DISCUSSION
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{8}) and Metoprolol tartrate (Metolar\textsuperscript{8}), have been selected for the study.

**GENERAL CONSIDERATIONS**

The preservation of integrity of DNA as a genetic blue print 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 then 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 he 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 mirconucleated 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 Isoxsuprine 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 Isoxsuprine 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 BNMN cells again the drug Isoxsuprine 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. Hypodiploidy 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.