

# Genotoxicity evaluation of certain Bhasmas using Micronucleus and Comet assays

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## Abstract

Bhasmas, herbal preparations of ayurvedic origin, contain heavy metals in traces. Very little/ no published information is available on the preclinical toxicity or mutagenicity of these Bhasmas. Considering the recent controversy over the risk of toxic heavy metals in ayurvedic herbo-mineral preparations, we studied the genotoxic potential of few such preparations. Most popular Bhasmas were investigated - Ras Manikya Ras, Lauha Bhasma, Tamra Bhasma and Kajjali Bhasma. A single dose (200mg/kg b.w) was administered orally to Wistar rats. Peripheral blood leukocytes and bone marrow samples were collected. Micronucleus assay and the comet assay were employed to study the endpoint of chromosomal damage and single / double-strand DNA breaks. The results revealed lack of induction of Micronuclei or DNA damage as evidenced by the Comet assay, despite the presence of traces of transformed toxic heavy metals.

## INTRODUCTION

Rasa shastra (Vedic chemistry), a branch of Ayurveda, describes the use of metals, gems, minerals and even poisons for manufacturing special formulations to combat chronic and difficult diseases. Several metallic preparations with organic macromolecules termed "Bhasmas", in Ayurvedic literature, are employed in the treatment of a variety of disorders. Bhasma preparations involve the conversion of the metal into its mixed oxides, during which, the zero valent metal state is converted to a higher oxidation state. The significance of this "Bhasmikarana" is that the toxic nature of the resulting metal oxide is completely destroyed while introducing the medicinal properties into it<sup>18</sup>.

Traditionally, many Indians have been using the Ayurvedic herbo-mineral preparations (Bhasmas) for the treatment of chronic ailments. Although no systematic pre-clinical and clinical studies on the efficacy and toxicity of these preparations are published, they are considered to be safe in view of clinical experience as recorded in the ancient Indian documents<sup>7</sup>. Recently, Saper et al<sup>89</sup> documented significantly high levels of heavy metals, beyond permissible limits, in some Ayurvedic herbal preparations exported from India and those manufactured in the US. Following his publication, Government of India through AYUSH, Central Council for Research in Ayurveda and Siddha, began scientific studies to characterize the herbo-

mineral preparations, (Bhasmas and other heavy metal containing formulations) being used in India and exported and also to evaluate their toxicity in standard battery of animal investigations. Preliminary data gathered indicated no evidence of increased heavy metal content and the lack of adverse effects in animal studies<sup>414</sup>.

For sometime now, we have been involved in gathering preclinical and genotoxicity information on some commonly used Bhasmas. As a part of this, we report here our findings of genotoxicity with respect to four Bhasmas - Ras Manikya Ras, Lauha Bhasma, Tamra Bhasma and Kajjali Bhasma. We selected these Bhasmas for our studies due to their extensive clinical use in India. Further, we thought that these Bhasmas would be more pertinent for toxicity and genotoxicity investigations since they contain metal ions of arsenic, mercury etc which are intentionally incorporated during their preparation and since some of these metals are known genotoxic agents.

## MATERIALS AND METHODS

### SAMPLES

The Bhasmas - Ras manikya ras, Lauha Bhasma, Tamra Bhasma and Kajjali Bhasma were obtained commercially.

### CHEMICALS

Fetal Bovine Serum (Gibco), Cyclophosphamide (Sigma), Low melting agarose (Sigma), Normal melting agarose

(Sigma), Ethidium Bromide (Sigma), Tris base (Hi Media), Triton X 100 (Hi Media), DMSO (Sigma).

**ANIMALS**

Six to eight week old Wistar rats weighing 100-150gm were obtained from the animal house – breeding facility, International Institute of Biotechnology and Toxicology (IIBAT), where the study was conducted. Animals were acclimatized for seven days and maintained on a standard feed and water ad libitum. The study was approved by the Institutional Animal Ethics Committee of IIBAT.

**EXPERIMENTAL GROUPS**

Animals were divided into 6 groups of 10 animals each (5 males and 5 females). Group 1 served as vehicle control receiving the vehicle alone (Corn oil) orally, Group 2 animals were dosed with the positive control chemical, Cyclophosphamide at the concentration of 25mg/kg b.w intraperitoneally. Group 3, 4, 5 and 6 received the Bhasmas- Ras Manikya Ras, Lauha Bhasma, Tamra Bhasma and Kajjali Bhasma, respectively, at the dose of 2000mg/kg b.w. suspended in corn oil by oral intubation. (Dose selected based on pilot study, data not presented here).

**EXPERIMENTAL PROCEDURE**

**MICRONUCLEUS ASSAY**

After 24 hrs of dosing, the animals were sacrificed by cervical dislocation. The femurs were exposed, cut just above the knee and the bone marrow was aspirated into tubes containing Fetal Bovine Serum (FBS). The cells were centrifuged and the supernatant was drawn off. The cells were re-suspended in minimal volume of FBS. The bone marrow suspension was spread onto a clean glass slide. The slides were fixed in Methanol, stained with Giemsa and mounted. With the oil-immersion objective, 2000 immature Poly Chromatic Erythrocytes were scored for the presence of “Micronucleus”, which are defined as round, darkly staining nuclear fragment indicating chromosome damage. To evaluate any cytotoxicity induced by the administered substances at the dose selected, the proportion of Polychromatic Erythrocytes to Normo Chromatic Erythrocytes was determined in 200 cells for each animal in all treatment groups.

**COMET ASSAY**

The comet assay was performed under alkaline conditions essentially following the procedure of Singh et al<sub>13</sub> with slight modifications. The peripheral blood lymphocytes obtained from each animal in all groups was mixed with

0.7% low melting point agarose dissolved in Phosphate-Buffered Saline and casted to frosted microscope slides pre-coated with 1% normal melting agarose. The cells were then lysed for 1 h at 4°C in a buffer consisting of 2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, 10 mM Tris, pH 10. After lysis, the slides were placed in an electrophoresis unit, allowing DNA to unwind for 20 min, in the electrophoretic buffer consisting of 300 mM NaOH, 1 mM EDTA, pH > 13. Electrophoresis was conducted at ambient temperature of 4°C for 25 min at electric field strength 0.56 V/cm (300 mA). The slides were then neutralized with 0.4 M Tris, pH 7.5, stained with 2 µg/ml Ethidium Bromide and covered with cover slips. To prevent an additional damage all the steps described above were conducted under dimmed light.

The objects were observed at 400x magnification in a Carl Zeiss Axiophot fluorescence microscope (Carl Zeiss, Germany). For DNA damage analysis, 100 cells were scored per slide (Singh and Stephens, 1997). Cells were assigned to various damage degrees, visually, based on their tail intensities. The percentage of damaged cells was calculated manually. An arbitrary unit (AU) was used to express the extent of DNA damage.

**Figure 1**

$$AU = \sum_{i=0}^4 n_i \times i$$

Where n<sub>i</sub> is the number of cells with damage degree i (0, 1, 2, 3 or 4)

**RESULTS**

**MICRONUCLEUS ASSAY**

Table 1 shows that the ratio of Poly Chromatic Erythrocytes and the Normo Chromatic Erythrocytes (Fig 1a) did not show any remarkable change in the treated versus the control groups indicating that the dose (2000mg/kg body weight) was non-cytotoxic. There was a significant increase in the number of MN-PCEs (Fig 1b) at the non-cytotoxic dose of Cyclophosphamide administered in the positive control group. No statistically significant increase in MN frequency with respect to the control values was recorded.

Figure 4

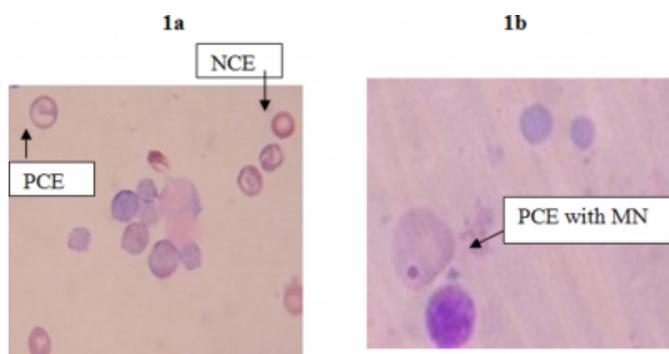
Table 2: Results of comet assay

C - CONTROL, PC- POSITIVE CONTROL, B1- RAS MANIKYA RAS, B2- LAUHA BHASMA, B3- TAMAR BHASMA, B4-KAJJALI BHASMA

Parameter	Sex	Control	PC	B1	B2	B3	B4
Mean PCE: NCE in 200 Cells	Male	1.41	1.34	1.40	1.20	1.41	1.36
		± 0.17	± 0.21	± 0.23	± 0.18	± 0.21	± 0.24
	Female	1.39	1.31	1.27	1.31	1.36	1.38
		± 0.26	± 0.20	± 0.24	± 0.26	± 0.23	± 0.15
Mean frequency of MN – PCEs	Male	1.9	27.6	1.8	1.8	0.5	0.5
		± 0.56	± 11.10	± 1.62	± 1.62	± 0.85	± 0.70
	Female	2	26.8	1.3	1.9	0.6	0.4
		± 0.47	± 4.61	± 0.48	± 1.67	± 0.85	± 0.52

Figure 3

Figure 1: Micronucleus assay



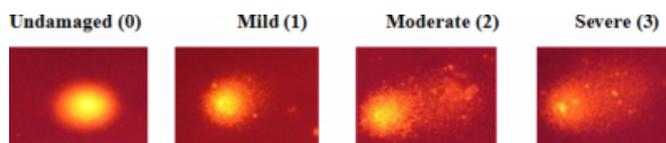
COMET ASSAY

The DNA damage percentage in the different groups (Fig 2) (Table 2) show that the background frequency is around 7.8% in Males and 8.8% in Females. In the control group (Group 1), the damages recorded were in the category of 1 and 2, which are mild and moderate. No third or fourth degree damages were observed. The positive control group (Group 2) has remarkable DNA damage accounting to 65.8% in Males and 62.6% in Females, covering all the categories of DNA damage in both the sexes. This confirms the sensitivity of experimental conditions employed. Group 3- Ras Manikya Rasa induced 7.6% and 5.6% DNA damage in Males and Females respectively. Group 4 (Lauha Bhasma) animals had an average of 6.2 % damage in both Males and Females, which is comparable to the control group. Group 5 animals treated with Tamra Bhasma had 5.6% and 4.4% DNA damage in Males and Females respectively, which is on par with the control values. In Group 6 animals treated with the Kajjali Bhasma, the DNA damage percentage was 5% and 6.8% respectively. The values are comparable to the control group. The DNA damage recorded in the control group is accredited to have occurred due to spontaneity and / or environmental factors.

None of the damages seen in the treated groups seem to be related to the Bhasmas administered as the values are not statistically significant and are concordant with the control group. The Arbitrary Unit (AU) calculated also complies with the fact the Bhasmas investigated do not cause any remarkable DNA damage in the animals tested.

Figure 5

Figure 2: Comet assay – Various degrees of DNA damage



{image:5}

DISCUSSION

Metallic herbal preparations offer advantages over plant drugs by virtue of their stability over a period, lower dosage, easy storability and sustained availability and contain minerals and metals as integral part of the formulations. They are being used with an intention to give therapeutic efficacy to the product of the designated illness. Use of metals in medicine is often associated with toxicity. It is to remove those toxic qualities of the metals that the preparations undergo various processes of purification (Shodhana, Marana, and Samskara). The metals and minerals are mixed with herbs because they are considered non-living and by treating them with herbs they are converted to a living state thereby becoming bio-compatible. The same metal processed with different herbs acts on different organs in the human body.

Ras manikya ras is used as an antiperiodic, expectorant and alterative. It is also indicated in case of cough, asthma and chronic fever. As an herbal formulation containing arsenic, it was tested for its genotoxic potential, as chronic exposure to arsenic is associated with the risk of cancer. Though arsenic is considered as the king of poisons, it is considered as an essential element in traditional medicine with analgesic activity and pro-convulsant effect. In the present study, there was no incidence of genotoxicity induced by this Bhasma in the Micronucleus assay or the Comet assay. The ready absorption of arsenic in the gut and its excretion in the urine might have contributed to this effect.

Lauha Bhasma is an iron-based ayurvedic preparation. Iron nourishes blood, enhances vigor and its astringency prevents blood from becoming too hot or too fluid. It is an essential element of hemoglobin, playing an important role in oxygen

transport<sup>5</sup>. Lauha bhasma is used in anaemia similar to iron tablets/capsules/syrup prescribed as iron supplementation in allopathy. These bhasmas are prepared from purified iron filings/ferric oxide or magnetic iron incinerated with decoction of Trifala, Ghritkumari ras, vinegar and sesame oil<sup>2</sup>. Animal studies state that Lauha Bhasma has haematinic activity and is non-toxic to animals. The present study shows that it is also safe in terms of genotoxicity, since it did not cause any increase in the percentage of micronucleus or DNA damage.

Tamra Bhasma is derived from metallic copper that is recommended for different ailments of liver and spleen, abdominal pains, colitis, heart problems, anaemia, tumors, loss of appetite, eye troubles and tuberculosis. Copper is an integral part of several enzymes and influences the immune system. Pharmacological investigations have reported the use of tamra bhasma for treating gastric ulcers and secretion, the management of lipid peroxidation in the liver of albino rats and as an antioxidant<sup>111516</sup>. It also participates in lipid peroxidation with no detectable adverse effects<sup>3</sup>. Tamra Bhasma did not induce micronuclei formation or an increase in the percentage of DNA damage, showing its safety upon consumption.

Kajjali Bhasma is a Mercury based preparation. Mercury is an environmental and industrial contaminant known for its toxicity causing Minimata disease<sup>5</sup>. Having said that, it is used in Ayurveda, as it is considered as a marvel drug as a nervine tonic and for restoring normalcy to collapsing patients. In a radioisotope tracer analysis to study the bioavailability and bio-distribution of Kajjali Bhasma no ill effects were observed in any of the organs in Wistar rats including the brain<sup>10</sup>. Its toxic effects were said to be neutralized in the presence of sulfur<sup>2</sup>. Kajjali was found to promote growth and facilitate learning process in animal studies<sup>17</sup>. In the present investigation, no incidences of genotoxicity, in terms of micronuclei induction or DNA damage was recorded in animals treated with Kajjali Bhasma, which re-emphasizes its safety for human consumption despite its trace mercury content.

The results reveal that the four Bhasmas investigated are not genotoxic in the Micronucleus and the Comet assays. The micronucleus assay used in this investigation is a powerful technique that detects chromatid / whole chromosome loss. The comet assay is presently used world wide in pharmaceutical industries for preliminary screening of drugs and genotoxicity assessment of heavy metal contaminations

in various test species. This assay requires fewer samples and is cost-effective. It detects DNA damages in the form of Single and double-strand breaks and alkali-labile sites (ALS).

The results reiterate the fact, Bhasmas, despite their trace heavy metal content, are safe, when appropriately manufactured and consumed as per directed instructions. It also re-emphasizes that the mere presence of a chemical compound of metallic origin does not contribute to the toxicity of the finished product as the standard manufacturing process inflicts intense changes and components of herbal origin after sequential reactions with diverse components of processing is responsible for the therapeutic action.

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