Estimation of *in vitro* frequency of micronuclei in binucleated cells of short-term cultured peripheral blood lymphocytes from cancer patients on remission by Cytokinesis blocked micronuclei assay

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Superna Thakur

09MBC018

Under the guidance of

Dr. Sonal. R. Bakshi

Dedicated to My mother

My mother's love for me was so great I have worked hard to justify it....

She is my teacher, my inspiration at every step...

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Learning: *"Process of progressive change from ignorance to knowledge, from inability to competence, and from indifference to understanding. The means by which we systematize the situations, conditions, tasks, materials, and opportunities by which learners acquire new or different ways of thinking, feeling, and doing."*

Above words reflects my journey of learning during my project. For all the knowledge and experience which I acquired would not have been possible without my teachers and my friends whom I would like to thank here.

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LIST OF ABBREVIATIONS

DNA: Deoxyribonucleic acid

MNi: Micronuclei

MNT: Micronucleus Test

BNC: Binucleated cells

AT: Ataxia Teleangiectasia

Cyt-B: Cytochalasin-B

BN: Binucleated cells

NDI: Nuclear Division Index

HPRT: Hypoxanthine Phospho-ribosyl Transferase

Hrs: Hours

mM: millimolar

CBMN: Cytokinesis Blocked Micronuclei Assay

EBRT: External Beam Radiotherapy

CA: Chromosome Aberrations

SCE: Sister Chromatid Exchange

PBL: Peripheral Blood Lymphocytes

µm: micrometer

PHA: Phytohemagglutinin

mm:milimeter

DMSO: Dimethyl sulfoxide

RPMI: Roswell Park Memorial Institute

KCl: Potassium Chloride

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

mg: milligram

mm: millimeter

FBS: Fetal Bovine Serum

FAC: 5-Fluorouracil, Adriamycin, Cyclophophamide

FEC: 5-Fluorouracil, Epirubicin, Cyclophophamide

BOD: Biological Oxygen Demand

CO_2: Carbon Dioxide

rpm: rotation per minute

min: minutes

DPX: mixture of Distyrene, a plasticizer, and xylene

SD: Standard Deviation

MN: Mononucleated cell

MNBN: Micronuclei in binucleated cell

MPF: Metaphase Promoting Factor

ABSTRACT

ABSTRACT:

Cytokinesis-block Micronuclei assay is among the various cytogenetic assays that have been used as a short-term test for any genotoxic assessment. The method has also gained attention as a tool for cancer risk assessment in lieu of chromosomal aberration test. We have studied this biomarker in known exposed groups retrospectively i.e., cancer patients on remission following treatment to evaluate if this reflects the genotoxic effects.

The aim of the study is to evaluate the *in vitro* micronucleus frequency in circulated peripheral blood lymphocytes of the patients who have received the cancer therapy at least 2 years back. In this study, we used the nuclear division index and micronucleus frequencies to evaluate the cytotoxic and genotoxic effects respectively. The analyzed samples included treated breast cancer patients on remission with history of cancer therapy (5 females with mean age 52.4± 5.12) and control group included 5 healthy donors (3 females and 2 males with mean age 47.0± 5.70). We did not observe any significant difference in micronucleus frequencies of exposed group and controls. The percentage reduction of nuclear division index in exposed group was also not significant. These findings need to be substantiated with a larger population and longer follow up grouped according to the therapy.

I. INTRODUCTION

1. GENETOXICITY ASSESSEMENT:

Genetic toxicity assessment is the evaluation of agents for their ability to induce genetic changes i.e. mutations in the genetic material (DNA) that can be detected at various levels viz, molecular level or chromosome level. In organisms such as humans, the genes are composed of DNA, which consists of individual units called nucleotide bases. The genes are arranged in discrete physical structures called chromosomes.

Genetic toxicology is the study of genetic damage, agents that induce the damage, its consequences and mechanisms involved. Genotoxicity can result in significant and irreversible effects upon human health. Genotoxic damage is a critical step in the induction of cancer and it can also be involved in the induction of birth defects and fetal death. The knowledge that many environmental agents are associated with human cancer development and that the genetic alterations are the basis for neoplasia underscored the need for testing genotoxic potential of chemicals. As a result many short term assays were developed. Assays that measure gene mutation are those that detect the substitution, addition or deletion of nucleotides within a gene. Assays that measure chromosomal mutation are those that detect breaks or chromosomal rearrangements involving one or more chromosomes. Assays that measure genomic mutation are those that detect changes in the number of chromosomes, a condition called aneuploidy. Chromosome alterations are of two types:

1. Structural chromosomal aberrations (chromosome or chromatid breaks, rearrangements)

2. Numerical chromosomal aberrations (aneuploidy, polyploidy).

Numerous *in-vivo* and *in-vitro* cytogenetic alterations can result from exposure to chemicals, and ionizing or non-ionizing radiations. Cell lines with defects in DNA repair have been exploited to increase the sensitivity for detecting effects of chemicals and radiations.

The association between specific cytogenetic alterations and tumorigenesis is strong (Mitelman, 1994) and it is this relationship that is used as one justification for including cytogenetic endpoints in toxicological evaluations of industrial chemicals

and new pharmaceutical and therapeutic compounds. Cytogenetic toxicity data are also used for other purposes, including ecological and environmental monitoring, assessment and cleanup and for workplace hazard evaluations (Tucker *et al*., 1996). Cytogenetic assays have the important advantage that they enumerate damage at the level of the individual cell (Geard CR., 1992).

Cytogenetic assays are divided into two test types:

1. *In vivo* tests: Chromosomal aberrations in rodent bone marrow cells (metaphase analysis) in which rats are used as model system or the mouse bone marrow micronucleus test in which mice are used as model system.
2. *In vitro* tests: Mammalian cells in culture.

There are four genetic endpoints most frequently used in hazard identification assays for risk assessment process. These are structural chromosome aberrations, micronuclei, aneuploidy and sister chromatid exchanges.

2. MICRONUCLEI:

Micronuclei (MNi) are small, round; DNA/chromatin-containing interphase structures occasionally found in the cytoplasm of cells. They are morphologically similar to but much smaller than the corresponding main nuclei. MNi have been used successfully as a cytogenetic endpoint to evaluate genotoxic effects of radiation, chemical agent or heavy metals in cells in vivo and in vitro. They have also been used as a marker for chromosome instability. Recently, MNi have been shown to be a valuable predictor of cancer risk and cardiovascular mortality. Numerous publications have shown that MNi may contain either chromosomes, or acentric chromosomal fragments (Rao *et al*., 2008).

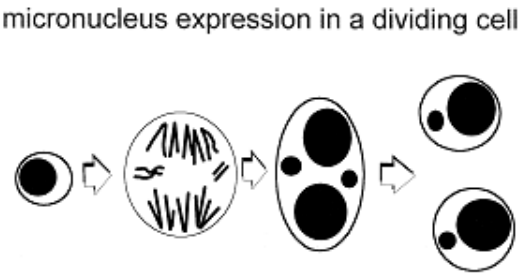


Fig. 1: The origin of micronuclei from lagging whole chromosomes and acentric chromosome fragments in a dividing cell at anaphase. Source: *M.Fenech/Mutation Research 428 (1999)271-283*

MNi, also known as Howell–Jolly bodies, were originally identified and described in erythrocytes by the hematologists William Howell and Justin Jolly and they were later found to be associated with deficiencies in vitamins such as folate and vitamin B12. The application of micronuclei as an *in vivo* test for mammalian chromosomal damage was first described in a test system using mouse bone marrow by Heddle (1973). The application of the micronucleus test in human lymphocytes was first explored by Countryman and Heddle (1976).

The formation of MNi is attributed to a variety of insults to genetic materials, which could be classified as exogenous factors and endogenous factors. Exogenous factors include radiation, chemical agents, microorganism invasion, etc. Endogenous factors include genetic defects, pathological changes, deficiency of essential nutritional ingredients (e.g. folic acid) and injuries induced by deleterious metabolic products (such as reactive oxygen species) (Huang *et al*., 2011).

MNi can originate during anaphase from lagging acentric chromosome or chromatid fragments caused by misrepair of DNA breaks or unrepaired DNA breaks. Malsegregation of whole chromosomes at anaphase may also lead to MN formation as a result of hypomethylation of repeat sequences in centromeric and pericentromeric DNA, defects in kinetochore proteins or assembly, dysfunctional spindle and defective anaphase checkpoint genes (Fenech *et al*., 2011). Entire chromosomes are more frequent in spontaneously occurring MNi or after induction by spindle poisons without any clastogenic treatment, as was demonstrated by anti-kinetochore antibody staining (Fenech.M and Morley, 1989; Tucker and Eastmond, 1990).

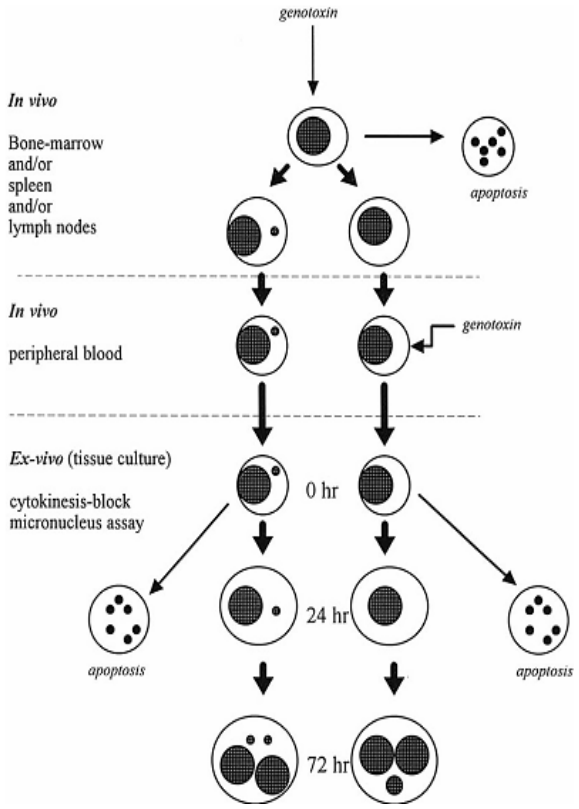


Fig 2: Schematic diagram illustrating the expression of micronuclei MNi in lymphocytes following in vivo nuclear division or ex vivo nuclear division in culture. Source: *M.Fenech/Mutation Research 428 (1999)271-283.*

2.1 Mechanism of Origin of Micronuclei:

It is now well-established that MNi mainly originate from acentric chromosome fragments, acentric chromatid fragments or whole chromosomes that fail to be included in the daughter nuclei at the completion of telophase during mitosis because they did not attach properly with the spindle during the segregation process in anaphase. These displaced chromosomes or chromosome fragments are eventually enclosed by a nuclear membrane and, except for their smaller size, are morphologically similar to nuclei after nuclear staining (Fenech *et al*., 2011).

1. *MNi from acentric chromosome or chromatid fragments*: Acentric chromosome fragments originate via multiple mechanisms. Radiation biology studies over several decades have shown that misrepair of DNA double-strand breaks can lead to

symmetrical and asymmetrical chromatid and chromosome exchanges as well as chromatid and chromosome fragments. A small proportion of acentric chromosome fragments may simply arise from unrepaired double-stranded DNA breaks, but this is only likely when DNA damage load exceeds the repair capacity of the cell within a specified time frame. Other mechanisms that could lead to MNi formation from acentric fragments include simultaneous excision repair of damaged (e.g. 8-oxo-deoxyguanosine) or inappropriate bases incorporated in DNA (e.g. uracil) that are in proximity and on opposite complementary DNA strands. Such simultaneous excision repair events, particularly if the gap-filling step is not completed, leads to DNA double-strand breaks and MNi formation.

2. *MNi from malsegregated whole chromosomes*: Lymphocyte MNi in healthy people, not abnormally exposed to genotoxins usually originate from either acentric chromosome fragments or whole chromosome loss events at a ratio ranging between ~30:70% at one extreme to 70:30% at the other extreme depending on age and gender. In lymphocytes, MNi increase with age and are generally higher in females relative to males. Sex chromosomes contribute the majority of chromosome loss events with increasing age. In females, the X chromosome can account for up to 72% of the observed MNi of which 37% appear to be lacking a functional kinetochore suggesting that defects may be present in kinetochore assembly possibly due to X chromosome inactivation. There are a range of possible molecular mechanisms that could cause chromosome malsegregation at anaphase resulting in MNi formation. One of the mechanisms that may lead to MNi from chromosome loss events is hypomethylation of cytosine in centromeric and pericentromeric repeat sequences such as classical satellite repeats at pericentromeric regions and higher order repeats of satellite DNA in centromeric DNA.

Kinetochore proteins play an important role in the engagement of chromosomes with the spindle; it is probable that mutations leading to defects in kinetochore and microtubule interaction dynamics could also be a cause of MN formation due to chromosome loss at anaphase. Other variables that are likely to increase MN from chromosome loss are defects in mitotic spindle assembly, mitosis check point defects and abnormal centrosome amplification. A recent study suggests that dicentric chromosomes resulting from telomere end fusions may often be involved in missegregation events; this may occur when the centromeres of the dicentric

chromosome are pulled towards opposite poles of the cell during anaphase with forces that are sufficient to detach the chromosome from the spindle. Pancentromeric DNA probes are used to distinguish between MN originating from any whole chromosome loss event and MNi containing acentric chromosome fragments.

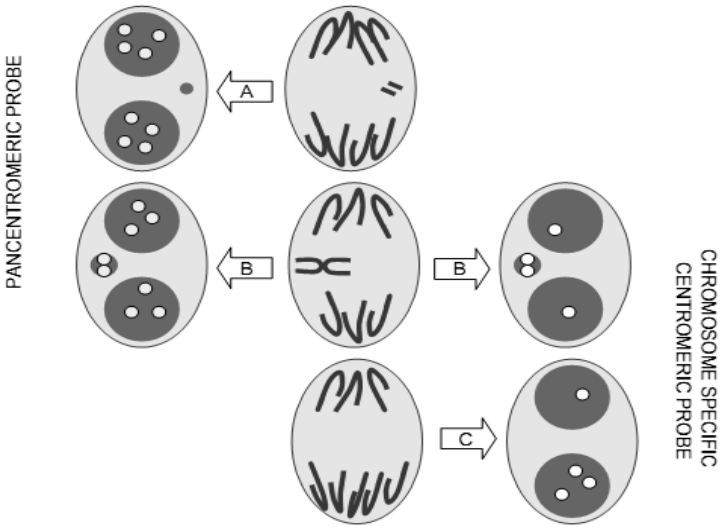


Fig 3: The use of molecular techniques for identifying (A) a MNi originating from a lagging acentric chromosome fragment, (B) a MNi originating from a lagging whole chromosome and (C) non-disjunction of a specific chromosome leading to aneuploid daughter nuclei. The spots in the nuclei and MNi of the binucleated cells on the left of each panel show the centromeric or kinetochore pattern of staining when pancentromeric probes or kinetochore antibodies are used. The nuclei and MNi of the binucleated cells on the right of each panel show the pattern of centromeric staining when a centromeric probe specific to the chromosomes involved in MNi formation or non-disjunction events is used. The example shown is for a hypothetical cell with only two pairs of chromosomes. Pancentromeric probes should be used only for distinguishing between micronuclei originating from chromosome breaks (centromere negative) and chromosome loss (centromere positive). Chromosome-specific centromere probes should be used only to measure malsegregation (due to non-disjunction or chromosome loss) involving unique chromosomes. It is important to

note that pancentromeric probes cannot be used to determine non-disjunction because of difficulty in reliably counting all the centromeres within the nuclei. Source: *M.Fenech et al/ Mutagenesis 26(2011)125-132.*

3. CYTOKINESIS BLOCKED MICRONUCLEUS ASSAY:

There is current interest in adopting the micronucleus test instead of metaphase analysis of chromosomes to assess the in vitro genotoxic potential of chemical and physical agents.

The in vivo bone-marrow micronucleus MNi test is well established as a standard assay for genotoxicity assessment at the chromosomal level (Heddle *et. al*., 1991). However, the trend for avoiding animal use *in vivo* testing has created the requirement and opportunity to further develop current in vitro systems for assessing chromosome damage. Traditionally, this has been done using a mammalian cell line and metaphase analysis of chromosomes (Evans, 1988). The Micronucleus Test (MNT) in Binucleated Cells (BNC) is a well-established assay, especially for mutagenicity testing (Kalantzi *et al*., 2003; Palus *et al*., 2003) and for human population monitoring (Bonassi *et al*., 2003; Neri *et al*., 2003). It has also been used to investigate chromosomal instability in humans who have mutations in genes which are needed for the repair of DNA damage, as in the case of Fanconi anemia (Zunino et al., 2001) and ataxia teleangiectasia (AT) (Gutierrez-Enriquez and Hall, 2003). It has also been shown to be an effective tool to measure cytogenetic damage by agents with different mechanisms of genotoxicity in vitro (Fenech and Morley, 1985c; Eastmond and Tucker, 1989; Norppa *et al*., 1993). *In vitro* micronuclei (MNi) test is a scientifically valid alternative to the in vitro chromosome aberration assay for genotoxicity.

A cell that has suffered a DNA-damaging event can only express such damage as a MNi if it completes at least one round of nuclear division after such an event *in vitro* (Fenech and Morley, 1985a). This effectively means that chromosome damage will not be expressed as MNi when cells are not dividing and that the level of MNi observed in a dividing cell population is dependent on the proportion of cells that are dividing. It has also been noted that the MNi frequency declines as cells proceed through more than one nuclear division *in vitro* after a DNA damaging insult (Fenech and Morley, 1985b). An absolute value for MNi frequency can only be obtained if

MNi is scored only in cells that have divided once only. It became evident after scoring hundreds of slides of lymphocyte cultures for MNi that the ideal stage to score MNi was the binucleated telophase stage (Fenech and Morley, 1985a, c). Cells at this stage are easily recognized by their binucleated appearance and there can be no doubt that they had completed one nuclear division and were therefore capable of expressing MNi. The binucleated telophase cell is the ideal stage to measure such events because it is only at this stage that one can ascertain whether the chromosome imbalance was due to non-disjunction or chromosome loss into a micronucleus. It is important to count MNi in binucleated cells (BNC) for several reasons: (i) cells have to pass through one cell cycle and mitosis after irradiation in order to form MNi; (ii) MNi may be lost in the second to the third cycle; (iii) new MNi may arise in the second to the third cycle.

The main task was then to devise a procedure that could block cells in this stage after completing one nuclear division only. As is well known, this was achieved by adding cytochalasin-B (Cyt-B) to cultured cells before the first mitotic wave after induction of DNA damage (Fenech and Morley, 1985a, b, c). Cells that complete nuclear division are then accumulated as binucleated cells because Cyt-B can inhibit cytokinesis without interfering with nuclear division (Carter, 1967). Scoring of MNi in cytokinesis-blocked binucleated BN cells has since become a standard procedure in genetic toxicology.

The cytokinesis-block technique has not only optimized the micronucleus technique, but also enabled new parameters of genotoxicity and cell division kinetics to be exploited.

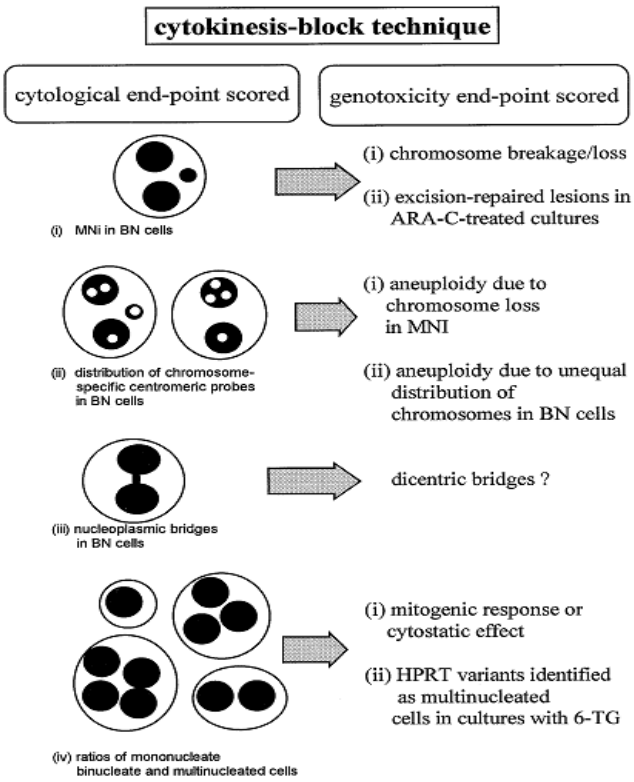


Fig 4: Schematic diagrams illustrating the various end-points that can be scored using the cytokinesis block technique. Source: *M.Fenech/Mutation Research 392(1997)11-18*

An important benefit of the cytokinesis-block method is that one can readily measure the extent and progression of nuclear division in a dividing cell population. This is achieved by measuring the frequency of mononucleate, binucleate and multinucleate (>2 nuclei) cells after a defined time point following the addition of cyt-B. The Nuclear Division Index (NDI) is readily measured using the following formula put forward by (Eastmond and Tucker 1989):

$$NDI= (MI+2MII+3MIII+4MIV) /N$$

where MI to MIV represents the number of cells with one to four MNi and N represents the total number of cells scored. Measurement of NDI provides important data on the cytostatic effect of a particular chemical or physical agent, helps identify molecules that can stimulate cell division and could be used to assess immunocompetence by measuring the mitogenic response of lymphocytes. All this can be done on the same slide that is used to score MNi. Such information cannot be readily derived without performing a cytokinesis-block.

There are a lot of factors which influence the frequency of MNi. The most important and interesting one is the biological differences between individuals. Other points concern the culture time, the cell harvesting methodology and the fixation and preparation of the slides (Bonassi *et al.*, 2001). Another source of variation is the scoring procedure for MNi, which is done visually. The criteria for scoring have been standardized in order to minimize non-biological variation and to allow comparison between laboratories. Visual scoring of MNi is very time consuming and the results depend on subjective interpretation of nuclei and MNi. The parameters considered for selection of binucleated cells and micronuclei in scoring can result in differing MNi frequencies between observers and laboratories (Fenech.M *et al*., 2003).

The unique advantage of the micronucleus assays over metaphase analysis is the capacity to detect chromosome loss events reliably because the presence of MNi, unlike the absence of a chromosome in a metaphase spread, is unlikely to be due to increased aberration. Recognition of whole chromosomes in MNi is achieved using either anti-kinetochore antibodies or chromosome-specific centromeric probes (Fenech and Morley, 1989; Lynch and Parry, 1993; Parry *et al*., 1995). MNi containing whole chromosomes is then identified by the staining of a centromeric region or kinetochore. The use of chromosome-specific centromeric probes allows not only the detection of whole chromosomes in MNi within binucleated cells, but also enables the distribution of chromosomes between the daughter nuclei within a binucleated cell to be scored (Farooqi *et al*., 1993; Hando *et al*., 1994). This is a unique feature of the cytokinesis-block method that cannot be readily achieved by alternative techniques and permits the detection of malsegregation of chromosomes within binucleated cells even when no MNi is produced (Zijno *et al*., 1994).

After assessing the MNi induction in human lymphocytes following exposure to a variety of genotoxins, it became evident that the extent of micronucleus formation in relation to cytotoxicity was low for chemicals and ultraviolet radiation which mainly induce base-lesions and adducts on DNA rather than strand breakage or spindle damage (Fenech, 1985; Fenech and Neville, 1992).

Norman *et al*. 1988 showed that it was possible to use the cytokinesis-block technique to measure the frequency of HPRT (Hypoxanthine phopho-ribosyl transferase) variant lymphocytes that were resistant to 6-thioguanine. Such cells were identifiable by their

ability to divide at least once during a 72-hrs incubation period in the presence of 0.2 mM 6-thioguanine: these cells are recognized by their binucleated or multinucleate appearance by using a Cyt-B block 30 hrs after mitogen stimulation.

Cyt-B may inhibit cytokinesis by binding to high molecular weight complexes in the plasma membrane that have the ability to induce actin polymerization and therefore microfilament assembly; the later is required for the formation of the cleavage furrow (Lin and Lin, 1979). The efficiency with which Cyt-B inhibits this process is dependent on the concentration used.

It has been shown that the MNi frequency observed in binucleated cells may depend on the efficiency with which the cultures have been cytokinesis-blocked (Surralles *et al.*, 1992).

The possibility that Cyt-B might induce MNi in binucleated cells has been raised several times, but in each case, studies have shown that there is no dose–response effect for MNi induction in binucleated cells in the concentration range (1–6 µg/ml) and the cell types (e.g., human lymphocytes, mouse spleen lymphocytes, mouse fibroblasts, Chinese hamster fibroblasts and human fibroblasts) that are normally used (Fenech.M and Morley, 1985c;Wakata and Sasaki, 1987; Prosser *et al.*, 1988; Linholm *et al.*, 1991).

The cytokinesis-block technique has been shown to be considerably versatile, enabling the detection of chromosome breakage, chromosome loss, non-disjunction, excision repair events as well as allowing the proportion of dividing cells to be measured. The current interest in employing the CBMN assay as a method of predicting the radio sensitivity of cells also highlights the importance of deriving as much information from a slide of cytokinesis-blocked cells. It is increasingly becoming evident that measurement of the MNi frequency together with identification of apoptotic cells (simply by morphological examination) may provide a more reliable identification of cellular sensitivity to genotoxins (Abend *et al.*, 1995).

The cytokinesis-blocked micronucleus assay could be considered as a multi-endpoint test for genotoxic responses to clastogens/aneugens.

4. CANCER:

The body is made up of hundreds of millions of living cells. Cancer begins when cells in a part of the body start to grow out of control. Cancer cell growth is different from normal cell growth.

Cells become cancerous because of damage to DNA. In a normal cell, when DNA gets damaged either the damage is repaired or the cell dies. In cancer cells, the damaged DNA is not repaired, but the cell does not die; instead, the damaged cell multiplies that leads to neoplastic conditions..

In addition to inherited DNA damage, most DNA damage can be caused by mutations occurring while replication or induced by the external environmental agents. The link between DNA damage and cigarette smoking is well known; however, often the etiology remains obscure.

Cancer is a group of diseases in which cells are *aggressive* (grow and divide without respect to normal limits), *invasive* (invade and destroy adjacent tissues), and sometimes *metastatic* (spread to other locations in the body).

4.1 Cancer causing mutations:

Genes responsible for causing cancer falls into three distinct classes:

1. *Proto-oncogenes*: products of these genes are components of signaling pathway regulating proliferation status of the cells. Any mutation will result in dominant oncogenes.
2. *Tumour suppressor genes:* are generally check points of cell cycle progression, cellular adhesion etc. These genes exhibit recessive effects.
3. *DNA repair enzymes:* any mutation in these genes will reduce the stability of the genome.

4.2 Cancer classification:

Cancers are classified by the type of cell that resembles the tumor and, therefore, the tissue presumed to be the origin of the tumor. Examples of general categories include:

1. **Carcinoma:** Malignant tumors derived from epithelial cells. This group represents the most common cancers, including the common forms of breast, prostate, lung and colon cancer.
2. **Sarcoma:** Malignant tumors derived from connective tissue, or mesenchymal cells.
3. **Lymphoma** and **leukemia:** Malignancies derived from hematopoietic (blood-forming) cells
4. **Germ cell tumor:** Tumors derived from totipotent cells.

4.3 Treatment:

Cancer can be treated by surgery, chemotherapy, radiation therapy, immunotherapy, monoclonal antibody therapy, and many other methods evolving due to research in this field. The choice of therapy depends upon the location and grade of the tumor and the stage of the disease, as well as the general state of the patient.

(i) **Surgery:** Complete removal of the cancer without damage to the rest of the body is the goal of the treatment. But the propensity of cancers to invade adjacent tissues or to spread to distant sites by microscopic metastasis often limits its effectiveness.

(ii) **Radiation therapy:** (also called radiotherapy, X-ray therapy, or irradiation) is the use of ionizing radiation to kill cancer cells and shrink tumors. Radiation therapy can be administered externally via external beam radiotherapy (EBRT) or internally via brachytherapy. The effects of radiation therapy are localized and confined to the region being treated. Radiation therapy injures or destroys cells in the area being treated (the "target tissue") by damaging their genetic material, making it impossible for these cells to continue to grow and divide. Although radiation damages both cancer cells and normal cells, most normal cells can recover from the effects of radiation and function properly. The goal of radiation therapy is to damage as many cancer cells as possible, while limiting harm to nearby

healthy tissue. Hence, it is given in many fractions, allowing healthy tissue to recover between fractions.

Radiation therapy may be used to treat almost every type of solid tumor, including cancers of the brain, breast, cervix, larynx, lung, pancreas, prostate, skin, stomach, uterus, or soft tissue sarcomas. Radiation is also used to treat leukemia and lymphoma. Radiation dose to each site depends on a number of factors, including the radio sensitivity of each cancer type and whether there are tissues and organs nearby that may be damaged by radiation. Thus, as with every form of treatment, radiation therapy is not without its side effects.

(iii) **Chemotherapy:** Chemotherapy is the treatment of cancer with drugs ("anticancer drugs") that can destroy cancer cells. In current usage, the term "chemotherapy" usually refers to *cytotoxic* drugs which affect rapidly dividing cells in general, in contrast with *targeted therapy*. Chemotherapy drugs interfere with cell division in various possible ways, e.g. with the duplication of DNA or the separation of newly formed chromosomes. Most forms of chemotherapy target all rapidly dividing cells and are not specific for cancer cells, although some degree of specificity may come from the inability of many cancer cells to repair DNA damage, while normal cells generally can. Hence, chemotherapy has the potential to harm healthy tissue, especially those tissues that have a high replacement rate (e.g. intestinal lining). These cells usually repair themselves after chemotherapy.

Because some drugs work better together than alone, two or more drugs are often given at the same time. This is called "combination chemotherapy"; most chemotherapy regimens are given in a combination.

Rationale of the study

To estimate the *in vitro* frequency of micronuclei in binucleated cells in short term cultured whole blood from patients who have received cancer treatment at least two years back.

The retrospective study of exposed group with respect to in vitro frequency of MNi in short term cultured blood lymphocytes can give an idea regarding its usefulness as a possible biomarker of genotoxic exposure. The MNi assay offers advantage of faster scoring of more number of cells and is amenable for automation, hence has been tested for various aspects in order to replace or at least in addition to the CA test.

Development and validation of biomarkers provides the most promising strategies for the prevention of cancers. There are various candidate cancer risk biomarkers of which classic cytogenetic endpoints: chromosome aberrations (CAs), sister chromatid exchanges (SCE) and MNi, measured in peripheral blood lymphocytes (PBL), are of great interest. In 1914, Boveri pointed to the relationship between numerical chromosome abnormalities and cancer. Tumors cells have been examined in detail and in almost all cases they contain structural and/or numerical chromosome alterations (Mitelman, 1994). The results concerning the role of mutations in cancer initiation, promotion, invasion and metastasis led to the development of a battery of in vitro and in vivo (essentially in rodents) mutagenicity tests to assess the carcinogenicity of a compound that included essentially the tests aiming at the detection of gene mutations and structural chromosome changes.

CAs is the first biomarker of chromosome damage that has been consistently associated with the overall cancer risk (Murgia *et al*., 2008). Although there are various positive evidences that link CAs with cancer risk, the method is labor intensive and time consuming. Therefore it is suitable to use simpler and quicker risk biomarkers.

As MNi origin is either from chromosome fragment or whole chromosome, it may reveal several genomic instability events which are associated with malignant cell transformations (Murgia *et al*., 2008).

Frequency of chromosomal aberrations in PBLs is a relevant biomarker for cancer risk in humans, reflecting both early biological effects of exposure to genotoxic carcinogens and individual cancer susceptibility. (Bonassi *et al*, 2000).

It has been reported that increased MNi frequencies occur in peripheral blood lymphocytes of subjects exposed to mutagenic agents at the workplace or in their environment (Fenech *et al*., 1993), and cancer patients or other patients receiving a cytostatic therapy or radiation therapy generally revealed the clearest effects (N.S. Arsoy *et al*., 2009). Increased frequencies of MNi can only be expected if lymphocytes with persistent damage are obtained and cultured, if damage is not removed during culture before the lymphocytes start to proliferate and is fixed as a MNi during cell division. If damage is efficiently repaired, increased MNi frequencies cannot be expected (N.S. Arsoy *et al*., 2009).

II. MATERIALS

AND

METHODS

1. Materials:

All chemicals used in this study were of analytical grade and were obtained from HIMEDIA, MERCK and s.d. fine-chem. limited. All water used in the preparation of solutions was either double distilled or filtered through a milli-Q (0.22µm). Solutions were sterilized either by autoclaving (at 15 psi on liquid cycle at 121˚C for 15 to 20 minutes as per the requirement) or by filtration through a 0.22µm filter. All solutions were stored at appropriate temperature. All glass wares and plastic wares were also sterilized by autoclaving and baking.

1.1 REAGENTS:

1. RPMI-1640 culture medium: HiGlutaXL™ RPMI-1640 (8 ml medium in 15 ml round bottom centrifuge tube). Cell culture tested, **Phytohemagglutinin:** HIMEDIA PHA-M (PHAM, 25mg), **Cytochalasin-B:** HIMEDIA, **Potassium chloride:** MERCK, **Methanol:** MERCK, **Acetic Acid:** MERCK, **Giemsa Staining solution:** HIMEDIA, **DPX:** s.d. fine-chem. Limited **(**Mounting medium).

1.2 EQUIPMENTS:

1. Evacuated tubes: [C.D RICH® (3 ML)] sterile vacutainer blood tubes with sodium heparin as anticoagulant.

2. Blood collection needle: [Greiner bio-one] sterile multi drawing needle, 22G×1".

3. Graduated sterile pipettes: [Borosil] 10 ml, 5 ml, and 2 ml.

4. Bench top centrifuge: [nuve] capable of spinning at 1000g.

5. 0.22µm filter units: [Axygen] single- use, syringe driven.

6. Microscope slides: [GC-1 microslides, Blue label Scientific Pvt. Ltd.] frosted end, 75×25 mm (± 0.05 mm), and 1.3 mm thick, cleaned with chromic acid treatment.

7. Cover slips: [HIMEDIA] Microscope cover slips, 24×60 mm.

8. Filter papers [Whatman]

9. Microscope: [LABOMED VISION 2000 or Nikon Eclipse 600] bright field microscope.

10. Water Bath: [Serological bath] for maintaining hypotonic solution at 37^{o}C.

1.3 REAGENTS SETUP:

1. RPMI-1640 culture medium: pre supplemented with L-Alanyl-L-Glutamine, HEPES buffer, 60 mg per litre Penicillin, 100 mg per litre Streptomycin, 15% FBS and Sodium bicarbonate.

2. Phytohemagglutinin (PHA): Dissolve 25 mg in 25 ml double distilled water. Its aliquots were prepared at a final concentration of 1mg/ml using 0.22µm filter.

3. Cytochalasin-B: 5 mg of solid was dissolved in 8.33 ml DMSO to give a Cyt-B solution concentration of 600µg/ml as follows:

- Cyt-B vial was removed from -20^{o}C and allowed to reach room temperature. Top of the rubber seal was sterilized with ethanol.
- 8.33 ml of DMSO was pipette into a 50 ml sterile falcon tube. Using a 5 ml sterile syringe and another needle, 4 ml of 8.3 ml DMSO was injected into the vial through the seal using 0.22µm sterile filter.
- 4 ml was removed from the vial and ejected into another sterile 15 ml tube labeled as '1'.
- Remaining 4.3 ml of DMSO was aspirated as before into the vial and again ejected into the sterile tube labeled as '1'.
- All the contents were mixed properly and 500µl was dispensed into sterile 1.5 ml eppendorfs. Aliquots were then stored at -20 ^{o}C.

4. Hypotonic solution: To the 100 ml of distilled water 0.56 g of KCl was added and mixed well to prepare 0.56 % KCl.

5. Fixative: Methanol and acetic acid were mixed in the ratio of 3:1 to prepare fixative.

6. 10% Giemsa Stain: 5.0 ml Giemsa stock solution was added to Sorenson's buffer (pH 7.0) and stored in coupling jar.

2. Methods:

A. Sampling and Blood collection:

5 unexposed, healthy subjects (3 women and 2 men), 40-60 years of age donated blood for control studies and 5 breast cancer patients (40-60 years) on remission with history of cancer therapy donated blood for the subject study. The cancer patients had received chemotherapy that included combination of drugs FAC (5-Fluorouracil, Adriamycin, Cyclophosphamide) or FEC (5-Fluorouracil, Epirubicin, Cyclophosphamide) followed by Paclitaxel.

3 ml of blood was collected using venipuncture taking proper aseptic conditions in sterile heparinised vacutainer and mixed gently to avoid clotting, which was used to set up cultures in sterile conditions.

B. Culture Procedure:

Day 1 (0 hour):

Complete RPMI-1640 culture tubes were taken and PHA was added at a final concentration of 30µl/ml. To each of the culture tubes, 1 ml of whole blood was added. Duplicate cultures were set up for each subject and control. Culture tubes were allowed to incubate for 44 hrs at 37^{o}C in BOD/ CO_2 incubator.

Day 3 (at 44 hours):

After 44 hrs of setting up of blood culture, 90 µl of cytochalasin-B (6µg/ml) was added to arrest cytokinesis in each tube. Culture tubes were again allowed to incubate for 24-28 hrs at 37^{o}C in BOD/ CO_2 incubator.

C. Harvesting of culture:

Day4 (at 72 hours):

The culture tubes were centrifuged at 1000 rpm for 10 min after 72 hrs of culture setup. The supernatant medium was discarded. The cells in the pellet were suspended in 5 ml of pre-warmed 37^{o}C hypotonic solution for 1-2 min. Approximately 1 ml of fixative was added to stop the hypotonic treatment and was stored at 4^{o}C for 2 hrs.

D. Washing:

Culture tubes were centrifuged at 1000rpm for 10 min after 2 hrs. Supernatant was discarded and the cells in the pellet (brown-black in color) were re-suspended in fresh pre-chilled fixative. Fixative wash at an interval of 10 min were given (until clear pellet obtained). After the final change of supernatant was discarded and small amount of fixative was added to the pellet to adjust the cell concentration.

E. Slide Preparation:

Two to three drops of cell suspension were dropped on grease free pre-chilled cleaned slides and were allowed to dry.

F. Staining:

Slides were stained in 10% Giemsa stain for 15 min. They were thoroughly rinsed in distilled water and allowed to dry. The slides were then mounted in DPX and observed under microscope.

G. Scoring for Micronuclei:

Criteria for selecting binucleated cells which can be scored for micronucleus frequency:

1. Cells should be binucleated.

2 The two nuclei in binucleated cell should have intact nuclear membranes and situated within the cytoplasmic boundary.

3. The two nuclei in a binucleated cell should be approximately equal in size, staining pattern and staining intensity.

4. The two nuclei within a binucleated cell may be unconnected or may be attached by one or more fine nucleoplasmic bridges, which are not wider than $1/4^{th}$ of the nuclear diameter.

5. The two main nuclei in binucleated cell may touch but should not ideally overlap each other. A cell with two overlapping nuclei can be scored only if the nuclear boundaries of either nucleus are distinguishable.

6. The cytoplasmic boundary or membrane of binucleated cell should be intact and clearly distinguishable from the cytoplasmic boundaries of adjacent cells.

Criteria for scoring Micronuclei:

1. Micronuclei should be morphologically identical and smaller than the main nuclei.

2. The diameter of micronuclei in human lymphocytes usually varies between $1/16^{th}$ and$1/3^{rd}$ of the mean diameter of the main nuclei.

3. Micronuclei should be scored in binucleated cells with well preserved cytoplasm.

4. It must show no refractility in contrast with nuclear particles.

5. Micronuclei should not be linked or connected to the main nuclei. It may touch but should not overlap the main nuclei.

6. Ideally micronuclei should have the same staining intensity as the main nuclei but occasionally may be more intense.

H. Nuclear Division Index Calculation:

Nuclear Division Index was calculated using following formula:

NDI= (MI+2MII+3MIII+4MIV) /N

where MI to MIV represents the number of cells with one to four MNi and N represents the total number of cells scored.

III. RESULTS

AND

DISCUSSION

1. Results:

1.1 Nuclear Division Index (NDI): NDI was calculated according to the method of Eastmond and Tucker (Eastmond and Tucker, 1989). To determine the frequency of viable cells with 1, 2, 3 or 4 nuclei, 500 cells were scored and NDI was measured using the following formula:

NDI= (MI+2MII+3MIII+4MIV) /N

where MI to MIV represents the number of cells with one to four MNi and N represents the total number of cells scored.

Duplicate cultures were set for each individual and for each culture 500 cells were scored in each slide. Mean± SD was calculated for all types of cells viz. mononucleated, binucleated and multinucleated cells as shown in Table 1 and 2. To calculate Average NDI for each sample and control, the above mentioned formula was used for each slide per individual, average was calculated. The graphs were plotted for Average NDI per individual for controls and patients as shown in Fig. 5 and Fig. 6 respectively.

Table 1: Table showing score sheet for Controls

Control A						
	Slide 1	Slide 2	Slide 3	Slide 4	MEAN	STDEV
MN cells	187	183	162	166	174.5	12.34
BN cells	270	276	300	296	285.5	14.73
Multi Nu	43	41	38	36	39.5	3.11
N D I	1.75	1.75	1.83	1.77	1.77	0.03
Control B						
MN cells	257	251	248	241	249.2	6.65
BN cells	232	235	243	248	239.5	7.32
Multi Nu	11	14	9	11	11.2	2.06
N D I	1.51	1.54	1.53	1.55	1.5	0.02
Control C						
MN cells	278	273	255	254	265	12.3
BN cells	216	218	233	238	226.2	10.9
Multi Nu	9	11	8	9	9.2	1.25
N D I	1.48	1.49	1.49	1.52	1.49	0.01
Control D						
MN cells	397	391	481	470	434.7	47.33
BN cells	92	99	18	25	58.5	42.91
Multi Nu	11	10	1	5	6.7	4.64
N D I	1.23	1.24	1.04	1.07	1.14	0.10
Control E						
MN cells	155	126	161	157	149.7	16.02
BN cells	270	267	254	280	267.7	10.71
Multi Nu	75	107	80	93	88.7	14.33
N D I	1.94	2.07	1.89	2.09	1.9	0.09

Table 2: Table showing score sheet for Patients

Patient A						
	Slide 1	Slide 2	Slide 3	Slide 4	MEAN	STDEV
MN cells	407	403	312	315	359.2	52.86
BN cells	90	96	185	183	138.5	52.6
Multi Nu	3	1	3	2	2.2	0.95
N D I	1.18	1.19	1.38	1.37	1.28	0.10
Patient B						
MN cells	383	379	335	331	357	27.8
BN cells	112	117	160	166	138.7	28.18
Multi Nu	5	4	5	3	4.25	0.95
N D I	1.25	1.25	1.34	1.34	1.3	0.05
Patient C						
MN cells	265	260	278	269	268	7.61
BN cells	226	228	218	226	224.5	4.43
Multi Nu	9	6	4	5	6	2.16
N D I	1.49	1.45	1.45	1.49	1.4	0.02
Patient D						
MN cells	144	139	213	215	177.75	41.91
BN cells	300	309	263	265	284.25	23.68
Multi Nu	56	52	24	20	38	18.61
N D I	1.76	1.88	1.64	1.63	1.72	0.11
Pateint E						
MN cells	244	247	267	263	255.2	11.44
BN cells	256	234	201	209	225	24.99
Multi Nu	13	19	32	28	23	8.60
N D I	1.6	1.56	1.57	1.55	1.57	0.021

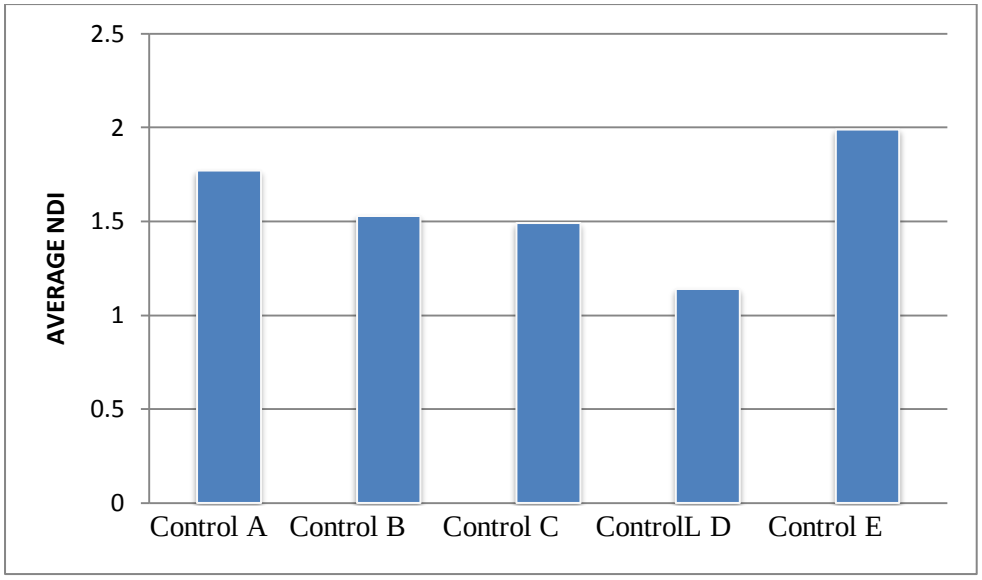


Fig 5: Graph showing Average NDI for each control.

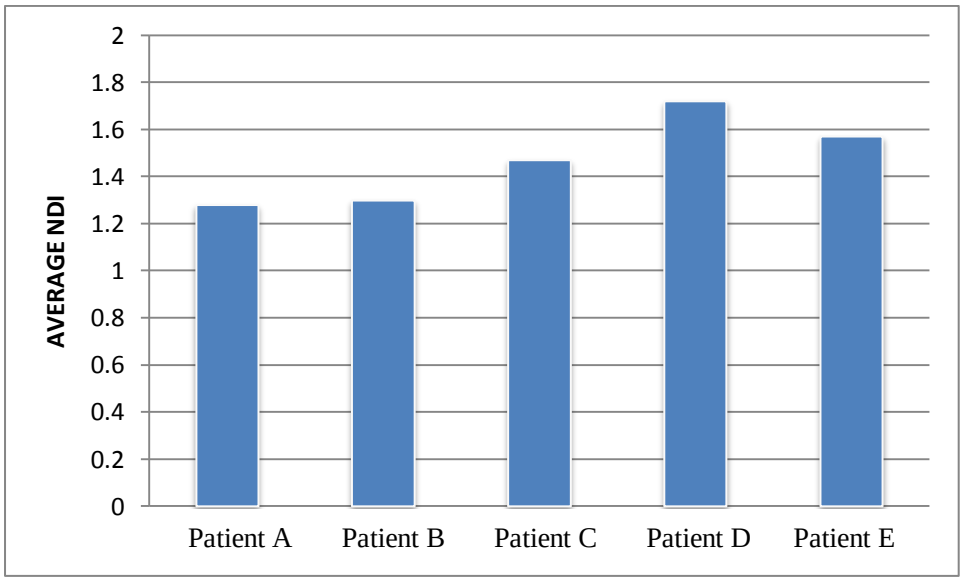


Fig 6: Graph showing average NDI for each Patients.

Individual values of the NDI in the peripheral blood lymphocytes of exposed group were in the range from 1.28 to 1.72.

The percentage reduction on NDI was calculated and it was found to be 3.9% in exposed group as compared to controls.

1.2. Micronuclei Scoring: Total 2000 BN cells were scored for estimation of MNi frequency per individual. MNi formation was observed in both BN cells and multinucleated cells but the number is very low. In comparison to BN cells, MNi frequency was found to be more in multinucleated cells. Further, the frequency of mononucleated cells were more in comparison to BN and multinucleated cells which contributed to lower frequencies of BN cells with MNi.

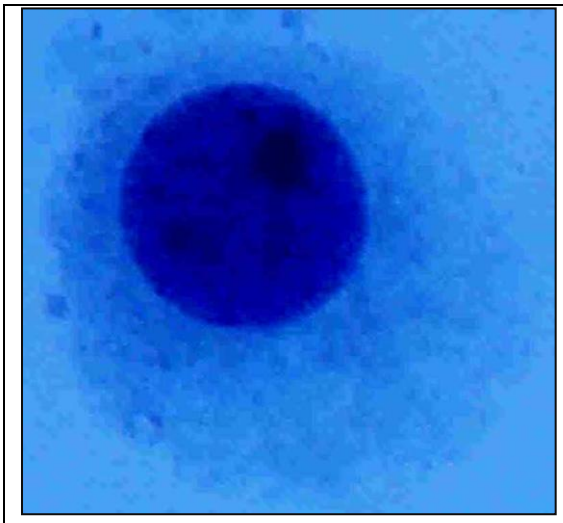
There was no significant difference between the samples and controls as there was no significant number of binucleated cells carrying the micronuclei.

Table 3: MNi scoring sheet for Controls

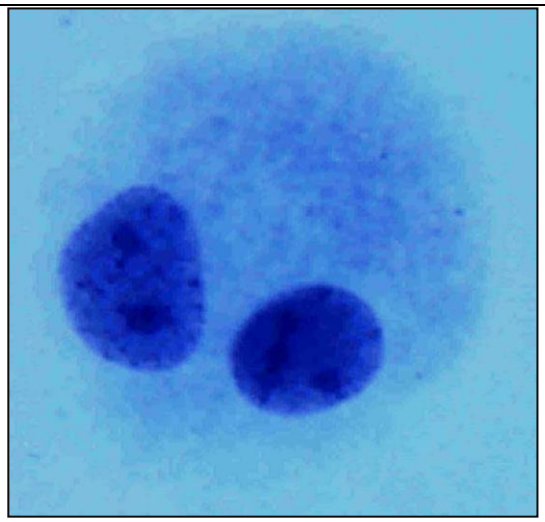
Controls	Total No. of BN cells	BN cells with MNi	Multinucleated cells with MNi	Multinucleated cells without MNi
A	2000	2	3	155
B	2000	-	-	45
C	2000	-	2	35
D	2000	-	-	17
E	2000	3	5	350

Table 4: MNi scoring sheet for Patients

Samples	Total No. of BN cells	BN cells with MNi	Multinucleated cells with MNi	Multinucleated cells without MNi
A	2000	1	-	9
B	2000	-	1	16
C	2000	1	3	21
D	2000	2	4	148
E	2000	1	3	89



A



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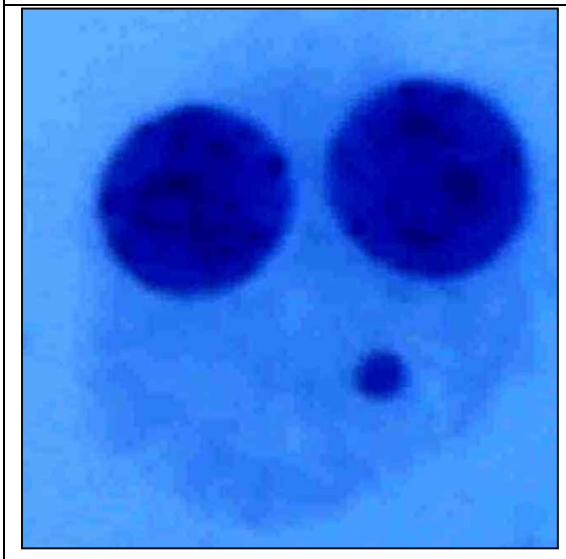
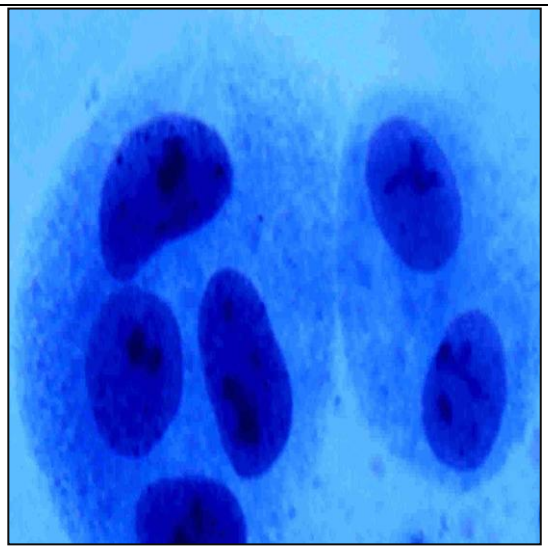
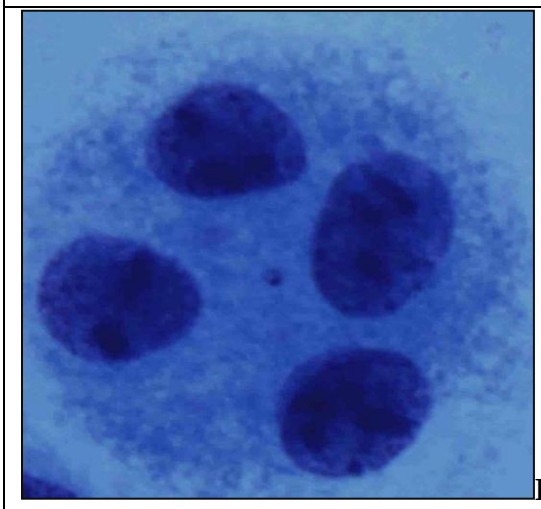


C



D



E

Fig. 7: Representative images of;

A: A mononucleated cell

B: A binucleated cell

C: A binucleated cell with a micronucleus

D: A multinucleated and a binucleated cell

E: A multinucleated cell with a micronucleus

2. Discussion:

The present study involving the breast cancer patients earlier exposed to chemotherapy and currently on remission did not show significant rise in baseline MNi frequency in binucleated cells *in vitro* compared to the controls. Although MNi formation was observed in both the cases, it was more in multinucleated cells, which indicates that the observed MNi induction was due to the *in vitro* culture conditions and factors which affects the baseline frequency of MNi like age, gender, diet and other lifestyle factors (Fenech and Bonassi, 2011). Further, the mode of action of therapy may be such that the damage is not persistent or has got repaired before going for division in *in-vitro* cell culture. In terms of its effect on the cell proliferation status, no significant difference was found between the controls and samples, as only 3.9% reduction in NDI was observed in exposed group.

Different combinations of drugs were used in the treatment. Chemotherapy given was either FAC or FEC following *Paclitaxel*. Mechanism and mode of action is as follows:

- **FAC Chemotherapy:** Here the drugs used to treat node-negative as well as node-positive breast cancer are a combination of three drugs: *Adriamycin* or *doxorubicin, 5-fluorouracil (5FU)* and *cyclophosphamide.*
- **FEC Chemotherapy:** Here the drugs used are same as that of FAC but in place of *Adriamycin*, *Epirubicin* is used.

2.1 Mode of Action of drugs used:

1. ***Fluorouracil/5-Fluorouracil/5-FU:*** It is a fluorinated pyrimidine antimetabolite that is metabolized intracellularly to its active form, fluorouridine monophosphate (FdUMP). The active form inhibits DNA synthesis by inhibiting thymidylate synthetase and the normal production of thymidine. Effects on RNA (incorporation into RNA and RNA inhibition) occur especially with bolus administration. Fluorouracil is cell cycle phase-specific (S-phase).

2. ***Doxorubicin/14-hydroxydaunorubicin:*** Its trade name is *Adriamycin. Daunorubicin* and its 14-hydroxy derivative, *doxorubicin*, are anthracycline antibiotics. Doxorubicin damages DNA by intercalation of the anthracycline portion, metal ion chelation, or by generation of free radicals. Doxorubicin has also been shown to inhibit DNA topoisomerase II which is critical to DNA function. Cytotoxic activity is cell cycle phase non-specific.

3. ***Cyclophosphamide:*** Cyclophosphamide is an inactive cyclic phosphamide ester of mechlorethamine. It is transformed via hepatic and intracellular enzymes to active alkylating metabolites, 4-hydroxycyclophophosphamide, aldophosphamide, acrolein and phosphoramide mustard. Cyclophosphamide causes prevention of cell division primarily by cross-linking DNA and RNA strands. It is considered to be cell cycle phase-nonspecific.

4. ***Epirubicin/ 4'-epi-doxorubicin:*** It is a stereoisomer of *doxorubicin* in which the hydroxyl group in the C-4' position of the amino sugar is epimerized. Like other anthracyclines, the precise mechanism of action of *epirubicin* is unknown, but is primarily related to intercalation of the planar ring with DNA and subsequent inhibition of DNA and RNA synthesis. *Epirubicin* appears to have less cardiotoxicity as compared to *doxorubicin*. It is cell cycle phase-nonspecific.

5. ***Paclitaxel:*** Unlike other antimicrotubule agents in clinical use (e.g., vincristine, colchicine) that inhibit mitotic spindle formation, paclitaxel promotes assembly of microtubules and stabilizes them against depolymerization. It also inhibits cell replication by blocking cells in the late G2 and/or M phases of the cell cycle.

6. ***Docetaxel:*** It acts by disrupting the microtubular network in cells that is essential for cell division. It promotes the assembly of tubulin into stable microtubules, while simultaneously inhibiting their disassembly. This leads to the production of microtubule bundles without normal function and to the stabilisation of microtubules, resulting in the inhibition of mitosis in cells.

Table 5: The chemotherapeutic agents administered for the treatment of breast cancer to the patients enrolled in the current study

Patient No.	Treatment	Drugs Used	Mode of Action
A.	Adjuvant Chemotherapy	(i) 5-Fluorouracil (ii) Doxorubicin (iii) Cyclophosphamide	(i) Antimetabolite (ii) Anti tumor antibotics (iii) Alkylating agent
B.	Adjuvant Chemotherapy	(i) 5-Fluorouracil (ii) Doxorubicin (iii) Cyclophosphamide	(i) Antimetabolite (ii) Anti tumor antibotics (iii) Alkylating agent
C.	Chemotherapy and Paclitaxel	(i) 5-Fluorouracil (ii) Epirubicin (iii) Cyclophosphamide (iv) Paciltaxel	(i) Antimetabolite (ii) Anti tumor antibotic (iii) Alkylating agent (iv) Mitotic Inhibitor
D.	Adjuvant Chemotherapy	(i) 5-Fluorouracil (ii) Doxorubicin (iii) Cyclophosphamide (iv) Epirubicin	(i) Antimetabolite (ii) Anti tumor antibotics (iii) Alkylating agent (iv) Anti-tumor antibiotic
E.	Adjuvant Chemotherapy	(i) 5-Fluorouracil (ii) Epirubicin (iii) Cyclophosphamide	(i) Antimetabolite (ii) Anti tumor antibotic (iii) Alkylating agent

Table 6: The genotoxic effect of chemotherapeutic drugs administered to the breast cancer patients enrolled in the current study

S. No.	Drug Name	Mode of Action	MNi inducibility	Chromosome breakage inducibility	Sister Chromatid Exchange
(i)	5-Fluorouracil	Inhibits DNA synthesis by inhibiting Thymidylate synthetase	Yes	Yes	Yes
(ii)	Doxorubicin	damages DNA by intercalation of the anthracycline portion, metal ion chelation, or by generation of free radicals and also inhibit DNA topoisomerase II	Yes	Yes	Yes
(iii)	Epirubicin	intercalation of the planar ring with DNA and subsequent inhibition of DNA and RNA synthesis	Yes	Yes	Yes
(iv)	Cyclo-phosphamide	causes prevention of cell division primarily by cross-linking DNA and RNA strands	Yes	Yes (but only in presence of metabolic activation system)	Yes (but only in presence of metabolic activation system)
(v)	Paclitaxel	promotes assembly of microtubules and stabilizes them against de-polymerization, also inhibits cell replication by blocking cells in the late G2 and/or M phases of the cell cycle.	Yes	Yes	Yes
(vi)	Docetaxel	Disrupts the microtubular network in cells that is essential for cell division. It promotes the assembly of tubulin into stable microtubules, while simultaneously inhibiting their disassembly. This leads to the production of microtubule bundles without normal function and to the stabilisation of microtubules, resulting in the inhibition of mitosis	Yes	Yes	Yes

Among all these drugs, *paclitaxel* and *docetaxel* act as aneugens (D. Cavallo et al., 2007). *Epirubicin* induces various types of chromosome aberrations (like breaks, gaps, deletions, and fragments) as well as numerical aberrations in the form of hypodiploidy or hyperdiploidy (O. El-Mahdy Sayed Othman, 2000). *Adriamycin, 5-fluorouracil*, and *cyclophosphamide* are known to cause increased SCE frequency (Tucker et al., 1990).

Earlier assessment the MNi induction in human lymphocytes following exposure to a variety of genotoxins revealed that the extent of micronucleus formation in relation to cytotoxicity was low for chemicals and ultraviolet radiation which mainly induce base-lesions and adducts on DNA rather than strand breakage or spindle damage (Fenech.M, 1985; Fenech and Neville, 1992).

Cell cycle regulator p53 when triggered by physical or chemical mutagens or other cellular stress factors results in different fates of cell depending on the cell cycle phase, and the type of lesion. Major alternatives of escaping the damage are toleration of DNA damage, generation of an abnormal base sequence for facilitating cell survival, cell cycle arrest to allow DNA repair, apoptosis/necrosis. Binding of toxic substances to non-DNA targets may induce apoptosis, necrosis, or mitotic slippage. These responses of cells to environmental factors can significantly modify the frequencies of cells which undergo mitosis at a given time in culture and therefore the frequencies of BN cells at harvest.

2.2 Various possible mechanisms are responsible for the escape of genetic damage.

1. *Division delay for repair and mitotic block*: DNA repair can be defined in a general sense as a range of cellular responses associated with restoration of the genetic instructions as provided by the normal primary DNA sequence (Lindahl and Wood, 1999). Cell cycle regulation is closely coupled with DNA damage responses (Yu *et al.*, 1999).

Cells containing damaged genomic DNA are arrested at the G_1/S and G_2/M transitions, so as to gain time for repair and to avoid fixing mutations during replication and cell division. DNA damage induces p53 which plays a central role in:

(1) Arresting cells in G_1.

(2) Arresting cells in G_2 in an indirect and in an unknown way.

(3) Induction of DNA repair, and

(4) Apoptosis.

High level expression of some repair enzymes can delay growth in G_1 phase and stop the cells from progressing into S phase (Dosanjh et al., 1994). These observations demonstrate the close relationship between DNA repair and cell cycle regulation.

Cell cycle block can also be induced by mutagens/aneugens which have non DNA targets, e.g. spindle poisons. Therefore repair and apoptotic processes are likely to influence both the expression of MNi *in vivo* during in vivo nuclear division and MNi expression *in vitro*. It is this expected division delay in cells with damaged DNA which is likely to cause MNBN cells to appear at a later time in culture then the non damaged cells. It has been observed that MNi frequency in BN cells increased steadily between 68 and 76 hrs, with the plateau level thereafter up to 96 hrs (Scott *et al*., 1998). These studies suggest that harvesting BN cells at a later time than the standard 72 hrs may better ensure complete nuclear division of all DNA damaged cells before harvest.

2. *In vitro apoptosis and necrosis of damaged cells*: The genotoxic events which may induce apoptosis or necrosis *ex vivo/ in vitro* may include DNA adducts, DNA breaks and/or protein adducts which accumulated during the *in vivo* exposure and/or to genotoxins present in the donor serum (if whole blood cultures are used for biomonitoring) and therefore in the culture media. The events like necrosis and apoptosis may be expected to be triggered either directly after the start of *in vitro* cultivation or that the lymphocyte requires stimulation/cycling to respond to necrotic/apoptotic stimuli. The latter case is more probable, otherwise the accumulated adducts could have induced cell death earlier *in vivo,* in resting G_0 cells. It is therefore clear that both apoptosis and necrosis can modify the number of cells which reach the first mitosis (on average 48 hrs after start of culture) and progress to the metaphase/anaphase transition to give rise to a BN cell in the presence of cyt-B.

3. *Mitotic slippage:* Even in the absence of mitotic spindle a consequent failure of chromatid migration to the poles, Metaphase Promoting Factor (MPF) can undergo spontaneous inactivation; this process is known as mitotic slippage which yields 4N cells. It has been concluded that some lymphocyte with a deficient microtubule apparatus pass mitosis without chromatid segregation to daughter nuclei do not undergo cytokinesis and therefore remain mononucleated cells even in the presence of cyt-B (Fenech and Volders., 2001) exposure to spindle fibers thus may induce mitotic slippage and contribute to lower frequencies of MNBN.

4. *Chronic low level exposure and adaptive response*: Adaptive responses are observed when cells become resistant to a high dose of cytotoxic agent after low dose exposure to that agent or another genotoxic agent. The induction of an adaptive response in human lymphocytes from workers occupationally exposed to mutagens has been observed for several genotoxic end points, including chromosome aberrations (Barquinero *et al*., 1995) and MNi formation (Gourabi and Mozdarani, 1998), and seems to depend on the nature of the challenge, the total dose of the pretreatment and the dose rate for ionizing radiation (Shadley and Wiencke, 1989)

5. *Some exposure may produce mainly adducts and few strand breaks:* It has been observed that lymphocytes exposed to genotoxins in G_0 mainly induce DNA adducts and not strand breaks and thus do not efficiently induce MNi in once divided cells (Fenech and Volders, 2001).

IV. SUMMARY

SUMMARY:

Genotoxicity is the study of the adverse effects of physical and chemical agents on the genetic material of cell (DNA or Chromosome) and the subsequent expression of these changes. Genotoxicity testing involves the study of those compounds which are either mutagenic or clastogenic. The knowledge that many environmental agents are associated with human cancer development and that the genetic alterations are the basis for neoplasia underscored the need for testing genotoxic potential of chemicals. Many short term assays have been developed as a result. Cytokinesis blocked micronuclei (CBMN) assay is among the various short term cytogenetic assays used to evaluate the genetic risk caused by known or unknown chemical agents. In this assay, Micronuclei (MNi) induction is used as an endpoint for genotoxicity. Earlier, the assay was performed using mouse bone marrow. However, the trend for avoiding animal use for *in vivo* testing has created the requirement and opportunity to further develop an alternative *in vitro* system. Hence, in 1985 M. Fenech put forward a modified form of the assay i.e. CBMN whereby peripheral blood lymphocytes is used as a surrogate tissue and cells were allowed to block at cytokinesis stage of cell cycle to monitor the *in vitro* cell division. MNi are small, round, DNA/ chromatin containing interphase structures occasionally found in the cytoplasm of cells. They are morphologically similar to but smaller than the main nuclei. The two basic phenomena leading to the formation of MNi in mitotic cells are chromosome breakage and dysfunction of the mitotic apparatus.

MNi frequency in PBL is a predictive biomarker of cancer risk within a population of healthy subjects (Bonassi *et al*., 2006). Increased frequencies of MNi can only be expected if lymphocytes with persistent damage are obtained and cultured, if damage is not removed during culture before the lymphocytes start to proliferate it is fixed as MNi during cell division. If damage is efficiently repaired, increased MNi frequencies cannot be expected (N.S. Arsoy *et al*., 2009). The retrospective study of exposed group with respect to *in vitro* frequency of MNi in short term cultured blood lymphocytes can give an idea regarding the long term persistence genetic damage as well as recurrence of second cancer.

The present study involving the breast cancer patients that are earlier exposed to chemotherapy and currently on remission did not show significant rise in baseline

MNi frequency in binucleated cells *in vitro* as compared to the controls. Although MNi formation was observed in both the cases, it was more in multinucleated cells. The multinucleated cells are the ones that have undergone more than one cell cycle *in vitro* hence, it is more likely that the observed MNi induction may be due to the *in vitro* culture conditions aided by effects of other factors affecting the baseline frequency of MNi like age, gender, diet and other lifestyle factors (Fenech and Bonassi, 2011). Further, the mode of action of some of the chemotherapy agents is involving MNi induction, it is revealed from the present study that the damage is not persistent, i.e. has got repaired before going for division in *in-vitro* cell culture. In terms of its effect on the cell proliferation status, no significant difference was found between the controls and samples, as only 3.9% reduction in NDI was observed in exposed group. These findings need to be substantiated with a larger population and longer follow up grouped according to the therapy. It may be recommended that such a study can be undertaken including more parameters of genotoxicity assessment to cover possible other modes of action

V. REFERENCES

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