ORIGINAL ARTICLE

Assessment of genotoxic potential of *Tamra Bhasma* (incinerated copper)

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Introduction: The presence of metallic content in Ayurvedic drugs became an important burning issue in present days. The usefulness of *Bhasmas* (incinerated metals/minerals) in therapeutics, their safety or toxicity is frequently being raised on different platforms. Considering this, there is a need to develop toxicity profiles of different metals/minerals. *Tamra Bhasma* (incinerated copper) one such metallic formulation is widely used in cardiac and lipid disorders by Ayurvedic Physicians. The present study is aimed to evaluate the genotoxic potential of *Tamra Bhasma*. **Materials and Methods:** It was prepared as per classical guidelines and administered to Swiss albino mice for 14 consecutive days. Chromosomal aberration and sperm abnormality assay were studied. **Results:** All treated groups exhibited significant body weight gain in comparison to cyclophosphamide (CP) group. Results revealed no structural deformity in above parameters in comparison to CP group. **Conclusion:** Reported data showed that both tested samples of *Tamra Bhasma* were not genotoxic and can be used safely.

Key words: Bhasma, chromosomal aberration, cyclophosphamide, genotoxicity, sperm abnormality assay, *Tamra Bhasma*

INTRODUCTION

Ayurveda, an ancient medical system of Indian subcontinent utilizes natural resources including herbs, metals, minerals, etc., and formulations in therapeutics. *Rasashastra*, an integral part of Ayurveda, exclusively deals with different types of metals, minerals, their origin, processing techniques, properties, therapeutic uses, possibilities of developing adverse effects and their management in a comprehensive way. Seers emphasized on following classical principles, while converting these metals into *Bhasmas* (incinerated metals/minerals) to facilitate in therapeutics and to eliminate possibilities of untoward effects if any. Classics of *Rasashastra* exclusively deal with wide range of *Bhasmas*, their therapeutic effects, and method of administration. *Tamra Bhasma* (incinerated copper), one among such *Bhasmas* is recommended for various ailments like ascites (*Udara*), anemia (*Pandu*), bronchial asthma (*Svasa*), hyperacidity (*Amlapitta*), etc. Though the metallic preparations are effective therapeutically, the presence of heavy metals always became a point of concern in the current scenario.[1-3] Huge number of studies have been carried out in recent past that evaluated the safety of metallic preparations.[4,5] The studies concluded that the metals or minerals when converted into the medicines by strictly following classical guidelines as specified in ancient texts are devoid of toxicity even when administered at higher doses than the specified therapeutic doses. In addition to the existing toxicity studies, Government of India expressed the need on conducting genotoxicity studies of different metal- or mineral-based drugs.[6] Sperm abnormality assay and chromosomal aberration (CA) assay are one of commonly used methods to evaluate the genotoxic potential of drugs.[7] Till date, very few studies have been carried out in this direction, and there is a need to extend such studies with all drugs of metal or mineral in origin. Genotoxic evaluation of *Tamra Bhasma* using micronucleus (MN) and comet assay was reported.[8] Considering the significance of this aspect, it is planned to study possibilities of genotoxic potentials of *Tamra Bhasma* using CA and sperm abnormality assay.

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MATERIALS AND METHODS

Test Drugs

Both the trial drugs (*Tamra Bhasma* with and without *Amritikarana* [TB and TBA]) were prepared in the Department Laboratory by following standard guidelines as prescribed in the classical Ayurvedic literature.

Copper scraps with 99.89% pure copper were procured from Bharat Bridge Plate, Jamnagar. It was subjected to general, and specific purification procedure followed by incineration after mixed with purified sulfur, *Kajjali* (black sulfide of mercury), and juice of *Citrus jambhiri* Lush. In *Amritikarana*, it was mixed with half part purified sulfur and juice of *C. jambhiri* Lush., kept in corm of *Amorphophallus campanulatus* Linn. It was subjected to heat treatment and labeled as TBA. Another sample was subjected to the process of *Marana* and labeled as TB. These two coded samples were subjected to evaluate genotoxic potential using CA and sperm abnormality assay.

Chemicals

Cyclophosphamide (CP) was procured from Getwell Pharmaceuticals, Gurgaon, Haryana (Batch No 3GCYOZ). Colchicine (Batch No T8371720), methanol, acetic acid, and potassium chloride were obtained from Sisco Research Laboratory, Mumbai, India.

Animals

Adult Swiss albino mice of either sex, weighing 30 ± 5 g were used in the study. Animals were obtained from animal house attached to the Pharmacology Laboratory, SSR College of Pharmacy, Silvassa and were exposed to natural day and night cycles, with ideal laboratory conditions in terms of ambient temperature and humidity. The temperature during the time of carrying out the experiment was between 24° ± 2° and humidity 50–60%. Animals were fed *ad libitum* with Amrut brand mice feed supplied by Pranav Agro Industries and reverse osmosis purified water. The experiment was carried out after obtaining the permission from Institutional Animal Ethics Committee (Approval number: IAEC/2013/04) and care of animals was taken as per the CPCSEA guidelines.

Dose Fixation

Clinical dose of *Tamra Bhasma* is 30 mg twice a day for human being.[9] The suitable dose for mice was calculated by referring to table of Paget and Barnes and was found to be 7.8 mg/kg.[10] The test drugs were administered in the form of a suspension made in honey orally with the help of rubber catheter attached to a disposable syringe. For the preparation of stock solution, both the test drug samples were taken in requisite quantity in small porcelain mortar and honey10 ml/kg mice was added, the formed mixture was further grounded for 5 min and homogenous mixture was formed.

Experimental Design

The animals were randomized into five groups consisting of five animals in each group for evaluating the influence of TB and TBA on CA and sperm morphological abnormality. Group I served as normal control (NC) receiving tap water and normal food. Group II served as positive control and treated with CP single dose 25 mg/kg intra-peritoneally 24 h prior to termination.[7,11] Group III served as vehicle control (VC) and treated with honey 10 ml/kg body weight. Groups IV and V were treated with both test drugs 7.8 mg/kg body weight along with honey for 14 consecutive days and sacrificed on the 15th day [Table 1].

Body Weight

Animals were examined throughout the experimental period for signs of gross toxicity. Body weight was recorded initially and at the time of sacrifice on the 15th day.

Chromosomal Aberration Assay

Animals were injected colchicine intra-peritoneally at the dose of 4 mg/kg body weight, on the 15th day to arrest dividing cells in metaphase[12] and sacrificed by cervical dislocation, 90 min after the colchicine treatment. Bone marrow cells from both femurs were extracted, subjected to hypotonic shock treatment (KCL 0.075M), for about 30 min, at room temperature and then centrifuged at 1000 rpm for 10 min. The cells were fixed 5 times using freshly prepared methanol-acetic acid (3:1). The cells were spread on clean glass slides that were dried on the hot plate at 40°C. One more drop of fixative was added on slides to see more reliable pictures of chromosomes and then the slides were air-dried at room temperature and finally stained with 5% dilution of Giemsa reagent in phosphate buffer (pH 6.8) for 15 min.[13] The chromosomes of 100 metaphase cells per group and 20 metaphases per animal were analyzed with a ×100 oil immersion objective, using a Trinocular Research Carl Zeiss Microscope (Germany). Metaphases with chromosomes and chromatid breaks, gaps, rings,

Table 1: Posology

Group	Number of animals	Drug	Dose	Duration
I	5	WC	-	14 days
II	5	CP	25 mg/kg	
III	5	VC	10 ml/kg	
IV	5	TB	7.8 mg/kg	
V	5	TBA	7.8 mg/kg	

CP – Cyclophosphamide control, VC – Vehicle control, TB – *Tamra Bhasma* without *Amritikarna*, TBA – *Tamra Bhasma* with *Amritikarana*, WC – Water control

stickiness, dicentrics, centric fusion, and deletion (if any) were recorded.[14]

Sperm Abnormality Assay

The method of Wyrobek and Bruce was used for investigating sperm morphology abnormality assay.[15] The test preparations were administered for 14 days to correlate the results with positive control group. On the 15th day, the overnight fasted animals were sacrificed by cervical dislocation and dissected out. Both the cauda epididymis were removed and placed in watch glass containing 1 ml phosphate buffered saline (pH 7.2) then minced and teased carefully well with fine scissors and forceps to release the spermatozoa. After gentle pipetting, the suspension was separated from the tissue fragments and filtered through double layers of muslin cloth to remove the tissue debris. A drop of eosin Y solution (10:1) was added to this suspension and kept for 30 min. air-dried smears were prepared on clean, grease-free glass slides, and a uniform smear was made. About 1000 sperms per animal were examined at ×400 from each treatment and control groups for the presence of sperm morphological abnormalities.

Statistical Analysis

Statistical methods were carried out to assess the change in body weight by applying paired *t*-test. The results were presented as mean ± standard error of mean for five mice in each group by using SigmaStat Software (version 3.1) for all the treated groups with the level of significance set at $P < 0.05$. Statistics were not applied in CA assay and sperm abnormality assay.

OBSERVATIONS AND RESULTS

The effect of TB and TBA in body weight, CA, and sperm abnormality assay are shown in Tables 2-4.

Table 2: Effect on body weight of Swiss albino mice during study

Group	Treatment	Body weight (g)		*t*	*P*
		Before treatment	On 15th day		
I	NC	32.00±0.73	37.16±0.70	10.826	<0.001
II	CP	31.00±0.51	32.66±0.80	3.953	0.01
III	VC	31.83±0.79	38.00±0.85	12.921	<0.001
IV	TB	31.83±0.70	37.50±0.84	17.000	<0.001
V	TBA	31.50±0.80	37.83±0.65	15.021	<0.001

Data: Mean±SEM, NC – Normal control, CP – Cyclophosphamide control, C – Vehicle control, TB – *Tamra Bhasma* without *Amritikarna*, TBA – *Tamra Bhasma* with *Amritikarana*, SEM – Standard error of mean

Table 3: Effect on sperm abnormality assay of Swiss albino mice during study

Groups	Sperm abnormality assay					
	Head abnormalities				Tail abnormalities	
	Amorphous shape	Hookless	Banana shaped	Folded	Double tailed	coiled
NC	-	-	-	-	-	-
CP	+	+	+	+	-	+
VC	-	-	-	-	-	-
TB	-	-	-	-	-	-
TBA	-	-	-	-	-	-

NC – Normal control, CP – Cyclophosphamide control, VC – Vehicle control, TB – *Tamra Bhasma* without *Amritikarna*, TBA – *Tamra Bhasma* with *Amritikarana*

DISCUSSION

Seers of Ayurveda were well aware about the toxicity and documented possible untoward toxic effects that can occur with the improper usage of metals or minerals. They have documented specific processing techniques, therapeutic dosage, and concurrent diet advice to avoid such ill effects. Being used for over a long period, these medicines are acknowledged as safe, which is the ultimate proof for their non-toxic beneficial effects. However, the use of metallic preparations has raised concerns and debate in the scientific community in the past couple of decades. *Tamra Bhasma* and STB, one of such herbo-metallic preparations is proven to be safe through acute and sub-chronic toxicity studies.[16,17] Antibacterial activity against enteric pathogens and antihyperlipidemic activity of *Tamra Bhasma* was also reported.[18,19] Incinerated copper is reported to have a role in hepato-protection and lipid peroxidation and exhibiting free radical scavenging activity suggesting its strong antioxidant potential.[20] The study concludes that *Tamra Bhasma* did not induce micronuclei formation or an increase in the percentage of DNA damage. The present study was undertaken to address reports of genotoxic

Table 4: Effect on chromosomal aberration of Swiss albino mice during study

Groups	Chromosomal aberration									
	Chromatid		Chromosomal		Deletion	Exchange	Fragments	Pulverization and stickiness	Ring	Dicentric
	Gap	Break	Gap	Break						
NC	-	-	-	-	-	-	-	-	-	-
CP	+	+	+	+	+	+	+			+
VC	-	-	-	-	-	-	-	-	-	-
TB	-	-	-	-	-	-	-	-	-	-
TBA	-	-	-	-	-	-	-	-	-	-

NC – Normal control, CP – Cyclophosphamide control, VC – Vehicle control, TB – *Tamra Bhasma* without *Amritikarna*, TBA – *Tamra Bhasma* with *Amritikarana*

effect due to administration of *Tamra Bhasma* prepared TB and TBA for 14 days in mice.

In vivo, CA assay in metaphase cells is one among simplest and most sensitive test to assess genotoxic profile of drugs in which direct visualization of the deleterious effects at the genetic level can be studied by microscopic observations. CA are based on the structural unit involved that is whole chromosome or the single chromatid and the type of morphological alterations like breaking or rearrangements, etc.[21] It is also suggested that CAs are early predictors of cancer risk.[22] Colchicine acts on sub cellular level and used to arrest metaphase when chromosome structure is to be seen evidently. Specifically, it interferes with microtubule growth and, therefore, affects mitosis so known as mitotic poison or spindle poison. It inhibits microtubule polymerization into microtubules by binding to intracellular protein tubulin, leading to the inhibition of leukocyte migration and phagocytosis.[23] CP is cytotoxic bifunctional alkylating agent widely used in neoplastic therapy and other non malignant diseases such as rheumatoid arthritis, systemic lupus erythematosus, and vasculitis. It is also used as immunosuppressive in higher doses prior to organ transplantation.[24] The cytotoxic effects of CP and other chemotherapeutic drugs result in part from their interaction with DNA leading to defective DNA, abnormal cell function and cell death. In somatic cells, CP has been shown to produce gene mutations, chromosome damage, and micronuclei in rats, mice, and Chinese hamster.[25] It is usually used as a standard positive control in CA, sister chromatid exchanges, and MN formation *in vitro* and *in vivo* tests for a short duration.[26]

In the present study, a 14-day genotoxic profile of TB and TBA was evaluated by employing CA assay and sperm abnormality assay *in vivo*. Body weight of mice was recorded after 14 days of drug administration and compared with CP group. It showed significant normal

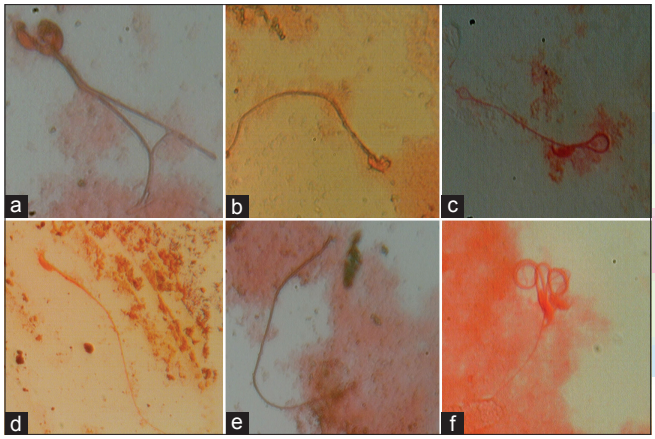

Figure 1: (a) Cyclophosphamide-abnormal sperm, (b) cyclophosphamide-amorphous, (c) cyclophosphamide-folded tail, (d) cyclophosphamide-banana shaped, (e) cyclophosphamide-headless, (f) cyclophosphamide-intermingled (×400)

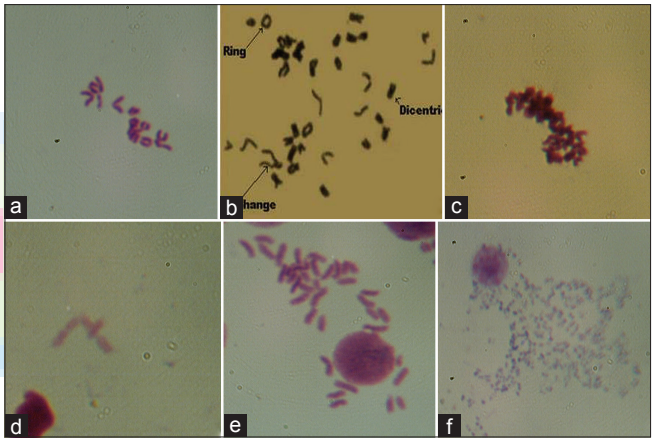

Figure 2: (a) cyclophosphamide-ring, (b) cyclophosphamide-ring, exchange and dicentric, (c) cyclophosphamide-stickiness, (d) cyclophosphamide-translocation, (e) cyclophosphamide-gap, (f) cyclophosphamide-pulverization (×100)

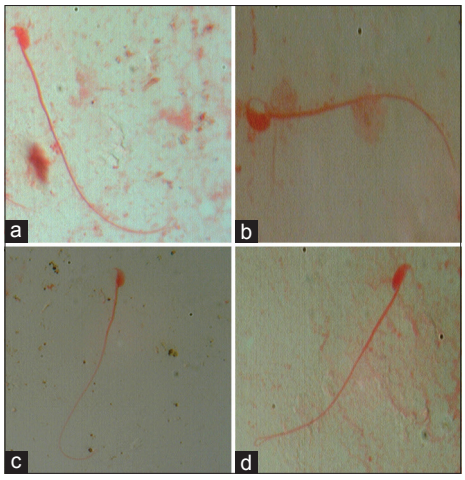

Figure 3: (a) Normal sperm, (b) vehicle control treated sperm, (c) *Tamra Bhasma* treated sperm, (d) *Tamra Bhasma* with *Amritikarana* treated sperm (×400)

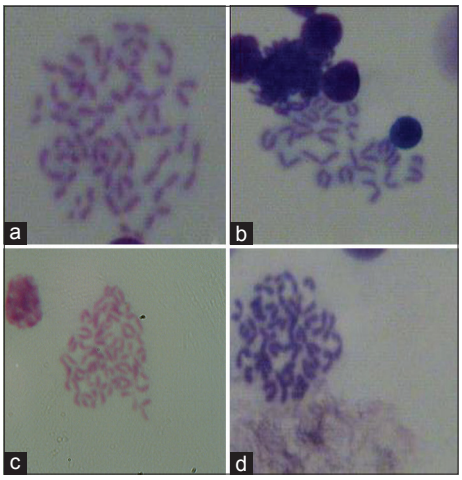

Figure 4: (a) Normal chromosome, (b) vehicle control treated chromosome, (c) *Tamra Bhasma* treated chromosome, (d) *Tamra Bhasma* with *Amritikarana* treated chromosome (×100)

progressive weight gain in all treated groups. This indicates that test drug did not have any toxic degenerative potential [Table 2].

Morphological abnormalities of sperm like head abnormalities (banana-shaped, hookless, amorphous, and folded head) and tail deformities (doubled tailed and coiled) were studied. CP treated group showed the maximum number of sperm abnormalities in both head and tail as shown in Table 3 [Figure 1a-f]. Increased frequency of CAs like chromatid break, gap, stickiness, and ring was also found in CP treated group as shown in Table 3 [Figure 2a-f]. Both the test drugs do not show any aberration in sperm and chromosomal assay [Figures 3 and 4].

CONCLUSION

Results obtained in the present study revealed that there is a lack of deformity in CA and sperm morphological abnormality assay. Hence, it can be concluded that therapeutic use of *Tamra Bhasma* prepared TB and TBA is safe at the genetic level.

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Conflicts of Interest

There are no conflicts of interest.

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