In vitro assessment of genotoxicity of Zinc oxide nanoparticles; Engineered & conventional (*Bhasma*) in comparison with the bulk counterpart on human cultured peripheral blood lymphocytes in terms of Chromosomal Aberration assay & Cytokinesis Blocked Micronuclei assay

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ABSTRACT

Invention of nanoparticles has proved to be next to a miracle in terms of its potential benefits in various areas including industrial and biomedical applications. With the rapid expansion in the nanoparticle industry, it is essential that the safety of engineered nanoparticles must be understood. One of the area for regulatory health risk assessment is genotoxicology (the study of genetic aberrations following exposure to test agents), as DNA damage may initiate and promote carcinogenesis, or impact fertility. A considerable attention has been given to the toxicity of engineered nanomaterials, but the importance of their genotoxic potential on human health has been overlooked. The phenomenon taking place at nanoscale is likely to be different from those occurring at larger dimensions and may be exploited for beneficial aspects.The present study focuses on the abilities of ZnO engineered nanoparticles to damage or interact with DNA. Ethnomedicines have been in use for treatment since ancient times in India including *Bhasma* which is reported to be in nanometer dimension. The toxicity studies reveal that the *Bhasma* may not have any deleterious effect on the body if proper methodology of preparation and dosage supplementation is followed. The data of toxicity studies of *Bhasma* are very scarce, and assessment of genotoxic potential is yet to be done to the best of our knowledge. *Yasada* i.e. zinc has been an essential component of several important Ayurvedic preparations. It is being known by the name of *Yasada*, a Sanskrit word. Unfortunately some metals and minerals have the potential to produce adverse effects. Therefore, during transformation of metals and minerals to drugs, it is essential to evaluate the margin of safety between the dose level that produce the therapeutic effect, and that produce adverse effect. Thus, the aim of the study is: ***In vitro assessment* of genotoxicity of Zinc nanoparticles; Engineered & conventional (*Bhasma*) in comparison with bulk counterpart on human cultured peripheral blood lymphocytes in terms of Chromosomal Aberration assay (CA) & Cytokinesis Blocked Micronuclei assay(CBMN).** The two cytogenetic endpoints used for the present study are referred from OECD guideline 473 and 487 respectively. Results showed than ZnO engineered nanoparticles are clastogenic compared to control while Bhasma did not showed any significant chromosomal aberration compared to the same. The CBMN assay indicated that aneugenic activity is absent in all the three test compounds.

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List of abbreviation

µg: microgram

µm: micrometre

^{0}C: Degree Celsius

BN: Binucleated

CA: Chromosome Aberrations

CBMN: Cytokinesis Blocked Micronuclei Assay

CO_2: Carbon Dioxide

Cyt-B: Cytochalasin-B

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DPX: mixture of Distyrene, a plasticizer, and Xylene

EDTA: Ethylene diamine tetra acetic acid

ENM: Engineered Nanomaterial

ENP: Engineered Nanoparticles

FBS: Fetal Bovine Serum

Hrs: Hours

KCl: Potassium Chloride

mg: milligram

min: minutes

mM: milli molar

mm: millimetre

MMC: Mitomycin C

MN: Mononucleated

MNi: Micronuclei

MNT: Micronucleus Test

NDI: Nuclear Division Index

NP: Nanoparticle

OECD: Organisation for Economic Co-operation & Development

PBL: Peripheral Blood Lymphocytes

PBS: Phosphate buffer saline

PHA: Phytohemagglutinin

RBC: Red Blood Cells

RPM: rotation per minute

RPMI: Roswell Park Memorial Institute

SE: Standard Error

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1.

Introduction

The nanoparticles can be defined as the particles having diameter in the range of 1 to 100 nanometers (nm) (1). The engineered nanoparticles are materials designed and produced to have structural features with at least one dimension of 100 nm or less (2). ZnO is an inorganic compound that is usually in the form of white powder and is nearly insoluble in water; only 1.6 to 5 mg/L is dissolved. The powder has variety of applications; as an additive in products including plastics, ceramics, glass, cement, rubber (e.g. car tyres), lubricants, paints, ointments, adhesives, sealants, pigments, foods (source of Zn nutrient), batteries, ferrites and fire retardants.

The nanotechnology presents the possibility of revolutionizing many aspects of our lives. People in many settings (small and large industry) are either developing or using engineered nanomaterials (ENMs) or ENM-containing products (1). Currentestimates indicate that there are already over 800 consumer products containing nanomaterials (3). The global production of nanoparticles was over 230,000 tons in 2011 which is expected to increase to 350,000 tons in 2016 (4).

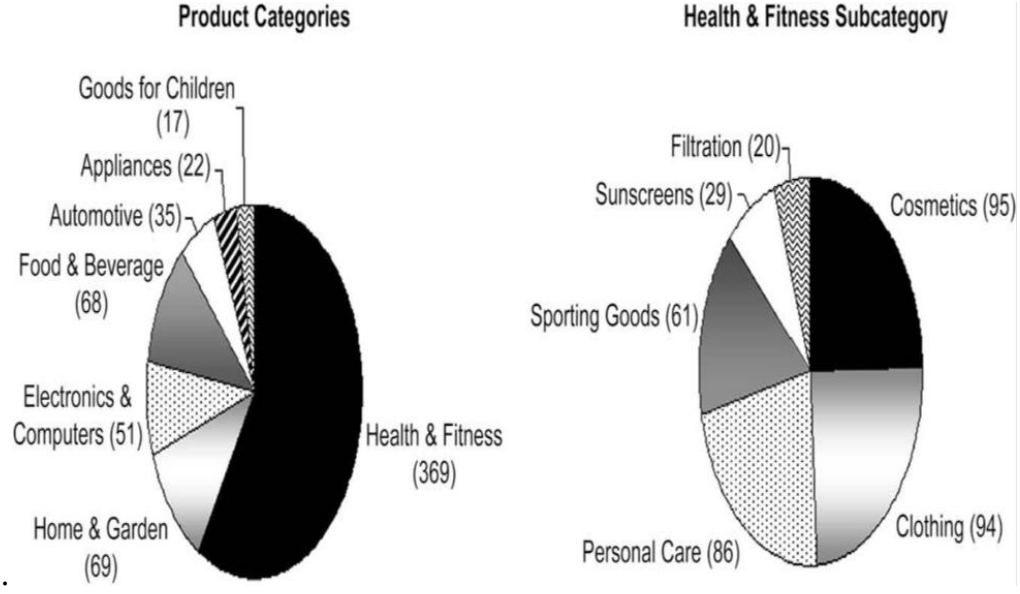

Fig 1.1: Consumer products currently available that contain nanomaterials. Adapted from the Woodrow Wilson Database in Jan 2009 (3)

This means that nanotechnology industry is growing substantially which promises benefits, not only commercially but also scientifically. The large increase in demand and production could lead to enormous exposure of humans and other organisms to engineered nanoparticles. Our understanding of the occupational health and safety aspects of ENMs is still in its formative stageand hasnot been sufficiently questioned. The concerns have been raised however and reports of various toxicity studies are now available. Globally various regulatory agencies have formed working groups to focus on safety evaluation of engineered nanoparticles in terms of human use, ecosystem, and biological systems at large.

Ethnomedicines have been in use for treatment since ancient times in India including *Bhasma* which is reported to be in nanometer dimensions. Metallic preparations are mainly termed as *Bhasma* and obtained by repeated incineration of metal with herbal extracts or juice (5). *Yasada* i.e. zinc has been an essential component of several important Ayurvedic preparations termed as *YasadaBhasma*. The toxicity studies reveal that the *Bhasma* may not have any deleterious effect on the body if proper methodology of preparation and dosage supplementation is followed (6). But the data of toxicity studies of *Bhasma* are very scarce, and assessment of genotoxic potential is yet to be done to the best of our knowledge. However lot of research has been carried out to provide additional evidence of its safety and efficacy profile, the quantity and quality of the safety and efficacy data on Ayurvedic medicines are far from sufficient to meet the criteria needed to support its use worldwide.

X-ray diffraction, TEM and particle size analysis revealed that *Bhasma* are in nanometer dimension, hence may be considered as nanomedicine (7). Therefore we have also included *Yasada Bhasma* in our genotoxicity study along with engineered ZnO nanoparticles.

The possible effect of nano form of zinc was studied using native i.e. non-nano or bulk form of zinc in order to see the effect of chemical as well as nano form.

The physical characterization of *Bhasma* and engineered nanoparticles was done as per the guidelines of ayurveda rasayanashstra to evaluate if both fulfill the criteria for *Bhasma*. In other words, if ENM and *Bhasma* both are in nano form, certain tests should be applicable to both. The

in vitro genotoxicity assessment of *Bhasma* has been carried out for the first time along with ENM.

The toxicity studies in human have to be extrapolated from animal models, which is also becoming harder due to regulatory reasons, extrapolation problems etc. The *in vitro* assays using short term cultures of human blood lymphocytes have been widely used and recommended by the OECD and are preferred due to following reasons.

- Simulation of *in vivo* condition due to the presence of RBCs, serum, etc.
- Cells of human origin hence extrapolation is not required.
- Cells cultured in the presence of test compound for limited duration i.e. two to three cell cycles hence, the effect of test compound on the cells measured are mainly free from other *in vitro* effects due to culture.

The *in vitro* culture of cells with NPs require special technical considerations due to the tendency of NPs to get agglomerated and thus losing nano form which can affect the bio-availability *in vitro*. The aqueous suspension of ENM was sonicated to obtain stable and dispersed solution to inoculate in blood culture. Dose finding experiment for all three test compounds i.e. engineered nanoparticles of ZnO, *Yasada Bhasma* (incinerated zinc) and bulk ZnO using *in vitro* cell culture was done in order to select concentration of the compound such that the mitotic index was not inhibited more than 50% and sufficient metaphases are obtained for analysis. The effect of these three test compounds on genetic material of the human peripheral blood lymphocytes was evaluated using two cytogenetic techniques namely chromosomal aberration assay and cytokinesis blocked micronuclei assay.

2.

Review of literature

2.1 Engineered Nanoparticle

Engineered nanoparticles are manufactured at the nanoscale by the process known as **nano-manufacturing**.There are two basic approaches to nanomanufacturing, either **top-down**or **bottom-up**. Top-down fabrication reduces large pieces of materials all the way down to the nanoscale. The bottom-up approach to nanomanufacturing creates products by building them up from atomic- and molecular-scale components, which can be time-consuming. Structures and properties of materials can be improved through these nanomanufacturing processes. Such products containing nanomaterials can be stronger, lighter, more durable, water-repellent, anti-reflective, self-cleaning, ultraviolet- or infrared-resistant, antimicrobial, scratch-resistant, or electrically conductive, among other traits. Taking advantage of these properties, the current nanotechnology-enabled products range from baseball bats and tennis rackets to catalysts; for refining crude oil and ultrasensitive detection and identification of biological and chemical toxins (8).

Toxicological concerns of engineered nanoparticles

Engineered nanoparticles exhibit novel properties such as self-assembling, small size, and greater surface area to volume ratio, due to which it shows high reactivity (9). Thus not only physico-chemical properties but also their electrical, magnetic, structural, and morphological properties also change; making them capable of interacting with biomolecules in unique ways and enable their physical transport into the interior structures of cells (10).

The unique size-dependent properties of nanomaterials mean that in some ways they behave like a new chemical substance. For example, ZnO is considered to be a “GRAS” (generally

recognized as safe) substance by the Food and Drug Administration (FDA). However, the GRAS designation most commonly refers to materials in the micron to larger size range, these substances when reduced to the nanoscale can acquire toxicity (11).

Globally various regulatory agencies have formed working groups to focus on safety evaluation of engineered nanoparticles in terms of human use, ecosystem, and biological systems at large e.g. Asia Pacific Nanotechnology Forum 2005, Governmental bodies within the European Union (European Commission 2004), National Institutions (e.g. De Jong et al 2005, Roszek et al 2005, US National Science and Technology Council 2004, IEEE 2004, US National Institute of Environmental Health Sciences 2004), Non-governmental organizations (e.g. UN-NGLS 2005), learned institutions and societies (e.g. Institute of Nanotechnology 2005, Australian Academy of Sciences 2005, METI 2005, UK Royal Society and Royal Academy of Engineering 2004) and individuals (e.g. Oberdörster et al 2005, Donaldson and Stone 2003) have published reports on the current state of nanotechnology, which indicates need for a thorough risk analysis (12).
The concern for human health risk requires considering various routes of internalization of NPs i.e. dermal, nasal, ocular, oral in order to understand the mechanism of risk due to NPs (1).

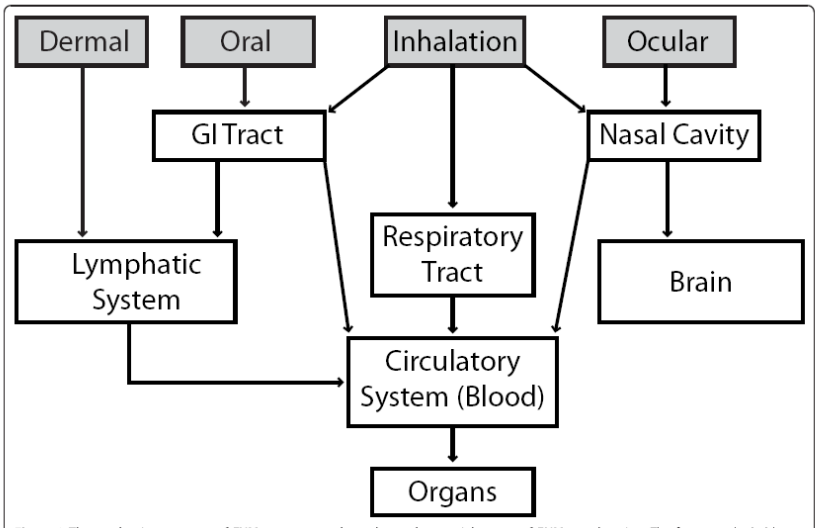

Figure 3 The predominant routes of ENM exposure and uptake, and potential routes of ENM translocation. The four gray shaded boxes indicate the primary routes of ENM exposure. The arrows down from these uptake sites show potential translocation pathways. The translocation pathways are described in more detail in **Section II, D. Clearance of ENMs, their translocation to distal sites, and persistence.** For example, the lung might be the primary route of exposure or might be a distal site after uptake from another route and translocation to the lung. ENMs might enter the brain from the nasal cavity or from blood, across the blood-brain barrier.

Fig 1.2: Route of exposure

Some of the published reports are as follows:
The liver, heart, spleen, pancreas, and bone were found to be the sites of ZnO nanoparticles in mice (13), and inhalation of these particles in rats produced potent yet reversible pulmonary inflammation (14). The dermal route includes application of sunscreens and other cosmetic products which contain ZnO NPs due to their UV absorption property.

Zinc nanoparticle exhibited toxicity towards Neuro-2A cells (15) and vascular endothelial cells (16), and induce apoptosis in neural stem cells (17).

It has also been reported that nanoparticle size influences cell viability (18). However, to date, the evaluation of cytotoxicity of ZnO nanoparticles on mammalian cells has been reported with no clear consensus among scientists on the significance of published results. A report from Food & Water Watch titled "Unseen Hazards: from Nanotechnologyto Nanotoxicity" linked nanoparticles to: (19).

- Damage to DNA
- Disruption of cellular function and production of reactive oxygen species
- Asbestos-like pathogenicity
- Neurologic problems (such as seizures)
- Organ damage, including significant lesions on the liver and kidneys
- Destruction of beneficial bacteria in wastewater treatment systems
- Stunted root growth in corn, soybeans, carrots, cucumber and cabbage
- Gill damage, respiratory problems and oxidative stress in fish

Our literature survey revealed that, there is little information about genotoxicity of nanoparticles. Hence, we report here the study of genotoxic potential of engineered nanoparticles and conventional nanoparticles (*Bhasma*) in comparison with ZnO bulk in terms of chromosome aberrations (CA) and micronucleus (MN) assay. This study was carried out in compliance with the Test Guidelines of the Organization for Economic Cooperation and Development (OECD-473, 487-1997, 2004).

2.2 Conventional Nanoparticles *(Ayurvedic Bhasma)*

Historically India probably was the first to maintain records of useful drugs. The Ayurvedic system of medicine is the only one, out of all traditional medicine systems of various civilizations, where importance of metals for curing ailments was emphasized. Therapeutic effectiveness of the Ayurvedic drugs have been established and well documented by the great *acharyas*. *Acharya Charaka*, the great Ayurvedic scholar of 1500 B.C. has mentioned the types of drugs on the basis of source, various formulations and surprisingly the pharmacology of drug with considerable precision. The later treaties described the medicinal properties of various metals like Mercury, Gold, and Lead etc. The metal used in Ayurvedic system of medicine include Mercury (*parade*), Gold (*Swarna*), Silver (*Rajata*), Copper (*Tamra*), Iron (*Lauha*), Tin (*Vanga*), Lead (*nag*a), Zinc (*Yasada*) etc. The metallic *Bhasmas* are result of range of physic-chemical processes. Various medicinal herbs are used. These are considered to be easily absorbed in the human body and produce optimum benefit in a minimum dose (6). *Bhasma*, which are unique Ayurvedic metallic/mineral preparations, treated with herbal juices or decoction and exposed for certain quantum of heat as per "*Puta"* system of Ayurveda are known in Indian subcontinent since seventh century AD and widely recommended for treatment of a variety of ailments. *Bhasma* are claimed to be biologically produced nanoparticles prescribed with several other medicines of Ayurveda (7).

Metallic preparations mainly termed as *Bhasma* and obtained by repeated incineration of metal with herbal extract of juice (6). In certain circumstances *Bhasma*; "*Vibhuti"* (*Sanskrit*) and "*Thiruneeru*" (*Tamil*) are synonymous (22).

2.2.1 Steps involved in preparation and contribution to properties specific to *Bhasma*

"Bhasmikaran" is a process by which a substance which is otherwise bio-incompatible is made biocompatible by certain "*samskaras"* or processes. The objectives of "*samskara"* are;

a) Elimination of harmful matters from the drug

b) Modification of undesirable physical properties of the drug
c) Conversion of some of the characteristics of the drug
d) Enhancement of the therapeutic action.

Various steps involved in the preparation of *Bhasma (*or B*hasmikaran*) are;
1) "*Shodhan"* i.e. Purification,
2) "*Maran*" i.e. Powdering,
3)" *Chalan*" i.e. Stirring,
4) "*Dhavan*" i.e. Washing,
5) "*Galan*" i.e. Filtering,
6) *"Putan*" i.e. Heating,
7) "*Mardan*" i.e. Triturating,
8) "*Bhavan*" i.e. Coating with herbal extract,
9) "*Amrutikaran*" i.e. Detoxification and
10) "*Sandharan*" i.e. Preservation.

Selection of these steps depends on the specific metal. Sometimes there is an overlapping of the steps e.g. "*marana"* is achieved by "*puttan"* (22), brief introduction and significance of some of the important steps are described here.

"Marana"

Generally, *Marana* is a process performed using fire and the ash obtained as a result of this incineration is known as *Bhasma*. The relation between the death and ash may be reason behind the acceptance of the word *Marana* for incineration in "*Rasasastra"*. In "*Rasasastra"*, the process by which metal, mineral or any other hard substance is converted to smooth, soft and ash like substance is known as *Marana*. *Marana* processes have been developing since centuries to attain the important position in formularies of medicaments.

Importance of "*Marana*"

Marana process is not just a chemical change. In "*Bhasmikarana"*, "*Bhavana"* as well as "*Mardana"* plays agreaterrole. Herbal drugs as juice, decoction or paste have been used in most of the *Marana* processes along with the sulphur group and mercury etc. Hence, in these processes mixing of organic substances from herbals induce assimilability in the human body. So that *Bhasma* practices not act as a foreign body for any counter-indications.

Bhasma played an important role in the medical field. Metals converted into minute particles by *Marana*. It brings the qualities of "*Nirindri*" substances vary near to that of "*sendriya*" or converts it to an assimilable form. The *Bhasama* attain the properties like "*Laghuta"* etc. Incinerated metals mix with*"Rasa Dhatu"* and come in contact with circulatory system and function properly. The *Bhasma* required less doses and helps in quicker relief. It enhances the properties of herbals and can be utilized for a longer time.

***"Marana"* Process**

The efficacy and other specialties of *Bhasmas* influenced the field of human ailments and various experiments are conducted to find out easy and more effective processes of *Marana*. Therefore nowadays different types of methods are found for *Marana* of each metal. All these processes come under the formerly quoted definition of *Marana* by Yadavji Acharya.

The total result of a series of processes constitutes the preparation of *Bhasma. "Sodhana"* (Purification), "*Jarana"* , "*Bhavana"* (impregnation) of liquid media, "*Mardana"* (trituration), "*CakrikaNirmana"* (cakes preparation), "*Sosanam"* (dessication), "*Samputanam"* (sealing inside saucers),"*Putanam"* (incineration), "*Svangasitalam"* (self-cooling), "*Kuttanam"* (pounding) are the stages of "*Marana"* process in the sequence.Among these processes "*Sodhana"* process is very significant and hence described here in brief.

***"Sodhana"*(Purification)**

"Sodhana" means to purify the metal and mineral before administrating them in alchemical and therapeutical purposes. "*Sodhana"* is a term for one of the "*Samskara"* in "*Rasa Sastra"*. Most of the raw materials used in*"Rasa Sastra"* are extracted from earth. So there is every chance of impurities, toxicity, heterogeneous qualities, mixing of other substances and unwanted qualities to a large extent. Certain drugs are poisonous like mercury, copper, arsenic etc. So, "*Sodhana"* is indicated to eliminate all such toxic qualities and to induce certain qualities, which are essential for the metabolism of the material in living body.

Aims of "*Shodhana"*:

- To eliminate impurities from metal
- To make the metal free from blemishes
- For direct therapeutic uses in some cases
- To increase the therapeutic values of the drug

2.2.2 Attributes of *Bhasma*

All *Bhasm*a have some common properties like *Rasayana*, *Yogavahi*, etc. *Rasayana* indicates immunomodulation and anti-aging quality; and *yogavah*i indicates ability of drug carry and targeted drug delivery by *Bhasma*. These are prescribed in very minute dose(15-250 mg/day).Under *Rasibhavana*, properly prepared *Bhasma* must be readily absorbable, adaptable and assimilable in the body, and will be non-toxic. *Shighravyapti* indicates that after *Marana, Bhasma* becomes easily absorbable and assimilable in the body and spreads quickly in the body. Under *Agnideepana, Bhasma* increases metabolism at cellular level and acts as catalyst (5).

2.2.3 *Bhasma* as Nanoparticles

These attributes of *Bhasma* are comparable with the action of NPs in the body. These are biodegradable, biocompatible and non-antigenic in nature. NPs, in general can be used to provide

selective/targeted/controlled delivery of drug to specific site of action in the body even across the blood brain barrier. These can be used to extend time window of bioavailability and to protect drug from chemical and enzymatic decomposition. These can also result in reduction of peripheral side effects of drug by decreasing overall dose of drug in the body (5).

2.2.4 *Yasada Bhasma* (Incinerated Zinc)

Yasada is a metallic compound known to Indian Physicians since the beginning of medicine. It is being known by the name of *Yasada* since 14th century (25). Sufficient description is available in ancient text books about its usage in many diseases (27). *Yasada* is a *Sanskrit* word and has been derived from the Persian word *Jasta*. In some of the ancient Ayurvedic literatures it has been mentioned under the name *kharpara* and *rasaka*. Various synonyms have been given for *Yasada* like *jasta*, Zinc, *Kharparaja, Ritihetu, Netrarogari*etc. *Yasada* was included for the first time in 14th century in *MadanpalNighantu* and then in 16th century it was dealt independently in two Ayurvedic texts *Ayurveda Prakash* and *Bhava Prakash*. But no description on *Yasada* is found in literatures prior to 14th century (20,28).

Clinical use of *Yasada Bhasma*

Yasada has been widely used in the treatment of several diseases like conjunctivitis, Spermatorrhoea, Gonorrhea, Jaundice, wound healing diabetes (28). It has moderate cytoprotective activity by promoting the formation of anti-stress proteins, by inhibiting lipid peroxidation and DNA fragmentation in tissues (29). *Yasada* has been an essential component of several important Ayurvedic preparations, some of the popular preparations which contain *Yasada* along with their uses are; *Pramehavati* used in *prameha*, *mahakanakasindoor* used in tuberculosis, *vrihatsomnathras* used as antidiuretic, *ratnaprabhavati* used in fever (28). *Yasada Bhasma* also used as *Chakshushya* (ophthalmic nourishes), *Rasayana* (immunomodulator), and to increases strength, potentiality and intellect. It is used in the treatment of (*prameh*a) diabetes; (*Pandu*) anemia; (*Shwasa*) dyspnoea; (*Kasa*) cough; (*Kshaya*) emaciation; (*Vrana*) ulcer;

(*Avasada*) depression; (*Kampavata*) tremor; *Chaksuroga* (opthalimic disorderlike visual disorders, conjunctivitis etc.) (5, 6). *Yasada Bhasma* prepared from $ZnCO_3$ (zinc carbonate) and ZnO is reported to have antimicrobial activity against Streptococcus (1).

2.2.5 Concerns

The *Bhasma* containing heavy metal salts are currently studied mainly from the toxicological point of view. The ancient Ayurvedic scholars have mentioned all ill effects of improperly prepared metallic preparations and emphasis has been given to rational preparation methods through various pharmaceutical processing like "*shodhana"* (purification), "*Marana"* (incineration)etc. The art of preparing the *Bhasmas* require certain amount of expertise and so much information is available about the likely impact of improper preparation on the expression of biological activity including possibility of production of undesirable effects (6). Long historical use of practices of Ayurvedic systems of medicine, including experience passed on from generation to generation, has demonstrated the safety and efficacy of this system. However lot of research work has been carried out to provide additional evidence of its safety and efficacy profile. The quantity and quality of the safety and efficacy data however on Ayurvedic medicines are far from sufficient to meet the criteria needed to support its use worldwide. The latest exclusive article published in 'Journal of American medical association' entitled "heavy metal content of Ayurvedic herbal medicinal product" has mentioned some Ayurvedic formulations containing heavy metals and permissible limits of these in human being and presented some case report on adverse effect of some Ayurvedic herbal formulation containing heavy metal. Metals and minerals that are transformed into drug must have excellent therapeutic efficacy and must be safe. Unfortunately some metals and minerals have the potential to produce adverse effect. Therefore, during transformation of metals and minerals to drugs, it is essential to evaluate the margin of safety between the dose level that produce the therapeutic effect and that produce adverse effect. *Yasada Bhasma* has no serious deleterious effect on body function as a whole; the epithelial proliferation may be indicative of androgenic activity and may not be considered as pathological change (29).

2.4 Genotoxicity Assessment

Genotoxins are substances that cause heritable changes in the genetic material in germ cells i.e. spermatocytes or oocytes (UNECE, 2004; ECVAM, 2002). Genotoxins are substances that induce changes to the structure or number of chromosomes by interacting with DNA and/or non-DNA targets chemically. Genotoxins are classified as follows;

- Category 1 chemicals are "known to induce heritable mutations or to be regarded as if they induce heritable mutations in the germ cells of humans",
- Category 2 chemicals are those that "cause concern for humans owing to the possibility that they may induce heritable mutations in the germ cells of humans" (UNECE, 2004)

There are different assays that can measure genotoxicity;

- 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 genetic mutation are those that detect changes in the number of chromosomes i.e. aneuploidy

Chromosome alterations are of two types;

1. Structural chromosomal aberrations (chromosome or chromatid breaks, rearrangements)
2. Numerical chromosomal aberrations (aneuploidy, polyploidy)

Cytogenetic assays are divided into two test types;

1. *In vivo* test- Study of chromosomal aberrations in rodent bone marrow cells 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* test- Mammalian cells in culture are assessed for genotoxicity by chromosomal aberration (CA), cytokinesis blocked micronuclei assay and comet assay

2.4.1 Chromosomal Aberration

The chromosomal aberration assay in human lymphocyte is a valuable assay. This test system uses primary cultures of lymphocytes from freshly drawn blood. These primary cells have a normal chromosome number and reliably detect clastogenic activity. Structural aberrations may be of two types, chromosome or chromatid. With the majority of chemical mutagens, induced aberrations are of the chromatid type, but chromosome type aberrations also occur. An increase in polyploidy may indicate that a chemical has the potential to induce numerical aberrations. Chromosome mutations and related events are the cause of many human genetic diseases and there is substantial evidence that chromosome mutations and related events causing alterations in oncogenes and tumor suppressor genes of somatic cells are involved in cancer induction in humans and experimental animals. This assay is used to screen for possible mammalian mutagens and carcinogens. Many compounds that are positive in this assay are mammalian carcinogens; however, carcinogenicity is not by only this mode of action. Correlation is dependent on chemical class and there is increasing evidence that there are carcinogens that are not detected by this test because they appear to act through mechanisms other than direct DNA damage. (OECD 473 guideline)

2.4.2 Mitotic index

Mitotic index is a measure of the proliferation of cell population. It is defined as the ratio of the number of cells in mitosis and the total number of cells scored. The mitotic index is only an indirect measure of cytotoxicity.

Calculation: [Cells in metaphase/total no. of cell count]

2.4.3 Cytokinesis Blocked Micronuclei assay (CBMN)

Micronucleus (MN) is a small additional nucleus and is readily identifiable by light microscopy. Biologically, micronuclei are the chromosome fragments or whole chromosomes that lag behind at anaphase during nuclear division. MN scoring is the indicator of the genetic damage.

Mechanism of origin of MN:

There are two predominant mechanisms leading to the formation of MNi in a mitotic cell;
(1) Chromosomal breakage
(2) Dysfunction of the mitotic apparatus.

Clastogens induce chromosome breaks and yield acentric fragments. These chromosomal fragments are directly included into micronuclei. In the other mechanism, aneugenic agents prevent the formation of the spindle apparatus during mitosis. As a result, the whole chromosomes lag behind at anaphase. The chromosome is surrounded by the nuclear envelope, forming micronuclei. Therefore the daughter cells have micronuclei containing whole chromosomes. Besides these two important mechanisms, MNi may be formed due to broken anaphase bridges. This may be because of dicentric chromatids, intermingled ring chromosomes, or union of sister chromatids.

DNA double strands break is a physiological phenomenon when a cell commits from G0 to G1 of the cell cycle and may occur in normal healthy cells. So MNi may also be noted in normal healthy individual but the numbers of micronucleated cells may be less. It has been shown from various studies that the prevalence and frequency of spontaneous occurrence of MNi in human lymphocytes increases with age. The micronucleation of human lymphocytes is nonrandom and there is increased frequency of age dependent micronucleation of X and Y chromosomes.The higher micronucleation of X chromosome in female may be due to inactive X chromosome.

The use of micronuclei (MNi) as a measure of chromosome damage in peripheral blood lymphocytes (PBL) was first proposed by Countryman and Heddle (1976) and subsequently developments of the cytokinesis-block micronucleus (CBMN) method were reported.

It became evident after scoring hundreds of slides of lymphocyte cultures for MNi that the ideal stage to score MNi was the binucleated stage. The development of the cytokinesis-block (CB) technique has made the human lymphocyte micronucleus assay (MN) a reliable and precise method for assessing chromosome damage.

It is important to count MNi in binucleated cells (BNC) for several reasons;

- Cells have to pass through one cell cycle and mitosis after treatment in order to form MNi
- MNi may be lost
- New MNi may arise when passing from second to the third cycle.

To block cells in this stage after completing one nuclear division and to observe binucleated cells cytochalasin-B (Cyt-B) is added. Cells that complete nuclear division are then accumulated as binucleated cells because Cyt-B can inhibit cytokinesis without interfering with nuclear division. Scoring of MNi in cytokinesis-blocked binucleated cells has since become a standard procedure in genetic toxicology.

3.

Materials and Methods

3.1 Equipments

Blood collection vials	B.D.Biosciences
Blood collection needles	Greiner bio-one
Micropipettes	Eppendorf
Bench top centrifuge	CM 101 REMI
Incubator	EIE Instruments
Filtration assemblies, disposable (0.22µm, DMSO resistant, and normal)	Axygen
Microscope slides	Himedia
Microscope	Nikon, Labomed
Refrigerator	Samsung
Freezer (-20^{o}C)	Blue star
Glasswares	Borosil
Gloves	Kimberley clark
Serological bath	EIE serological water bath (Wiswo instruments)
Weighing balance	Shimadzu
UV-Visible spectrophotometer	Shimadzu
Sonicator	Ningbo Haishu – Sklon
Autoclave	Yorco

3.2 Reagents

Acetic Acid: MERCK

Chromic acid: MERCK

Colchicine: HIMEDIA

Cytochalasin-B: SIGMA

DPX: S.D. Fine-chem. Ltd. (Mountingmedium)

Giemsa Staining solution: HIMEDIA

Methanol: MERCK

Mitomycin-C: SIGMA

Phytohemagglutinin: HIMEDIA PHA-M (PHA-M, 25mg)

Potassium Chloride: MERCK

RPMI-1640 culture medium: HiGlutaXL^{TM}RPMI-1640 (8 ml medium in 15ml round bottom centrifuge tube), Cell culture tested

Sodium hypo chloride: S.D. fine chem. Limited

Yasada Bhasma: Baidyanath

ZnO bulk: Sigma-Aldrich

ZnO nanoparticles: ALDRICH

3.3 Reagent Preparation

1. Culture Media: RPMI-1640 supplemented with 15% fetal calf serum, L-alanyl-L-Glutamine, HEPES buffer, Penicillin (60 mg/ litre), streptomycin (100 mg/litre), and 2% $NaHCO_3$.

2. KCl (Hypotonic): 0.56 g of KCl was dissolved in 100 ml of sterile distilled water.

3. Fixative: Methanol and acetic acid were freshly added in 3:1 ratio respectively and chilled.

4. Stock Giemsa stain: 1 g Giemsa powder was added in 54 ml glycerol mixed and kept at 60^0C water bath for overnight, 84 ml of methanol was added, filtered, and kept in dark bottle.

5. Working Giemsa stain: 4 ml Giemsa stain was added in 25 ml Sorenson's buffer with pH 7 and 25 ml water, mixed well and prepared freshly whenever required.

6. Sorenson's buffer: 0.345 g of monobasic sodium phosphate (NaH_2PO_4) and 0.454 g dibasic sodium phosphate (Na_2HPO_4) was added in 250 ml of sterile distil water for obtaining pH 7.

7. Chromic acid: 10 % $K_2Cr_2O_7$ was added with 25 % of H_2SO_4 for slide washing.

8. Colchicine: 10 mg absolute powder was dissolved in 10 ml of autoclaved deionized water and aliquots were dispensed (1 mg/ml). It was used to block the cells at metaphase stage.

9. Phytoheamagglutinin (PHA): 25 mg of absolute powder of PHA was added to 25 ml autoclaved deionized water and aliquots were dispensed (1 mg/ml). Phytohemaglutinin (PHA), derived from extracts of *Phaseolus vulgaris* seeds, on account of its twin properties of causing erythroagglutination and of stimulating progressive lymphocyte mitosis is used as mitotic inducer in the culture. PHA-M stimulates T-lymphocytes.

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

Cyt-B vial was removed from -20^0C 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 0.22 µm sterile filter.

- 4 ml was removed from the vial and ejected into another sterile 15 ml tube labelled as "1".

- Remaining 4.3 ml of DMSO was aspirated as before into the vial and again ejected into the sterile tube labelled as "1".
- All the contents were mixed properly and 500 µl was dispensed into sterile 1.5 ml eppendorfs. Aliquots were then stored at -20^{0}C.

11. MMC (Mitomycin–C): 2 mg of solid was dissolved in 2 ml of sterile deionized water to give a mitomycin-C solution concentration of 1 mg/ml.

3.4 Test Compounds

3.4.1 ZnO Nanoparticles

Zinc compound is sparingly soluble in water, its water solubility is 1.6 to 5 mg/L, and has strong tendency to form agglomerates. An agglomerate is a "collection of loosely bound particles or mixtures of the two where the resulting external surface area is similar to the sum of the surface areas of the individual components," while an aggregate is defined as a "particle comprising strongly bonded or fused particles where the resulting external surface area maybe significantly smaller than the sum of the calculated surface areas of the individual components" (British Standards Institution, 2007). This means that agglomerates might be easily separated by small amount of energy (e.g. vortex or short sonication), while further dispersion of aggregates is unlikely. Therefore sonication was applied to ZnO ENPs suspension in water.

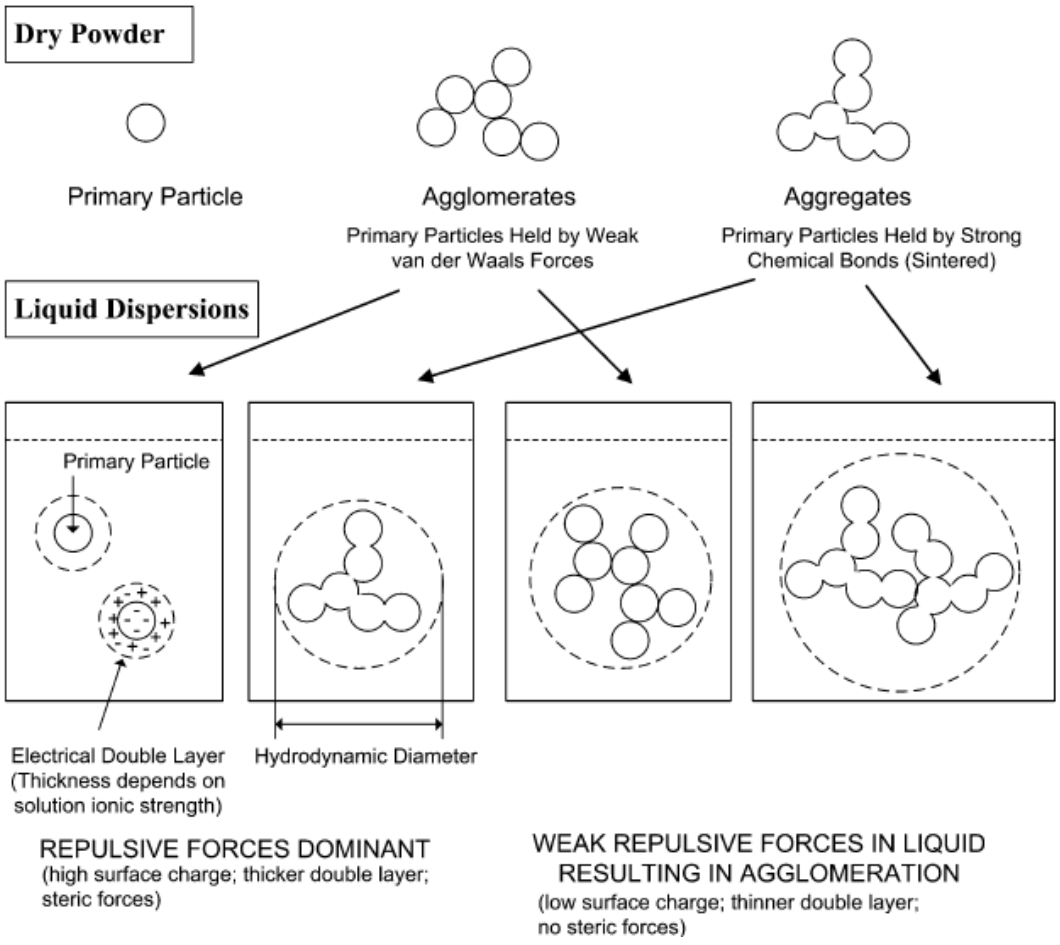

Fig 3.1: **Behavior of NPs in solution**

In order to make a suspension of zinc nanoparticles in water following protocol was followed. This protocol of sonication was standardized for the use of our experiment as under;

Table 3.1: Standardized sonication protocol for dispersion of zinc nanoparticle

Power (W)	Amount (mg)	Volume (ml)	Sonication time (min)	Total time(Min)
100	1	10	13.5	20
200	1	10	13.5	20
300	1	10	13.5	20
400	1	10	13.5	20

This solution was sterilized by exposing to UV light for 10-20 minutes. Sterility was tested by inoculating solution on N-agar and Potato Dextrose Agar (PDA) and incubated at 37^{o}C and room temperature to detect the presence of bacteria or fungus respectively. From this sterile solution, particular volume was added to the three culture tubes so as to make final concentration of 5, 10, and 15 µg/ml of culture volume respectively.

3.4.2 *Yasada Bhasma*

10 mg of *Yasada Bhasma* was suspended in 10 ml of deionized water to make the stock solution of 1 mg/ml. This solution was sterilized by exposing to UV light for 10 minutes. Sterility was tested by inoculating solution on N-agar and Potato Dextrose Agar (PDA) and incubated at 37^{o}C and room temperature to detect the presence of bacteria or fungus respectively. From this sterile solution, particular volume was added to the three culture tubes such as to make final concentration of 5, 10, &15 µg/ml of culture volume respectively.

3.4.3 Zinc Bulk

Zinc powder bottle was opened in Laminar Air flow and some amount of zinc powder was added to pre-weighed glass vial, then the glass vial was weighed again and the difference between the preweighed and post addition weighed was considered as weight of the powder. Thus a stock solution of 0.1 mg/ml was prepared. This solution was sterilized by exposing to UV light for 10 minutes. Sterility was tested by inoculating solution on N-agar and Potato Dextrose Agar (PDA) and incubated at 37^{o}C and room temperature to detect the presence of bacteria or fungus respectively. From this sterile solution, particular volume was added to the three culture tubes such as to make final concentration of 5, 10, and 15 µg/ml of culture volume respectively.

Physical Characterization

The physical characterization of *Bhasma* and engineered nanoparticles was done as per the guidelines of "*ayurveda rasayanashstra*" to evaluate if both fulfill the criteria for *Bhasma*. In other words, if ENM and *Bhasma* both are in nano form, certain tests should be applicable to

both. The *in vitro* genotoxicity assessment of *Bhasma* has been carried out for the first time along with ENM. (Appendix I)

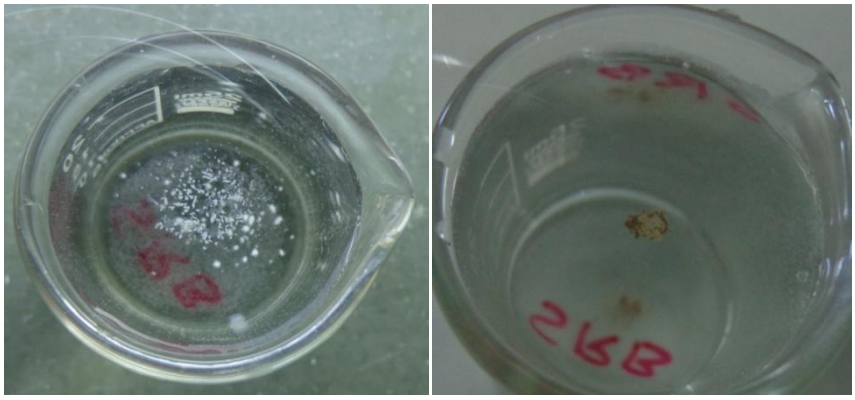

Fig 3.2

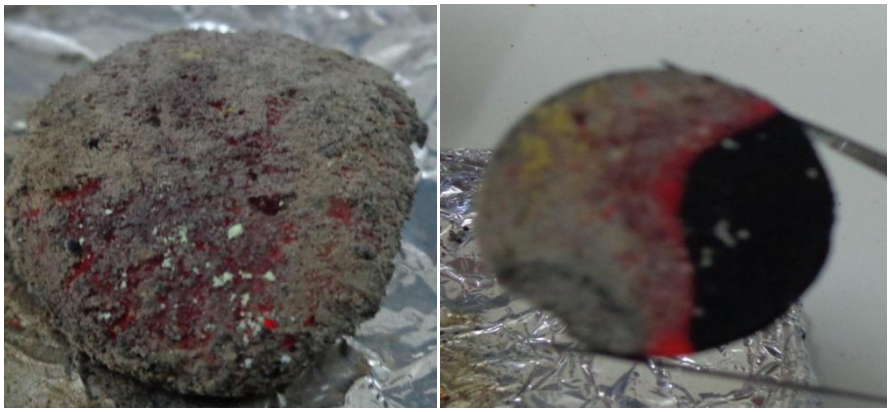

Fig 3.3

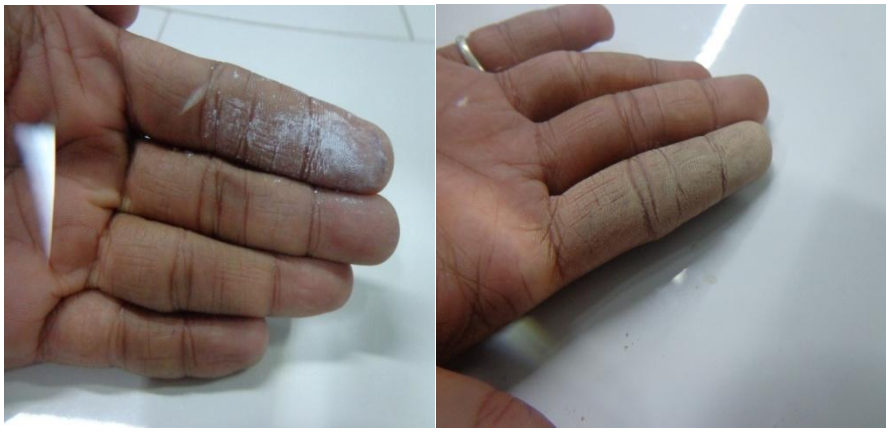

Fig 3.4

Fig 3.2 depicts *varitaratva* of Zno ENPs (left side) and *yasada Bhasma* (right side)

Fig 3.3 depicts *nirdhumatvam* of Zno ENPs (left side) and *yasada Bhasma* (right side)

Fig 3.4 depicts *rekhapuratvam* of Zno ENPs (left side) and *yasada Bhasma* (right side)

Table 3.2: Findings of physical characterization of bhasma and nanoparticle

Standard	Finding	
	"Bhasma"	**Nanoparticle**
Varitaratva	Floats on water	Floats on water
Rekhapuratvam	Partial	Partial
Slanksanatva	Smooth	Smooth
Gatarasatva	Tasteless	-
Nishchandratvam	Lusterless	Lusterless
Nirdhumatvam	No smoke	No smoke

3.5 Methods

3.5.1 Sampling and Blood Collection

Venous blood from healthy donor was collected using venipuncture taking proper aseptic precautions in sterile heparinised vacutainer and mixed gently to avoid clotting, which was used to set up cultures in sterile conditions.

3.5.2 Mitotic Index (MI)

At 0^{th} hr: Culture tubes containing 8ml RPMI-1640 were taken and kept in the incubator to set 37^0C temperature. PHA was added at a volume of 200 µl to each of the culture tubes, with100 µl of L-Glutamine and 500 µl of whole blood was added in sterile condition under laminar air flow (LAF). Culture tubes were allowed to incubate for 48 hrs at 37^0C in BOD/ CO_2 incubator.

At 48^{th} hr: After 48 hrs of culture set up test compound was added in various concentrations i.e. 5 µg/ml, 10 µg/ml and 15 µg/ml, with one culture tube untreated as negative control, and positive

control was set by adding MMC (Mitomycin-C) 45 µl.The culture tubes were allowed to incubate for 22 hrs at 37^0C in BOD/ CO_2 incubator.

At 70^{th} hr: After 70hrs of culture set up and 2 2hrs of test compound treatment to culture addition of colchicine is done to stop the cell division at metaphase. The culture tubes were allowed to incubate for 2 hrs at 37ºC in BOD/CO_2 incubator.

Harvesting of cultures:

At 72^{nd} hour: The culture tubes were harvested after 24 hrs from starting of culture treatment. The culture tubes were centrifuged at 2000 rpm for 10 min after 2 hrs of colchicine treatment. The supernatant medium was discarded. The cells in the pellet were suspended in 9 ml of pre-warmed 37^0C hypotonic solution (0.56% KCl) mixed vigorously and kept for 20 min at 37^0C in water bath. After 20 min 3 ml of chilled fixative (3:1- methanol:glacial acetic acid) was added to each culture tubes and kept at 4ºC for 15 min. Then centrifuged at 2000 rpm for 10 min. Supernatant was discarded and the cells in the pellet (brown-black in colour) were re-suspended in fresh pre-chilled fixative (7 ml). Pellet was washed with 6 ml of fixative and centrifuged at 2000 rpm for 10 min at least twice (until clear pellet obtained). After the final change, supernatant was discarded and small amount of fixative was added to the pellet to adjust the cell concentration.

Slide Preparation and staining: Three to five drops of cell suspension were dropped on grease free pre-chilled cleaned slides and were allowed to dry. Slides were stained in 4% Giemsa stain for 10 min. They were thoroughly rinsed in distilled water in a coplin jar and allowed to dry. The slides were then mounted with DPX and observed under the microscope.

Scoring for mitotic index: At least 100 well spread metaphases were scored for the chromosomal aberration per treatment group. Only a well spread metaphase containing number of chromosomes equal to 46 were considered. Cells with more than or less than 46 chromosomes,

with chromosomal break & gap, with chromatid gap & break, fragments ring were recorded on score sheet.

Calculation: [Cells in metaphase/total no. of cell count]

3.5.3 Chromosomal Aberrations:

Scoring of chromosomal aberrations was done for long term culture treatment to check the effect of test compound on cultured human peripheral blood lymphocytes without metabolic activation system.

For continuous treatment:

At 0^{th}hr: Culture tubes containing 8ml RPMI-1640 were kept in incubator before 30 min of culture set up to bring culture at 37^0C temperature. PHA was added at a final concentration of 200 µl to each of the culture tubes, with 500 µl of whole blood was added in sterile condition under laminar air flow (LAF). Culture tubes were allowed to incubate for 48 hrs at 37^0C in BOD/ CO_2 incubator.

At 48^{th} hr: The test compound was added in various concentrations of 5 µg/ml,10 µg/ml and 15 µg/ml with one culture tube as negative control, and positive control was set by treating with MMC (Mitomycin-C) 45 µl. Culture tubes were allowed to incubate for 22 hrs at 37^0C in BOD/ CO_2 incubator.

At 70^{th} hr: The culture tubes were harvested at 70^{th} hr of the initiation of culture treatment.

The harvesting and **slide preparation** were performed as same as mentioned above.

Slide scoring: At least 100 well spread metaphases were scored for the chromosomal aberration per treatment group. Only a well spread metaphase containing number of chromosomes equal to

46 were considered. Cells with more than or less than 46 chromosomes, with chromosomal break & gap, with chromatid gap & break, fragments ring were recorded on score sheet.

Evaluation and Interpretation criteria

There were several criteria for determining a positive result, such as a concentration-related increase or a reproducible increase in the number of cells with chromosomal aberrations at a single test concentration. Statistical methods were used as an aid in evaluating the test results. An increase in the number of polyploid cells may indicate that the test substance has the potential to interfere with the mitotic processes and to induce numerical chromosomal aberrations. Positive results from the *in vitro* chromosomal aberration test indicate that the test substance induces structural chromosomal aberrations in cultured mammalian somatic cells. Negative results indicate that, under the test conditions, the test substance does not induce chromosomal aberrations in cultured mammalian somatic cells.

3.5.4 Cytokinesis Blocked Micronuclei assay(CBMN)

At 0^{th} hr: Culture tube containing 8ml RPMI-1640 were kept in incubator before 30 min of culture set up to bring culture at 37^0C temperature. PHA was added at a final concentration of 200 µl to each of the culture tubes, 500 µl of whole blood was added in sterile condition under laminar air flow (LAF). Culture tubes were allowed to incubate for 48 hrs at 37^0C in BOD/ CO_2 incubator.

At 48^{th} hr: After 48 hrs of culture set up, test compound was added in various concentrations of 5 µg/ml, 10 µg/ml, and 15 µg/ml with one culture tube untreated as negative control and positive control was set by treating with MMC (mitomycin -C) 45 µl. Culture tubes were allowed to incubate for 6 hrs at 37^0C in BOD/ CO_2 incubator.

At 54^{th} hr: After 6hrs of culture treatment 90 µl Cyt-B was added. Culture tubes were incubated at 37^0C in BOD/ CO_2 incubator for 18 hrs.

At 78th hr: The culture tubes were harvested at 78th hr from the initiation of culture. The culture tubes were centrifuged at 2000 rpm for 10 min. The supernatant medium was discarded. The cells in the pellet were suspended in 5 ml of pre-warmed 37^{0}C hypotonic solution (0.56% KCL) for 2 min. Approximately 2 ml of pre-chilled fixative was added to stop the hypotonic treatment and was stored at 4^{0}C for 2 hrs. The culture tubes were centrifuged at 2000 rpm for 10 min after 2 hrs. The supernatant medium was discarded and the cells in the pellet (brown-black in colour) were re-suspended in fresh 6ml of pre-chilled fixative. Pellet was washed with 6 ml of pre-chilled fixative at least twice (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.

The harvesting and **slide preparation** were performed as same as mentioned above.

Scoring of micronuclei: Total 1000 cells (Mononucleated and Binucleated) were counted, 500 binucleated and binucleated with micronuclei were counted per treatment and locations of binucleated cells with micronuclei were noted separately.

Criteria for selecting binucleated cell which can be scored for micronucleus frequency

1. Cells should be binucleated
2.The two nuclei in binucleated cell should be approximately equal in size,staining pattern and intensity.
3. 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 1/4th of the nuclear diameter.
4. 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.
5. 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.

4.

Results and Discussion

Table 4.1: Effect of ZnO NPs on Mitotic Index (MI) of *in vitro* short term cultures of peripheral blood lymphocytes

No.	Treatment Groups	No. of Totalcells scored	No. of Total metaphase	Mitotic Index	% MI	% Reduction in MI(compared to PHA)
1	Control (PHA)	1000	98	0.098	100	0.00
2	Positive control (MMC)	1000	79	0.079	80.61	19.38
3	5 µg/ml	1000	88	0.088	89.79	10.21
4	10 µg/ml	1000	136	0.136	138.77	(-38.77)
5	15 µg/ml	1000	138	0.138	140.81	(-40.81)

Figure 4.1: Graphical representation of effect of ZnO NP on % Mitotic index of *in vitro* short term cultured peripheral blood lymphocytes as depicted in Table-4.1

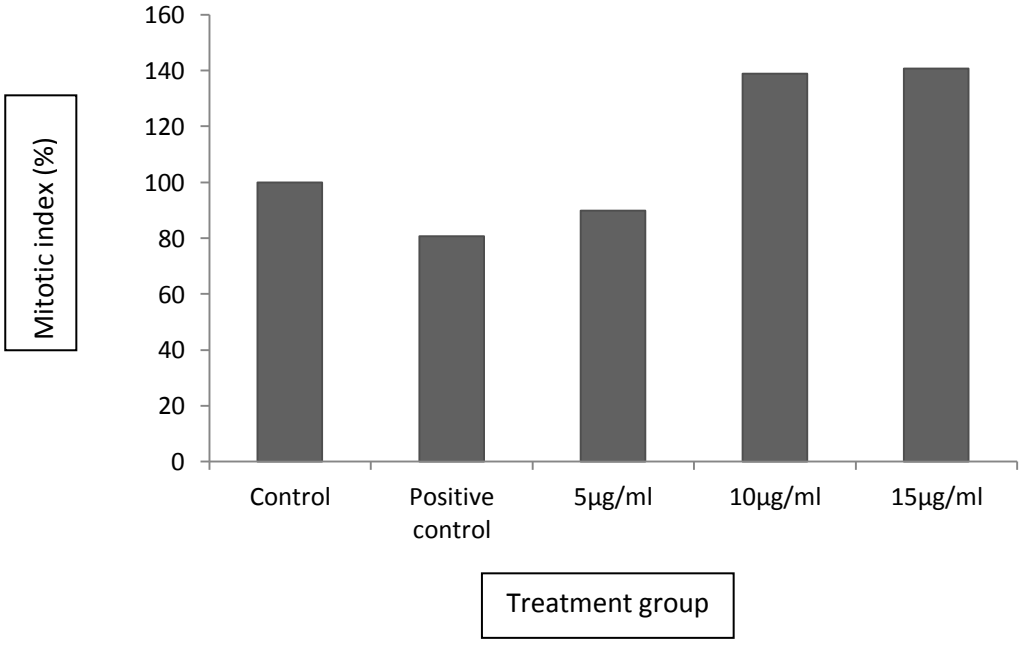

Table 4.2: Effect of ZnO NPs on mean CA/cell values of *in vitro* short term cultures of peripheral blood lymphocytes

No.	Treatment Group	No. of well spread metaphase scored	No. of cells observed with aberration	Mean ± SE
1	Control (PHA)	100	1	0.01 ± 0.01
2	Positive control (MMC)	100	13	0.13 ± 0.033*
3	5 µg/ml	100	26	0.26± 0.044**
4	10 µg/ml	100	21	0.21± 0.040**
5	15 µg/ml	100	23	0.23± 0.042*

***: Significant (P value<0.05);**: highly significant (P value< 0.01)**

Figure 4.2: Graphical representation of effect of ZnO NP on mean CA/cell values of *in vitro* short term cultured peripheral blood lymphocytes as depicted in Table-4.2

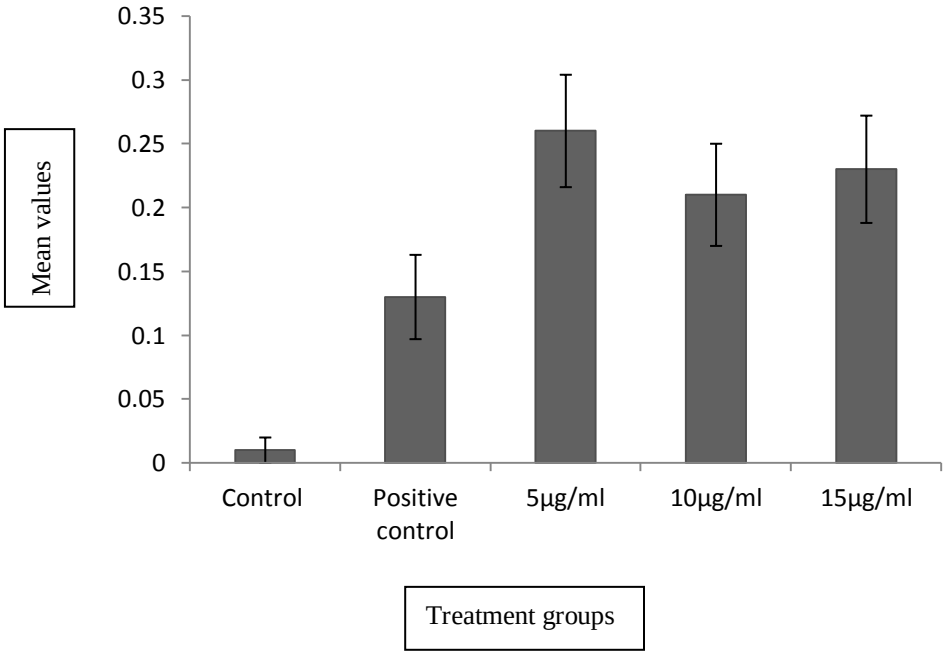

Table 4.3: Effect of ZnO NPs on % binucleated cells with micronuclei in cytokinesis blocked micronucleus assay

Treatment group	No. of Mononucleated cells	No. of Binucleated (BN) cells	No. ofBNC with micronuclei	NDI	% BNC with MNi	Mean ± SE
PHA	650	350	2	1.35	0.2	0.002±0.00141
MMC	600	400	11	1.40	1.1	0.011 ±0.0033*
5 µg/ml	720	280	6	1.28	0.6	0.006±0.00244#
10 µg/ml	600	400	4	1.46	0.4	0.004±0.00199#
15 µg/ml	720	280	6	1.28	0.6	0.006±0.00244#

***: Significant (P value<0.05), #: Non-significant (P value>0.05)**

Figure 4.3: Graphical representation of effect of ZnO NPs on % binucleated cells with micronuclei in cytokinesis blocked micronucleus assay as depicted in Table 4.3

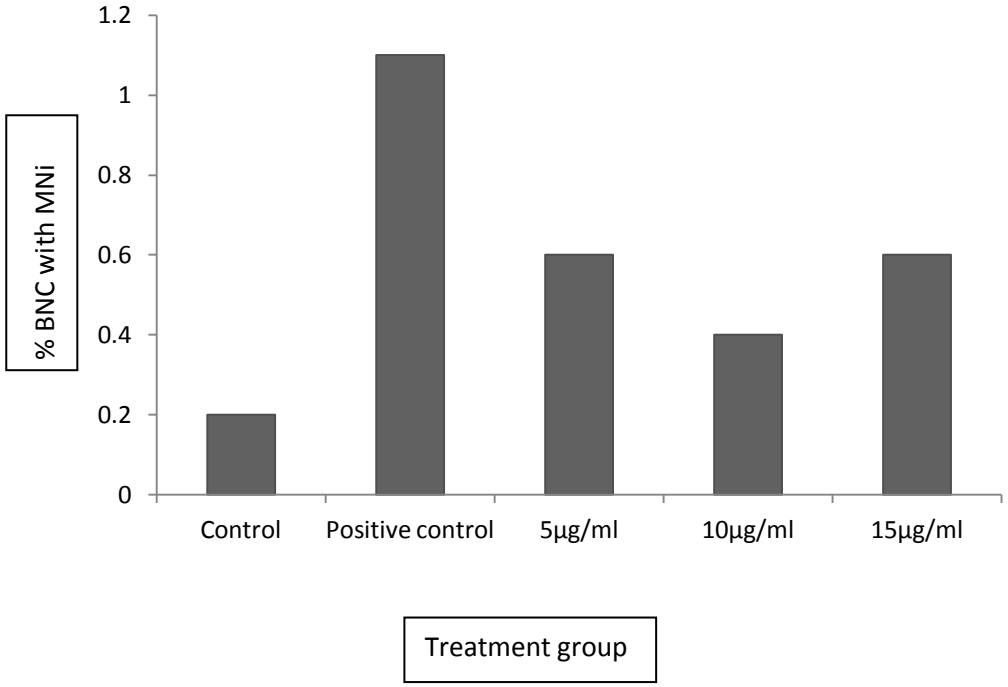

Table 4.4: Effect of *Yasada Bhasma* on Mitotic Index (MI) of *in vitro* short term cultures of peripheral blood lymphocytes

No.	Treatment Groups	No. ofTotal cells scored	Total No. of metaphase	Mitotic Index	% MI	% Reduction in MI
1	Control (PHA)	1000	70	0.070	100	0
2	Positive control (MMC)	1000	61	0.061	87.14	12.86
3	5 µg/ml	1000	53	0.053	75.71	24.39
4	10 µg/ml	1000	79	0.079	112.85	(-12.85)
5	15 µg/ml	1000	46	0.046	65.71	34.39

Figure 4.4: Graphical representation of effect of *Yasada Bhasma* on % Mitotic index of *in vitro* short term cultured peripheral blood lymphocytes as depicted in Table-4.4

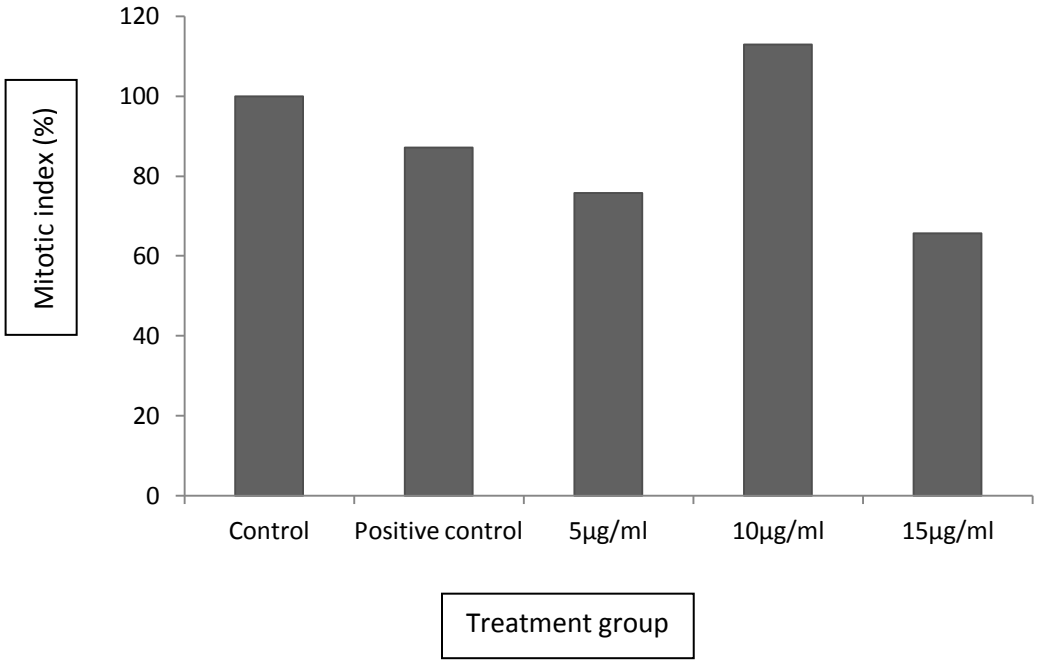

Table 4.5: Effect of *Yasada Bhasma* on mean CA/cell values of *in vitro* short term cultures of peripheral blood lymphocytes

No.	Treatment Group	No. of well spread metaphase scored	No. of cells observed with aberration	Mean ± SE
1	Control (PHA)	100	0	0 ± 0
2	Positive control (MMC)	100	20	0.2± 0.040*
3	5 µg/ml	100	1	0.01±0.01#
4	10 µg/ml	100	0	0±0#
5	15 µg/ml	100	1	0.01±0.01#

***: Significant (P value<0.05), #: Non-significant (P value>0.05)**

Figure 4.5: Graphical representation of effect of *Yasada Bhasma* on mean CA/cell values of in vitro short term cultured peripheral blood lymphocytes as depicted in Table-4.5

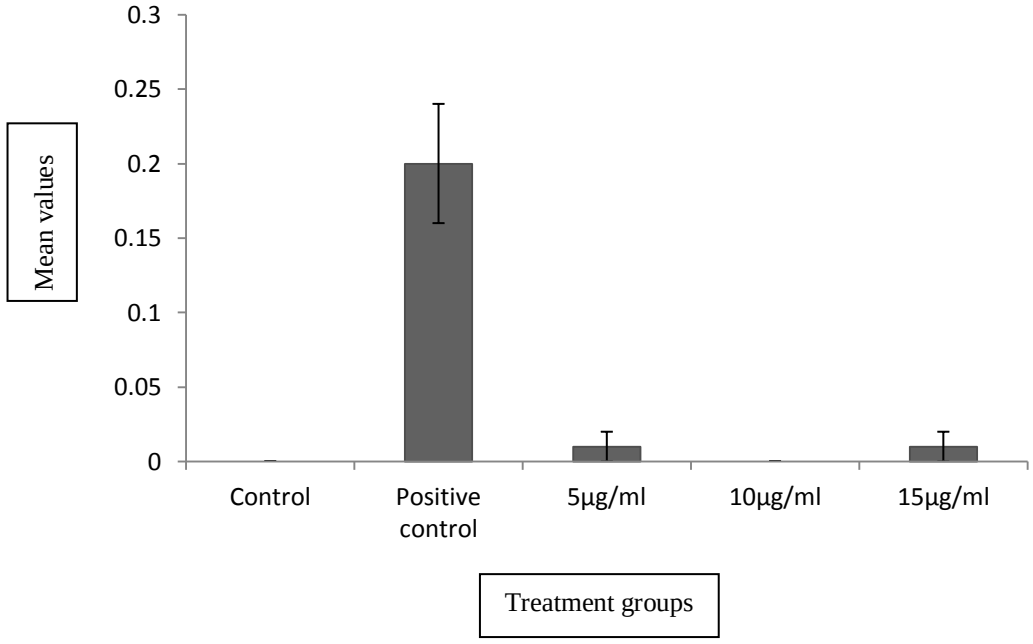

Table 4.6: Effect of *Yasada Bhasma* on % binucleated cells with micronuclei in cytokinesis blocked micronucleus assay

Treatment group	No. of Mononucleated cells	No. of Binucleated cells	No. of BNC with micronuclei	NDI	% BNC with MNi	Mean ± SE
PHA	654	346	2	1.34	0.2	0.02±0.014
MMC	700	300	16	1.30	1.6	0.2±0.0402*
5 µg/ml	594	406	2	1.40	0.2	0.02±0.014#
10 µg/ml	779	221	2	1.22	0.2	0.02±0.014#
15 µg/ml	608	392	4	1.39	0.4	0.04±0.019*

***: Significant (P value<0.05), #: Non-significant (P value>0.05)**

Figure 4.6: Graphical representation of effect of *Yasada Bhasma* on % binucleated cells with micronuclei in cytokinesis blocked micronucleus assay as depicted in Table 4.6

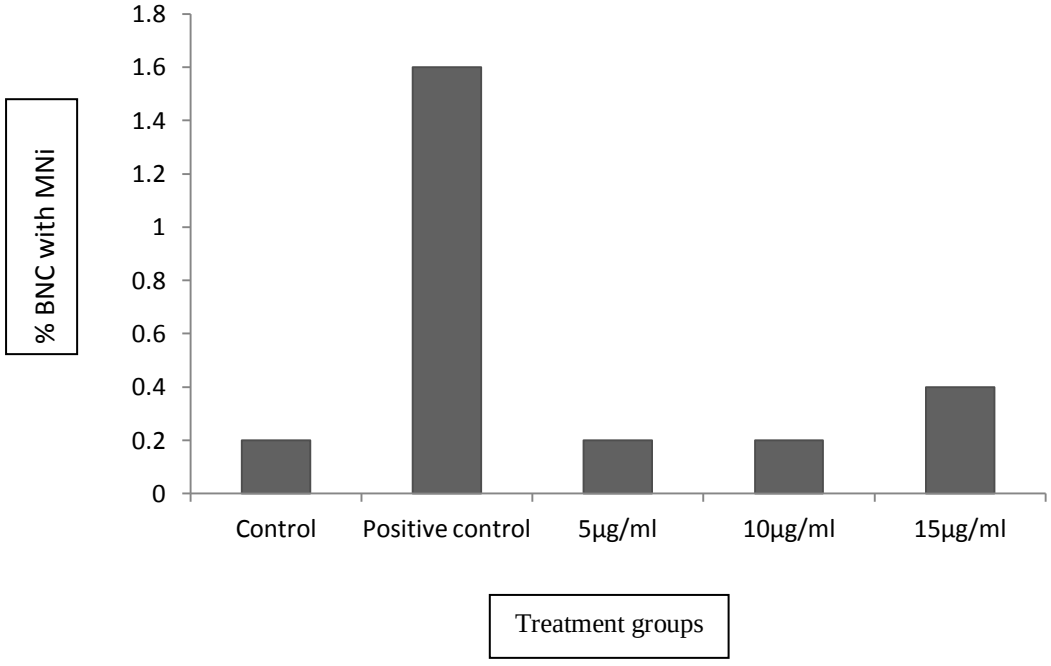

Table 4.7: Effect of ZnO bulk on Mitotic Index (MI) of *in vitro* short term cultures of peripheral blood lymphocytes

No.	Treatment Groups	No. ofTotal cells scored	Total No. of metaphase	Mitotic Index	% MI	% Reduction in MI
1	Control (PHA)	1000	66	0.066	100	0.00
2	Positive control (MMC)	1000	98	0.098	148.4	48.4
3	5 µg/ml	1000	63	0.063	95.45	4.54
4	10 µg/ml	1000	33	0.033	34.84	65.16
5	15 µg/ml	1000	0	0	0	0

Figure 4.7: Graphical representation of effect of ZnO bulk on % Mitotic index of *in vitro* short term cultured peripheral blood lymphocytes as depicted in Table-4.7

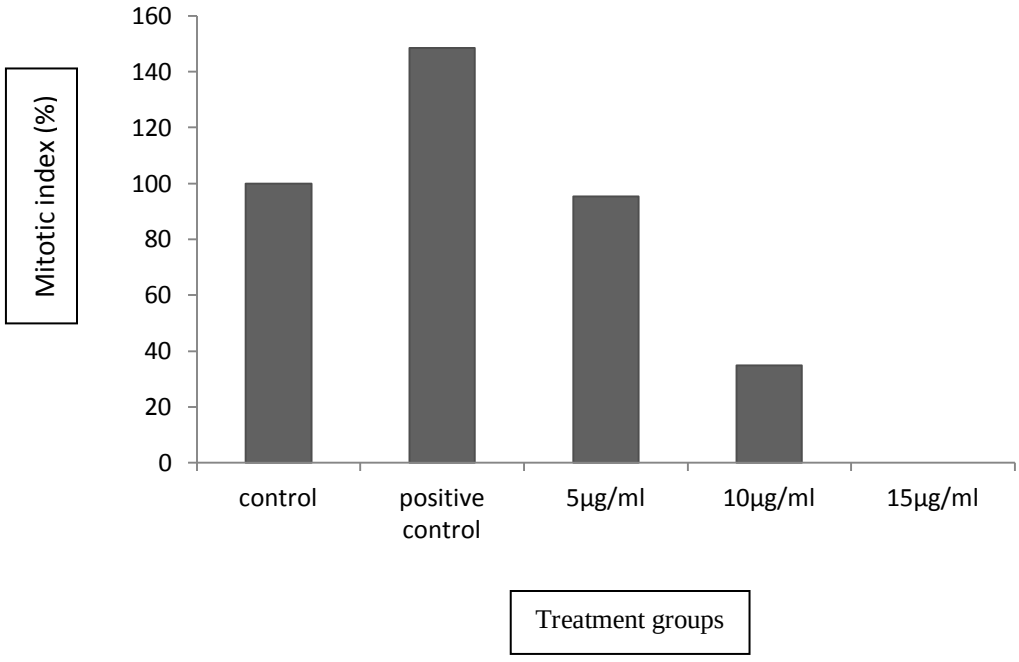

Table 4.8: Effect of ZnO bulk on mean CA/cell values of *in vitro* short term cultures of peripheral blood lymphocytes

No.	Treatment Group	No. of well spread metaphase scored	No. of cells observed with aberration	Mean ± SE
1	Control (PHA)	100	2	0.02 ± 0.014
2	Positive control (MMC)	100	9	0.09 ± 0.028*
3	5 µg/ml	100	2	0.02 ± 0.014#
4	10 µg/ml	100	1	0/01 ± 0.01#
5	15 µg/ml	-	-	-

***: Significant (P value<0.05), #: Non-significant (P value>0.05)**

Figure 4.8: Graphical representation of effect of ZnO bulk on mean CA/cell values of in vitro short term cultured peripheral blood lymphocytes as depicted in Table-4.8

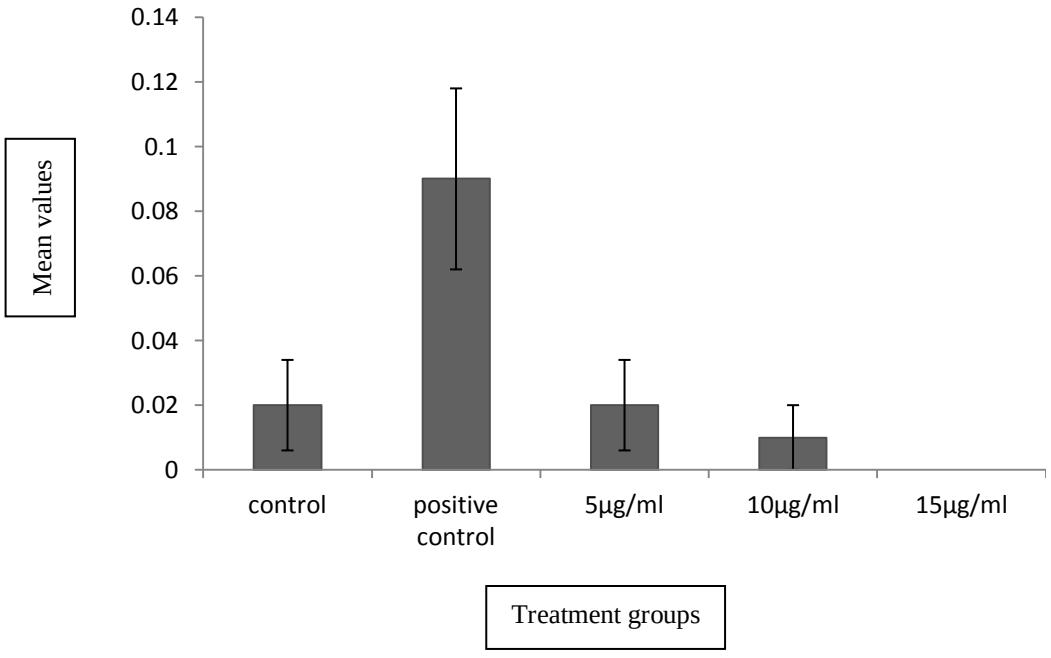

Table 4.9: Effect of ZnO bulk on % binucleated cells with micronuclei in cytokinesis blocked micronucleus assay

Treatment groups	No. of Mononucleated cells	No. of Binucleated cells	No. of BNC with micronuclei	NDI	% BNC with MNi	Mean ± SE
PHA	876	124	3	1.12	0.3	0.003±0.0017*
MMC	926	74	14	1.07	1.4	0.014±0.0037#
5 µg/ml	690	310	1	1.31	0.1	0.001±0.001#
10 µg/ml	760	240	3	1.24	0.3	0.003±0.0017#
15 µg/ml	876	124	0	1.12	0.0	0±0#

***: Significant (P value<0.05), #: Non-significant (P value>0.05)**

Figure4.9: Graphical representation of effect of ZnO bulk on % binucleated cells with micronuclei in cytokinesis blocked micronucleus assay as depicted in Table 4.9

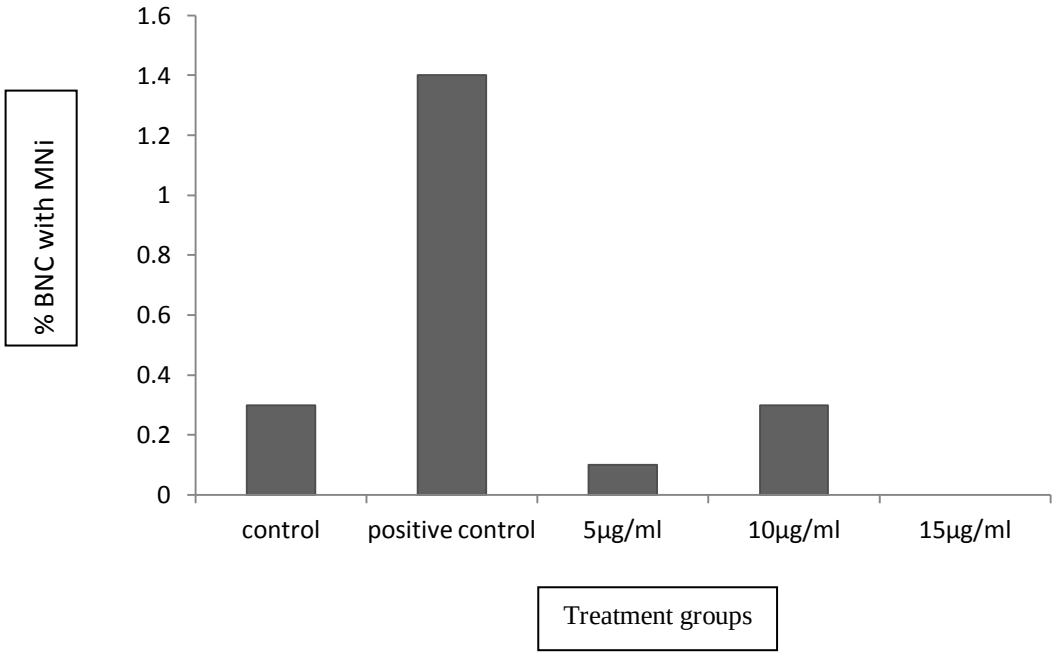

Table 4.10: Comparative data of number of chromosomal aberrations in controls and ENP of ZnO, *Yasada Bhasma*, and bulk ZnO in terms of significance using Student's t test

Chromosomal aberration assay for ZnO nanoparticles	No	Group	Significance
	1	Control v/s positive control	Significant
	2	Control v/s 5 µg/ml	Highly significant
	3	Control v/s 10 µg/ml	Highly significant
	4	Control v/s 15 µg/ml	Significant
Chromosomal aberration assayfor *Yasada Bhasma*	1	Control v/s positive control	Significant
	2	Control v/s 5 µg/ml	Non-significant
	3	Control v/s 10 µg/ml	Non-significant
	4	Control v/s 15 µg/ml	Non-significant
Chromosomal aberration assay for ZnO bulk	1	Control v/s positive control	Significant
	2	Control v/s 5 µg/ml	Non-significant
	3	Control v/s 10 µg/ml	Non-significant
	4	Control v/s 15 µg/ml	Non-significant

Table 4.11 Comparative data of % micro nucleated cells in controls and ENP of ZnO, *Yasada Bhasma*, and bulk ZnO in terms of significance using Student's t test

	No	Groups	Significance
CBMN assay for zinc nanoparticle	1	Control v/s Positive control	Significant
	2	Control v/s 5 µg/ml	Not significant
	3	Control v/s 10 µg/ml	Not significant
	4	Control v/s 15 µg/ml	Not significant
CBMN assay for *Yasada Bhasma*	1	Control v/s positive control	Significant
	2	Control v/s 5 µg/ml	Non-significant
	3	Control v/s 10 µg/ml	Non-significant
	4	Control v/s 15 µg/ml	Significant
CBMN assay for ZnO bulk	1	Control v/s positive control	Significant
	2	Control v/s 5 µg/ml	Non-significant
	3	Control v/s 10 µg/ml	Non-significant
	4	Control v/s 15 µg/ml	Non-significant

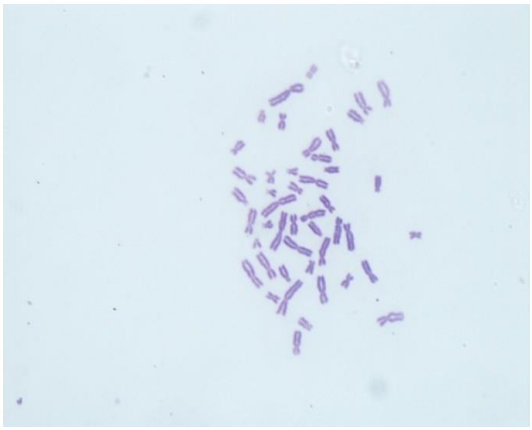

Figure 4.10

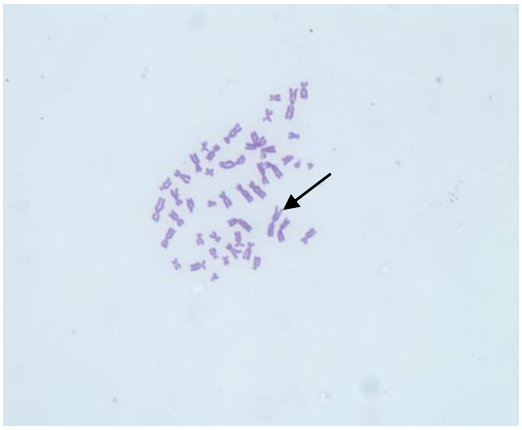

Figure 4.11

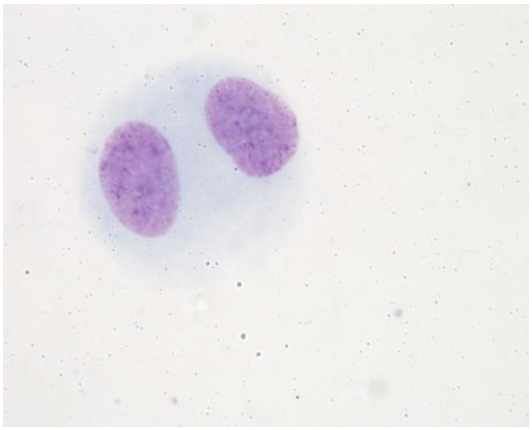

Fig 4.13

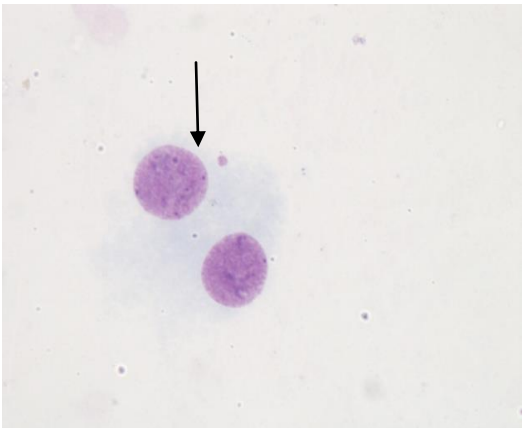

Fig 4.14

[Magnification 1000X digital zoom]

Figure 4.10: A Giemsa-stained normal metaphase

Figure 4.11: Arrow depicts a fragment in Giemsa stained metaphase plate

Figure 4.12: Giemsa stained Cytokinesis blocked binucleated cell

Figure4.13: Arrow depicts Cytokinesis blocked binucleated cell with micronuclei

The results of dose finding experiment were expressed in terms of mitotic index. The MI of lymphocyte treated with ZnO engineered nanoparticles, was reduced in the positive control by 20%, and in 5 µg/ml i.e. lowest concentration treated group by 10%, whereas at higher i.e. 10 µg/ml and 15 µg/ml the MI was increased by about 40% suggesting non-inhibition on mitotic activity of ZnO ENPs. The results indicate that ZnO engineered nanoparticle may act as a

clastogen, and the proliferation rate of cell was significantly accelerated. The possible genotoxic effect of all the three compounds were evaluated by chromosome aberration test using cultured human blood lymphocytes. The results of group treated with ZnO ENPs are given in the Tables-4.2 and Figure-4.2. Treatment with all the three concentration of ZnO ENPs suspension for 24 hrs induced significant number of structural chromosomal aberrations Table-4.2. The aberration rates at all the tested concentrations of ZnO ENPs suspension were higher than the control. Suggesting the possible clastogenic activity of ZnO engineered nanoparticles. The frequency of binucleated cells and binucleated cells with micronuclei obtained in micronucleus test is shown in Table-4.3 and Figure-4.3. Results revealed (as shown in table-4.11) that the test compound did not produce significant aneugenic activity after treatment with any of the tested concentrations when compared to control. Therefore, our results indicate that zinc engineered nanoparticle is not aneugenic. Our study may be used as a reference to support the hypothesis that ENPs are in real a new chemical, with different physio-chemical properties as compared to its native compound. This is very evident, as the concentration at which ZnO bulk particle inhibits the growth of cell, at same concentration ZnO ENPs accelerates the proliferation rate of the lymphocytes. By comparing the results of CA of ENPs and ZnO bulk, it can be derived that the adverse biological properties is an attribute of nano-size of the particle.

In case of *Yasada Bhasma* the maximum concentration i.e. 15 µg/ml showed inhibition of MI by 34%, no significant difference was seen in 5 µg/ml, & 13% increase in 10 µg/ml, So the reduction in MI was not dose dependent. The results of group treated with *Yasada Bhasma* are given in the Table-4.5 and Figure-4.5. Treatment with all three concentration of *Yasada Bhasma* for 24 hrs did not induced significant number of structural chromosomal aberrations. This suggests that *Yasada Bhasma* do not have clastogenic activity. The frequency of binucleated cells and binucleated cells with micronuclei obtained in micronucleus test is shown in Table-4.6 and Figure-4.6 at different concentration of "*Yasada Bhasma*". Results revealed (as shown in table-4.6) that the test compound did not produce significant aneugenic activity after treatment with any of the tested concentrations when compared to the control. But significant results are obtained for the same when compared with positive control. Therefore, our results indicate that "*Yasada Bhasma*" is neither clastogenic nor aneugenic.

The possible reason for this outcome may be the process involved in the preparation of "*Ayurvedic Bhasma"* which includes the interaction of metal with herbal extract, which may cap the particles of metal which are of nanosize. This capping may change the properties of metal particle contributed by its nanosize.

Whereas, in group treated with ZnO bulk, 5 µg/ml reduced the MI least, and as the concentration was increased the reduction in MI also increased. This indicates that ZnO bulk may hinder the proliferation rate of the lymphocytes. The possible genotoxic effect of ZnO bulk particle was evaluated using chromosome aberration test using cultured human blood lymphocytes and the results are given in the Table-4.8) and Figure-4.8. The results showed that the treatment groups5 µg/ml and 10 µg/ml were comparable to control group, whereas at the 15 µg/ml the mitotic index was inhibited, hence CA scoring was not applicable. The frequency of binucleated cells and binucleated cells with micronuclei obtained in micronucleus test is shown in Table-4.6 and Figure-4.6. As shown in table-4.11 the test compound did not produce significant aneugenic activity after treatment with any of the tested concentrations when compared to the control. Therefore, our results indicate that bulk zinc particle is neither clastogenic nor aneugenic.
From these results, it could be inferred that the ZnO ENPs can be considered as new chemical compound.

5.

Summary and conclusion

Summary

With the rapid expansion in the nanomaterial industry, it is essential to ensure the safety of engineered nanomaterials. However considerable attention has been given to the toxicity of engineered nanomaterials, possibility of their genotoxic potential and human health has been largely overlooked. Many of the engineered nanomaterials assessed were found to cause genotoxic effects such as chromosomal fragmentation, DNA strand breakages, point mutations, oxidative DNA adducts and alterations in gene expression profiles. However, from the published literature it is difficult to draw conclusions whether nanoparticles promote genotoxicity. ZnO NPs are used in a variety of applications viz., cosmetics, paints, as drug carriers, etc. Though ZnO NPs are believed to be nontoxic and biocompatible there are currently a small number of reports in the literature demonstrating that they could exert negative cellular responses. Exposure to ZnO NPs has been associated with inflammatory responses and cytotoxicity, but there is only one study that has considered the DNA damaging potential of ZnO NPs. Dufour and colleagues used the chromosome aberration test on CHO cells (31).

Bhasma are nearer to nanocrystalline materials. NP size of A*yurvedic Bhasma* has been confirmed in one study where it is proposed that NPs are responsible for its fast and targeted action. Subsequent action upon DNA/RNA molecules & protein synthesis within the cell are further hypothesized as possible mechanism for rapid onset of therapeutic action of *Bhasma* preparation. A study demonstrated the effect of *Yasada Bhasama* on intracellular DNA and proteins of treated human lung adenocarcinoma cell line, with the help of Raman spectroscopy (5).

The present study focuses on ***In vitro assessment*** **of genotoxicity of; Engineered & conventional (*Bhasma*) nanoparticles in comparison with bulk counterpart on human cultured peripheral blood lymphocytes in terms of Chromosomal Aberration assay (CA) & Cytokinesis Blocked Micronuclei assay (CBMN).**

The purpose of the *in vitro* chromosome aberration test is to identify agents that cause structural chromosome aberrations in cultured mammalian cells. Structural aberrations may be of two types, chromosome or chromatid. With the majority of chemical mutagens, induced aberrations are of the chromatid type, but chromosome-type aberrations also occur. Chromosome aberrations are the cause of many human genetic diseases including cancer when occur in somatic cells. The *in vitro* micronucleus assay is a genotoxicity test system used for the detection of micronuclei in the cytoplasm of interphase cells. These micronuclei may originate from acentric fragments (chromosome fragments lacking a centromere) or whole chromosomes that are unable to migrate with the rest of the chromosomes during the anaphase of cell division. The assay detects the activity of bothclastogenic and aneugenic chemicals in cells that have undergone cell division after exposure to the test substance.

A short term *in vitro* cell culture protocol was followed for chromosomal aberration, by taking guidelines from OECD 473. Test compounds were added at 48^{th} h rof initiation of cell culture and continuous exposure was given to the cells. The process of cell growth was stopped at metaphase stage by addition of colchicine. After harvesting the cells chromosomal aberration was observed.

The CBMN was performed according to OECD guideline 487.Test compound were added at 48^{th} hr of initiation of cell culture. The cell cycle was stopped at interphase by addition of cytochalasin-B. After harvesting the binucleated cells were observed.

Statistical analysis for both the assay was done by using Student's t test. The results showed that the ZnO ENPs induced significant number of chromosomal aberrations as compared to its control, while *Bhasma* and bulk particles did not show any significant rise in number of aberrations. The bulk particles have been observed to inhibit the proliferation rate of the blood lymphocytes with increase in concentration, whereas the ZnO ENPs was observed to accelerate the rate of proliferation of lymphocytes.

The CBMN assay results indicate that none of the three test compounds showed considerable increase infrequency of micronuclei in binucleated cells compared to their respective controls.

Conclusion

Statistical analysis showed that ZnO ENPs may have clastogenic, but no aneugenic effect on blood lymphocyte, while *Yasada bhasma* and ZnO bulk particle did not show any adverse biological effect on human peripheral blood lymphocytes

Test compounds	Clastogenic activity	Aneugenic activity
ZnO ENPs	*	#
Yasada Bhasma	#	#
ZnO bulk particles	#	#

#: Non-significant; *: Significant

The objective of this paper was to summarize the knowledge about the genotoxic effect of ENPs, *Yasada Bhasma* in comparison to its counterpart ZnO bulk particles. The results of two cytogenetic endpoints are summarized above

- ZnO ENPs are clastogenic and significantly different from ZnO bulk particle, thus raising the question on its unmonitored use and its safety towards human and environment.
- *Yasada Bhasma* did not show any clastogenic or aneugenic effects on the cultured blood lymphocytes, it may be because of the procedure followed in its preparation.
- ZnO bulk particles also were not found to be clastogenic or aneugenic to the cultured blood lymphocytes which could be because of its particle size.

In May 2006 a petition was received by FDA which indicated requirement of laboratory data to confirm toxicity potential or otherwise of nanoparticles in reply to concerns raised by various organizations. Thus, the toxicity studies on nanoparticles need to be substantiated in terms of genotoxic potential also.

Further detailed study should be carried out for the better understanding of the adverse biological effects of ENPs. Some of our suggestions are;

1. Standardization of comet assay for cultured blood lymphocytes.
2. Genotoxic assessment of all the three test compounds at lower concentration to avoid mitotic index inhibition.
3. Characterization of ENPS in culture by Dynamic Light Scattering technique.

6.

Appendix

Appendix I: Tests for Physical Characterizationof *Bhasma (5)*

Physical characterization of *Bhasma* and nanoparticle was done by criteria mentioned in ayurveda to evaluate the degree of similarity between *Bhasma* and engineered nanoparticle and to check the quality of *Bhasma*.

(1) "*Varna"* (color): A specific color is mentioned for each *Bhasma*. Alterations in specific color suggest that *Bhasma* is not prepared properly. Because a particular metallic compound is formed during *Bhasma* preparation and every chemical compound possesses specific color.

(2) "*Nishchandratvam*": The *Bhasma* must be "*nishchandra*" (lusterless) before therapeutic application. "*Chandratva"* (luster) is a character of a metal. For this test *Bhasma* is observed under sun light. If luster or shining is still present it requires further incineration.

(3) "*Varitaratva*": This is applied to study lightness and fineness of *Bhasma*. To float on stagnant water surface is a character of *Bhasma*. This test is based on water surface tension. Little amount of *Bhasma* is taken and sprinkled slowly on stagnant water surface from a short distance. Properly incinerated *Bhasma* will float on water surface.

(4) "*Unama*" test: it is further assessment of "*varitara"* test. A grain of rice is to be kept carefully on the layer of floated *Bhasma*. Observe whether grain floats or sinks. If grain remains as it is on layer, then *Bhasma* can be considered as excellent (properly prepared).
(5) "*Rekhapurnata*": this test is applied to study fineness of *Bhasma*. *Bhasma* particles should be of minimum size for easy absorption and assimilation in body. *Bhasma* should be so fine that it

can fill furrows of finger tips. A little amount of *Bhasma* is rubbed in between index finger and thumb to observe whether particle can fill furrow of finger tips.

(6) "*Slakshnatvam*": It is tactile sensation produced by *Bhasma* by simple touch with finger tips. Properly incinerated *Bhasma* attain this quality. "*Slakshna*". *Bhasma* can be absorbed and assimilated in the body without producing any irritation to mucous membrane of gastrointestinal tract.

(7) "*Susukshma*": It indicates fineness of *Bhasma* preparation. This character can be perceived by "*varitara" and "Rekhapurnata*". *Bhasma* must be "*sukshma*", so that it can be absorbed in the body easily.

(8) Particle size: prepared *Bhasma* should be in "*Churna*" (powder) form, size of particles of *Bhasma* will be similar to pollen grains of Pendants odoratissimus flower (ketaki Rajah).

(9) "Gatarasatvam: Properly incinerated *Bhasma* of a metal should be of particular taste. It indicates transformation of particular metallic taste to compounds of specific taste.

7.

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