Journal of Ayurveda and Integrative Medicine 14 (2023) 100598

Contents lists available at ScienceDirect

Journal of Ayurveda and Integrative Medicine

journal homepage: http://elsevier.com/locate/jaim

Original Research Article (Experimental)

Understanding the effects of *Abhraka Bhasma* on genotoxicity and its DNA repair potential in mouse model

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ARTICLE INFO

Article history:
Received 5 February 2022
Received in revised form
24 May 2022
Accepted 27 May 2022
Available online 13 August 2022

Keywords:
Genotoxicity
DNA repair
Abhraka Bhasma
Chromosomal aberrations
reproductive toxicity

ABSTRACT

Background: Metal toxicity is of major concern to human health. The metals may modulate molecular mechanisms of various pathways. *Rasashastra*, the branch of Ayurveda, narrates the properties, unique preparation, processing techniques, and therapeutic uses of minerals. The use of herbal metallic preparations has evoked concern for their potential to produce toxicity, interest in efficacy as therapeutic agents and safety related issues. *Abhraka Bhasma*, is one such incinerated herbo-metallic preparation of mica, widely used by traditional medicine practitioners. Although there are reports of *Abhraka Bhasma* on beneficial effects, clear evidence is lacking on the effect of *Abhraka Bhasma* on genotoxicity and DNA repair.

Objective: The present study aims to understand the effects of *Abhraka Bhasma* on geno toxicity, DNA repair, and other mechanisms in the mice test model.

Material and methods: The experiments were conducted in *in vivo* Swiss albino mice. The acute oral toxicity was performed as per the OECD guidelines. The mice were treated with *Abhraka Bhasma* (120 or 360 mg/kg body weight) for 7 days. They were then challenged with ethyl methanesulfonate and the DNA repair was analyzed.

Results: The data obtained indicated that the *Abhraka Bhasma* is not a genotoxic and reproductive toxic formulation. The selected higher concentration of *Abhraka Bhasma* showed a protective role against ethyl methanesulfonate induced chromosomal damages and enhanced constitutive DNA base excision repair in mice.

Conclusion: The anti-oxidant, potentiation of DNA repair and hematinic properties of *Abhraka Bhasma* may be attributed to the synergistic actions of its bioactive components.

1. Introduction

Metal preparations are widely used for centuries in traditional medicinal systems as therapy for several health conditions under *Rasashastra*. *Rasashastra*, a branch of Ayurveda the Indian traditional medicinal system also deals with the study of minerals/metals and their properties, unique preparation and processing techniques, and their therapeutic uses. *Bhasmas* are the distinctive herbo-metallic formulations which are in fact an incinerated metal formulations prepared by a systematic procedure known as '*Bhasmikarana*'. These are considered as safe medications, used extensively by Ayurvedic practitioners to treat various disorders [1]. However, there is a need for evidence on the diverse effects of *Bhasma* which limits their wide applicability. *Abhraka Bhasma* is one such herbo-metallic formulation, prepared by treating biotite form of mica with different natural products. The process includes heating at very high temperatures and quenching in liquid media containing *Triphala* decoction, *Zizyphus jujuba* decoction, cow milk and cow urine. This is further incinerated repeatedly to reduce pre-oxidized ions in mica [2]. The repeated incinerations result in reducing the size of *Bhasma* to nano-scale for better absorption in

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Peer review under responsibility of Transdisciplinary University, Bangalore.
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https://doi.org/10.1016/j.jaim.2022.100598

the human body. The *Abhraka Bhasma* thus obtained is a red colored powder of approximately the particle size of 100–1000 nm which is rich in iron, calcium, potassium, silica, magnesium, and aluminum oxides. *Abhraka Bhasma* is used to treat various diseases such as gastritis, anorexia, renal diseases, respiratory disease, hepatic dysfunction, anemia, stroke, asthma, bone marrow depletion, leukemia, ascites, and diabetes [1–3]. It is also considered as *rasayana* and comprehensively used as a rejuvenator, aphrodisiac, anti-ageing and health promoter. There are evidence on hepatoprotective [4–6], anti-diuretic [7], anti-stress, anti-inflammatory and immunomodulatory [8], adaptogenic [9], memory-enhancing [10], anti-cancer [11], and anti-diabetic [3] role of *Abhraka Bhasma*.

Metal toxicity is of significant concern to human health. The use of metals as medicines or as an ingredient of the medicines are linked with toxicity [12]. Albeit the traditional metallic formulations including *Abhraka Bhasma* are used for centuries, the evidence on their toxicity is sparse. In the recent years, the use of such herbal metallic preparations has evoked concerns on potential toxicity, efficacy, safety and other modulatory effects. Besides, there are reports on mica poisoning [13]. The other elements present in *Abhraka Bhasma* although beneficial, the improper processing of mica during the *Bhasma* preparation may lead to the presence or generation of toxic trace elements which may result in toxic effects [2,14]. Furthermore, *Abhraka Bhasma* as a *rasayana* postulated to be a rejuvenator, anti-ageing and promotor of health [15]. It is known that the proper cellular functions which are mainly dependent on genome integrity, play an important role in the health of the individual. The genome is often under the threat of DNA damaging agents. The defective or inefficient DNA repair machinery leads to the accumulation of DNA damages which are eventually responsible for multifactorial disorders. The protection of the genome or enhancement of DNA repair capacity thus plays an essential role in cellular functions including cellular regeneration [16]. Although, there are few reports on the beneficial effects of *Abhraka Bhasma* including cytotoxicity, to our knowledge there is no clear information available on potential genotoxicity and reproductive toxicity [3,4,11]. Furthermore, there is no evidence on the potentiation of DNA damages or protection of DNA integrity by *Abhraka Bhasma*. Hence, the present study is focused on the understanding of effects of *Abhraka Bhasma* on genotoxicity, reproductive toxicity, and influence on DNA repair. The present investigations were thus undertaken to assess the effects of *Abhraka Bhasma* on chromosomal aberrations induced by ethyl methanesulfonate a known clastogenic/mutagenic agent *in vivo* mouse model. Further, the effect of *Abhraka Bhasma* was also investigated on constitutive base excision repair as a repair enhancer.

2. Materials and methods

2.1. Animals

Inbred male Swiss albino mice (*Mus musculus*) of 6–8 weeks of age (weighing 30–40 g) were used for the experiments. Animals were procured from the Institutional Animal house and they were housed in polypropylene cages with a grill top. The mice were maintained in a spacious cage that is well-lit with 12 h of light and dark cycles and provided with food and water. Care was taken to maintain the hygienic conditions along with proper ventilation in the facility where the mice are housed. All the experiments were performed following the standard Organization for Economic Co-operation and Development (OECD) guidelines. The ethics committee approval was obtained from the Kasturba Medical College Institutional animal ethics committee (No: IAEC/KMC/68/2019) for carrying out the experiments.

2.2. Chemicals

Sahasraputi Abhraka Bhasma a commercially available product manufactured by Shree Dhootapapeshwar Ltd (Mumbai India) was purchased from local Ayurvedic vendors and the date of manufacture and expiry was noted for each batch. Ethyl methanesulfonate (EMS) (CAS No. 62-50-0) served as standard clastogen and was procured from Sigma Aldrich (St. Louis, USA).

2.3. Quality control

The standard tests for the safety index of the formulations as explained in the literature [1,17] were analyzed. These tests are related to the particle size, density, chemical, and physical ability of *Bhasma*. The characteristics of purchased *Abhraka Bhasma* were tested in the laboratory. The particle size measurement of *Abhraka Bhasma* was carried out at 25 °C by dispersing *Bhasma* particles in Milli-Q water and measured using Zetasizer (Malvern; Nano-ZS90). Quality control of *Abhraka Bhasma* preparations were assessed by liquid chromatography-mass spectrometry analysis and the elemental analysis was performed using Atomic Absorption Spectrometry (Varian; AA240). In addition, the quality assessment of purchased *sahasraputi Abhraka Bhasma* was performed as per the Ayurveda concept of testing the safety index of the formulation such as *nishchandratva, rekhapurnatvam, varitaratvam, nisvadutam, amla pariksha, nirdhoom, slakshnatvam*, and *dantagreaka-chikachitatva* properties.

2.4. Analysis of metabolites

LC-MS analysis for *Abhraka Bhasma* was carried in Agilent 1200 liquid chromatography coupled 6520 time-of-flight mass spectrometer (LC-QTOF) from Agilent technologies, CA, USA. The *Bhasma* powder was constituted in methanol comprising of 0.1% formic acid and 8 μL of this sample was injected into C18 reverse phase HPLC column (0.1% formic acid in water-mobile phase A; 0.1% formic acid in 90% acetonitrile - mobile phase B) and eluted at a gradient of 5–35% B in 35 min and then up to 75% B in 45 min at 400 μL/min flow rate in Accurate Mass Q-TOF LC/MS. The mass analyzer was scanned over the range 50–1700 *m/z* and the LC-MS profile was processed employing Agilent Mass Hunter Qualitative Analysis software (version B.04.01) and the constituents were identified based on PubChem and METLIN databases.

2.5. Analysis of genotoxicity

The acute toxicity studies were executed by following the OECD guidelines 423, where the mice were orally administered with a single dose of *Abhraka Bhasma* (2 g and 5 g/kg body weight). The mice were observed for 14 days from the day of administration of *Abhraka Bhasma* for the changes in behavioral pattern, body weight and physical appearances such as hair fall, redness of ear lobe, eye color, mucus secretion, salivation, convulsions, diarrhea and lethargy. Further, the dose of 120 mg–360 mg/kg body weight was employed to treat various disease condition in humans. And hence, in the present studies, these human equivalent doses were used. These two doses of *Abhraka Bhasma* were orally administered to the mice for 7 days and they were observed for a period of 14 days. The physical parameters including the body weight were monitored throughout the experimental period. The genotoxicity was

analyzed in these mice by examining the chromosomal aberrations, sperm count and sperm abnormalities and mitotic index.

2.6. DNA repair analysis

In the present experiments, the mice were grouped into control, *Abhraka Bhasma* (120 mg and 360 mg/kg body weight), combined treatment of *Abhraka Bhasma* and Ethyl methanesulfonate (EMS), and EMS groups. The Control group of mice were fed with a normal diet and water and were injected with 0.9% saline (0.5 mL) intraperitoneally on the 8th day of feeding. *Abhraka Bhasma* treatment was given to two groups of mice of doses 120 mg/kg body weight and 360 mg/kg body weight orally for 7 days. Another group of mice were intraperitoneally injected with EMS (240 mg/kg body weight) on the 8th day of feeding and were fed with a normal diet every day. *Abhraka Bhasma* and ethyl methanesulfonate treatment (combined treatment group) were given to two groups of mice which were fed with 120 mg/kg body weight and 360 mg/kg bodyweight for 7 days respectively and on the 8th day they were injected with 240 mg/kg body weight of ethyl methanesulfonate. Each treatment group consists of three animals and the experiments were repeated twice. *Abhraka Bhasma* treatment was given according to their body weight and it weighed and dissolved in sterile saline. The solution was then fed orally to the animals once a day. EMS was injected intraperitoneally on the 8th day. The mice were sacrificed at intervals of 24, 48, and 72 h. The bone marrow was processed for chromosomal analysis. The liver tissue was dissected out for analysis of base excision repair. Another set of reproductive toxicity experiments using the same group of animals was also conducted where the animals were left undisturbed for 34 days after the last treatment regimen. On the 35th day, the animals were sacrificed by cervical dislocation and the epididymis is dissected out and processed for sperm count and sperm shape abnormalities.

2.7. Analysis of chromosomal aberrations

Chromosomal aberrations analysis was carried out as per the method described by Guruprasad et al. [18]. The mice were sacrificed at intervals of 24 h, 48 h and 72 h by cervical dislocation. One and half hours before sacrifice, each animal was injected with Colchicine (1 mL: 0.05%). The bone marrow was processed, and the slides were prepared by air-drying technique [19]. In brief, the bone marrow from the dissected femur bones was dispensed into a tube containing hypotonic solution (0.56% potassium chloride), the obtained cell suspension was thoroughly mixed and incubated for 30 min at 37 °C. The sample was centrifuged at 1500 rpm for 7 min. The supernatant was discarded and the pellet formed was suspended in chilled methanol/acetic acid fixative in the ratio of 3:1 and this was repeated thrice. The pellet was finally suspended in freshly prepared fixative, and the cell suspension obtained was dropped on a pre-chilled, clean and non-greasy slide using a Pasteur pipette. The slides were air-dried and stained with Giemsa stain. The slides were assessed for the presence of chromosomal aberrations including chromatid breaks, intrachromatid deletions, triradials, chromatid exchanges, dicentrics, rings, chromosome breaks, and minutes, microscopically. In each group, at least 100 well-spread metaphase plates were scored.

2.8. Assessment of mitotic index (MI)

It was used to analyze the potential mitotic toxicity of *Abhraka Bhasma*. The mitotic rates in different treatment groups were analyzed by scoring the number of mitotic cells in 2000 cells per animal. And hence a total of 12,000 cells per treatment group and controls. The mitotic index was calculated using % MI = (Total number of mitotic cells/Total number of cells counted) x (100).

2.9. Analysis of base excision repair

The assay was performed by following the protocol of Krishna et al. [20]. In brief, approximately 100 mg of liver tissues from all the groups were weighed and were then homogenized using protein extraction buffer. Then the samples were centrifuged at 16000 g for 20 min. The supernatant was collected and the protein present in it was estimated by Bradford's method. The oligomer duplex with one nucleotide gap possessing fluorescence tag on the strand with gap was made. The required concentrations of extracted protein were then mixed with the Cy3-labeled DNA duplex containing one nucleotide gap. This was then incubated at 37 °C for 90 min in dark. The reaction was then stopped by adding an equal volume of formamide. The samples were maintained at a temperature of 90 °C for 10 min and then they were snap cooled in ice. The sample was loaded onto the 7 M urea gel along with the tracking dye on the side and the gel was electrophoresed for 2 h at 250 V. The gel was then scanned with Phosphor-imager and analysed.

2.10. Analysis of reproductive toxicity by sperm abnormality and sperm count

Sperm counts were carried out according to the method described by Guruprasad et al. [18]. In brief, the paired capita was dissected out and macerated in 1–2 mL of 1% (w/v) trisodium citrate. The resulting solution is diluted by adding 2–4 mL of trisodium citrate, which is based on the weight of the caput. The sperms were mixed thoroughly and were counted utilizing a Neubauer hemocytometer. The sperm morphological abnormalities were analyzed by diluting the sperm suspension in the ratio of 10:1 and stained using 1% Eosin Y stain for 30 min. The sperm suspension was smeared on the slides and air-dried. The slides were microscopically examined and about 2000 sperms were scored in each animal for the presence of different sperm morphology abnormalities including amorphous, pinhead, triangle, sword head, hookless, double-tailed, doubled head and others.

2.11. Autophagy detection by Cadaverine uptake

To understand the effects of *Bhasma* on autophagy, approximately 50 mg of liver tissue from control and treatment groups was weighted and then homogenized using 0.5 mL of homogenization buffer. The homogenate obtained is then centrifuged to remove cell membranes and other debris. The supernatant (243.75 μL) was incubated with 6.25 μL of Alexa Flour 488™ Cadaverine (25 μM) for 10 min. The tubes were then centrifuged at 14,000 rpm for 20 min at 4 °C. The obtained pellet was washed twice with resuspension buffer and was suspended in 350 μL of the same buffer. 250 μL of this solution was then transferred into 96 well plate. The fluorescence intensity was noted using an excitation/emission wavelength of 495/519 nm [21]. The relative fluorescence units were then normalized to each sample protein concentration by Bradford assay [22].

2.12. Histological analysis

The liver, kidney, and testis tissues were dissected out from the *Abhraka Bhasma* treated and control animals. They are processed for histological analysis. The routine H and E staining was performed.

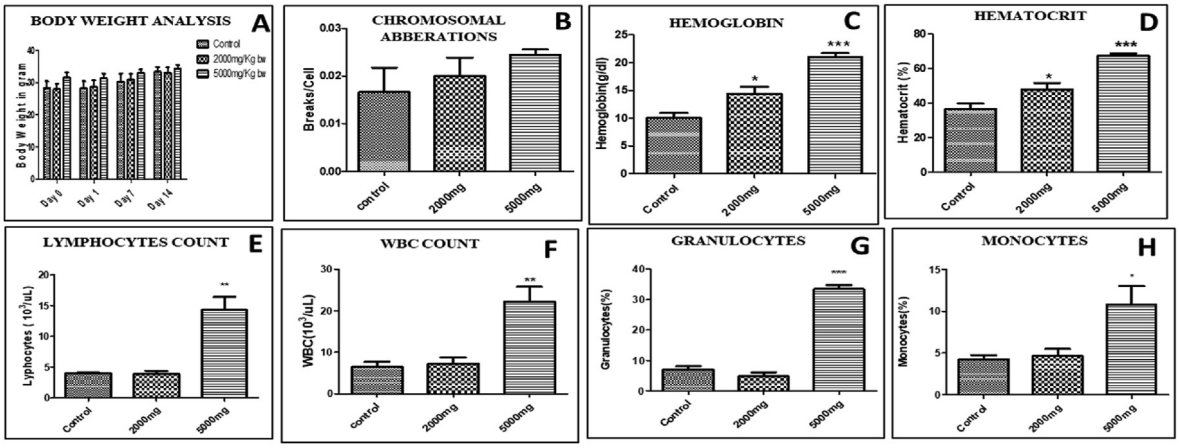

Fig. 1. Analysis of acute oral toxicity in mice model. The data showed that *Abhraka Bhasma* did not elicit significant change in the body weight (A) and Chromosomal aberrations (B). The significant increase of Hemoglobin (C), Hematocrit (D), Lymphocyte Count (D), Total WBC count (E), Granulocyte Count (G) and Monocytes (H) was observed at highest dose (5000 mg/kg body weight) of *Abhraka Bhasma* treatment. A total of three animals for each group were used for the analysis.

2.13. Measurement of antioxidant enzyme activities

Abhraka Bhasma is considered as *rasayana* and the *rasayana* are effective antioxidants which in turn may play a role in DNA repair. In this connection, the *Abhraka Bhasma* was also assessed for its antioxidant activities in the mouse test model. The antioxidant enzymes viz., superoxide dismutase (SOD) [23] and catalase [24] activities were studied employing the liver tissue of the *Abhraka Bhasma* treated and control animals.

2.14. Statistical analysis

The statistical analysis was performed using the GraphPad Prism software (version 5.01). The data are expressed as mean ± SEM. Statistical significance was determined using Student's t-test and the p-value <0.05 was considered as statistically significant.

3. Results

The present investigations focus on genotoxicity and reproductive toxicity elicited by *Abhraka Bhasma* and its effects on potentiation/protection of EMS-induced DNA repair in the mouse test model.

Initially, the quality assessment of purchased *sahasraputi Abhraka Bhasma* was performed as per the Ayurveda concept of evaluation and the results showed that the *Bhasma* qualified the Ayurvedic safety index properties. The results of particle size measurement of *Abhraka Bhasma* showed two size groups of particles with a peak value of 62.56 nm and 199.9 nm for micro filtered (0.2 μm) and a peak value of 102.9 nm and 486.5 nm for unfiltered *Bhasma* samples (Supplementary Fig. 1). Furthermore, the representative LC chromatograms of water extracted *Abhraka Bhasma* preparations run in positive mode of ionization in ESI/QTOF are provided as Supplementary Fig. 2. The elemental analysis of *Abhraka Bhasma* showed the presence of iron, magnesium and calcium (Supplementary Table 1).

3.1. Toxicity studies

The initial pilot toxicity studies were performed in *in vivo* Swiss albino mice using a single dose of 5000 and 2000 mg/kg body weight. The results indicated these doses did not induce any abnormal behavior within 24 h after the administration and there was no death till 14 days. The data obtained clearly indicate that there was no change in the body weight between control and *Bhasma* treated animals. The analysis of chromosomal aberrations showed that there was an insignificant increase of chromosomal aberrations by *Abhraka Bhasma* when compared to chromosomal aberrations observed in control ($p > 0.05$) mice. The analysis of blood parameters such as hemoglobin, hematocrit value, white blood corpuscles and lymphocytes showed a significant ($p < 0.05$) increase at the 5000 mg/kg bodyweight concentration when compared to control animals (Fig. 1). The mice treated with selected doses of 120 mg and 360 mg/kg bodyweight for 7 days, showed no significant ($p > 0.05$) differences in the analyzed blood parameters, and chromosomal aberrations in comparison to control mice. However, there was a decreasing trend with increasing the dose was observed for induction of chromosomal aberrations (data not shown). The reproductive toxicity of the *Abhraka Bhasma* has been assessed by using sperm shape abnormalities and sperm count. The results have shown that there were no significant changes ($p < 0.05$) in the number of sperms and sperms with abnormalities when compared to control mice indicating that *Abhraka Bhasma* did not induce reproductive toxicity in mice (Table 1). The study thus signifies that *Abhraka Bhasma* is not a toxic agent. The histological analysis (data not shown) of liver, kidney and testis of animals treated with *Abhraka Bhasma* and their comparison with controls did not show significant toxicity.

3.2. Analysis of constitutive and EMS induced DNA repair

Next, our study addressed the influence of *Abhraka Bhasma* on EMS-induced chromosomal aberrations and constitutive DNA repair *in vivo* mice. The results obtained after treatment of *Abhraka Bhasma* are presented in Fig. 2. The data clearly indicate that the

Table 1
Analysis of Sperm count and Sperm abnormalities in *Abhraka Bhasma* treated mice.

Treatments	Mean Sperm Count/Caput (x 10^{-6})	% of Sperm abnormalities
Control	2.9	1.9 ± 0.072
Abhraka Bhasma - 2000 mg	3.1	1.87 ± 0.412
Abhraka Bhasma - 5000 mg	3.0	1.78 ± 0.446
Abhraka Bhasma - 120 mg	3.2	1.72 ± 0.482
Abhraka Bhasma - 360 mg	3.0	1.81 ± 0.321

Note: The data is from a total of six animals from each group.

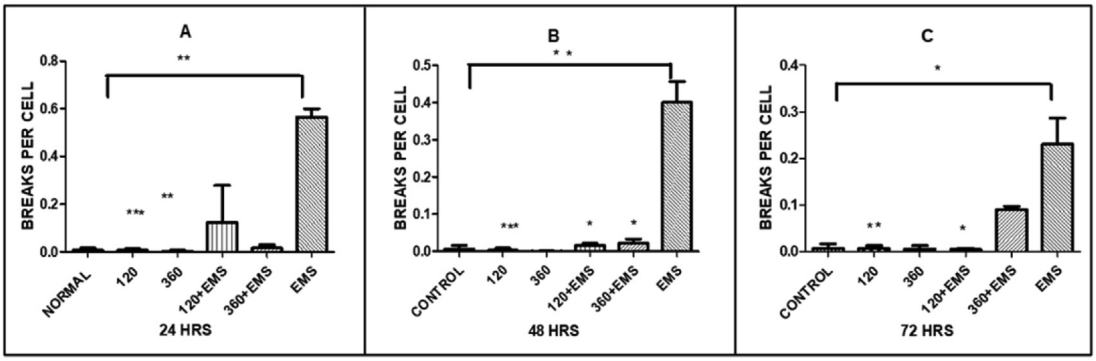

Fig. 2. Analysis of chromosomal aberrations in *Abhraka Bhasma* treated and EMS challenged mouse bone marrow cells at 24 h (A), 48 h (B), and 72 h (C) recovery times. The data is from a total of six animals from each group. The EMS induced high frequencies of chromosomal aberrations at all recovery times tested. The low and high dose of *Abhraka Bhasma* did not induce significant number of breaks. The treatment of *Abhraka Bhasma* for 7 days and challenged with EMS showed significantly reduced number of breaks when compared to EMS alone.

Table 2
Mitotic indices in mouse bone marrow cells of *Abhraka Bhasma* and EMS treated group of animals at all recovery times.

Treatment Groups	24 h	48 h	72 h
Control	5.07 ± 0.38	5.07 ± 0.41	4.63 ± 0.26
Abhraka Bhasma (120 mg)	5.20 ± 0.35	4.90 ± 0.23	4.97 ± 0.34
Abhraka Bhasma (360 mg)	4.73 ± 0.23	5.00 ± 0.21	4.93 ± 0.32
EMS	2.27 ± 0.20[a]	2.63 ± 0.44[a]	3.00 ± 0.15[a]
Abhraka Bhasma (120 mg)+EMS	4.40 ± 0.31	4.57 ± 0.09	4.93 ± 0.19
Abhraka Bhasma (360 mg)+EMS	4.77 ± 0.37	4.73 ± 0.38	5.37 ± 0.12

[a] Significant when compared to control ($p < 0.05$); The data is from a total of six animals from each group.

Abhraka Bhasma did not induce significant ($p > 0.05$) chromosomal aberrations in comparison to control animals at all the recovery times tested. The chromosomal aberrations induced by the clastogen ethyl methanesulfonate (EMS; 240 mg/kg body weight) were significantly ($p < 0.05$) higher at 24, 48 and 72 h of recovery times (Fig. 2). The mice treated with *Abhraka Bhasma* for 7 days and the challenged with EMS showed significantly reduced chromosomal aberration when compared to a positive control (EMS). Furthermore, the reduction was observed in 360 mg/kg body weight treated mice when compared to 120 mg/kg body weight treated mice. The data suggests protective role of *Abhraka Bhasma* against clastogenicity of EMS.

The analysis of Mitotic index showed that there was a reduced number (2.27–3.00 at 24–72 h recovery times) of mitotic cells by the treatment of EMS. However, the number of mitotic cells was restored in the combined treatment group of mice (*Abhraka Bhasma* + EMS) at all recovery times. On the other hand, no significant differences were noticed in other treatment groups (Control- 4.63 to 5.07; *Abhraka Bhasma* 120 mg- 4.97 to 5.20; *Abhraka Bhasma* 360 mg–4.93 to 5.00) (Table 2).

The base excision repair was tested in all the treatment groups of animals to understand the influence of *Abhraka Bhasma* by employing cell-free extracts of liver tissues. The results showed that there was an increase in the constitutive base excision repair by *Abhraka Bhasma* at the concentration of 360 mg/kg body weight when compared to control mice. The combined treatment of *Abhraka Bhasma* and EMS although showed an increase in the repair capacity when compared to EMS alone, it was insignificant at all the recovery times (Fig. 3).

3.3. Assessment of reproductive toxicity

In another set of experiments, the sperm shape abnormalities and sperm count were analyzed in all the treatment groups of animals to understand the effects of *Abhraka Bhasma*. The results showed that there was a reduced number of sperms (1.31 ± 0.05) and increased sperm shape abnormalities (4.27 ± 0.22) by the treatment of EMS (Table 3). The number of sperms was increased insignificantly (2.73 ± 0.12 and 3.13 ± 0.09) and the sperm shape abnormality was reduced (3.13 ± 0.09 and 1.75 ± 0.16) in the combined treatment group

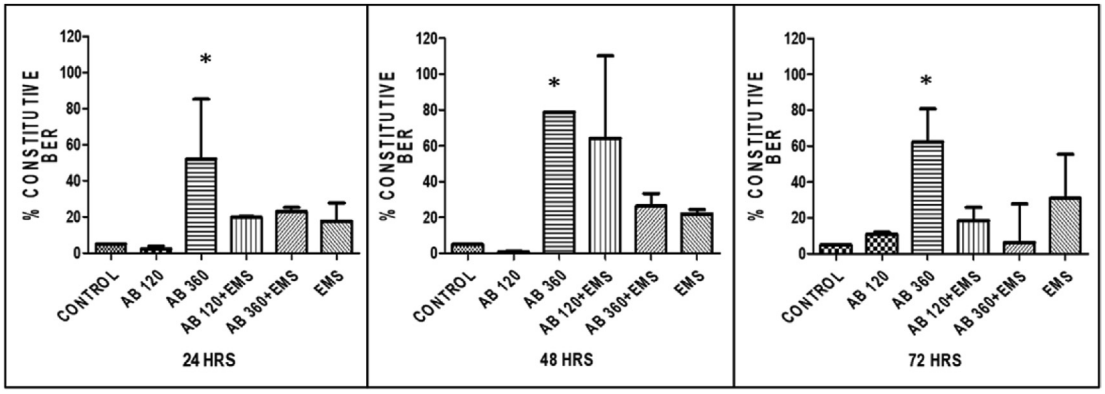

Fig. 3. Modulation of Constitutive and EMS induced Base excision repair in liver tissues of mice (n = 3 per each group). The treatment of high dose equivalent to 360 mg of human dose of *Abhraka Bhasma* enhanced the base excision repair at 24, 48 and 72 h of recovery times. No significant effects of *Abhraka Bhasma* were observed against EMS challenge.

Table 3
The percentage of sperm abnormalities and mean sperm count in different groups of treatment regimen.

	Amor-phous	Triangle	Pin head	Hookless	Sword shape	Banana Shaped	Double Headed	Double tailed	Total Sperm Abnormalities	% of sperm abnorm alities	Mean Sperm Count
Control	18.67 ± 2.03	17.67 ± 4.06	39.00 ± 3.46	15.33 ± 1.45	2.67 ± 0.88	5.00 ± 1.73	1.33 ± 0.88	0.67 ± 0.33	100.33 ± 6.23	**1.67 ± 0.10**	**3.10 ± 0.07**
AB 120	18.67 ± 1.45	18.00 ± 3.51	36.00 ± 2.52	15.67 ± 2.85	1.00 ± 0.58	15.00 ± 1.73	0.67 ± 0.33	0.00 ± 0.00	105.00 ± 10.26	**1.75 ± 0.17**	**3.29 ± 0.03**
AB 360	13.33 ± 1.45	11.67 ± 0.88	21.67 ± 1.45	8.00 ± 0.58	3.67 ± 0.67	6.00 ± 1.15	0.33 ± 0.33	0.00 ± 0.00	64.67 ± 2.73	**1.08 ± 0.05**	**3.21 ± 0.11**
EMS	51.67 ± 7.69	30.00 ± 2.65	82.33 ± 4.26	33.67 ± 3.53	17.00 ± 2.65	29.33 ± 3.76	11.00 ± 1.00	1.33 ± 0.33	256.33 ± 13.38	**4.27 ± 0.22**[a]	**1.31 ± 0.05**[a]
AB 120 + EMS	29.67 ± 2.03	28.00 ± 3.21	49.33 ± 2.85	17.33 ± 2.91	14.67 ± 2.60	21.33 ± 3.48	2.00 ± 1.15	0.33 ± 0.33	162.67 ± 4.26	**2.71 ± 0.07**	**2.73 ± 0.12**
AB 360 + EMS	22.67 ± 1.86	22.00 ± 3.79	20.33 ± 5.24	22.00 ± 2.31	5.33 ± 1.45	11.67 ± 1.45	0.67 ± 0.67	0.33 ± 0.33	105.00 ± 9.54	**1.75 ± 0.16**	**3.13 ± 0.09**

[a] Significant when compared to control ($p < 0.05$); The data is from a total of six animals from each group.

of animals when compared to EMS alone indicating the protective role of *Abhraka Bhasma*.

3.4. Measurement of antioxidant enzyme activities and autophagy

The antioxidant enzymes activity was also performed in all the treatment groups. The results obtained indicate that the catalase and SOD enzyme activity was increased by a selected higher dose of *Abhraka Bhasma* when given alone or in combination with EMS in comparison to control and EMS alone (Fig. 4). The data thus indicates antioxidant defense elicited by *Abhraka Bhasma*.

An attempt was also made to understand the effects of *Abhraka Bhasma* on autophagy. The results have shown that the level of autophagy was not significantly altered at 48 h and 72 h recovery times. However, at 24 h recovery times, there was an increase in the levels of autophagy in the animals treated with EMS with or without *Abhraka Bhasma* (Fig. 5). These need to be further evaluated.

4. Discussion

The traditional medicines generally prepared from plants, minerals or metals; and may contain an array of chemicals/pharmacological ingredients which may show antagonistic effects. An area of Ayurveda, the '*Vishagarvajrodhika Tantra*' is described with respect to toxicity [25]. It emphasizes on side effects of therapeutically useful drugs and its minimization. Furthermore, it indicates the time, condition, and dose of administration of drugs and also the relationship of drugs with the lifestyle [25]. Ayurvedic literature describes improperly prepared or administered drug as toxic [25].

Abhraka Bhasma, a herbo metallic formulation of mica, was used for treating various diseases [2–5]. Besides, it is considered as *rasayana* and used as a rejuvenator and health promoter. However, the evidence of *Abhraka Bhasma* on toxicity in general and genotoxicity, reproductive toxicity in particular is not clear. Hence, in the present investigations, the commercially available *Abhraka Bhasma*, was employed to test its genotoxicity and reproductive toxicity, DNA repair and other parameters in *in vivo* mouse model. Initially, the quality parameters of the *Abhraka Bhasma* were also tested as per the Ayurveda concept of testing (*Bhasma Pareeksha*) the formulation [1] and it qualified in the parameters tested. The analysis of particle size by Zeta-sizer of the *Abhraka Bhasma* is in the range of 60–900 nm which is on par with that of the characterization data of the manufacturer and also the available evidence [3]. Our quality analysis of aqueous extracts of the *Abhraka Bhasma* by LC-MS showed the existence of different components in the *Abhraka Bhasma* (Supplementary Fig. 2).

The pilot acute oral toxicity studies were conducted as per the Organization for Economic Cooperation and Development (OECD) 423 guidelines. The toxicity studies using 2000 and 5000 mg/kg body weight of *Abhraka Bhasma* showed no death of the animals, no change in the body weight and also these doses did not induce any abnormal behavior. Further, the 5000 mg/kg body weight also showed enhancement in the blood parameter values (Fig. 1). The histological analysis of liver, kidney, and testis did not show changes. The data thus indicates that *Abhraka Bhasma* is not the toxic agent. In agreement with this, Gopinath and Shivashankar [3], reported *Abhraka Bhasma* is not toxic to rats. The OECD and other international bodies including Environmental protection agencies (EPA), Food and Drug Administration (FDA), have suggested the guidelines for testing reproductive toxicity of chemicals, pesticides, food additives, pharmaceuticals and others in the laboratory animals [26]. The reduction in the sperm count and increase of sperm abnormalities which may result in fertility related issues are among the standard criteria to identify toxic agents [27]. Hence, the reproductive toxicity of *Abhraka Bhasma* was performed and the results of the studies showed no significant differences between *Abhraka Bhasma* treated and control animals suggesting that it may

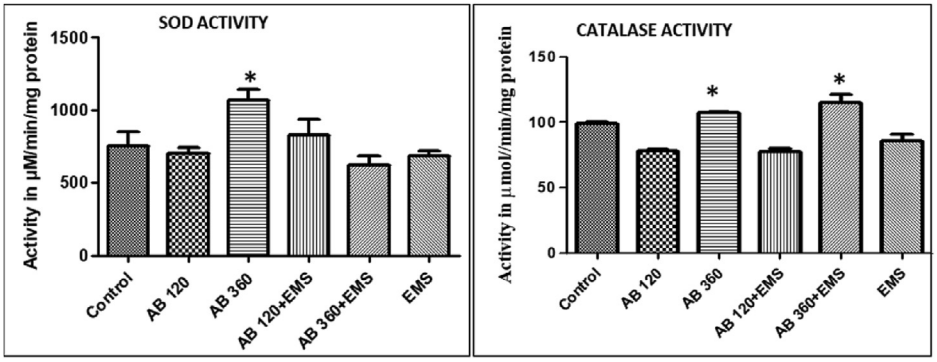

Fig. 4. The effect of *Abhraka Bhasma* on antioxidant enzyme activities was observed at 24 h recovery times in liver cells from animals (n = 6). The high dose of *Abhraka Bhasma* showed more SOD and Catalase activity. Catalase activity is also increased in combined treatment group when compared to EMS alone.

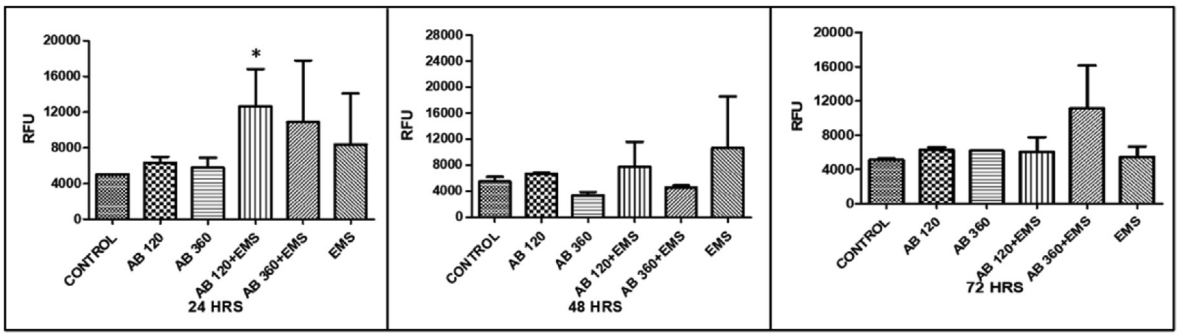

Fig. 5. Effect of *Abhraka Bhasma* on autophagy in different treatment groups of animals (n = 6). The data showed increased autophagy in *Abhraka Bhasma* and EMS combined treatment groups of animals compared to control and *Abhraka Bhasma* groups alone at 24 h recovery times. No significant differences were observed between the groups at 48 h and 72 h recovery times.

not be a reproductive toxic agent. Long term studies are needed to further understand the effects.

DNA damage may induce apoptosis, hinder regeneration and may influence cancer progression [28,29]. When exposed to damaging agents, DNA undergoes genetic changes including chromosomal aberrations and point mutations. These induced and persistent alterations may result in degeneration and cell death. In this context, whether *Abhraka Bhasma* damages the DNA or possesses other genotoxic effects is not clearly understood. Hence, the present studies were undertaken to understand and effect of *Abhraka Bhasma* on the genotoxicity in *in vivo* mice systems. The data demonstrated no evidence for *Abhraka Bhasma* induced chromosomal aberrations suggesting its non-toxic nature. Vardhini et al. [30] also reported insignificant induction of micronucleus and DNA strand breaks by different *Bhasma* including *Abhraka Bhasma* in Wistar rats.

The constituent or induced DNA damages in the cells are repaired by several repair mechanisms including base and nucleotide excision repair, DNA single and double-strand break repair, mismatch repair and others which play a major role in the maintenance of the integrity of the genome [29]. However, there is several evidences stating that the intrinsic fidelity and activity of such repair systems in various species might be influencing the age-associated decline [31]. Therefore, any defect in DNA repair would lead to the accumulation of DNA damages which may culminates in cellular senescence and death. However, no data is available to our knowledge on the potentiation or protection of DNA repair by *Abhraka Bhasma*. Thererfore, we attempted to evaluate the effect of *Abhraka Bhasma* on chromosomal aberrations induced by ethyl methanesulfonate, a known mutagenic and clastogenic agent. In addition, the constitutive base excision repair was also carried out to understand its role in DNA repair. The two human equivalent doses of 120 mg and 360 mg/kg body weight of the *Abhraka Bhasma* were employed in the studies and the animals were treated for 7 days. Our data showed the presence of an insignificant number of chromosomal aberrations by both the doses when compared to control. On the other hand, EMS induced significant chromosomal aberrations. It is interesting to note that the *Abhraka Bhasma* treated animals, when challenged by EMS, showed a reduced number of chromosomal aberrations compared to EMS alone at all the recovery times tested (Fig. 2). The data thus suggests that the *Abhraka Bhasma* either alleviated the EMS-induced DNA damages or enhanced the DNA repair capacity of the repair. Similarly, the *Swarna makshika Bhasma* and *Hridayarnava rasa* (composed of *Tamra Bhasma*, *Kajjali* and decoction of three myrobalans and juice *of Solanum nigrum*) where the number of breaks in the treated group was significantly lower when compared to the positive control, the cyclophosphamide for 15 days [32,33]. The mitotic indices also indicate recurrence of several mitotic cells in the combined treatment of *Abhraka Bhasma* and EMS as compared to that of EMS alone alluding the protective role of *Abhraka Bhasma* against the cell proliferation inhibition. Our results of base excision repair using liver cell-free extracts from a different group of animals showed that the constituent base excision repair capacity was increased when higher dose (human equivalent dose of 360 mg/kg body weight) when compared to that of control and lower dose of *Abhraka Bhasma*. However, the effects of *Abhraka Bhasma* against EMS induced DNA repair although higher, was not statistically significant indicating the enhancement of constitutive BER and little protection of EMS induced BER. The EMS is a monofunctional alkylating agent and capable of inducing DNA lesions such as DNA adducts, cross-links and strand breaks and pose clastogenic/mutagenic risks at the higher concentrations. It mainly interacts with N7G to form an unstable N7 ethylguanine which subsequently leads to deprotonation, decomposition, depurination and also the formation of DNA strand breaks [34]. The N-ethyl guanine adducts are recognized by the N methyl purine glycosylase enzyme and initiate BER machinery to repair the EMS-induced adducts. The clearance of EMS-induced damages depends on the amount of the BER glycosylase enzymes/machinery [35]. Our data of enhanced constitutive BER and reduced chromosomal aberrations by *Abhraka Bhasma* may be due to enhanced activities of mainly BER and other repair machinery, the molecular mechanism of action of which needs to be evaluated further. It is also known that chromatin alteration by EMS affects reproduction [36,37]. The results of the present studies also showed recurrence of EMS-induced sperm count and sperm abnormalities to the level of controls by *Abhraka Bhasma*. Consistent with this, the hyperthermia-induced testicular damage and reduced spermatogenesis was mitigated by *Abhraka Bhasma* [38]. An increase of blood parameters including hemoglobin, and WBCs, was observed by the high dose of *Abhraka Bhasma* explaining cellular proliferative and the antioxidant nature of the *Bhasma* which plays a role in DNA damages/repair also.

Oxidative stress occurs when higher free radicals are produced when compared to antioxidant capacity. Therefore, antioxidant enzyme activities such as SOD and catalase were analyzed in different groups and our data suggests increased catalase enzyme activities by *Abhraka Bhasma*. Our findings of such anti-oxidant nature of *Abhraka Bhasma* are aligned with the data of Subedhi et al. [39,40] where they have shown that *Abhraka Bhasma* enhances the catalase activity and reduced glutathione content upon oxidative stress in the *Drosophila* model. Further, they have reported an enhancement of heat shock protein 70, and catalase

genes by *Abhraka Bhasma* supplement. Balakrishna et al. [8] showed dose-dependent increased immunomodulatory effects of *Abhraka Bhasma*.

Autophagy, is a lysosome-mediated catabolic process present in eukaryotic cells, responsible for the degradation of long-lived macromolecular complexes and organelles. It is also a major determinant of cellular homeostasis and essential for the renewal of cytosolic materials and the degradation of defective cytoplasmic proteins. Autophagy is involved in housekeeping, control of growth, cellular differentiation and tissue remodeling [41]. Evidence are also available on the association of autophagy with DNA damage/repair and oxidative stress [42,43]. In this connection, we studied the effects of *Abhraka Bhasma* on autophagy and the overall data showed no significant changes in the level of autophagy by *Abhraka Bhasma*. The increase of autophagy at 24 h in combined treatments may be due to the EMS-induced DNA damage response. The subsequent reduction at 48 and 72 h recovery times may be due to the enhancement of DNA repair. Autophagy a cellular environment-dependent pathway is responsible for both cell survival and autophagy-induced cell death [41]. The decrease and increase of autophagy than the basal level and the rate at which the defective molecules/organelles are removed influence the survival of cells suggesting its protective role [41]. However, the pattern of molecular mechanism of action of *Abhraka Bhasma* in relation to autophagy needs to be evaluated further.

5. Conclusion

The *Abhraka Bhasma* preparation employed nearly 72 herbs and 1000 *puta* is considered to enables bioavailability. This contains multiple bioactive components including many elements such as iron, magnesium, calcium, zinc et cetera. The net effects of the *Bhasma* on various intricate pathways including anti-oxidant, potentiation of DNA repair and hematinic properties may be attributed to the synergistic activities of these bioactive components. The future work thus requires to focus on the bioactive components and unraveling the molecular mechanisms of action of *Abhraka Bhasma*. Furthermore, our study results suggest that the *Abhraka Bhasma* is non-toxic metal formulation which may be further tested for therapeutic purposes.

Author contributions

Divya S. Kulala: Validation, Formal analysis, Investigation, Data curation, Writing – review and editing, Visualization. **Keshava Prasad:** Validation, Formal analysis, Investigation, Data curation, Writing – review and editing, Visualization. **Poojitha S. Reddy:** Investigation, Data curation, Writing – review and editing, Visualization. **Swathi Maruthiyodan:** Validation, Investigation, Data curation, Writing – review and editing. **Manjunath B. Joshi:** Validation, Writing – review and editing. **Kapaettu Satyamoorthy:** Conceptualization, Validation, Writing – original draft, Writing – review and editing, Visualization, Supervision, Project administration. **Kanive P. Guruprasad:** Conceptualization, Methodology/Study design, Validation, Resources, Writing – original draft, Writing – review and editing, Visualization, Supervision, Project administration, Funding acquisition.

Funding

The study was funded by Vision Group of Science and Technology (VGST), Government of Karnataka [No: CESEM-GRD 179).

Declaration of competing interest

The authors declare there are no conflict of interests with the content in the article.

Acknowledgements

Authors thank TIFAC-CORE, Government of India and Manipal Academy for Higher Education, Manipal for infrastructure and Centre for Ayurvedic Biology for valuable inputs. We gratefully acknowledge the financial support from Vision Group of Science and Technology (VGST), Government of Karnataka (Grant No: CESEM-GRD 179). KP received the Joint CSIR-UGC fellowship under UGC scheme.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaim.2022.100598.

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