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Pharmacological Study

Evaluation of subchronic genotoxic potential of *Swarna Makshika Bhasma*

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Abstract

Extremely diminutive published information is available on the mutagenic activity of Ayurvedic *Bhasmas*. Genotoxicity of few *Bhasmas* were reported on single maximum dose, but no reference is available on the sub-chronic level. Hence the present study was carried to generate and evaluate genotoxic potentials of *Swarna Makshika Bhasma* (mineral preparation) administered at therapeutic dose for 14 days. Chromosomal aberrations and abnormal sperm assay parameters were taken in this study. Cyclophosphamide (CP) was taken as positive group and results were compared. The results revealed a lack of generation of structural deformity in above parameters by tested drugs compared to CP treated group. Observed data indicate that the *Bhasmas* tested were non-genotoxic under the experimental conditions.

Key words: Abnormal sperm assay, chromosomal aberrations, cyclophosphamide, genotoxicity, *Swarna Makshika Bhasma*

Introduction

Ayurveda is known and carried forward as an ancient Indian heritage. It is a traditional medical system used by a majority of Indian population.[1] The drugs known as "*Bhasmas*" are well-known in the traditional Indian Ayurveda and these are chemically mixed oxides of one or more metals.[2] Their traditional preparation involves conversion of a pure metal into its oxide form following a typical procedure, available in the ancient literature of Ayurveda.[3] Recently, doubts have been raised about the safety of Ayurvedic preparations using *Bhasma* and concerns were expressed regarding the metal toxicity of traditional preparations containing *Bhasmas*. Ayurveda fraternity claims that these medicines, if properly prepared and administered are safe and therapeutic.[4] According to them toxicity can arise only from a metal in its free form, and that a *Bhasma* prepared according to the classical methods never contains a metal in free form. Despite these theories claiming *Bhasmas* are non-toxic, documented case reports of poisoning was noted.[5,6] Hence the current study of *Swarna Makshika Bhasma* (SMB) would serve as a database of baseline information for genotoxicity, since there is apparently no literature on this aspect of mineral preparations.

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Materials and Methods

Test drugs

Three different samples of *Swarna Makshika* (SM) were collected from different mines across India. *Bhasmas* were prepared according to classical reference.[7] Coded as mentioned below:

1. SMB prepared from samples collected from Khetri Mine, Rajasthan (SMBKR).
2. SMB prepared from samples collected from Hatti Gold Mine, Karnataka (SMBHK).
3. SMB prepared from Malharjkhand Mine, Madhya Pradesh (SMBMM).

Chemicals

Colchicine was obtained from Hi-media, Mumbai. Cyclophosphamide (CP) procured as Cyphos vial from Intas Pharmaceuticals, Mumbai. Potassium chloride, methanol, acetic acid, giemsa stains were obtained from Sissco Research Laboratory, Mumbai, India.

Study design

Animals

Adult Swiss albino mice (weighing 25 ± 5 g) procured from

an institutional animal house attached to SSR College of Pharmacy, Silvasa, where the study was conducted. Animals were maintained with controlled temperature (23 ± 2°C), relative humidity of 50 ± 10% and 12 h light/dark photo-period. The animals were acclimatized for 7 days prior to the experiment and provided with standard mice feed and distilled water *ad libitum*. Study was carried out after obtaining approval from Institutional Animal Ethics Committee (IAEC/2011/01).

Animals were randomly divided into five groups (5 mice per group) after an acclimatization period. Group I served as positive control and challenged with CP single-dose of 25 mg/kg body weight intra-peritoneally 24 h prior to termination. Group II served as vehicle control. Vehicle was prepared in combination of honey and deionized water (with a ratio of 1:1.5) and administered in the dose of 0.5 ml/kg body weight as per CCRAS/NIN guidelines. Group III, IV, and V received SMBKR, SMBHK and SMBMM at therapeutic dose 4.5 mg/kg body weight respectively along with vehicle for 14 consecutive days and sacrificed on the 15th day. The doses of test drugs were calculated as per the reference of Paget and Barnes (1969).

Body weight

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

Experimental procedure

Chromosomal aberration assay

Animals were injected colchicine intra-peritoneally at the dose of 4 mg/kg body weight, on the 15th day in order to arrest dividing cells in metaphase[8] 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 0.075 M), 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 a 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 a 5% dilution of Giemsa reagent in a phosphate buffer (pH 6.8) for 15 min. The chromosomes of 1000 cells in metaphase abnormalities were analysed with a ×100 oil immersion objective, using a Trinocular Research Carl Zeiss Microscope (Germany). Metaphases with chromosomes and chromatid breaks, gaps, rings, stickiness, centric fusion, and deletion were recorded.[9]

Sperm abnormality assay

The method of Wyrobek and Bruce[10] was used for investigating sperm morphology abnormality assay. The test preparations were administered for 14 days, to correlate the results with positive control group. On 15th day, the overnight fasted animals were sacrificed by cervical dislocation and dissected out. Both the cauda epididymus were removed and placed in a 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 magnifications from each treatment and control groups for the presence of sperm morphological abnormalities.

Statistical analysis

Statistical methods were carried out only to assess change in body weight by applying paired *t*-test. Statistics were not applied in CA assay and sperm abnormality assay. Different kinds of morphological changes were observed in CAs and sperm abnormal aberrations.

Observations and Results

The effect of SMBs on body weight, chromosomal aberration and sperm abnormality assay are shown in Tables 1-3.

Discussion

In vivo CA assay is one of the most frequently used and sensitive tests for the detection of the genotoxic profiles of drugs. The test has been recommended for routine analysis and data obtained are considered highly relevant in human context.[11,12] In the present study a 14-day sub-chronic genotoxicity of SMBs prepared by different samples are evaluated by employing *in vivo* CA assay and abnormal sperm assay (ASA). Although the genotoxic profile of some of *Bhasmas* have been evaluated in various studies,[13] Till date no reports of sub-chronic genotoxicity studies on *Bhasmas* and SMB are available. With this view body weight of animals also recorded after 14 days of drug administration and compared with CP group [Table 1]. All treated groups exhibited significant gain in body weight in comparison to CP. Body weight loss is an indicator of marked tissue loss in the body protein degradation. Gain of body weight is indicating that test drugs are not bearing degenerative potentials.

Colchicine is effective in causing metaphase stasis in cell dividing matrix.[14] Thus used to arrest metaphase, when chromosome structure seen noticeably. It inhibits microtubule polymerization by binding to tubulin, one of the main constituents of microtubules. Availability of tubulin is essential to mitosis, and therefore, colchicine effectively functions as a "mitotic poison" or spindle poison. Hypotonic solution (KCl) causes the cells to swell and enhances eventual separation of the chromosomes to facilitate visual analysis.

Table 1: Effect of SMBs on body weight

Groups	Body weight		
	Before treatment	On 15th day	Actual % changes
CP	25.20±00.86	27.30±00.68↑	06.58±02.09
VC%	25.60±01.08	30.30±00.73↑	20.92±01.29***
SMBKR	26.00±00.89	30.50±00.67↑	20.13±01.65***
SMBHK	25.80±01.07	30.00±00.62↑	21.28±02.36**
SMBMM	26.60±00.81	29.60±00.78↑	20.16±01.66**

Data: Mean±SEM; ↓: Decrease; ↑: Increase; **P<0.01; ***P<0.001 (unpaired *t* test in comparison to CP group). CP: Cyclophosphamide; VC:Vehicle control; SMBKR: *Swarna Makshika Bhasma* prepared from Khetri mine, Rajasthan; SMBHK: *Swarna Makshika Bhasma* prepared from Hatti gold mine, Karnataka; SMBMM: *Swarna Makshika Bhasma* prepared from Malharjkhand mine, Madhya Pradesh; SEM: Standard error of mean

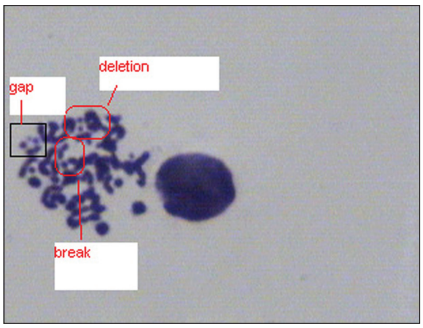

Figure 1a: CP-chromosomal and chromatid break and gap

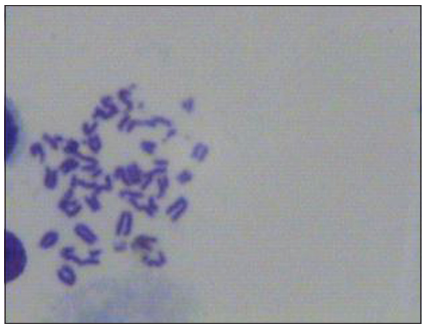

Figure 1b: CP-Deletion

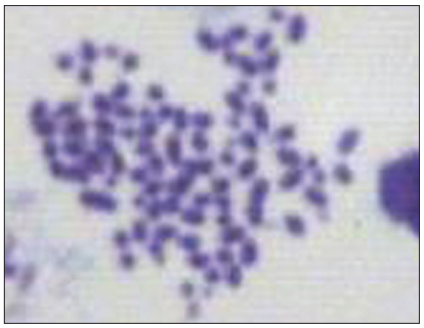

Figure 1c: CP- Pulverization

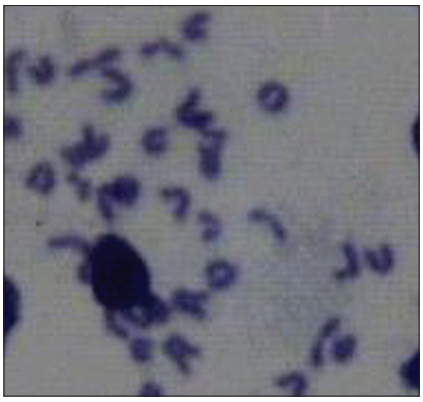

Figure 1d: CP-ring

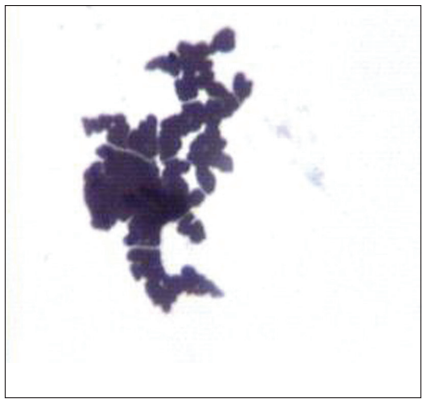

Figure 1e: CP-stickiness

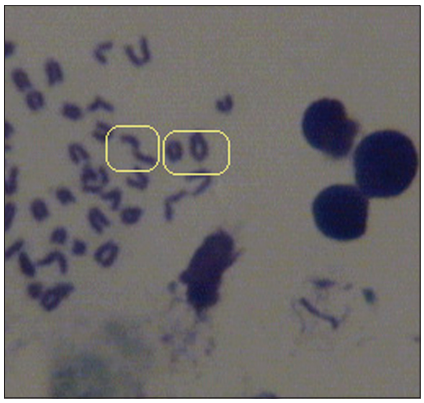

Figure 1f: CP-Translocation and ring

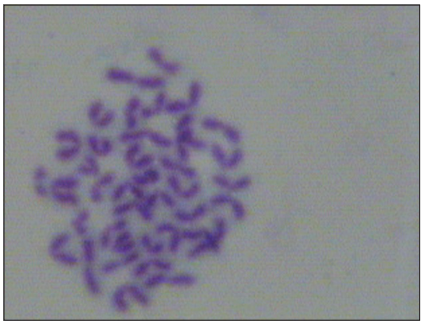

Figure 1g: Normal chromosomes

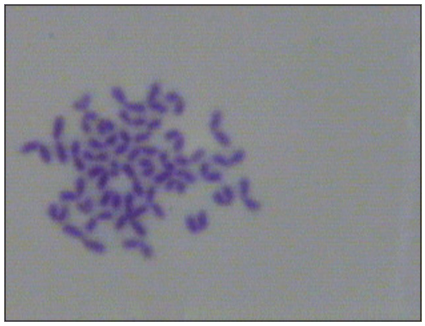

Figure 1h: Normal chromosomes – SMBKR

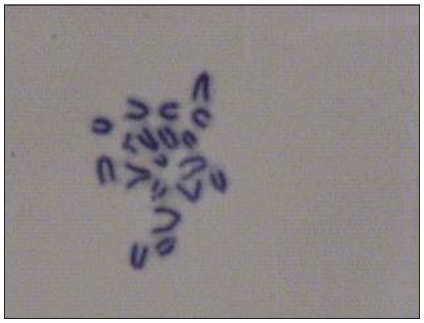

Figure 1i: Normal chromosomes – SMBHK

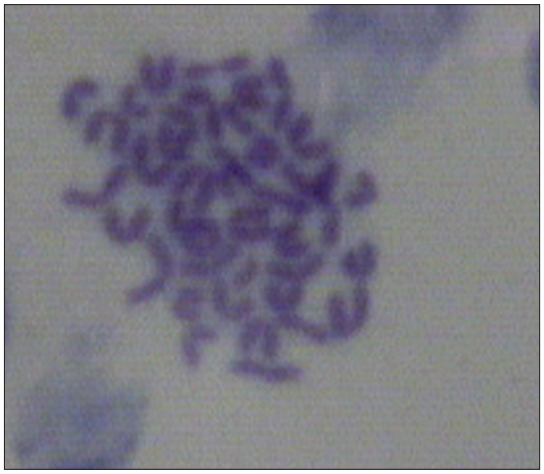

Figure 1j: Normal chromosomes – SMBMM

CP is an anticancer drug that is widely used in anti-neoplastic therapy as well as in the treatment of some non-malignant diseases like rheumatoid arthritis. It is also used as an immunosuppressive agent prior to organ transplantation.[15] In somatic cells, CP has been shown to produce gene mutations, chromosome aberrations, micronuclei and sister chromatid exchanges in a variety of cultured cells in the presence of metabolic activation as well as sister chromatid exchanges without metabolic activation. The compound also produced chromosome damage and micronuclei in rats, mice and Chinese hamster.[16] Its use as a positive control chemical in genotoxicity tests has been recommended.[17] It increased the number of chromosome aberrations in the given dose with relatively high frequencies of chromosome breaks, centric fusion compared with other types of chromosome abnormalities. Gap, ring formation as well as stickiness were also frequent in CP treated group [Figure1a-f]. This may be

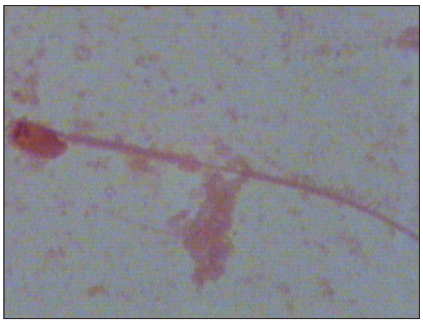

Figure 2a: CP – Hook less head

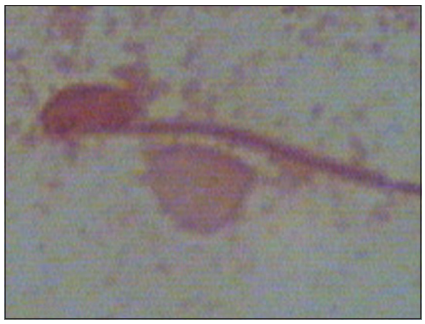

Figure 2b: CP – Deletion

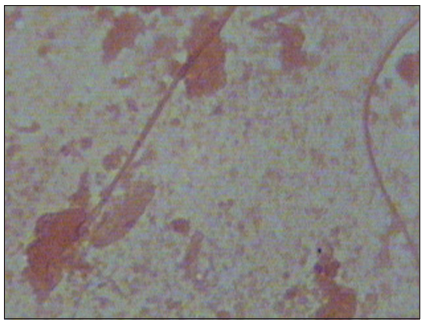

Figure 2c: CP – Banana shaped head

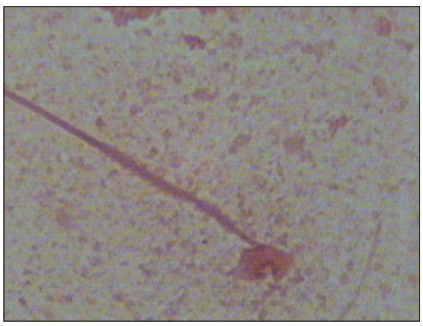

Figure 2d: CP – Amorphous shaped head

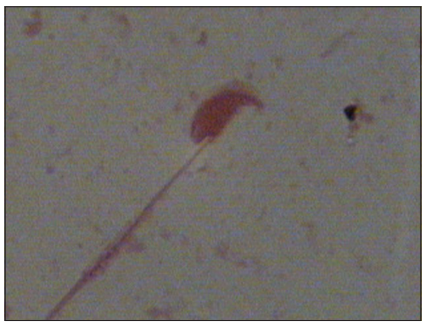

Figure 2e: Vehicle Control treated

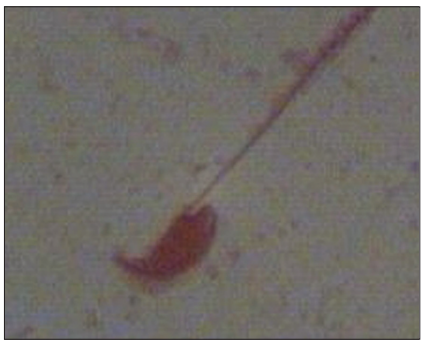

Figure 2f: SMB KR treated

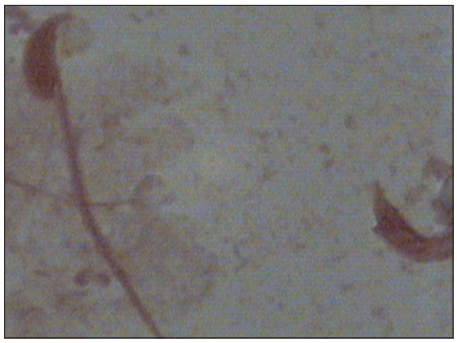

Figure 2g: SMB HK treated

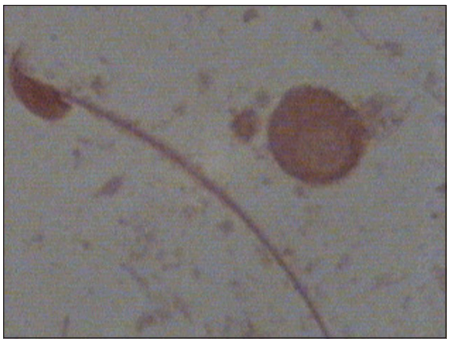

Figure 2h: SMB MM treated

Table 2: Effect of SMBs on chromosomal aberration

Groups	Chromosomal aberration									
	Cromatid		Chromosomal		De	Ex	Fg	PS	R	Dc
	Gap	Break	Gap	Break						
CP	+	+	+	+	+	+	+	+	+	+
VC	--	--	--	--	--	--	--	--	--	--
SMBKR	--	--	--	--	--	--	--	--	--	--
SMBHK	--	--	--	--	--	--	--	--	--	--
SMBMM	--	--	--	--	--	--	--	--	--	--

+: Presence; - Absence; De: Deletion; Ex: Exchange; Fg: Fragmentation; PS: Pulverization and stickiness; R: Ring; Dc: Dicentric; CP: Cyclophosphamide; VC: Vehicle control; SMBKR: *Bhasma* prepared from *Swarna Makshika* collected from Khetri mine, Rajasthan; SMBHK: *Bhasma* prepared from *Swarna Makshika* collected from Hatti gold mine, Karnataka; SMBMM: *Bhasma* prepared form *Swarna Makshika* collected from Malharjkhand mine, Madhya Pradesh; SEM: Standard error of mean

Table 3: Effect of SMBs on sperm abnormality assay

Groups	Sperm abnormality assay					
	Head abnormalities				Tail abnormalities	
	Amorphous shape	Hook less	Banana shaped	Folded	Double tailed	Coiled
CP	++	++	+	+	+	++
VC	--	--	--	--	--	--
SMBKR	--	--	--	--	--	--
SMBHK	--	--	--	--	--	--
SMBMM	--	--	--	--	--	--

--: Absence; +: Mild degree presence; ++: Moderate degree presence; CP: Cyclophosphamide; VC: Vehicle control; SMBKR: *Bhasma* prepared from *Swarna Makshika* collected from Khetri mine, Rajasthan; SMBHK: *Bhasma* prepared from *Swarna Makshika* collected from Hatti gold mine, Karnataka; SMBMM: *Bhasma* prepared form *Swarna Makshika* collected from Malharjkhand mine, Madhya Pradesh; SEM: Standard error of mean

because almost all mouse chromosomes are acrocentric. These types of chromosomes have the exceptional facility to merge with each other. Only structural aberrations were enumerated in all treated groups [Figure 1g-j] against CP treated group, with special emphasis on chromosome and chromatid gap, breaks and centric diffusions placed in Table 2.

Morphological abnormalities of sperms are described as two types as head and tail abnormalities. The head abnormality included amorphous shape, without hook, banana shaped and folded head. CP treated group observed maximum number of abnormalities in both head and tail as shown in Table 3. Amorphous shaped head, hook less head and coil-tailed abnormalities were more frequent than other abnormalities of head and tail of CP treated group [Figures 2a-d]. The test preparations observed negative results in sperm abnormality showing non-toxic to sperms [Figures 2e-h]. Wyrobek[18] reported that large reductions in sperm number or mortality or large increases in sperm with abnormal shapes are associated with reduced fertility.

Conclusion

Present study revealed that SMB prepared from different samples were found to be safe after the administration for 14 days at the therapeutic doses. No abnormality was noticed in CA and sperm abnormal aberrations in all trial groups. Further, above findings provide new information that may be more imperative for the use of *Bhasmas*.

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हिन्दी सारांश

स्वर्ण माक्षिक भस्म–एक हर्बो मिनरल योग का जीन–विषाक्तता अध्ययन

पवन बी. सावलगी, बिस्वाज्योति पटगिरी, जलाराम एच. ठक्कर, बी. रविशंकर, वरुण बी. गुप्ता

आयुर्वेदिक भस्मों का म्युटाजेनिक कर्म पर बहुत कम सन्ज्ञान लिया गया है । प्रस्तुत अध्ययन में स्वर्ण माक्षिक भस्म का सब क्रोनिक विषाक्तता के अन्तर्गत चिकित्सकीय मात्रा में १४ दिन चूहों पर क्रोमोसोमल् विपथन एवम् असामान्य शुक्राणु विश्लेषण का मूल्यांकन किया गया । सायक्लोफोस्फमाइड उपचारित समूह से तुलना करने पर उपरोक्त प्रचाल में सरंचनात्मक विकृति अत्यल्प प्राप्त हुयी । प्रस्तुत मूल्यांकन के अनुसार उपरोक्त भस्म प्रायोगिक रूप से जीन–विषाक्तता में निर्दोष पायी गयी ।