifylline.

Fig. 2. Dose-response curve of aberrations per cell in human lymphocytes \textit{in vitro} treated with different concentrations of Pentoxifylline.
Table 2: Estimation of frequencies of Pentoxifylline induced micronuclei in human lymphocytes in vitro.

<table>
<thead>
<tr>
<th>Drug concentration (mg/ml)</th>
<th>Total BN cells</th>
<th>Total BNMN cells</th>
<th>Distribution of MN in BN cells</th>
<th>Total MN</th>
<th>Frequency of MN (%)± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
<td>&gt;2</td>
</tr>
<tr>
<td>0.15</td>
<td>1000</td>
<td>28</td>
<td>28</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.30</td>
<td>1000</td>
<td>51</td>
<td>51</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.60</td>
<td>1000</td>
<td>56</td>
<td>56</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1.00</td>
<td>1000</td>
<td>67</td>
<td>67</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2.00</td>
<td>1000</td>
<td>100</td>
<td>50</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>1000</td>
<td>16</td>
<td>16</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

BN- Binucleated cells, BNMN- Binucleated Mirconucleated cells, MN- Micronuclei, SE- Standard error.
* All values are significant at P < 0.005, ** All values are highly significant at P < 0.005, Student’s t – test.
Table 3: Frequencies of binucleated cells with nucleoplasmic bridges, micronuclei and nuclear buds observed after the treatment of Pentoxifylline at different concentrations in human lymphocytes *in vitro*.

<table>
<thead>
<tr>
<th>Drug Concentration (mg/ml)</th>
<th>BN Total</th>
<th>BN + NPB</th>
<th>Frequency (%)+SE</th>
<th>BN + MN</th>
<th>Frequency (%)+SE</th>
<th>BN + NB</th>
<th>Frequency (%)+SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15</td>
<td>1000</td>
<td>129</td>
<td>12.9±1.05**</td>
<td>28</td>
<td>2.8±0.52</td>
<td>26</td>
<td>2.6±0.50</td>
</tr>
<tr>
<td>0.30</td>
<td>1000</td>
<td>118</td>
<td>11.8±1.02**</td>
<td>51</td>
<td>5.1±0.69**</td>
<td>30</td>
<td>3.0±0.53</td>
</tr>
<tr>
<td>0.60</td>
<td>1000</td>
<td>187</td>
<td>18.7±1.23**</td>
<td>56</td>
<td>5.6±0.72**</td>
<td>40</td>
<td>4.0±0.61*</td>
</tr>
<tr>
<td>1.00</td>
<td>1000</td>
<td>143</td>
<td>14.3±1.10**</td>
<td>67</td>
<td>6.7±0.78**</td>
<td>66</td>
<td>6.6±0.78**</td>
</tr>
<tr>
<td>2.00</td>
<td>1000</td>
<td>286</td>
<td>28.6±1.4**</td>
<td>100</td>
<td>10.0±0.94**</td>
<td>90</td>
<td>9.0±0.90**</td>
</tr>
<tr>
<td>Control</td>
<td>1000</td>
<td>18</td>
<td>1.8±0.42</td>
<td>16</td>
<td>1.6±0.39</td>
<td>15</td>
<td>1.5±0.38</td>
</tr>
</tbody>
</table>

* All values are significant at P < 0.005, ** All values are highly significant at P < 0.005, Student’s t – test.
Fig. 3. Dose-response curve of micronuclei frequencies in human lymphocytes *in vitro* treated with different concentrations of Pentoxifylline.

\[
Y = 2.05 + 6.15X
\]

Fig. 4. Frequencies of binucleated micronucleated cells, nucleoplasmic bridges and nuclear buds in human lymphocytes *in vitro* plotted against different concentrations of Pentoxifylline.
Table 4: Estimation of Nuclear Division Index (NDI) for 500 viable cells after the treatment of different concentrations of Pentoxifylline in vitro.

<table>
<thead>
<tr>
<th>Drug Concentration (mg/ml)</th>
<th>Mono</th>
<th>Nucleated Cells</th>
<th>Tetra</th>
<th>Nuclear Division Index (NDI)</th>
<th>2X3 Chi-square test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bi</td>
<td>Tri</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.15</td>
<td>378</td>
<td>115</td>
<td>7</td>
<td>0</td>
<td>1.25</td>
</tr>
<tr>
<td>0.30</td>
<td>452</td>
<td>44</td>
<td>4</td>
<td>0</td>
<td>1.10</td>
</tr>
<tr>
<td>0.60</td>
<td>486</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>1.03</td>
</tr>
<tr>
<td>1.00</td>
<td>465</td>
<td>35</td>
<td>0</td>
<td>0</td>
<td>1.07</td>
</tr>
<tr>
<td>2.00</td>
<td>469</td>
<td>31</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Control</td>
<td>399</td>
<td>87</td>
<td>9</td>
<td>5</td>
<td>1.24</td>
</tr>
</tbody>
</table>

Mono- Mononucleated cells, Bi- Binucleated cells, Tri- Trinucleated cells, Tetra- Tetranucleated cells.
Table 5: Estimation of Nuclear Division Cytotoxicity Index (NDCI) for 500 (viable + nonviable) cells after the treatment of different concentrations of Pentoxifylline in vitro.

<table>
<thead>
<tr>
<th>Drug Concentrations (mg/ml)</th>
<th>Mono</th>
<th>Viable Cells (Nucleated)</th>
<th>Non viable Cells</th>
<th>NDCI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bi</td>
<td>Tri</td>
<td>Tetra</td>
</tr>
<tr>
<td>0.15</td>
<td>389</td>
<td>99</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>0.30</td>
<td>442</td>
<td>43</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>0.60</td>
<td>470</td>
<td>11</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1.00</td>
<td>450</td>
<td>29</td>
<td>0</td>
<td>0</td>
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<tr>
<td>2.00</td>
<td>466</td>
<td>26</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>399</td>
<td>71</td>
<td>8</td>
<td>3</td>
</tr>
</tbody>
</table>

Mono- Mononucleated cells, Bi- Binucleated cells, Tri- Trinucleated cells, Tetra- Tetranucleated cells

* All values are significant at P< 0.005, **All values are highly significant at P< 0.005, 2X3 Chi-square test.
Fig. 5. Dose-response curve of nuclear division indices in human lymphocytes \textit{in vitro} treated with different concentrations of Pentoxifylline.

Fig. 6. Dose-response curve of nuclear division cytotoxicity indices in human lymphocytes \textit{in vitro} treated with different concentrations of Pentoxifylline \textit{in vitro}. 

\[ Y = 1.16 + (-0.06) X \]

\[ Y = 1.13 + (-0.06) X \]

Drug concentrations (mg/ml)

Nuclear Division Index (NDI)

Nuclear Division Cytotoxicity Index (NDCI)
fit regression line determined by least squares method along with regression line equation is presented in Fig. 5.

(a) **Nuclear Division Cytotoxicity Index (NDCI):**

Table 5 reveals nuclear division cytotoxicity index (NDCI) for the drug Pentoxifylline. In this table mononucleated, binucleated, trinucleated, tetranucleated, apoptotic and necrotic cells were also scored. It is apparent from the table that the NDCI values decreased over all, as the drug doses got increased (1.20 at lowest concentration and 1.05 at highest concentration), thus indicating the cytotoxic effects and cell cycle delay which are indicated by a reduction in the NDCI values. The only exceptions were for 0.60 mg/ml and 1.00 mg/ml drug concentrations, where the values showed a gradual increase. The obtained range for NDCI values was 1.20, 1.10, 1.02, 1.05 and 1.05 respectively. Statistical analysis of the data, applying $\chi^2$ test, showed that all values were highly significant at $P<0.005$ compared with the control value (1.192).

Study of the best fit regression line showed a negative correlation between drug concentrations and NDCI values for Pentoxifylline (Correlation coefficient $R = 0.56$) (Fig. 6).

1. **ISOXSURPRINE HYDROCHLORIDE (DUVADILAN®)**

(a) **Chromosomal aberrations:**

Table 6 represents the results of chromosomal aberrations studied at five different concentrations (0.15 mg/ml, 0.30 mg/ml, 0.60 mg/ml, 1.00 mg/ml and 2.00 mg/ml) of the drug Isoxsurprine hydrochloride at 48 hour of incubation. According to the table, percentage of aberrant metaphases
increased gradually with the increasing concentrations of the drug. However, two of the values (at 0.30 mg/ml and 2.00 mg/ml drug respectively), showed contrasting results. Thus, to summarize the effect, 46.15% abnormal metaphases were found at 0.15 mg/ml drug concentration, while this percentage got increased to 71.4% at 2.00 mg/ml of drug concentration. Statistically, all values were highly significant at $P < 0.005$ when compared with negative control. The positive control also showed significant effect at $P < 0.005$ for 48 hour of incubation. Similarly, the study of aberrations per cell showed an over all increased effect of the drug, the values being 0.39, 0.61, 0.93, 1.01 and 1.00 at 0.15 mg/ml, 0.30 mg/ml, 0.60 mg/ml, 1.00 mg/ml and 2.00 mg/ml of drug concentrations respectively. All these readings along with the positive control reading were highly significant at $P < 0.005$.

Graphical presentation of aberrant metaphases per 100 normal metaphases (minus negative control) displayed the high frequency with increased drug concentrations, except for the two contrasting values (Fig. 7).

Regression analysis of aberrations per cell between increased drug concentration and increased aberrations per cell showed a positive relationship, (Correlation coefficient $R = 0.76$). The best fit regression line determined by least squares method and the equation of the line are shown in Fig. 8.

(b) **Micronucleus and Binucleated micronucleated cell frequencies**

Table 7 and table 8 summarize the results of the CBMN assay for Micronucleus (MN) and Binucleated Micronucleated (BNMN) cell frequencies respectively. As per Table 7, drug Isoxsuprine hydrochloride
induced MN frequencies displayed an increased pattern with increased drug concentrations respectively, except for one reading at 0.60 mg/ml drug dose showing a much lesser value than others. The range of the frequencies of MNi in percentage was from 2.0, 10.0, 2.8, 13.3 to 14.3 respectively. Statistically speaking, two values (at 0.15 mg/ml and 0.60 mg/ml drug concentrations) were not significant, while rest of the three values were highly significant at P < 0.005.

The observed frequency of BNMN cells increased gradually from 2.0, 6.0, 2.8, 13.3 and 14.3 in percent, with increasing drug concentrations (0.15 mg/ml, 0.30 mg/ml, .60 mg/ml, 1mg/ml and 2mg/ml) excepting one value at 0.60 mg/ml drug dose. The values at 0.30 mg/ml, 1mg/ml and 2mg/ml were found to be highly significant statistically at P < 0.005 compared to negative control value at 24 hour of incubation. Rest two values were not significant at P < 0.005, level.

Regression graph analysis of MN frequencies plotted against drug concentrations (minus control) revealed dose – response curve indicating increased MN induction with increasing drug doses. Correlation between the two parameters was positive with Correlation Coefficient, R being 0.73. The best fit regression line obtained by least squares method and regression line equation were shown in Fig. 9.

The bar diagram analysis of BNMN cell frequencies (minus control) drawn against drug concentrations also showed increased bar lengths (symbolizing BNMN cell frequencies) with increasing drug doses respectively (Fig. 10).

(e) **Nucleoplasmic bridge frequencies:**
The results of nucleoplasmic bridge (NPB) frequencies for the drug Isoxsuprine hydrochloride are presented in Table 8. This table shows relative occurrence of nucleoplasmic bridges in 1000 binucleated cells. NPB frequencies increased consistently with drug doses, but at one concentration (0.30 mg/ml), a contrasting value was obtained. However an over all increase in the effect was obtained comparing the lowest drug dose (0.15 mg/ml) and highest drug dose (2.00 mg/ml) respectively. At 0.15 mg/ml concentration the NPB frequency was 14.3 % while at 2.00 mg/ml concentration, the NPB frequency got increased to 28.6 % for the drug Isoxsuprine hydrochloride. Statistically, all the values were highly significant at P < 0.05 as compared with the negative control.

NPB frequencies plotted against drug concentrations showed a dose-dependant graph indicating elevated response shown by bars (Fig. 10).

(d) Nuclear bud frequencies:

Table 8 also summarizes the results of nuclear bud (NB) frequencies, for the drug Isoxsuprine hydrochloride. The observed range was 5.3%, 8.3%, 7.1 %, 6.3 % and 10.0 % at drug concentrations 0.15 mg/ml, 0.30 mg/ml, 0.60 mg/ml, 1.00 mg/ml and 2.00mg/ml respectively. According to the obtained series, the effect did not increase uniformly, however considering the lowest drug concentration and the highest drug concentrations, the increased response was obtained. Analyzing statistically, all the values were highly significant as compared with the negative control value at P < 0.005.

The bar diagram drawn for NB frequencies against drug doses also indicated the same result (Fig. 10). Here increased bar length pattern were
obtained with increased drug concentrations respectively. Control reading was not plotted in the graph.

(e) **Nuclear Division Index (Cell Division Kinetics):**

The data for nuclear/cell division kinetics of the drug Isoxsuprine hydrochloride is shown in Table 9. Cells with one, two, three and four nuclei were also scored in the table. Cell division kinetics involving nuclear division is studied through nuclear division index (NDI). According to the table, the NDI value reduced down for lowest drug concentration (0.15 mg/ml) to the highest drug concentration (2.00 mg/ml) gradually. The observed pattern of reduction in NDI values was 1.24, 1.21, 1.21, 1.22, and 1.16 respectively indicating a significant cell cycle delay due to drug exposure. Statistical analysis, applying 2X3 Chi-square test, indicated that these values were highly significant at P < 0.005 when compared with the control which has an NDI value of 1.24.

A negative correlation was observed between the two measures, i.e., drug concentrations and NDI (correlation coefficient, \( R = 0.88 \)). Regression graph analysis of the parameter NDI showed a best fit regression line determined by least squares method. The regression line and equation are presented in Fig. 10.

(f) **Nuclear Division Cytotoxicity Index (NDCI):**

A more accurate assessment of nuclear division status along with nuclear cytotoxicity measure can be obtained through Nuclear Division Cytotoxicity Index (NDCI). NDCI for Isoxsuprine hydrochloride is given in Table 10. Besides mononucleated, binucleated, trinucleated and
Table 6: Chromosomal aberrations induced by Isoxsuprine hydrochloride in cultured human peripheral blood lymphocytes.

<table>
<thead>
<tr>
<th>Drug Concentration (mg/ml)</th>
<th>Aberrant metaphase/100 metaphase (±SE)</th>
<th>Chromatid g</th>
<th>Chromatid b/f</th>
<th>Chromatid other</th>
<th>Aberrations/100 metaphases Isochromatid g</th>
<th>Isochromatid b/f</th>
<th>Isochromatid other</th>
<th>Exchange dic</th>
<th>Exchange tr</th>
<th>Total no. of CA (excluding gaps)</th>
<th>Aberrations/ cell± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15</td>
<td>46.15±4.9**</td>
<td>8</td>
<td></td>
<td></td>
<td>31</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>39</td>
<td>0.39± 0.04**</td>
</tr>
<tr>
<td>0.30</td>
<td>40±4.8**</td>
<td>27</td>
<td>7</td>
<td></td>
<td>7</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td>61</td>
<td>0.61± 0.04**</td>
</tr>
<tr>
<td>0.60</td>
<td>71.4±4.5**</td>
<td>57</td>
<td>7</td>
<td></td>
<td>29</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td>93</td>
<td>0.93± 0.02**</td>
</tr>
<tr>
<td>1.00</td>
<td>75±4.3**</td>
<td>17</td>
<td>67</td>
<td></td>
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<td>1.01± 0.01**</td>
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<td>71.4±4.5**</td>
<td>7</td>
<td>57</td>
<td>14</td>
<td>43</td>
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<td></td>
<td></td>
<td></td>
<td>100</td>
<td>1.00± 0.00**</td>
</tr>
<tr>
<td>Control</td>
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<td></td>
<td></td>
<td></td>
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<td>4.33±2.03</td>
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<td></td>
<td></td>
<td>1.33</td>
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<td></td>
<td></td>
<td></td>
<td>4.66</td>
<td>0.046±0.02</td>
</tr>
<tr>
<td>Positive</td>
<td>17.33±3.7*</td>
<td>31.33</td>
<td>20.33</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>51.66</td>
<td>0.52±0.05**</td>
</tr>
</tbody>
</table>


* All values are significant at P < 0.005, ** All values are highly significant at P < 0.005, Student’s t – test.
Fig. 7. Frequencies of aberrant metaphases plotted against different concentrations of Isoxsuprine HCl.

Fig. 8. Dose-response curve of aberrations per cell in human lymphocytes \textit{in vitro} treated with different concentrations of Isoxsuprine HCl.
Table 7: Estimation of frequencies of Isoxsuprine hydrochloride induced micronuclei in human lymphocytes
_in vitro._

<table>
<thead>
<tr>
<th>Drug concentration (mg/ml)</th>
<th>Total BN cells</th>
<th>Total BNMN cells</th>
<th>Distribution of MN in BN cells</th>
<th>Total MN</th>
<th>Frequency of MN (%)± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
<td>&gt;2</td>
</tr>
<tr>
<td>0.15</td>
<td>1000</td>
<td>20</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>1000</td>
<td>60</td>
<td>20</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>0.60</td>
<td>1000</td>
<td>28</td>
<td>28</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1.00</td>
<td>1000</td>
<td>133</td>
<td>133</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2.00</td>
<td>1000</td>
<td>143</td>
<td>143</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>1000</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td></td>
</tr>
</tbody>
</table>

BN- Binucleated cells, BNMN- Binucleated Micronucleated cells, MN- Micronuclei, SE- Standard error.
* All values are significant at $P < 0.005$, ** All values are highly significant at $P < 0.005$, Student's $t$-test.
Table 8: Frequencies of binucleated cells with nucleoplasmic bridges, micronuclei and nuclear buds observed after the treatment of Isoxsuprine hydrochloride at different concentrations in human lymphocytes in vitro.

<table>
<thead>
<tr>
<th>Drug Concentration (mg/ml)</th>
<th>BN Total</th>
<th>BN + NPB</th>
<th>Frequency (%)±SE</th>
<th>BN + MN</th>
<th>Frequency (%)±SE</th>
<th>BN + NB</th>
<th>Frequency (%)±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15</td>
<td>1000</td>
<td>143</td>
<td>14.3±1.10**</td>
<td>20</td>
<td>2.0±0.44</td>
<td>53</td>
<td>5.3±0.70**</td>
</tr>
<tr>
<td>0.30</td>
<td>1000</td>
<td>214</td>
<td>21.4±1.29**</td>
<td>60</td>
<td>6.0±0.75**</td>
<td>83</td>
<td>8.3±0.87**</td>
</tr>
<tr>
<td>0.60</td>
<td>1000</td>
<td>154</td>
<td>15.4±1.14**</td>
<td>28</td>
<td>2.8±0.52</td>
<td>71</td>
<td>7.1±0.81**</td>
</tr>
<tr>
<td>1.00</td>
<td>1000</td>
<td>231</td>
<td>23.1±1.33**</td>
<td>133</td>
<td>13.3±1.07**</td>
<td>63</td>
<td>6.3±0.76**</td>
</tr>
<tr>
<td>2.00</td>
<td>1000</td>
<td>286</td>
<td>28.6±1.42**</td>
<td>143</td>
<td>14.3±1.10**</td>
<td>100</td>
<td>10±0.94**</td>
</tr>
<tr>
<td>Control</td>
<td>1000</td>
<td>18</td>
<td>1.8±0.42</td>
<td>16</td>
<td>1.6±0.39</td>
<td>15</td>
<td>1.5±0.38</td>
</tr>
</tbody>
</table>

* All values are significant at P < 0.005, ** All values are highly significant at P < 0.005, Student’s t – test.
Fig. 9. Dose-response curve of micronuclei frequencies in human lymphocytes in vitro treated with different concentrations of Isoxsuprine HCl.

Fig. 10. Frequencies of binucleated micronucleated cells, nucleoplasmic bridges and nuclear buds in human lymphocytes in vitro plotted against different concentrations of Isoxsuprine HCl.
Table 9: Estimation of Nuclear Division Index (NDI) for 500 viable cells after the treatment of different concentrations of Isoxsuprine hydrochloride in vitro.

<table>
<thead>
<tr>
<th>Drug Concentration (mg/ml)</th>
<th>Mono</th>
<th>Nucleated Cells</th>
<th>Nuclear Division Index (NDI)</th>
<th>2X3 Chi-square test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bi</td>
<td>Tetra</td>
<td></td>
</tr>
<tr>
<td>0.15</td>
<td>403</td>
<td>81</td>
<td>16</td>
<td>0.124</td>
</tr>
<tr>
<td>0.30</td>
<td>408</td>
<td>79</td>
<td>13</td>
<td>0.121</td>
</tr>
<tr>
<td>0.60</td>
<td>395</td>
<td>105</td>
<td>0</td>
<td>0.121</td>
</tr>
<tr>
<td>1.00</td>
<td>392</td>
<td>108</td>
<td>0</td>
<td>0.122</td>
</tr>
<tr>
<td>2.00</td>
<td>436</td>
<td>48</td>
<td>16</td>
<td>0.116</td>
</tr>
<tr>
<td>Control</td>
<td>399</td>
<td>87</td>
<td>9</td>
<td>0.124</td>
</tr>
</tbody>
</table>

Mono- Mononucleated cells, Bi- Binucleated cells, Tri- Trinucleated cells, Tetra- Tetroneucleated cells.
Table 10: Estimation of Nuclear Division Cytotoxicity Index (NDCI) for 500 (viable + nonviable) cells after the treatment of different concentrations of hydrochloride *in vitro*.

<table>
<thead>
<tr>
<th>Drug Concentration (mg/ml)</th>
<th>Viable Cells (Nucleated)</th>
<th>Non viable Cells</th>
<th>NDCI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mono</td>
<td>Bi</td>
<td>Tri</td>
</tr>
<tr>
<td>0.15</td>
<td>400</td>
<td>73</td>
<td>13</td>
</tr>
<tr>
<td>0.30</td>
<td>378</td>
<td>67</td>
<td>11</td>
</tr>
<tr>
<td>0.60</td>
<td>382</td>
<td>88</td>
<td>0</td>
</tr>
<tr>
<td>1.00</td>
<td>337</td>
<td>87</td>
<td>0</td>
</tr>
<tr>
<td>2.00</td>
<td>400</td>
<td>34</td>
<td>11</td>
</tr>
<tr>
<td>Control</td>
<td>399</td>
<td>71</td>
<td>8</td>
</tr>
</tbody>
</table>

Mono- Mononucleated cells, Bi- Binucleated cells, Tri- Trinucleated cells, Tetra- Tetrnucleated cells
* All values are significant at P< 0.005, **All values are highly significant at P< 0.005, 2X3 Chi-square test.
Fig. 11. Dose-response curve of nuclear division indices in human lymphocytes *in vitro* treated with different concentrations of Isoxsuprine HCl.

Fig. 12. Dose-response curve of nuclear division cytotoxicity indices in human lymphocytes *in vitro* treated with different concentrations of Isoxsuprine HCl.
tetranucleated cells, apoptotic and necrotic cells are also included in
the table. Study of the table revealed that the NDCI values decreased (1.20,
1.18, 1.18, 1.17 and 1.17) as the drug doses increased gradually (from 0.15
mg per ml to 0.30, 0.60, 1.00 and 2.00). The gradual decrease in NDI range
was indicative of cellular toxicity of drug in vitro at the concentrations
tested. Statistical examination of the data, applying $\chi^2$ test, shows that all
values are highly significant at $P<0.005$, compared with the control value
(1.192) except for a single one which is not significant at lowest drug dose.

Regression line analysis showed a highly strong negative correlation
between drug concentrations and NDCI for Isoxsuprine hydrochloride
(correlation coefficient, $R = 0.97$). The best fit regression line along with the
equation of the line is shown in Fig. 12.

3. XANTHINOL NICOTINATE

(a) Chromosomal aberrations:

Data for the analysis of chromosomal aberrations for five different
concentrations (0.15 mg/ml, 0.30 mg/ml, 0.60 mg/ml, 1.00 mg/ml and 2.00
mg/ml) of the drug Xanthinol nicotinate at 48 hour of incubation has been
summed up in Table 11. According to the table, percentage of aberrant
metaphases did not increase linearly with the increased drug concentrations,
but the effect was low (58%) at lower drug concentration (0.15 mg/ml) and
was highest (61%) at the highest tested concentration (2.00 mg/ml). All the
values in the range were statistically highly significant as compared to the
negative control (4.33%) at $P < 0.005$. 

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Study of aberrations per cell also revealed similar results. According to the table, aberrations per cell were 0.83 at initial concentration (0.15 mg/ml) of the drug, while at final drug concentration of 2.00 mg/ml. aberrations per cell got increased to 1.16. Statistically all the values were highly significant as compared to negative control values. The results of the positive control were also significant at P < 0.005 at 48 hour of incubation.

The relationship between aberrant metaphase frequencies and applied concentrations of the drug is depicted in graphs where the increasing bar lengths (symbolizing increased aberrant metaphase frequencies) was observed with increasing drug concentrations respectively (Fig. 13).

Regression study of aberrations per cell revealed dose-response curve plotted for increasing drug concentrations versus increasing aberrations per cell. A positive correlation was found between above two measurements (correlation coefficient, R = 0.42). The best fit regression line determined by least squares method along with regression line equation is given in Fig. 14.

(b) Micronucleus frequency and Binucleated micronucleated cell frequency:

The results of the CBMN assay for Micronucleus (MN) frequencies and Binucleated Micronucleated (BNMN) cell frequencies are summarized in Table 12 and Table 13 respectively. According to the Table 12, MN frequency has increased gradually in percent (4.3, 5.3, 5.9, 6.7 and 15.0) with the increasing drug concentrations (from 0.15 mg/ml to 0.30, 0.60, 1.00 and 2.00 respectively). Statistical analysis, applying Student’s t – test, revealed that the last four MN frequency values were highly significant with respect to control (1.6%) at P < 0.005, the MN frequency value at lowest
drug concentration of 0.15 mg/ml, was found to be insignificant at P < 0.005 level of significance.

The frequencies of BNMN cells showed linear increase of the values in percentage as 4.3, 5.3, 5.9, 6.7 to 10.0 with increasing drug concentrations. These values were similar to the values obtained for MN frequencies. Only last readings of both the parameters differ from each other. Considering the statistical analysis of BNMN cell frequencies, the last four values were highly significant at P < 0.005 as compared to control values. The first value was found to be insignificant at P < 0.005.

Analysis of regression graph of micronucleus frequencies and drug concentrations plotted against each other revealed a dose – response curve. Graph shows a good positive correlation between observed MN frequencies and applied drug concentrations (correlation coefficient, R = 0.96). The best fit regression line for the relationship between the two measures and the equation describing it is presented in Fig. 15.

Analysis of bar diagram of BNMN frequencies for different drug concentrations shows increase as depicted in gradual elevation of bar lengths with increased drug doses respectively (Fig. 16).

(a) **Nucleoplasmic bridge frequencies:**

Table 13 indicates the results of nucleoplasmic bridge (NPB) frequencies induced by Xathinol nicotinate. The table shows relative occurrence of nucleoplasmic bridges in 1000 binucleated cells. As given in the table, NPB frequencies showed consistent increase in value with increased drug doses, except at 0.60 mg/ml drug concentration where the
value (50.0%) is very high as compared to the values (33.3%) at concentrations higher than this. But an over all increased response was observed comparing lowest drug dose (0.15 mg/ml) and highest drug dose (2.00 mg/ml) respectively. At 0.15 mg/ml concentration, the NPB frequency was 8.3 % while at 2.00 mg/ml concentration the NPB frequency increased to 33.3 %. All the values were greatly significant at P < 0.005 as compared to the control (1.8%).

The dose-dependant nucleoplasmic bridge frequencies are also shown in Fig. 16, where increased bar lengths (representing nucleoplasmic bridge frequencies) were observed with increasing drug doses (from 0.15 mg per ml to 0.30, 0.60, 1.00 and 2.00 respectively).

(b) **Nuclear bud frequencies:**

Table 13 presents the results of nuclear bud (NB) frequencies given against different concentrations of the drug Xanthinol nicotinate. The Table shows that, at the initial three drug concentrations, i.e., 0.15 mg/ml, 0.30 mg/ml and 0.60 mg/ml, nuclear buds were not at all scored. Where as there were 7.7% and 12.5% of NB frequencies scored for the last two drug doses (1.00 mg/ml and 2.00 mg/ml) respectively. Both these values were statistically highly significant as compared to the control values (1.5%) at P < 0.005.

The NB frequencies drawn against drug doses showed the increased bar length pattern with increased drug concentrations respectively (Fig. 16).

(c) **Nuclear Division Index (Cell Division Kinetics):**
The data for nuclear/cell division kinetics of the drug Xanthinol nicotinate is presented in Table 14. Viable cells with one, two, three and four nuclei are also scored in the table. The NDI values decreased gradually with increasing drug concentrations. The observed range was 1.10, 1.11, 1.21, 1.11 and 1.06 for the increased drug concentrations of 0.15 mg/ml, 0.30 mg/ml, 0.60 mg/ml, 1.00 mg/ml and 2.00 mg/ml respectively. A slightly higher value (1.21) was noticed in the middle of the series at 0.60 mg/ml drug concentration. All the values were statistically highly significant at P < 0.005 when compared to control which has an NDI value of 1.24. The study of the decline in NDI values revealed that Xanthinol nicotinate disturbed cell-proliferation kinetics producing a significant cell cycle delay.

Regression analysis of the NDI showed a negative correlation between the two variables, i.e. drug concentrations and NDI (correlation coefficient, R = 0.45). The best fit regression line determined by least squares method along with regression line equation was presented in Fig. 17.

(d) Nuclear Division Cytotoxicity Index (NDCI):

The data of nuclear division cytotoxicity index (NDCI) for the drug Xanthinol nicotinate has been given in Table 15. Mononucleated, binucleated, trinucleated, tetranucleated, apoptotic and necrotic cells were also included in the table. Analysis of the data showed that NDCI value was 1.07 at the lowest drug concentration (0.15 mg/ml) and gets decreased to 1.06 at highest drug concentration of 2.00 mg/ml. At drug concentrations 0.30 mg/ml and 0.60 mg/ml the NDCI values were increased showing a divergent mode from rest of the data. Analysis of the whole data of NDCI revealed that the drug Xanthinol nicotinate did not impose significant effect
Table 11: Chromosomal aberrations induced by Xanthinol nicotinate in cultured human peripheral blood lymphocytes.

<table>
<thead>
<tr>
<th>Drug Concentration (mg/ml)</th>
<th>Aberrant metaphase/100 metaphase (±SE)</th>
<th>Chromatid g</th>
<th>Chromatid b/f</th>
<th>Chromatid other</th>
<th>Isochromatid g</th>
<th>Isochromatid b/f</th>
<th>Isochromatid other</th>
<th>Exchange dic</th>
<th>Exchange tr</th>
<th>Total no. of CA (excluding gaps)</th>
<th>Aberrations/cell± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15</td>
<td>58±4.9**</td>
<td>8</td>
<td>25</td>
<td></td>
<td>42</td>
<td>8</td>
<td>8</td>
<td></td>
<td></td>
<td>83</td>
<td>0.83± 0.03**</td>
</tr>
<tr>
<td>0.30</td>
<td>25±4.3**</td>
<td></td>
<td>42</td>
<td></td>
<td>17</td>
<td>25</td>
<td></td>
<td></td>
<td></td>
<td>84</td>
<td>0.84± 0.03**</td>
</tr>
<tr>
<td>0.60</td>
<td>40±4.8**</td>
<td></td>
<td>70</td>
<td>10</td>
<td>20</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td>110</td>
<td>1.10± 0.03**</td>
</tr>
<tr>
<td>1.00</td>
<td>63±4.8**</td>
<td></td>
<td>125</td>
<td></td>
<td>26</td>
<td>25</td>
<td>13</td>
<td></td>
<td></td>
<td>189</td>
<td>1.89± 0.12**</td>
</tr>
<tr>
<td>2.00</td>
<td>61±408**</td>
<td></td>
<td>62</td>
<td></td>
<td>23</td>
<td>23</td>
<td>8</td>
<td></td>
<td></td>
<td>116</td>
<td>1.16± 0.04**</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>4.33±2.03</td>
<td>3.33</td>
<td></td>
<td>1.33</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.66</td>
<td>0.046±0.02</td>
</tr>
<tr>
<td>Positive</td>
<td>17.33±3.7*</td>
<td>31.33</td>
<td></td>
<td>20.33</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>51.66</td>
<td>0.52±0.05**</td>
</tr>
</tbody>
</table>


* All values are significant at P < 0.005. ** All values are highly significant at P < 0.005. Student’s t-test.
Fig. 13. Frequencies of aberrant metaphases plotted against different concentrations of Xanthinol nicotinate.

Fig. 14. Dose-response curve of aberrations per cell in human lymphocytes *in vitro* treated with different concentrations of Xanthinol nicotinate.
Table 12: Estimation of frequencies of Xanthinol nicotinate induced micronuclei in human lymphocytes *in vitro*.

<table>
<thead>
<tr>
<th>Drug concentration (mg/ml)</th>
<th>Total BN cells</th>
<th>Total BNMN cells</th>
<th>Distribution of MN in BN cells</th>
<th>Total MN</th>
<th>Frequency of MN (%)± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
<td>&gt;2</td>
</tr>
<tr>
<td>0.15</td>
<td>1000</td>
<td>43</td>
<td>43</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.30</td>
<td>1000</td>
<td>53</td>
<td>53</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.60</td>
<td>1000</td>
<td>59</td>
<td>59</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1.00</td>
<td>1000</td>
<td>67</td>
<td>67</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2.00</td>
<td>1000</td>
<td>100</td>
<td>50</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>1000</td>
<td>16</td>
<td>16</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

BN- Binucleated cells, BNMN- Binucleated Micronucleated cells, MN- Micronuclei, SE- Standard error
* All values are significant at P < 0.005, ** All values are highly significant at P < 0.005, Student’s t – test.
Table 13: Frequencies of binucleated cells with nucleoplasmic bridges, micronuclei and nuclear buds observed after the treatment of Xanthinol nicotinate at different concentrations in human lymphocytes *in vitro*.

<table>
<thead>
<tr>
<th>Drug Concentration (mg/ml)</th>
<th>BN Total</th>
<th>BN + NPB</th>
<th>Frequency (%)±SE</th>
<th>BN + MN</th>
<th>Frequency (%)±SE</th>
<th>BN + NB</th>
<th>Frequency (%)±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15</td>
<td>1000</td>
<td>83</td>
<td>8.3±0.87**</td>
<td>43</td>
<td>4.3±0.64*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.30</td>
<td>1000</td>
<td>143</td>
<td>14.3±1.10**</td>
<td>53</td>
<td>5.3±0.70**</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.60</td>
<td>1000</td>
<td>500</td>
<td>50.0±1.58**</td>
<td>59</td>
<td>5.9±0.74**</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1.00</td>
<td>1000</td>
<td>333</td>
<td>33.3±1.49**</td>
<td>67</td>
<td>6.7±0.79**</td>
<td>77</td>
<td>7.7±0.84**</td>
</tr>
<tr>
<td>2.00</td>
<td>1000</td>
<td>333</td>
<td>33.3±1.49**</td>
<td>100</td>
<td>10.0±0.94**</td>
<td>125</td>
<td>12.5±1.04**</td>
</tr>
<tr>
<td>Control</td>
<td>1000</td>
<td>18</td>
<td>1.8±0.42</td>
<td>16</td>
<td>1.6±0.39</td>
<td>15</td>
<td>1.5±0.38</td>
</tr>
</tbody>
</table>

* All values are significant at P < 0.005, ** All values are highly significant at P < 0.005, Student’s t – test.
Fig. 15. Dose-response curve of micronuclei frequencies in human lymphocytes *in vitro* treated with different concentrations of Xanthinol nicotinate.

Fig. 16. Frequencies of binucleated micronucleated cells, nucleoplasmic bridges and nuclear buds in human lymphocytes *in vitro* plotted against different concentrations of Xanthinol nicotinate.
Table 14: Estimation of Nuclear Division Index (NDI) for 500 viable cells after the treatment of different concentrations of Xanthinol nicotinate in vitro.

<table>
<thead>
<tr>
<th>Drug Concentration (mg/ml)</th>
<th>Nucleated Cells</th>
<th>Nuclear Division Index (NDI)</th>
<th>2X3 Chi-square test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mono</td>
<td>Bi</td>
<td>Tri</td>
</tr>
<tr>
<td>0.15</td>
<td>449</td>
<td>51</td>
<td>0</td>
</tr>
<tr>
<td>0.30</td>
<td>441</td>
<td>59</td>
<td>0</td>
</tr>
<tr>
<td>0.60</td>
<td>395</td>
<td>105</td>
<td>0</td>
</tr>
<tr>
<td>1.00</td>
<td>455</td>
<td>34</td>
<td>11</td>
</tr>
<tr>
<td>2.00</td>
<td>465</td>
<td>35</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>399</td>
<td>87</td>
<td>9</td>
</tr>
</tbody>
</table>

Mono- Mononucleated cells, Bi- Binucleated cells, Tri- Trinucleated cells, Tetra- Tetranucleated cells.
Table 15: Estimation of Nuclear Division Cytotoxicity Index (NDCI) for 500 (viable + nonviable) cells after the treatment of different concentrations of Xanthinol nicotinate in vitro.

<table>
<thead>
<tr>
<th>Drug Concentration (mg/ml)</th>
<th>Mono</th>
<th>Viable Cells (Nucleated)</th>
<th>Non viable Cells</th>
<th>NDCI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bi</td>
<td>Tri</td>
<td>Tetra</td>
</tr>
<tr>
<td>0.15</td>
<td>420</td>
<td>40</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.30</td>
<td>387</td>
<td>75</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.60</td>
<td>350</td>
<td>75</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1.00</td>
<td>398</td>
<td>23</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>2.00</td>
<td>430</td>
<td>30</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>399</td>
<td>71</td>
<td>8</td>
<td>3</td>
</tr>
</tbody>
</table>

Mono- Mononucleated cells, Bi- Binucleated cells, Tri- Trinucleated cells, Tetra- Tetraneucleated cells

* All values are significant at P< 0.005, **All values are highly significant at P< 0.005, 2X3 Chi-square test.
Fig. 17. Dose-response curve of nuclear division indices in human lymphocytes \textit{in vitro} treated with different concentrations of Xanthinol nicotinate.

Fig. 18. Dose-response curve of nuclear division cytotoxicity indices in human lymphocytes \textit{in vitro} treated with different concentrations of Xanthinol nicotinate.
over cell cycle kinetics and was thus not much cytotoxic. A lower cell cycle delay which was measured as a slight reduction in the NDCI values was noticed. Statistically speaking, all values were highly significant at P<0.005 compared with the control value (1.192).

Study of regression graph showed a negative correlation (Correlation Coefficient R = 0.51) between drug concentrations and NDCI values. The best fit regression line together with the line equation was presented in Fig. 18.

4. METOPROLOL TARTRATE (METOLAR®)

(a) Chromosomal aberrations:

The effects of different concentrations of the drug Metoprolol tartrate on chromosomal aberrations in human peripheral blood lymphocytes in vitro have been summarized in Table 16. The drug concentrations used were from 0.15 mg/ml to 0.30, 0.60, 1.00 and 2.00 mg/ml applied at 48 hours of incubation of culture. According to the table percentage of aberrant metaphases was found to be increased accordingly with three initial concentrations of the drug (0.15 mg/ml, 0.30 mg/ml, and 0.60 mg/ml) as 46.15, 50.0 and 61.5% respectively. Cell death was noticed for the last two drug concentrations. Aberrations per cell have also been calculated for the first three drug concentrations. These values also showed an increasing pattern (0.69, 0.83, and 1.01 respectively) with increasing concentrations. Statistical analysis of data revealed that the obtained values were highly significant at P < 0.005, compared with the negative value. Positive control also showed high significance at P <0.005.
Graphical observations also indicated the high frequencies of aberrant metaphases plotted against increased drug concentrations (Fig. 19).

Regression analysis of aberrations per cell for the drug Metoprolol tartrate against increased drug concentration showed a dose-response curve. A very strong positive correlation was found between above two measurements (correlation coefficient, R = 0.99). The best fit regression line determined by least squares method along with regression line equation is shown in Fig. 20.

(b) Micronucleus frequency and Binucleated micronucleated cell frequency:

The micronucleus (MN) frequencies and binucleated micronucleated (BNMN) cell frequencies results are given in Table 17 and Table 18 respectively. According to Table 17, exposure of peripheral blood lymphocytes (in culture) to drug Metoprolol tartrate for 24 hours induced elevated numbers of MN (from 1.5% to 4.3, 5.7, 8.3 and 9.1%) at concentrations (mg/ml) from 0.15 to 0.30, 0.60, 1.00 and 2.00 respectively. Analyzed statistically, the initial MN frequency value (at lowest drug concentration) was insignificant, MN frequency value at next concentration was found to be significant, whereas the last three MN frequency values were highly significant with respect to control at P < 0.005.

Table 18 shows that the frequencies of BNMN cells also increased in percentage from 1.5, 4.3, 5.7, 8.3 to 9.1 with increasing drug concentrations. This increase was similar with the increase of MN frequencies at all concentrations. The statistical result of BNMN cell frequencies, applying Student’s t – test, also showed the same significance level in case of MN
induction. Here also the last three values were highly significant at $P < 0.005$ as compared to control value at 24 hour of incubation. The first one was insignificant and second one was significant at $P < 0.005$.

Regression graph analysis of micronucleus frequencies plotted against drug concentrations revealed dose – response curve. The observed MN frequencies were correlated strongly and positively (Correlation Coefficient $R = 0.88$) with increased drug concentrations. The best fit regression line together with the line equation determined is presented in Fig. 21.

The bar diagram analysis of BNMN cell frequencies plotted against increasing drug concentrations also showed increased bar lengths (indicating BNMN frequencies) with increasing drug doses respectively (Fig. 22).

(a) Nucleoplasmic bridge frequencies:

The results of nucleoplasmic bridge (NPB) frequencies induced by the drug Metoprolol tartrate are summarized in Table 18. According to the table, the NPB frequencies did not show consistent increase with increasing drug doses, but an over all increase in the effect was observed when NPB frequency at lowest drug concentration was compared with the NPB frequency at highest concentration of the drug. At 0.15 mg/ml concentration, the NPB frequency was 13.3 % while at 2.00 mg/ml concentration the NPB frequency was increased to 28.6 %. Statistically the result was highly significant at $P < 0.005$ as compared with the control (with 1.8% NPB frequency).

The relative increase of nucleoplasmic bridge frequencies between lowest and highest concentrations is presented in Fig. 22. Here NPB
frequencies were plotted against different concentrations in the form of bar graph. Increased bar lengths (presenting elevated NPB frequencies) were obtained with increasing drug doses (mg/ml) (from 0.15 to 0.30, 0.60, 1.00 and 2.00).

(b) Nuclear bud frequencies:

The results of nuclear bud (NB) frequencies are also depicted in Table 18. Study of the table showed that nuclear bud frequencies increased with increasing drug concentrations respectively. A little drop in the value was noticed at 1.00 mg/ml drug concentration. The observed range, in percentage, was from 7.1 to 9.1, 10.3, 9.1 to 11.1 respectively. Observed data indicated the effect has increased with increased drug concentrations (0.15 mg/ml, 0.30 mg/ml, 0.60 mg/ml, 1.00 mg/ml and 2.00 mg/ml). Statistical analysis showed that all the values were highly significant at P <0.005 level, as compared with the control value.

The increasing bar lengths, symbolizing the increased NB frequencies, are noticed again with increasing drug concentrations as shown in graph (Fig. 22).

(c) Nuclear Division Index (Cell Division Kinetics):

The study of nuclear division index (NDI) results for the drug Metoprolol tartrate are summarized in Table 19 which also contains mononucleated, binucleated, trinucleated and tetrasnucleated cells. The extent and progression of nuclear division could be assessed by analyzing the data. According to the table a step wise increase and decrease in the NDI values has been noticed with increasing drug concentrations. These
values were 1.10, 1.01, 1.04, 1.01 and 1.05 obtained for 0.15 mg/ml, 0.30 mg/ml, 0.60 mg/ml, 1.00 mg/ml and 2.00 mg/ml drug concentrations respectively. The relative dose-response studies revealed a decrease in the NDI value from the lowest drug dose to the highest drug dose. At initial lowest concentration (0.15 mg/ml), the NDI value was 1.10, which decreased to 1.05 at the highest final drug concentration (2.00 mg/ml) demonstrating a cell cycle delay. All values were highly significant statistically when compared to the control value (1.24) at \( P < 0.005 \) level of significance.

Regression analysis showed a weak negative correlation between drug concentrations and NDI (correlation coefficient, \( R = 0.19 \)). The best fit regression line determined by least squares method along with regression line equation is presented in Fig. 23.

(d) **Nuclear Division Cytotoxicity Index (NDCI):**

The data of nuclear division cytotoxicity index (NDCI) for the drug Metoprolol tartrate is summed up in Table 20. Both, viable (mononucleated, binucleated, trinucleated, tetranucleated) and non – viable (apoptotic and necrotic) cell populations were included in the table. To study this parameter, a uniformly consistent result was not obtained in a decreasing pattern with increasing concentrations of the drug. The relatively decreasing effect was noticed when NDCI values were at lowest (0.15 mg/ml) and highest (2.00 mg/ml) drug doses. These values were 1.08 and 1.06 respectively at 24 hour of culture incubation. The relative decline in the values suggested a cell cycle delay along with cellular cytotoxicity of the
Table 16: Chromosomal aberrations induced by Metoprolol tartrate in cultured human peripheral blood lymphocytes.

<table>
<thead>
<tr>
<th>Drug Concentration (mg/ml)</th>
<th>Aberrant metaphase/100 metaphase (±SE)</th>
<th>Chromatid Aberrations/100 metaphases</th>
<th>Isochromatid Aberrations/100 metaphases</th>
<th>Exchange dic/tr</th>
<th>Total No. of CA (excluding gaps)</th>
<th>Aberrations/cell± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g b/f other</td>
<td>g b/f other</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.15</td>
<td>46.15±4.9**</td>
<td>8 38</td>
<td>16</td>
<td>15</td>
<td>69</td>
<td>0.69±0.04**</td>
</tr>
<tr>
<td>0.30</td>
<td>50±5**</td>
<td>33</td>
<td>50</td>
<td></td>
<td>83</td>
<td>0.83±0.03**</td>
</tr>
<tr>
<td>0.60</td>
<td>61.5±4.8**</td>
<td>54</td>
<td>39</td>
<td>8</td>
<td>101</td>
<td>1.01±0.01**</td>
</tr>
<tr>
<td>1.00</td>
<td>Cell death</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.00</td>
<td>Cell death</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>4.33±2.03</td>
<td>3.33</td>
<td>1.33</td>
<td></td>
<td>4.66</td>
<td>0.046±0.02</td>
</tr>
<tr>
<td>Positive</td>
<td>17.33±3.7*</td>
<td>31.33</td>
<td>20.33</td>
<td></td>
<td>51.66</td>
<td>0.52±0.05**</td>
</tr>
</tbody>
</table>

* All values are significant at P < 0.005, ** All values are highly significant at P < 0.005, Student’s t – test.
Fig. 19. Frequencies of aberrant metaphases plotted against different concentrations of Metoprolol tartrate.

Fig. 20. Dose-response curve of aberrations per cell in human lymphocytes *in vitro* treated with different concentrations of Metoprolol tartrate.
Table 17: Estimation of frequencies of Metoprolol tartrate induced micronuclei in human lymphocytes *in vitro*.

<table>
<thead>
<tr>
<th>Drug concentration (mg/ml)</th>
<th>Total BN cells</th>
<th>Total BNMN cells</th>
<th>Distribution of MN in BN cells</th>
<th>Total MN</th>
<th>Frequency of MN (%)± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15</td>
<td>1000</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>1.5±0.38</td>
</tr>
<tr>
<td>0.30</td>
<td>1000</td>
<td>43</td>
<td>43</td>
<td>43</td>
<td>4.3±0.64*</td>
</tr>
<tr>
<td>0.60</td>
<td>1000</td>
<td>57</td>
<td>57</td>
<td>57</td>
<td>5.7±0.73**</td>
</tr>
<tr>
<td>1.00</td>
<td>1000</td>
<td>83</td>
<td>83</td>
<td>83</td>
<td>8.3±0.87**</td>
</tr>
<tr>
<td>2.00</td>
<td>1000</td>
<td>91</td>
<td>91</td>
<td>91</td>
<td>9.1±0.90**</td>
</tr>
<tr>
<td>control</td>
<td>1000</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>1.6±0.39</td>
</tr>
</tbody>
</table>

BN- Binucleated cells, BNMN- Binucleated Micronucleated cells, MN- Micronuclei, SE- Standard error.
* All values are significant at P < 0.005, ** All values are highly significant at P < 0.005, Student’s t – test.
Table 18: Frequencies of binucleated cells with nucleoplasmic bridges, micronuclei and nuclear buds observed after the treatment of Metoprolol tartrate at different concentrations in human lymphocytes *in vitro*.

<table>
<thead>
<tr>
<th>Drug Concentration (mg / ml)</th>
<th>BN Total</th>
<th>BN + NPB</th>
<th>Frequency (%)±SE</th>
<th>BN + MN</th>
<th>Frequency (%)±SE</th>
<th>BN + NB</th>
<th>Frequency (%)±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15</td>
<td>1000</td>
<td>133</td>
<td>13.3±1.07**</td>
<td>15</td>
<td>1.5±0.38</td>
<td>71</td>
<td>7.1±0.81**</td>
</tr>
<tr>
<td>0.30</td>
<td>1000</td>
<td>43</td>
<td>4.3±0.64*</td>
<td>43</td>
<td>4.3±0.64*</td>
<td>91</td>
<td>9.1±0.90**</td>
</tr>
<tr>
<td>0.60</td>
<td>1000</td>
<td>143</td>
<td>14.3±1.10**</td>
<td>57</td>
<td>5.7±0.73**</td>
<td>103</td>
<td>10.3±0.96**</td>
</tr>
<tr>
<td>1.00</td>
<td>1000</td>
<td>83</td>
<td>8.3±0.87**</td>
<td>83</td>
<td>8.3±0.87**</td>
<td>91</td>
<td>9.1±0.90**</td>
</tr>
<tr>
<td>2.00</td>
<td>1000</td>
<td>286</td>
<td>28.6±1.42**</td>
<td>91</td>
<td>9.1±0.90**</td>
<td>111</td>
<td>11.1±0.98**</td>
</tr>
<tr>
<td>Control</td>
<td>1000</td>
<td>18</td>
<td>1.8±0.42</td>
<td>16</td>
<td>1.6±0.39</td>
<td>15</td>
<td>1.5±0.38</td>
</tr>
</tbody>
</table>

* All values are significant at P < 0.005, ** All values are highly significant at P < 0.005, Student’s *t* – test.
Fig. 21. Dose-response curve of micronuclei frequencies in human lymphocytes *in vitro* treated with different concentrations of Metoprolol tartrate.

Fig. 22. Frequencies of binucleated micronucleated cells, nucleoplasmic bridges and nuclear buds in human lymphocytes *in vitro* plotted against different concentrations of Metoprolol tartrate.
Table 19: Estimation of Nuclear Division Index (NDI) for 500 viable cells after the treatment of different concentrations of Metoprolol tartrate in vitro.

<table>
<thead>
<tr>
<th>Drug Concentration (mg/ml)</th>
<th>Mono</th>
<th>Nucleated Cells</th>
<th>Nuclear Division Index (NDI)</th>
<th>2×3 Chi-square test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bi</td>
<td>Tri</td>
<td>Tetra</td>
</tr>
<tr>
<td>0.15</td>
<td>460</td>
<td>29</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>0.30</td>
<td>495</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.60</td>
<td>476</td>
<td>24</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1.00</td>
<td>494</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>2.00</td>
<td>482</td>
<td>12</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Control</td>
<td>399</td>
<td>87</td>
<td>9</td>
<td>5</td>
</tr>
</tbody>
</table>

Mono- Mononucleated cells, Bi- Binucleated cells, Tri- Trinucleated cells, Tetra- Tetranucleated cells.
Table 20: Estimation of Nuclear Division Cytotoxicity Index (NDCI) for 500 (viable + nonviable) cells after the treatment of different concentrations of Metoprolol tartrate in vitro.

<table>
<thead>
<tr>
<th>Drug Concentration (mg/ml)</th>
<th>Viable Cells (Nucleated)</th>
<th>Non viable Cells</th>
<th>NDCI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mono</td>
<td>Bi</td>
<td>Tri</td>
</tr>
<tr>
<td>0.15</td>
<td>443</td>
<td>22</td>
<td>9</td>
</tr>
<tr>
<td>0.30</td>
<td>457</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>0.60</td>
<td>447</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>1.00</td>
<td>384</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>2.00</td>
<td>332</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>399</td>
<td>71</td>
<td>8</td>
</tr>
</tbody>
</table>

Mono- Mononucleated cells, Bi- Binucleated cells, Tri- Trinucleated cells, Tetra- Tetrnucleated cells
* All values are significant at P< 0.005, **All values are highly significant at P< 0.005, 2X3 Chi-square test.
Fig. 23. Dose-response curve of nuclear division indices in human lymphocytes \textit{in vitro} treated with different concentrations of Metoprolol tartrate.

Fig. 24. Dose-response curve of nuclear division cytotoxicity indices in human lymphocytes \textit{in vitro} treated with different concentrations of Metoprolol tartrate.
drug. Statistical analysis of the data, applying $\chi^2$ test, showed that all values were highly significant at $P < 0.005$ compared with the control value (1.192).

Best fit regression line graph plotted for increased concentrations of the drug against obtained respective NDCI values showed a very weak negative correlation between the two measurements (correlation coefficient, $R = 0.05$). The regression line and the equation representing it are both shown in Fig. 24.

**Comparative analysis of the Pentoxifylline, Isoxsuprine hydrochloride, Xanthinol nicotinate and Metoprolol tartrate**

The above four tested cardiovascular drugs were also analyzed together to assess their relative cytogenotoxic potential. The data is summarized in Tables 21, 22 and 23 respectively. Average mean values ($\pm$ Standard error of mean, SEM) of the measurements (percent aberrant metaphases, aberrations per cell, micronucleus frequencies, binucleated micronucleated cell frequencies, nucleoplasmic bridge frequencies, nuclear bud frequencies, nuclear/cell division kinetics and nuclear division cytotoxicity index) were studied for each drug, all the four drugs being grouped under one table.

Table 21 contains comparative analysis of average mean ($\pm$ SEM) frequencies of aberrant metaphases per 100 normal metaphases and aberrations per cell in four test drugs. According to this table, percent aberrant metaphases were highest in drug Isoxsuprine hydrochloride (60.79%) followed by Pentoxifylline (53.96%), Metoprolol tartrate (52.55%) and Xanthinol nicotinate (49.40%). A differing result was obtained for
aberrations per cell study where the effect was highest in drug Xanthinol nicotinate (1.16) followed by Pentoxifylline (0.98), Metoprolol tartrate (0.84) and Isoxsuprine hydrochloride (0.78). These results are also shown graphically in Fig. 25.

Studies of the structural aberrations induced by these drugs show mainly chromatid-type of aberrations consisting of terminal deletions, breaks etc in one arm of the chromosome. Some chromosome-type aberrations were also seen (rings, dicentric chromosomes) (Fig. 29 – Fig. 50).

Comparative analysis of average means (± SEM) of micronucleus frequency (%), binucleated micronucleated cell frequencies (%), nucleoplasmic bridge frequencies (%) and nuclear bud frequencies (%) of four test drugs is given in Table 22 and Fig. 26. Table showed contrasting outcomes considering all these parameters. Data for micronucleus frequency revealed highest percentage in drug Isoxsuprine hydrochloride (8.48%). The trail being followed by Xanthinol nicotinate (7.44%), Pentoxifylline (7.04%) and Metoprolol tartrate (5.78%).

Relative study results of binucleated micronucleated cell frequencies indicated highest frequency in drug Isoxsuprine hydrochloride (7.68%) followed by Xanthinol nicotinate (6.44%), Pentoxifylline (6.04%) and Metoprolol tartrate (5.78%). These results were similar to the results of the MN frequency parameter.

Results of the average of mean nucleoplasmic bridge frequencies demonstrated greatest effect by the drug Xanthinol nicotinate (27.84%), then Isoxsuprine hydrochloride (7.6820.56%), Pentoxifylline (17.26%) and lastly Metoprolol tartrate (13.76%).
Analysis of data for nuclear bud frequencies indicated greatest frequency in drug Xanthinol nicotinate (10.10%) followed by Metoprolol tartrate (9.34%), Isoxsuprine hydrochloride (7.40%) and Pentoxifylline (5.04%).

Data for comparative analysis of average mean nuclear division index (NDI) and average mean nuclear division cytotoxicity index (NDCI) values of four drugs is presented in Table 23. According to this table all the four drugs showed nearly the same NDI and NDCI values, with slight differences, indicating similar response to cell cycle delay and cellular cytotoxicity. For NDI the drugs were arranged accordingly for the response as Isoxsuprine hydrochloride (1.20), Xanthinol nicotinate (1.11), Pentoxifylline (1.10) and Metoprolol tartrate (1.04). Drugs with comparative NDCI values were arranged as Isoxsuprine hydrochloride (1.17), Xanthinol nicotinate (1.10), Pentoxifylline (1.08) and Metoprolol tartrate (1.05). All the four cardiovascular drugs demonstrated nearly same cell division kinetics results (Fig. 27 and Fig. 28).

Fig. 51 – Fig. 66 represent results of the CBMN assay, obtained in the form of affected cell/nuclear shapes like binucleated, trinucleated and tetranucleated cells, micronuclei, cytoplasmic bridges, nuclear buds, apoptotic and necrotic cells etc.
Table 21: Comparative analysis of Averages of means (± SEM) of % aberrant metaphases and aberrations / cell in four test drugs.

<table>
<thead>
<tr>
<th>Test Drugs</th>
<th>% Aberrant metaphases (average of mean±SEM)</th>
<th>Aberrations / Cell (average of mean±SEM)</th>
<th>Type of principle Aberration (Chromatid / Chromosome)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentoxifylline (Trental)</td>
<td>53.96±4.54</td>
<td>0.98±0.13</td>
<td>Chromatid</td>
</tr>
<tr>
<td>Isoxsuprine HCl (Duvadilan)</td>
<td>60.79±7.32</td>
<td>0.78±0.12</td>
<td>Chromatid</td>
</tr>
<tr>
<td>Xanthinol Nicotinate (Complamina)</td>
<td>49.4±7.33</td>
<td>1.16±0.19</td>
<td>Chromatid</td>
</tr>
<tr>
<td>Metoprolol tartrate (Metolar)</td>
<td>52.55±4.61</td>
<td>0.84±0.09</td>
<td>Chromatid</td>
</tr>
</tbody>
</table>
Table 22: Comparative analysis of averages of means (± SEM) of % MN Frequency, % BNMN cell Frequency, % BNNPB cell Frequency and % BNNB cell Frequency in four test drugs.

<table>
<thead>
<tr>
<th>Name of the Drug</th>
<th>Average MN Frequency (%)</th>
<th>Average BNMN cell Frequency (%)</th>
<th>Average BNNPB cell Frequency (%)</th>
<th>Average BNNB cell Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentoxifylline (Trental)</td>
<td>7.04±2.08</td>
<td>6.04±1.17</td>
<td>17.26±3.06</td>
<td>5.04±1.21</td>
</tr>
<tr>
<td>Isoxsuprine HCl (Duvadilan)</td>
<td>8.48±2.58</td>
<td>7.68±2.59</td>
<td>20.56±2.62</td>
<td>7.40±0.81</td>
</tr>
<tr>
<td>Xanthinol Nicotinate (Complamina)</td>
<td>7.44±1.93</td>
<td>6.44±0.97</td>
<td>27.84±7.46</td>
<td>10.10±2.4</td>
</tr>
<tr>
<td>Metoprolol tartrate (Metolar)</td>
<td>5.78±1.37</td>
<td>5.78±1.37</td>
<td>13.76±4.12</td>
<td>9.34±0.67</td>
</tr>
</tbody>
</table>
Fig. 25. Average of mean frequencies of aberrant metaphases and aberrations/cell in four cardiovascular drugs plotted against different concentrations of all 4 drugs.

Fig. 26. Average of mean frequencies of binucleated micronucleated cells, nucleoplasmic bridges and nuclear buds in 4 cardiovascular drugs plotted against different concentrations of all drugs.
Table 23: Comparative analysis of averages of mean nuclear division index (NDI) and mean nuclear division cytotoxicity index (NDCI) values of four test drugs.

<table>
<thead>
<tr>
<th>Name of the Drug</th>
<th>Average of mean NDI±SEM</th>
<th>Average of mean NDCI±SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentoxifylline (Trental)</td>
<td>1.10±0.04</td>
<td>1.08±0.03</td>
</tr>
<tr>
<td>Isoxsuprine HCl (Duvadilan)</td>
<td>1.20±0.01</td>
<td>1.17±0.01</td>
</tr>
<tr>
<td>Xanthinol Nicotinate (Complamina)</td>
<td>1.11±0.02</td>
<td>1.10±0.02</td>
</tr>
<tr>
<td>Metoprolol tartrate (Metolar)</td>
<td>1.04±0.02</td>
<td>1.05±0.008</td>
</tr>
</tbody>
</table>
Fig. 27. Dose-response curve of average mean nuclear division indices in human lymphocytes *in vitro* in four tested drugs treated with different drug concentrations.

Fig. 28. Dose-response curve of mean nuclear division cytotoxicity indices in human lymphocytes *in vitro* in four tested drugs treated with different drug concentrations.
DISCUSSION
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Present study has been done to evaluate and assess the *in vitro* cytogenotoxic effects of some clinically recommended cardiovascular drugs, mainly antihypertensive vasodilators. Herein different parameters used during the study including the selection of drugs of study, selection of test system, advantages and disadvantages of the chosen test system, the parameters assayed during the study and the cytogenotoxic responses and effects of the selected cardiovascular drugs evaluated on these parameters have been discussed.

SELECTION OF TEST DRUGS

A cardiovascular problem is considered as the second most dreaded challenge the world faces today. About a quarter of the world’s population has been estimated to have hypertension at the turn of the millennium (Kearney, *et al.*, 2005; Vaidya, *et al.*, 2007). The main factor leading to this widespread and chronic problem is hypertension (Staessen, *et al.*, 2001). Hypertension, being chronic, requires long-term drug therapy and thus interaction of antihypertensive drugs and their metabolites is continual with the human system. The continuing drug interaction may cause various types of side effects including those that affect human hereditary material, *i.e.*, DNA. Thus, in order to evaluate the possible cytogenotoxic response that the drugs may cause, four antihypertensive vasodilator drugs, Pentoxifylline (Trental®), Isoxsuprine hydrochloride (Duvadilan®), Xanthinol nicotinate
(Complamina\textsuperscript{\textregistered}) and Metoprolol tartrate (Metolar\textsuperscript{\textregistered}), have been selected for the study.

**GENERAL CONSIDERATIONS**

The preservation of integrity of DNA as a genetic blueprint is of prime importance for any living organism including humans. Damage inflicted upon the genetic material may lead to harmful effects in the cell or in the organism as a whole. Thus, human health safeguard is the main aim to study and evaluate the cytogenotoxic potentialities of cardiovascular drugs which are mostly used for long-term therapy. In order to assess adequately any expression of genotoxicity, the direct use of human system, exposed to these drugs, or the involvement of a system that has the same sensitivity of response as the human genome is preferred. This can be achieved by utilizing human peripheral blood lymphocyte culture system which provides a large number of dividing cells potentially yielding good enough amount of mitotic metaphase plates used for chromosome analysis. Peripheral blood lymphocytes represent a good indicator of exposure to genotoxic agents, carrying information on both doses and genotoxic effects (Morimoto, *et al.*, 1993; van Asten, *et al.*, 1998; Anderson, 1999). Circulating throughout the body, the peripheral blood lymphocytes provide an estimate of average whole body exposure (Tucker and Preston, 1996). The lymphocytes also contain some metabolizing enzyme systems that can be able, to some extent, to activate certain promutagens into a mutagenically active state (Buckton and Evans, 1982; Preston, 1999).

**EVALUATION OF TEST SYSTEMS**
The first stage in the evaluation of a chemical is to investigate the ability of the chemical to interact with DNA and produce a detectable change in the genetic material. Bacterial, yeast, plant, Drosophila, and in vitro mammalian cell assays are designed for this purpose (IPCS, WHO, 1985).

A large number of genotoxicity tests are presently available for use in hazard evaluation. These tests detect the two main categories of mutations, gene mutation and chromosomal aberration, as well as indications of DNA damage. Direct or indirect effects of DNA damage can be observed as cytogenetic end points, in the form of changes in chromosome structure, changes in chromosome numbers, micronuclei and sister chromatid exchanges (Obe, et al., 1982). Tests to assess these endpoints can be carried out both in vitro and in vivo, with in vivo tests being conducted in germ cells as well as in somatic cells. In order to assess adequately any expression of genotoxicity, a simplified systematic approach to the selection of these tests is required. Therefore, there must be an ordered approach using a limited number of well-defined tests that complement each other in terms of endpoints, and that permits a systematic assessment of genotoxicity (Environmental Health Directorate, Canada, 1993).

It is ideal to study the effects of chemicals directly on persons exposed to these chemicals or drugs (Gebhart, 1982, 1984). But due to ethical reasons the in vivo study is possible only in specific cases when individuals have been exposed to the agents either as a chemotherapeutic necessity or occupational inevitability or accidental reasons.
Besides, the *in vivo* testing systems have their own limitations. Standard *in vivo* tests are unable to provide additional useful information for some compounds. This includes compounds for which data from studies on toxicokinetics or pharmacokinetics indicate that they are not systemically absorbed and therefore are not available for the target tissues in standard *in vivo* genotoxicity tests. Examples of such compounds are some radioimaging agents, aluminum based antacids, and some dermally applied pharmaceuticals. In cases where a modification of the route of administration does not provide sufficient target tissue exposure, it may be appropriate to base the evaluation only on *in vitro* testing (Hutchinson. 2004). Other short coming of *in vivo* genotoxicity is that in case of exposed model the cytogenetic abnormalities studied represent the most drastic and obvious expressions of genetic damage, but the frequent and subtle genetic changes, like mutations, can not be detected.

To overcome the shortcomings of the *in vivo* testing systems, *in vitro* assays are preferred. *In vitro* cytogenetic tests are designed to demonstrate the induction of chromosome damage (aberrations), visible under the light microscope, in cultured cells. A physical or chemical agent is classified as a clastogen if it produces an increase in the number of breaks in chromosomes over that found in control samples. Cytogenetic tests therefore assess gross damage to the DNA involving at least one double-strand break. The experimental approach can be use and the effect of drugs/chemicals can be evaluated at a range of concentrations (including subtoxic and toxic levels) on lymphocyte cultures of healthy donors. Evaluation of the response of cells at different time exposures to the drugs and the fate of lesion after
consecutive cell division along with the drug effect during G₁ as well as S-phase of the cell cycle can also be evaluated in the in vitro cytogenetic assay.

However, in vitro cytogenetic test has its own shortcomings because of the absence of metabolizing system of drugs and chemicals. Lymphocytes can only metabolize some compounds. Some other compounds have also been metabolized due to the presence of erythrocytes which aid the metabolic process (Wilmer, et al., 1984)

EVALUATION OF THE PARAMETERS ASSAYED

To test whether pharmaceuticals are genotoxic or not, normally, two short-term, high precision, in vitro tests are considered to be sufficient. These include the chromosome aberration assay and the cytokinesis-blocked micronucleus (CBMN) assay, both utilizing the peripheral human lymphocytes as the base.

The chromosome aberration test using cultured mammalian cells is one of the most sensitive methods to predict environmental mutagens and/or carcinogens, and is a complementary test to the Salmonella/microsome assay (Ames test) (Ishidate, et al., 1998). The parameter usually assessed via this assay is chromosomal aberrations.

The study of DNA damage at the chromosome level is an essential part of genetic toxicology because chromosomal mutation is an important event in carcinogenesis. The micronucleus assays have emerged as one of the preferred methods for assessing chromosome damage because they enable both chromosome loss and chromosome breakage to be measured reliably. Because micronuclei can only be expressed in cells that complete
nuclear division, a special method was developed that identifies such cells by their binucleate appearance when blocked from performing cytokinesis by cytochalasin-B (Cyt-B), a microfilament-assembly inhibitor. This recognition prevents the confounding effects caused by differences in cell division kinetics (Kirsch-Volders, et al., 2001; Fenech, 2002). Thus, the cytokinesis-blocked micronucleus (CBMN) assay, more recently known as the CBMN “cytome” or CBMN-Cyt assay (Fenech, 2007), is a multi-endpoint assay that allows better precision because the data obtained are not confounded by altered cell division kinetics caused by cytotoxicity of agents tested or sub-optimal cell culture conditions. In its current basic form the CBMN assay provides, using simple morphological criteria, the different measures of genotoxicity and cytotoxicity. These measures involve chromosome breakage, chromosome loss, chromosome rearrangement (nucleoplasmic bridges), cell division inhibition, necrosis and apoptosis (Fenech, 2000).

**Chromosomal alterations/aberrations:**

Chromosomal aberration assessment serves as a good indicator of the clastogenic effects of agents (Buckton and Evans, 1982; Hsu, 1982). Aberrations are divided into chromatid-type and chromosome-type, the first involving only one chromatid, the latter, both chromatids at identical sites. The chromatid-type aberrations are caused in the $S_0$ or $G_2$ stage of cell cycle, and include chromatid gaps, breaks and deletions, while chromosome-type aberrations are found in $G_1$ or early S-phase of the cell cycle and include isochromatid gaps, ring chromosomes, acentric fragments and interstitial deletions (Hsu, 1982).
Breaks can be distinguished from exchange configurations by their physical appearance at metaphase rather than by their mode of formation. Breaks are true discontinuities with clearly dislocated fragments and also include fragments without obvious origin. They should not be confused with achromatic lesions (gaps), which do not represent true discontinuity in the DNA. It is generally assumed that gaps are sites of despiralization in the metaphase chromosome that render the DNA non-visible under light microscopy (IPCS. WHO, 1985). It has been proposed that an achromatic lesion may actually be a single-strand break in the DNA double helix as a result of incomplete excision repair and, thus, may represent a point of possible instability (Bender, et al., 1974). Therefore, gaps are always noted but reported separately from true chromosomal aberrations.

**Micronuclei (MNI):**

MNI are expressed in dividing cells that either contain chromosome breaks lacking centromeres (acentric fragments) and/or whole chromosomes that are unable to travel to the spindle poles during mitosis. At telophase, a nuclear envelope forms around the lagging chromosomes and fragments, which then uncoil and gradually assume the morphology of an interphase nucleus with the exception that they are smaller than the main nuclei in the cell, hence are known as "micronucleus". MNI, therefore, provide a convenient and reliable index of both chromosome breakage and chromosome loss. Enumeration of micronuclei in mitogen-stimulated lymphocytes provides a simpler and statistically more precise method for quantification of chromosomal damage (Fenech and Morley, 1985; Kirsch-Volders, et al., 1997).
Binucleated micronucleated cells:

In the CBMN assay, cells that have completed one nuclear division are blocked from performing cytokinesis using cytochalasin-B and are consequently readily identified by their binucleated appearance. MNi are scored in binucleated cells only, which enables reliable comparisons of chromosome damage between cell populations that may differ in their cell division kinetics.

Nucleoplasmic bridges:

These are considered as dicentric chromosomes in which the two centromeres were pulled to opposite poles of the cell and the DNA in the resulting bridge covered by nuclear membrane. They provide a measure of asymmetrical chromosome rearrangement (owing to misrepair of DNA strand breaks or possibly telomere end-joining) (Thomas, et al., 2003). Thus are scored together with the micronucleus count.

Nuclear buds:

Nuclear buds are the outgrowths from main nucleus which get attached to nucleus through nucleoplasmic connections. Nuclear buds are supposed to result due to the induction of gene amplification that may lead to extrusion of amplified genes during S-phase. These buds are eventually detached from the nucleus to form a micronucleus (Shimizu, et al., 1998; Fenech, 2002). The frequency of nuclei with nuclear bud formation is scored if gene amplification is suspected.

Cell/nuclear division kinetics:
Cell/nuclear division kinetics is assessed by scoring cells with one, two, three and four nuclei in CBMN assay. The index used for the calculation of division status is known as nuclear division index (NDI). The NDI provides a measure of the proliferative status of the viable cell fraction. It is therefore an indicator of cytostatic effects and, in the case of lymphocytes, it is also a measure of mitogenic response, which is useful as a biomarker of immune function (Chandra, 2002). The lowest NDI value possible is 1.0, which occurs if all of the viable cells have failed to divide during the cytokinesis-block period and are therefore all mononucleated. If all viable cells completed one nuclear division and are therefore all binucleated, the NDI value is 2.0 (Fenech, 2007). The calculation of the NDI when compared with the control, used during the study, reveals the significant cell cycle delay or progression detectable after exposing culture cells to varying concentrations of the drug.

**Nuclear division cytotoxicity index:**

The more accurate assessment of nuclear division status is obtained by including necrotic and apoptotic cells in the total number of cells scored. It is because at higher toxic doses of chemicals tested a very large proportion of cells became non-viable. In that case both binucleate cell ratio and the NDI became overestimated if necrotic and apoptotic cells were not included in scoring cells. Thus, at higher toxicity levels the precise estimate of nuclear division status and cell division kinetics is obtained using the nuclear division cytotoxicity index (NDCI) which takes into account both viable as well as necrotic and apoptotic cell fractions (Fenech, 2000). Any change in NDCI values following drug exposure compared to concurrent control, provides information on cell cycle progression and cellular cytotoxicity.
CYTOGENETIC EFFECTS OF THE VASODILATOR ANTIHYPERTENSIVE DRUGS IN VITRO

Antihypertensive vasodilators are drugs that are recommended for the treatment of peripheral vascular diseases, high blood pressure problems, cardiac problems like stroke and other cerebral vascular diseases etc. depending on the function and class of that particular antihypertensive drug.

These cardiac problems are, nowadays, commonly encountered. Thus, in the present investigation, keeping in mind the widespread, long-term and continual use of vasodilator antihypertensives, four such drugs are tested for their genotoxic potential in human peripheral lymphocytes in vitro. No such type of study has been done previously in three of these drugs, namely, Isoxsuprine hydrochloride (Duvadilan®), Xanthinol nicotinate (Complamina®) and Metoprolol tartrate (Metolar®). Whereas, some study has earlier been done (Bozsakyova, et al., 2001) over the genotoxicity of the third drug Pentoxifylline (Trental®).

In the present study, we have estimated the rate of chromosomal instability in the cultured human lymphocytes treated with four above cardiovascular drugs. Concerning the results obtained in our present in vitro studies, all the four drugs, at applied concentrations, are found to have statistically significant enhancement of variables of chromosomal aberration assay and cytokinesis-block micronucleus assay. Most of these effects have been reported for the first time in our study.

Considering the evaluation of these drugs through chromosomal aberration studies, in peripheral blood lymphocytes in vitro, it is noticed that the drugs significantly increase the drug induced frequencies of aberrant
metaphases as compared with their concurrent control. A much higher than normally obtained percentage of abnormal metaphases have been reported in present study in case of all the four test drugs.

Studied individually, out of the four drugs, the highest response was given by drug Isoxsuprine hydrochloride (Duvadilan®). This drug induced 75% aberrant metaphases at concentration of 1.00 mg/ml. Rest three drugs also induce significantly high percentage of abnormal metaphases at their respective concentrations demonstrating a positive correlation with increasing drug doses.

In case of the drug Metoprolol tartrate (Metolar®), the increasingly toxic effect of higher drug concentration resulted in cell death for last two drug doses.

Considering gross structural type of chromosomal abnormalities, chromatid-type of aberrations was more common in all the test drugs indicating a clastogenic response of the drugs towards human lymphocytes in vitro. The prevalence of -type of aberrations suggest S-dependency of the drugs. It is well known, that the type of aberrations induced by genotoxic agents are cell cycle dependent. Most of the chemically induced aberrations are formed only during the DNA synthesis phase (probably due to misreplication). Such chemical agents induce mainly chromatid-type aberrations and are also very efficient in inducing sister chromatid exchanges (Natarajan, 1993).

Structural Chromosomal aberrations are thought to be induced due to the double-stranded breaks (Obe, et al., 1982), although single-stranded breaks may also play a quantitatively minor role. Dicentric chromosomes
and acentric fragments have been originated as a result of asymmetrical recombinations relative to the centromere. These acentrics may give rise to the formation of micronuclei. Aberrations in chromosome number arise from disturbances of the mitotic cell division process (Baan, 1987).

Assessment of cytogenetic response of the drugs under study via variables of CBMN assay suggested their statistically significant positive involvement in the induction of chromosome loss and rearrangement.

Drug induced enhanced micronucleus frequencies were found in vitro for all the four drugs, the highest result being obtained in pentoxifylline and Xanthinol nicotinate. Both these drugs induce 15% micronucleus frequencies. Such a high percent induction suggested the involvement of aneuploidy (specifically chromosome loss) phenomena in the origin of drug-induced MN in vitro. The significantly increased frequencies of MN in vitro could be related to the lack of metabolic clearance of these drugs by lymphocytes in culture.

Other variables of the assay like binucleated mitconucleated cell too get positively affected by the increasing drug concentration. Increasingly high frequencies of these cell types indicate cytogenetic responsiveness of the drugs.

Studies of chromosomal rearrangements via nuclear bridge formation in binucleated cells in drug treated cultures at 24 hour of incubation show a significantly increasing dose-dependent response. All the four vasodilator antihypertensive drugs induce chromosomal rearrangements in cultured human lymphocytes.
Concerning the cytogenetic effect of the drugs in terms of the appearance of nuclear buds, the increasingly positive dose-dependent response show involvement of gene amplification induced by test drugs. The highest responsive drug being Xanthinol nicotinate. However, this drug shows no bud frequency at all at some initial concentration. Thus higher dose of the drug resulted in gene amplification.

More cytogenotoxic effects of the drugs under study were assessed by studying the cell/nuclear kinetics of the test drugs in human lymphocytes at varying concentrations of the drug. A significant data suggested that a very clear cell cycle delay and cellular cytotoxicity was caused by the test drugs. Remarkably reduced NDI and NDCI values were achieved at increasing concentrations of the drug, the exposure time being kept constant. The highest cell cycle delay was observed by the drug Pentoxifylline, very closely followed by Isoxsurprine HCl. Other drugs also follow the response. NDCI values also pointed out notable cell death response in the form of an increment in apoptotic and necrotic cells in human cultured lymphocytes in vitro. These results suggested a positive in vitro cytotoxic response of the drugs under concern.

In the present study, very high frequencies of some significantly positive responses including chromosomal aberration frequencies and greater number of nuclear bridges and nuclear buds which have been obtained as a results of the treatment of human peripheral blood lymphocytes with increasing concentrations of test drugs, may be justified due to the use of very high concentrations, in mg/ml, of the drugs.
The contrasting results, at different concentrations of treatment, for one or the other parameter analyzed, may appeared due to some handling mistakes and cross reactions. The overall estimates, otherwise, were consistent.

Concerning the comparative analysis of the data of all the four cardiovascular treatment drugs, taking each assayed parameter separately, it was noticed that no single drug was highly differentially responsive to any parameter. Comparison of average mean values (± SEM) of data for each variable gave an overall average assessment of obtained response for each drug. In case of mean percent aberration metaphases and mean aberrations per cell reports all the four drugs showed high response. Drug Isoxsaprine HCl gave a slightly more reactive response by inducing 75% aberrations, then the rest three. While aberration per cell data was more for the drug Xanthinol nicotinate. In case of induction of micronuclei and occurrence of BNMMN cells again the drug Isoxsaprine HCl showed more percentage. Taking nucleoplasmic bridges and nuclear buds into consideration, the highest response was given by drug Xanthinol nicotinate. Likewise for NDI and NDCI measures were more reduced for the drug Metoprolol tartrate, indicating the highest response of this drug in cell cycle delay and cellular cytotoxicity. Drug Pentoxifylline gave close rest to all these values. This suggested that all the four drugs show the positive cytogenetic effects over human lymphocytes in culture.

When the observed genotoxic potentialities of the test drugs were analyzed in comparison with the studies done for other antihypertensive vasodilator drugs, our data was in line with them. Although much studies have not been done for this class of drugs, but the available data show
similarities with our findings. Studying the genotoxicity of diuretic antihypertensive drug Hydrochlorothiazide, a clear chromosome delay was found (Andrianopoulos, et al., 2006). According to an extensive survey done over marketed antihypertensive drugs, 32 drugs were found to be positive in at least one genotoxicity assay (Brambilla and Martelli, 2006). Sodium nitroprusside, used for the ischaemic patients undergoing nitrate therapy, showed cytotoxicity and cell cycle delays when studied in vitro (Andreassi, et al., 2001). Telez, et al. (2001) found highly induced MN frequencies containing whole chromosomes for another antihypertensive drug nimodipine in vitro. Telez, et al. (2000) also found a significantly high frequency of MN in the lymphocytes of patients treated with a β-blocker antihypertensive drug Atenolol thus showing positive aneugenic responses in CBMN assay with human lymphocytes. Studies show that antihypertensive drugs Hydralazine and Dihydralazine can induce micronuclei in vitro, the effect depending on time of exposure and concentration of the drug (Chlopkiewicz, 1996). Drug Pentoxifylline was also tested positive to induce chromatid type aberrations in vitro (Slamenova, et al., 1995). Methyldopa, a widely used antihypertensive drug, was found to induce significant increase in the SCE frequency in human lymphocytes in culture (Cesar and Catarina, 1991). Also induction of mutation and chromosomal aberration was observed in experimental models following exposure to nitrogen oxides, nitrate and nitrite (Isomura, et al., 1984; Luca, et al., 1985, 1987; Nayak, et al., 1989) and an increase of MN formation by nitric oxide was observed in mammalian cells using Sodium nitroprusside as a drug donor of NO (Lin, et al., 1998). Thus, our data is in good agreement with these previous reports, the cytogenetic responses of the
three drugs, Isoxsuprine hydrochloride (Duvadilan®), Xanthinol nicotinate (Complamina®) and Metoprolol tartrate (Metolar®) being reported for the first time.

Summing up, the four test drugs indicate significantly positive results for their cytogenotoxic potentials (clastogenic and aneugenic effects) in cultured peripheral blood lymphocytes in vitro, employing various parameters of chromosomal assay and CBMN assay techniques. Their chronic use may impose negative side effects on the health of the patient. However, in vitro analysis alone is insufficient to identify these hazardous effects and thus, in vivo analysis also has to be carried out (Vlastos, et al., 1998) to observe and evaluate the chronic exposure of the drug among the patients.
PHOTOMICROGRAPHS
Metaphase plates showing chromosomal aberrations due to the effects of the test drugs
Fig. 29. A spread with fragments and breaks.

Fig. 30. Dicentric chromosomes, triradii, and terminal deletion.

Fig. 31. Endoreduplication, extreme chromatid separation.

Fig. 32. Terminal deletion.
Fig. 33. Chromatid end joining of two different chromosomes.

Fig. 34. Hypodyploidy of chromosomes.

Fig. 35. Different chromosome shapes, terminal deletion.

Fig. 36. Chromatid sticking and condensation.
Fig. 37. Terminal deletion, acentric fragment, and chromatid separation.

Fig. 38. Multiple and random chromosome anomalies.

Fig 39. Complete p- arm deletion.

Fig. 40. Terminal deletions and chromatid end sticking.
Fig. 41. Minutes and chromatid separation.

Fig. 42. Chromatid gaps and chromatid separation.

Fig. 43. Break at q-arm, and chromatid end sticking.

Fig. 44. Chromosome condensation and chromatid exchange.
Fig. 45. Chromosome rings.

Fig. 46. Dicentric chromosomes.

Fig. 47. Chromatid separation.

Fig. 48. Hypoploidy and, arm sticking and terminal deletion.
Fig. 49. q - Arm terminal deletion.

Fig. 50. Extreme chromosome condensation.
Plates showing nuclear changes due to the effects of the test drugs
Fig. 51. Cell population with budded, binucleated, necrotic and apoptotic cells along with some normal cells.

Fig. 52. Normal, necrotic and budded cells.

Fig. 53. A binucleated cell with a micronucleus.

Fig. 54. A binucleated cell with a micronucleus and a nucleoplasmic bridge connecting both nuclei.
Fig. 55. A binucleated cell with a micronucleus overlapping one nucleus.

Fig. 56. A binucleated cell with a nucleoplasmic bridge connecting both nuclei.

Fig. 57. A binucleated cell with two budded nuclei.

Fig. 58. A trinucleated cell with two budded nuclei.
Fig. 59. A binucleated cell with a nucleoplasmic bridge connecting both nuclei.

Fig. 60. A binucleated, an apoptotic and a necrotic cell.

Fig. 61. A mononucleate cell undergoing necrosis.

Fig. 62. One necrotic and one apoptotic cell.
Fig. 63. A mononucleated cell with an extruding nucleus.

Fig. 64. Plate showing a tetranucleated cell with one extruding nuclei.

Fig. 65. A trinucleated cell.

Fig. 66. A tetranucleated and mononucleate cell.
BIBLIOGRAPHY


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