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“Genotoxicity: *In Vivo* Cytogenetic Assays”

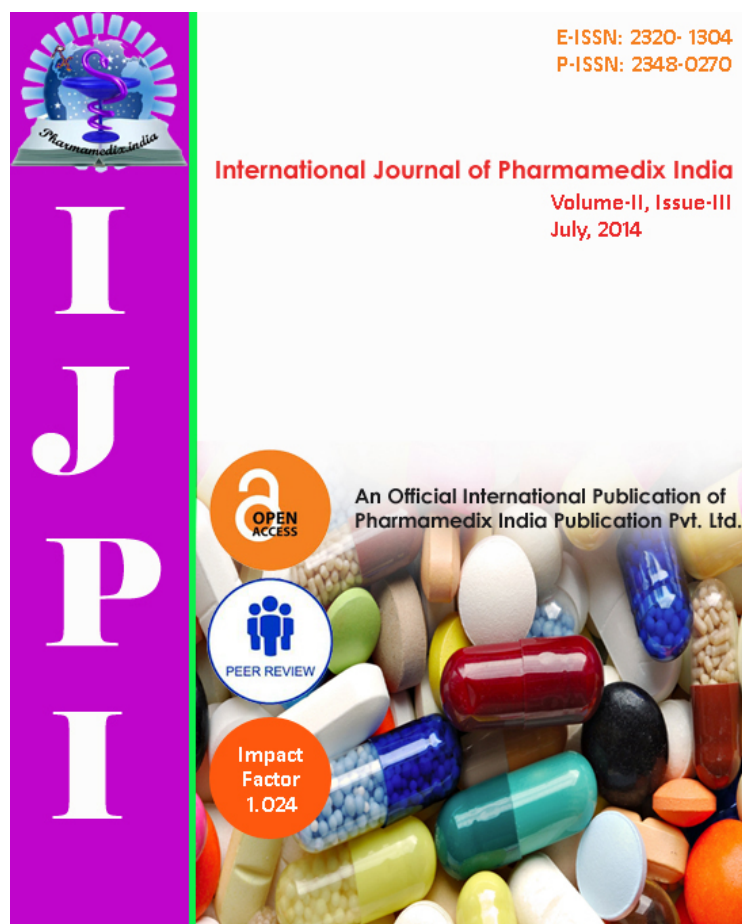
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Abstract:

The pre-clinical toxicity studies are generally conducted to obtain the basic toxicological profile of new chemical entities (NCE). The toxicological data are used to evaluate the safety and efficacy of NCE. One of the most important aspects of safety pharmacology is the evaluation of genotoxicity potential of the new chemical entities (NCE). Genotoxicity studies are conducted both at non-clinical and clinical levels. Regulatory authorities all over the world require data on the genotoxic potential of NCE as part of the safety evaluation process. Moreover, it is the requirement of OECD guidelines also that a drug molecule should be evaluated for the genotoxicity for its safety. For the evaluation of genotoxicity the bone marrow micronucleus test has been the most suitable test along with some other simultaneously used tests viz., chromosomal aberrations, comet assay and sperm morphology test. This battery of tests would ensure a 100% accurate genotoxicity testing. These assays 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. A 'negative' on these crucial tests takes the sample drug one step closer to the market.

Keyword: Genotoxicity, Chromosomal Aberration, Comet Assay, Sperm Morphology.

Introduction

Development of genetic toxicology began in the 1960s, in the midst of increasing awareness of human exposure to toxic chemicals in the environment. Early genetic toxicology studies were designed to detect reproductive toxicants. It was not until the 1970s that most of the current routines genetic toxicology tests were developed and test results were used for identification of carcinogens and risk characterization for cancer risk assessment.¹

Genotoxicity is a word in genetics defined as a destructive effect on a cell's genetic material(DNA,RNA) affecting its integrity.² Genotoxicity refers to interaction between various agents and DNA or the cellular machinery that regulates fidelity of the genome.³ The term "genotoxicity" refers either to a generic name regarding a chemical or a physical material's capability of inducing mutations (gene mutations, structural chromosome mutations, genome mutations) or indicator effects that have mechanistical relationship with the formation

of mutations (for example, inducing DNA modifications, DNA repair or recombination).⁴ Genotoxins are mutagens, they can cause mutations. Genotoxins include both radiation and chemical genotoxins. A substance that has the property of genotoxicity is known as a genotoxin.² A genotoxin is a chemical or agent that can cause DNA or chromosomal damage. Such damage in a germ cell has the potential to cause a heritable altered trait (germ line mutation). DNA damage in a somatic cell may result in a somatic mutation, which may lead to malignant transformation (cancer). Many in vitro and in vivo tests for genotoxicity have been developed that, with a range of endpoints, detect DNA damage.⁵

PURPOSE OF GENOTOXICITY STUDY

- To investigate the safety and efficacy of new chemical entities before their release into the market.
- To give quantitative estimate of the contribution of chemical agents to the incidence of genetic diseases and risk characterization for cancer.
- To detect the responsible for heritable effect on germ cells and impose risk to future generations.
- To detect the hazard identification with respect to DNA damage in the form of gene mutation, structural chromosomal aberration, recombination and numerical changes.⁶

GENOTOXICITY TESTING

Genotoxicity tests can be defined as *in-vitro* and *in-vivo* tests designed to detect compounds which induce genetic damage directly or indirectly by various mechanisms. These tests should enable the hazard identification with respect to damage to DNA and its fixation. Fixation of damage to DNA in the form of gene mutations, larger scale chromosomal damage, recombination and numerical chromosome changes is generally considered to be essential for heritable effects and in the multi-step process of malignancy, a complex process in which genetic changes may play only a part. Compounds which are positive in tests that detect such kinds of damage have the potential to be human carcinogens and/or mutagens, i.e. may induce cancer and/or heritable defects.⁷

Genotoxicity testing is an important part of the hazard assessment of chemicals for regulatory purposes.^{8,9}

It is undertaken for two main reasons.

- (a) To detect chemicals that might cause genetic damage including point mutations in germ cells and hence increase the burden of genetic disease in humans.
- (b) To detect chemicals that might be carcinogenic.¹⁰

CYTOGENETIC ASSAY

The need to include an *in vivo* cytogenetic assay in a battery of tests conducted to determine the genotoxicity of a compound has been clearly indicated by the large percentage (46%) of 'false positive' results obtained from *in vitro* cytogenetic assays.¹¹ Furthermore it has been recommended that all new *in vitro* genotoxins should be evaluated in the *in vivo* mouse bone marrow micronucleus test in an attempt to ascertain the relevance of the *in vitro* findings to carcinogenicity in mammals.¹²

PURPOSE OF GENOTOXICITY TEST ASSAYS

- Assays allow detection of a drug's potential for Genotoxicity early in drug development.
- Assays are inexpensive, have high statistical power, are generally reproducible and detect a variety of genotoxic end-points.
- Assays designed to be more sensitive to damage in order to enhance hazard identification.¹³

MICRONUCLEUS ASSAY IN BONE MARROW

The evaluation of micronuclei frequency *in vivo* is one of the primary genotoxicity test recommended internationally by regulatory agencies for product safety assessment.¹⁴ 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. The assay detects damage to the chromosomes or the mitotic apparatus of immature red blood cells found in the bone marrow. During cell division, undamaged chromosomes give rise to normal daughter nuclei, if chromosomes are broken or the mitotic apparatus of the cell is damaged, chromosomes or chromosome fragments may be incorporated in a second nucleus, instead of the main nucleus called micronucleus.¹⁵

In the adult rodent, the bone marrow is the hemopoietic organ. During proliferation, if a given genotoxic/ clastogenic agent is administered it may act during the cell division and cause chromosomal damage, such as breaks. Other agents may act on macromolecules, eg the tubulin, causing spindle dysfunction and these are called aneuploidy inducers.¹⁶ A fragment or a whole chromosome may lag behind during cell division and may not become integrated into the daughter nuclei, forming micronuclei, which can be seen in the cytoplasm. During maturation, when an erythroblast develops into polychromatic erythrocyte (PCE) or reticulocyte, the main nucleus is extruded, and micronucleus that has been formed as a result of chromosomal breakage may remain behind in the enucleated cytoplasm.

Visualization of micronuclei is possible in these cells because they lack a main nucleus.¹⁷

Micronuclei are acentric chromosomal fragments or whole chromosomes left behind during mitotic cellular division, appearing in the cytoplasm of interphase cells as small additional nuclei. The detection of micronuclei, manual or, as more recently described, automated and provides a readily measurable index of chromosome breakage and loss.¹⁸ Decrease in the percentage of PCEs and increase percentage of MNPCs and MNNCEs are the indicators of the chromosomal aberrations and these damages implicate as the major cause for the appearance of nuclei at the last stage of mitosis and as an indicator of genotoxic insult to the nuclei.¹⁹

BONE MARROW CHROMOSOME ASSAY

The mammalian *in vivo* chromosome aberration test is used for the detection of structural chromosome aberrations induced by test compounds in bone marrow cells of animals, usually rodents. Structural chromosome aberrations may be of two types, chromosome or chromatid. An increase in polyploidy may indicate that a chemical has the potential to induce numerical aberrations. With the majority of chemical mutagens, induced aberrations are of the chromatid-type, but chromosome-type aberrations also occur.²⁰

Chromosomal aberrations are the changes in chromosome Structure resulting from the breakage or an exchange of Chromosome material. Most of the chromosomal aberrations are lethal, but there are many corresponding aberrations that are viable and can cause genetic effects either somatic or inherited. High frequency of chromosomal aberrations is the indication of the possibility of the carcinogenesis.²¹

COMET ASSAY

The comet assay, also called the single cell gel electrophoresis assay, is a rapid and sensitive method for the detection of DNA damage in individual cells, induced by a variety of genotoxic agents. The assay is based on the embedding of cells in agarose, their lyses in alkaline buffer and finally subjection to an electric current. The resulting images named for their appearance as comets, are measured to determine the extent of DNA damage.²²

A single cell suspension is necessary for comet assay. For this reason, peripheral blood leukocytes, which are easily available, are quite suitable for operation. However, any animal or plant tissue that can be rendered into a single cell suspension can be used. The cells embedded in agarose gel are layered on a microscope slide. They are lysed in detergents with high salt concentrations and liberated DNA electrophoresed. Since

DNA is negatively charged, nucleoids with damage display migration of DNA towards anode. The slides are stained with a fluorescent dye, like ethidium bromide, and examined under a fluorescent microscope for quantification of DNA damage in each cell. Non-fluorescent staining technique for DNA, though somewhat cumbersome, is also available, which gives equally satisfactory results.²³

There are two major versions of comet assay. The first one is after Singh et al. (1988) who used alkaline lysing solution (pH~10) and alkaline electrophoresis buffer (pH>13). This version is capable of detecting single- strand breaks and alkali-labile lesions in the DNA of individual cells. This technique was called single cell gel (SCG) assay by Singh et al. (1998), although commonly referred to as comet assay. The second version is after Olive and her co workers who used mildly alkaline (pH 8) lysis followed either by electrophoresis at neutral (Olive et al. 1990a) or alkaline (pH 12.3) conditions (Olive et al. 1990b) to detect double or single strand breaks respectively. However, based on the respective ability of these two versions to detect DNA damage induced by ionizing radiation, the technique of Singh et al. (1998) is at least an order or two of magnitude more sensitive.²⁴

The concept of micro gel electrophoresis was first introduced in 1984 by Ostling and Johanson as a method to measure DNA single-strand breaks that caused relaxation of DNA supercoils. A modified version was published by Singh and colleagues in 1988, which used alkaline conditions. The idea was to combine DNA gel electrophoresis with fluorescence microscopy to visualize migration of DNA strands from individual agarose-embedded cells. If the negatively charged DNA contained breaks, DNA supercoils were relaxed and broken ends were able to migrate toward the anode during a brief electrophoresis. If the DNA was undamaged, the lack of free ends and large size of the fragments prevented migration. Determination of the relative amount of DNA that migrated provided a simple way to measure the number of DNA breaks in an individual cell. In 1990, a modification of the original method of Ostling and Johanson was introduced and named the "comet assay" after the appearance of the DNA from an individual cell. The comet head containing the high-molecular-weight DNA and the comet tail containing the leading ends of migrating fragments were measured in real time from digitized images using software developed for this purpose. Tail moment, a measure of both amount of DNA in the tail and distribution of DNA in the tail, became a

common descriptor along with tail length and percentage of DNA in the tail.²⁵

Olive Tail Moment (OTM), given by Olive 1990. The OTM expressed in arbitrary units and calculated by multiplying the percentage of DNA (Fluorescence) in the tail by the length of the tail in micrometer. A major advantage of OTM as an index of DNA damage is that both the amount of damaged DNA and the distance of migration of genetic material in the tail are represented by a single number.¹²

ADVANGAGES AND LIMITATIONS OF COMET ASSAY

The assay has applications in testing novel chemicals for genotoxicity, monitoring environmental contamination with genotoxins, human biomonitoring and molecular epidemiology, and fundamental research in DNA damage and repair.²⁶

With all the advantages of comet assay mentioned above there are a few limitations also:

- The comet assay can determine whether an agent produces DNA strand breaks but cannot measure the fidelity of repair of those breaks.²⁷
- There is lack of specificity for DNA damage and the possible effect DNA repair has on detection sensitivity.²⁴

SPERM SHAPE ABNORMALITY ASSAY

Sperm is the reproductive cell of the male, which contains genetic information and participates in the act of fertilization of an ovum. Male infertility can result from abnormal spermatogenesis, sperm motility, erection, ejaculation or failure of sperm egg interactions, genetic abnormalities, excessive heat or electrostatic electricity around the testicles, lifestyle problems such as excessive alcohol, smoking, stress and anti-cancer chemotherapy.²⁸ Genotoxic substances are potential inductors of sperm cell morphology alterations because they could modify the normal process of gametogenesis and/or the synchronization of the stages in the seminiferous epithelium.²⁹ Various reasons are given for the induction of abnormal sperms in rodents. Perhaps they are the results of the naturally occurring errors in differentiation process, or the consequence of an abnormal chromosome complement.³⁰ The characteristics controlling the sperm head shape are carried on the autosomes and sperm abnormality test identifies those agents which cause small alterations in testicular DNA.³¹ Furthermore, sperm mutations are induced by exogenous compounds via point mutations³² or due to the inimical nature of the compound on spermatogenesis.²⁹

DRUGS/CHEMICALS USED AS POSITIVE CONTROL IN CYTOGENETIC ASSAYS

CYCLOPHOSPHAMIDE

An anticancer drug that is widely used in anti-neoplastic therapy as well as in the treatment of some non-malignant diseases, such as rheumatoid arthritis. It is also used as an immuno-suppressive agent prior to organ transplantation.³³ Metabolic conversion of CP leads to the formation of three cytotoxic metabolites 4-hydroxycyclophosphamide (4-OHCP), phosphoramidate mustard and acrolein. CP metabolites targets rapidly dividing cells, disturbing cell growth, mitotic activity, differentiation, and functions via alkylation of DNA at the N7 position of guanine.³⁴ However, despite its wide spectrum of clinical uses, CP also possesses a wide spectrum of cytotoxicity to normal cells in humans and experimental animals.³⁵ The acute toxicities of CP are associated primarily with its genotoxicity.³⁶ In many genotoxicity assay systems to evaluate chemical safety, CP is often used as a positive control.³⁷ It has been shown to produce micronuclei and chromosomal aberrations in rat bone marrow and spleen cells using in vivo and in vivo/in vitro methodologies.³⁸ The types of chromosome aberrations induced by CP as a positive control were reported to be chromosome breaks, chromatid breaks, chromatid exchange, chromosomal exchange and ring chromosomes.^{39,40} In somatic cells, CP has been shown to produce gene mutations,

chromosome aberrations, micronuclei and sister chromatid exchanges in a variety of cultured cells in the presence of metabolic activation as well as sister chromatid exchanges without metabolic activation.^{41, 42} It also produces chromosome damage and micronuclei in the erythropoietic system of rats, mice and Chinese hamsters via production of highly active carbonium ions.^{43,44}

METRONIDAZOLE

MTZ, (1-[2-hydroxyethyl]-2-methyl-5-nitroimidazole), an antiparasitic and antibacterial Compound, can induce alterations in somatic and germinal cells. It has been reported that after 6 weeks of treatment of rats with MTZ (400 mg/kg/day), decreases testicular weight, testicular and epididymal spermatozoon counts and causes abnormal spermatozoon morphology with degeneration of seminiferous tubules.⁴⁵ Abnormalities in the flagellum and the head as well as decrease in the number of motile spermatozoon have been reported in the morphological analysis after MTZ treatment.^{46,47}

Mercury

Mercury induces DNA single-strand breaks at low concentrations in mammalian cells. A dose-dependent increase in comet tail length in wistar rat leucocytes after oral treatment with HgCl₂ was reported. Being more harmful than inorganic mercury compounds, organic mercury compounds cause damage at even lower concentrations.^{48,45}

USAGE OF GENOTOXICITY TESTS

- In vivo and in vitro genotoxicity tests may be used to provide information regarding the efficiency of the compound in directly or indirectly inducing mutations and an alert about the probability of the compound's carcinogenicity.
- Reveal the molecular mechanism underlying the chemicals genotoxic and carcinogenic effects.
- Identify hazards in risk assessment with in molecular epidemiologic research with regards to occupational and environmental chemicals.
- Determine toxicity profiles of chemicals.
- Monitor the diseases and effectiveness of clinical treatments.
- Develop regulations concerning medical, cosmetic and industrial chemicals according to international and national guidelines.⁴⁹

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