EVALUATION OF GENOTOXICITY PROFILE OF JASADA BHASMA (A ZINC-BASED MINERAL FORMULATION) IN SWISS ALBINO MICE

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ABSTRACT

Objective: Genotoxicity is regarded as one of the potential risk factors for causing pathological diseases. It was confirmed that many chemicals have the mutagenic activity which leads to cancer. A compound which interacts with genetic material DNA and shows adverse effects by altering its structure or function is referred to as genotoxic.

Aim of the Study: The present research was mainly focused to evaluate genotoxicity profile of classically prepared Jasada Bhasma (JB) by employing three crucial genotoxic testing protocols.

Methods: The present study involved 40 Swiss albino mice weighing between 25 and 30 g body weights categorized into four different groups. Group-I (normal control) received 0.5% carboxymethyl cellulose as vehicle. Group-II (toxicant control) received 40 mg/kg body weight cyclophosphamide on the 28th day. Group-III and IV received test drug JB 15.6 mg/kg and 78 mg/kg, respectively for 28 consecutive days. Blood samples were collected and processed for evaluating by comet assay. The animals were sacrificed and collected the bone marrow from both the femur for chromosomal aberration and micronuclei assay.

Results: JB administered at two different dose levels did not show any significant changes in the comet assay parameters, no micronucleus was found and did not produce any chromosomal aberrations both numerically and structurally when compared to positive test control group.

Conclusion: The genotoxicity evaluation of JB did not show any chromosomal aberrations and presence of micronucleus. Thus, the safety data will refine therapeutic utility of JB encouraging their rationale use and translate into greater and broader utilization of JB.

Keywords: Jasada Bhasma, Genotoxicity, Comet assay, Chromosomal aberrations.

INTRODUCTION

Zinc is considered as fourth most consumed metal after iron, aluminum, and copper. Besides, its widespread use in the industries, it also used in health-care products. It is considered an essential trace element and in the human growth and development, immunity, and neuronal development, zinc has an important role. An adult human body contains 2–3 g of zinc. The essential dietary sources are oysters, beef, and peanuts [1,2]. Zinc is used in Indian traditional systems of medicine, especially Ayurveda in the form of Bhasma. Bhasmas are the ash preparations. They are inorganic preparations produced by metals or minerals made into 200–300 nm sized particles by conversion into its compounds such as carbonates and oxides prepared by the traditional method [3,4]. Jasada Bhasma [JB] is one of the mineral based ash formulations. It is highly absorbable due to the decreased particle size. It has many therapeutic indications such as immune-modulatory, as a supplement in zinc deficiency, and as anti-inflammatory, anti-pyretic, anti-diabetic, hematric, and hematogenic agent [5].

According to some experts, there is a need to provide an experimental basis for their safety and efficacy. In this background, some studies have been done to assess them for toxicity. However, there are a few informative data related to the effect at the genetic level. In this study, zinc-based JB was assessed for potential genotoxicity considering its wide use for the treatment of diseases such as diabetes, anemia, ophthalmalic disorders, cough, ulcers, and skin diseases [6–10]. A chemical which interacts with genetic material DNA and shows adverse effects by altering its structure or function is referred to as genotoxic. Most of the drugs are genotoxins and produce an irreversible impact on the genetic map. Mutagenicity studies by in vivo tests in mammals with a close resemblance of metabolism to humans have been reported [11-13]. The standard laboratory tests available for genotoxicity evaluation are bacterial reverse mutation test (Ames Test), sex-linked recessive lethal test in Drosophila melanogaster, Escherichia coli, reverse assay, in vitro mammalian chromosome aberration test, in vitro mammalian cell gene mutation test, mammalian erythrocyte micronucleus test, mammalian bone marrow chromosome aberration test, rodent dominant harmful lest, in vitro sister chromatid exchange assay in mammalian cells, Saccharomyces cerevisiae, and gene mutation assay [14].

The present study was mainly focused to evaluate genotoxicity profile of classically prepared JB by employing three important genotoxic testing protocols such as chromosomal aberrations, micronuclei assay, and comet assay in Swiss albino mice.

METHODS

The present experimental study was performed using 40 Swiss albino mice of both sexes which were obtained from animal house attached to...
The extent of DNA migration was determined for each sample by bromide. The slides were scored without knowledge of the dose group. After fixation, the slides were air dried then stained with ethidium base and incubated in alkaline conditions (300 mM NaOH, 1 mM EDTA, lysing solution, two slides per sample) was washed with 0.4 M Trizma and 10% dimethyl sulfoxide). Following at least 1–2 h of incubation in cold lysis solution (2.5 M NaCl, 100 mM ethylenediaminetetraacetic acid (EDTA), and 10 mM Trizma base, with 1% Triton X-100 (Sigma), and 10% dimethyl sulfoxide). Following at least 1–2 h of incubation in cold lysis solution, two slides per sample was rinsed with 0.4 M Trizma base and incubated in alkaline conditions (300 mM NaOH, 1 mM EDTA, pH 13) for 20 min, followed by electrophoresis in the same buffer for 30 min at 0.7 V/cm (electrode to electrode) and 300 mA current. After electrophoresis to neutralize the alkali, slides were immersed in an excess amount of 0.4 M Trizma base and then fixed in 100% ethanol. After fixation, the slides were air dried then stained with ethidium bromide. The slides were scored without knowledge of the dose group. The extent of DNA migration was determined for each sample by simultaneous image capture and scoring of 100 cells (50 cells on each of two slides) at ×200 magnifications using an imaging system with comet assay software. The extent of DNA migration for all samples was evaluated according to the following endpoint measurements such as percentage tail DNA, tail length, and olive tail moment.

**Micronucleus assay**

It was carried out by the method suggested by Witt et al., 2008 [15]. The bone marrow was aspirated from the shaft of femurs into 5% bovine serum albumin (BSA) using 25 gauge needle. The homogeneous suspension was made and centrifuged at 1000 rpm for 5 min. The supernatant was removed after the centrifugation. With 0.5 ml of 5% BSA, the sediment was mixed thoroughly and smeared on a clean slide. Air-dried the prepared slides and fixed for 5 min in absolute methanol. The air-dried smears were first stained with 1:1 diluted May-Grünwald stain using phosphate buffer of pH 6.8 for 15 min. Then, the slides were transferred to Giemsa stain diluted with phosphate buffer for 10 min. Moreover, slides were washed in buffer solution for 5 min. Air-dried slides were observed under 100X oil immersion objective to score the micronucleated polychromatic erythrocytes and micronucleated normochromatic erythrocytes. About 1000 polychromatic erythrocyte (PCE) and the corresponding number of normochromatic erythrocyte (NCE) scored for the presence of micronuclei and the determination of PCE/NCE ratio. These ratios were used as a measure of the toxicity of test materials. Statistical analysis was done to find out the significance of micronucleus induction.

**Chromosomal aberration test**

It was carried out in bone marrow cells by the method suggested by Hayashi, 1992 [16]. Bone marrow cells from both femurs were collected and subjected to hypotonic shock (KCl 0.075 M) for about 20 min at room temperature. The suspension was centrifuged at 1000 rpm for 8 min. The cells were fixed with the freshly prepared methanol-acetic acid (3:1) fixative for 45 min at room temperature and centrifuged at 1000 rpm for 8 min. Again the cells were re-suspended in fresh fixative for 10 min and centrifuged for 8 min at 1000 rpm. The procedure was repeated for 2–3 times. Finally, 0.5 ml of the suspension was prepared using fresh fixative. Two to three drops of cell suspension were dropped from a distance using Pasteur's pipette on a clean pre-chilled glass slide and were dried on the hot plate at 40°C. Then, the slides were air dried at room temperature and finally stained with a 5% dilution of Giemsa stain in phosphate buffer (pH 6.8) for 30 min. The chromosomes of 1000 cells in metaphase were analyzed with a ×100 oil immersion objective, using a trinocular microscope. Metaphases with chromosomes, chromatid breaks, gaps, rings, stickiness, centric fusion, and deletion were recorded.

**Statistical analysis**

The data were collected and expressed as mean ± SEM and analyzed by one-way ANOVA followed by Dunnett's multiple tests using GraphPad InStat version 3.5. p<0.05 was considered statistically significant.

**RESULTS**

Genotoxic potential of JB analyzed by alkaline (pH>13) comet assay

Cyclophosphamide (40 mg/kg) dose caused significant damage to the genetic material by significant increase in the parameters, such as comet length, comet height, comet tail length, percentage DNA, tail movement, and olive moment as compared to standard control (p<0.01). JB administered for 28 consecutive days at two different dose levels did not show any significant changes in these parameters, such as comet length, comet height, comet tail length, percentage DNA, tail movement, and olive moment and values are comparable with that of normal control (Table 1 and Fig. 1).

**Micronuclei assay**

There was a significant increase in the number of micronuclei (MNi) in the cyclophosphamide group as compared to normal control (p<0.01). The micronucleus was not found at the two dose levels of JB as compared cyclophosphamide group, and micronucleus assay did not reveal any statistically significant difference between the groups (Table 2 and Fig. 2).
Table 1: Effect of JB on DNA damage in comet assay

| Parameters          | Normal control (mg/kg) | Positive control (mg/kg) | JB 15.6 mg/kg | JB 78 mg/kg |
|---------------------|------------------------|--------------------------|---------------|-------------|
| Comet length        | 132±12.98              | 233±0.0±12.97 **         | 182.3±25.63   | 104.7±3.15  |
| Comet height        | 113±1.0.94             | 180.0±9.39 **            | 154±22.04     | 106.6±4.13  |
| Comet tail length   | 1.7±0.42               | 18.7±5.89 **             | 2.9±0.64      | 6.8±2.19    |
| % DNA               | 0.8±0.51               | 22.3±1.44 **             | 5.4±1.41      | 10.7±3.87   |
| Tail movement       | 0.002±0.0016           | 6.3±2.15 **              | 0.9±0.64      | 2.5±1.26    |
| Olive movement      | 7.3±2.18               | 19.3±2.04 **             | 6.1±2.24      | 2.4±1.38    |

JB: Jasada Bhasma

Table 2: Effect of JB on bone marrow cells in micronuclear test

| Group                 | MNPCEs/2000 PCEs |
|-----------------------|-----------------|
| Normal control        | 1.1±0.65        |
| Cyclophosphamide (40 mg/kg) | 60.6±6.86 **    |
| JB 15.6 mg/kg         | 1.6±0.33        |
| JB 78 mg/kg           | 1.8±0.30        |

Data expressed in mean ± SEM. *p<0.05, **p<0.01 in comparison to normal control. MNPCEs: Micronucleated polychromatic erythrocytes, PCE: Polychromatic erythrocyte, JB: Jasada Bhasma

Chromosomal aberration assay

The bone marrow cells were arrested in the metaphase stage and microscopically examined for both numerical and structural chromosomal aberrations such as chromosomal gap, chromatid gap, exchange, fragments, pulverization, ring, and deletion. It is observed that repeated administration of JB at both therapeutic and 5 times of dose levels did not produce any chromosomal aberrations both numerically and structurally (Table 3 and Fig. 3).

DISCUSSION

Genotoxicity is considered to be one of the potential risk factors for causing diseases such as cancer, cardiovascular, aging-related disorders, cystic fibrosis, sickle cell anemia, and diabetes. The incidence of genotoxicity and mutagenicity is prevailing high in recent years due to industrialization, exposure to agricultural pesticides and a wide range of chemical substances and radiations. Thus, the regulatory authorities have made it mandatory to test any substances for its safety profile including genotoxicity and mutagenicity. In the present study, a battery of tests such as comet assay, chromosomal aberration tests, and micronucleus assay was conducted to evaluate the genotoxicity potential of JB.

The toxicant materials cause genotoxicity by direct interaction with the genetic materials such as DNA or RNA. This interaction might be either with single nucleotide base or a particular location on the DNA structure. DNA damage can be assessed based on a number of breakings, lesions, fusions, deletions, or mis-segregations in intact DNA. It may lead to mutagenesis and causes different malignancies. Oxidative stress also plays an important role in the genotoxicity. During the normal cellular metabolism, that is, mitochondrial respiratory activity, certain amounts of oxidative free radicals are produced, which are effectively managed or detoxified by either endogenous or dietary antioxidants. However, in case of any chronic or acute exposure to certain cytotoxic or noxious chemicals, the endogenous antioxidant fails to detoxify. It results in the generation of the enormous amount of reactive oxygen species (ROS) such as O_2, OH-, and H_2O_2. ROS can cause severe oxidative lesions in DNA and form 8-hydroxy deoxyguanosine, which is a potent mutagenic lesion [17,18]. Along with genetic material, it can also denature biologically essential lipids and protein molecules and leads to degenerative diseases such as atherosclerosis, neurodegenerative disorders, and cystic fibrosis. Zinc considered being one of the essential trace elements, and it is safer only at lower doses; however, it can exert system toxicity at higher doses, mainly by deposit in neuronal tissues of the brain, and slowly causes cellular damage [19]. The long-term use or acute intoxication of zinc interferes with the absorption of copper, and hence many of the toxic symptoms are due to copper deficiency in the body. It also plays an important role in the cellular apoptosis by interfering with the molecular regulators of programmed cell death [20]. A study report reveals a boy who has developed lethargy and focal neurological deficits after 3 days of 12g of metallic zinc ingestion [21]. It has been reported that the zinc oxide nanoparticle exerts dose-dependant genotoxicity and cytotoxicity [22].

Comet assay or single cell gel electrophoresis is a simple, inexpensive, and more sensitive technique used in the analysis of DNA damage. It has wide applications in testing genotoxic potentials of large-scale pharmaceutical products, agriculture pesticides, and industrial

Fig. 1: Photomicrograph of comet assay. (a) NC - normal control, (b) CYP - cyclophosphamide, (c) JB (TED) - Jasada Bhasma therapeutic dose (TED), and (d) JB (TED ×5) - Jasada Bhasma 5 times of TED

Fig. 2: Photomicrograph of micronucleus assay in bone marrow cell smear. (a) NC - normal control, (b) CYP - cyclophosphamide control, (c) JB (TED) - Jasada Bhasma therapeutic dose (TED), and (d) JB (TED ×5) - Jasada Bhasma 5 times of TED
Despite its extensive use, the genotoxicity profile of JB is unexplored. In this regard evaluating the genotoxic potential of JB provided experimental proof for its safety. In the present study, the genotoxicity of JB was assessed by comet assay, micronuclei, and chromosomal aberration tests. Results show normal intact DNA and chromosomal structure. Thus, providing safety data will refine therapeutic utility of JB encouraging their rationale use and translate into higher and broader utilization of JB.

ACKNOWLEDGMENT

The authors are highly grateful to Director and Dean, Krishna Institute of Medical Sciences Deemed University, Karad, Maharashtra, India, for their constant motivation to carry out this research work. The authors thank revered Sri. CAA. Raghavendra Rao President of Srinivas Institute of Medical Sciences and Research Centre, Mangaluru, Karnataka, India, for his constant support.

AUTHOR’S CONTRIBUTION

Ravi Bhaskar carried out the study and was the charge of overall performance and planning. Megha Doshi, Shaktuhalna R Pai and Ravishankar S have designed and supervised the study. Ravi M is responsible for study concept and manuscript draft. Savitha Hemalatha is responsible for data analysis and critical revision.

CONFLICTS OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflicts of interest.

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