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Original Article

Anatomy Section

Evaluation of Genotoxicity Profile of Tamra Bhasma (A Copper Based Metallic Preparation) in Swiss Albino Mice

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ABSTRACT

Introduction: Since, thousands of years we have been using natural medication in Indian system of medicine. Till today lots of work has been done to study their toxicity, but very few studies have been undertaken on genotoxicity. There is urgent requirement for taking initiative in this regard. Hence, interested in evaluating the genotoxicity potential of Tamra Bhasma well known metallic (Copper) preparation widely used in alternative system of medicine.

Aim: To evaluate the genotoxicity profile of Tamra Bhasma drug (a copper based metallic formulation in Indian system of Medicine) with main emphasis on comet assay, micronucleus assay and chromosomal aberration assay in Swiss albino mice.

Materials and Methods: Animals were randomly grouped into four different groups containing ten mice (five male and five female) in each group. Group I considered as normal control which received only vehicle (0.5% CMC). The Group II considered as positive control and on 28th day which received cyclophosphamide (40 mg/kg/body weight) through intra-peritoneal injection for comparative assessment. The Groups III and IV administered with test drug Tamra Bhasma 2.86 mg/kg and 14.3 mg/kg respectively. These groups were administered the test drug for 28 consecutive days. On 29th day blood was collected into heparinized tubes for comet assay analysis. Half of the animals were injected with colchicine (4 mg/kg IP) in order to arrest the cells into metaphase stage and then sacrificed the animals to collect the bone marrow from the both the femurs for chromosomal aberration analysis. The rest of the animals were also sacrificed and bone marrow was collected for micronuclei analysis.

Results: The repeated administration of test drug Tamra Bhasma of both the dose levels didn't produce remarkable genotoxicity, DNA damage or chromosomal abnormalities both numerically as well as structurally. However, Tamra Bhasma administered at both the dose level groups showed slight increase in comet height, comet tail length, % DNA and Tail movement. But increase was not significant as compare to normal control.

Conclusion: The repeated administration of test drug Tamra Bhasma at therapeutic dose level confirms its relative safety and did not produce any marked toxic effect or genetic material abnormalities. This work may inspire more efficacious and safer use in clinical set up and global acceptance of the test formulation.

Keywords: Chromosomal aberration, Comet assay, Micronuclei assay

INTRODUCTION

Genotoxicity testing of a drug is an integral component of regulatory requirement of different regulatory authorities around the world. Genotoxicity testing of a drug, hazardous identification with respect to DNA damage is generally applied. The DNA damage can be manifested in the form of gene mutation, chromosomal aberration and recombinant changes [1,2]. These structural and functional changes in DNA on germ cells may be responsible for heritable effect and it leads to sever health hazardous on future generation. In somatic cells the DNA damaged cells may also play an important role in development of different kinds of malignancies [3].

The preclinical data related to the safety and efficacy of a drug is very much essential to carry out the clinical trial and the data can be used to extrapolate to the human health risk. Hence, in the present scenario the toxicity assessment are carried out in parallel with that of pharmacological studies. According to regulatory requirements the information related to the mutagenic and carcinogenic testing are also required for clinical trials as well as to market the drug.

In India the genotoxicity testing for a drug is mandatory under Scheduled Y of Drug and Cosmetic Rules 1988, Central Drugs Standard Control Organisation, New Drug Division issued by Ministry of Health and Family Welfare, Government

of India [4]. The OECD guidelines are specifically important because of complying the same data one can ensure the acceptance of toxicity data of 30 member countries of OECD [5,6]. In India eighth amendment of Drug and Cosmetics Rules recommends mutagenecity and carcinogenicity testing under scheduled Y before clinical trials and no specific tests has been recommended for this purpose.

Tamra Bhasma is an important metallic preparation used in Indian System of Medicine. It has wide medicinal value and indicated in ailments such as anaemia, peptic ulcer, skin disorders, metabolic disorders, fever, tuberculosis, liver and spleen disorders, eye diseases, lipid metabolic disorders, dyspepsia and cardiac disorders etc., [7]. Besides its wide therapeutic value, the detailed preclinical toxicity profile was not documented. In the current scenario much attention has been given on the safety issues of drugs. Hence, the present study was aimed to evaluate the genotoxicity profile of Tamra Bhasma by employing three important genotoxicity testing such as chromosomal aberration, micro nuclear assay and comet assay in Swiss albino mice.

MATERIALS AND METHODS

Genotoxicity experiments were carried out in Swiss albino mice of both the sexes weighing from 25-30 gm body weight. Study was conducted between the years 2012-16, after getting approval from ethical committee. All the animals were procured from animal house facility attached to pharmacology laboratory at SDM Centre for Research in Ayurveda and Allied Sciences, Udupi, India. All the animals were acclimatized to standard laboratory conditions such as temperature of 25±2°C and 55-60% of humidity and 12 hours day and night cycle throughout study. The animals were fed with mice diet and water ad libitum. All experiments were carried out after getting an Institutional animal ethical committee approval bearing reference number SDMCA/IAEC/CPCEA/GI04/2011.

Experimental design: Healthy Swiss albino mice weighing 25-30 gm body weight were selected for the study. Animals were grouped into four different groups with ten mice each. The Group I considered as normal control which received only vehicle (0.5% CMC). The Group II considered as toxicant control which received 40 mg/kg cyclophosphamide on 28th day. The Group III received the test drug Tamra Bhasma therapeutic dose (2.86 mg/kg) and the Group IV receives five times of therapeutic dose (14.3 mg/kg). The test drug Tamra Bhasma was administered for 28 consecutive days. On 28th day the Group II (toxicant control) cyclophosphamide at dose of 40 mg/kg/body weight was injected through intra-peritoneal route for comparative assessment. On 29th day blood was collected into heparinized tubes from orbital plexus under mild ether anaesthesia from all the groups for comet assay analysis.

Test drug: Tamra Bhasma (a copper based metallic preparation) was used to assess the Genotoxicity profile. The test drug was manufactured by renowed expert in *Rasa Shastra* (a specialised branch of Ayurvedic Science). The Batch number of test drug Tamra Bhasma- 25/November, 2012 was used.

Dose fixation: The dose of Tamra Bhasma for the therapeutic purpose in humans is 22 mg/kg/body weight. The mice dose was calculated by referring the standard conversation table of Paget and Barne's (1964) [8]. Two dose ranges were selected for the genotoxicity studies i.e. therapeutic dose (TED) and five times of therapeutic dose (TED x 5). On the basis of this the mice dose was calculated as 2.86 mg/kg/body weight (TED) and 14.3 mg/kg/body weight (TED x 5). Tamra Bhasma was suspended in 0.5% Carboxy Methyle Cellulose (CMC) and administered orally at a dose of 1 ml/100 mg body weight with the help of oral catheter.

On 29th day, after blood withdrawal five mice from each group were injected with colchicine (4 mg/kg IP) in order to arrest the dividing cells into metaphase stage and then sacrifice the animals to collect the bone marrow from the both the femurs for chromosomal aberration test. And the rest of the animals were also sacrificed and bone marrow was collected for micronuclei assay [9].

Peripheral blood samples were processed for evaluating comet assay [10,11] and the bone marrow cells were processed for chromosomal aberration test [12] and micro nuclear assay [13].

1. Alkaline (pH>13) Comet Assay

The alkaline (pH>13) comet assay and was carried out according to Burlinson B et al., and Hartmann A et al., [10,11]. Aliquots of blood sample were immediately placed into micro centrifuge tubes. The slides for the comet assay were prepared. A 10 μL aliquot of suspension containing approximately 10,000 cells were mixed with 0.5% low melting point agarose (Sigma) and spread on standard microscope slides pre-dipped in agarose. prior to further processing slides were then allowed to harden on a cold surface. All slides were placed in cold lysis solution (2.5 M NaCl, 100 mM EDTA, and 10 mMTrizma base, with 1% Triton X-100 (Sigma) and 10% DMSO). Following at least 1-2 hour of incubation in lysing solution, two slides per sample were rinsed with 0.4 M Trizma base and incubated in alkaline conditions (300 mMNaOH, 1 mMEDTA, pH>13) for 20 minutes, followed by electrophoresis in the same buffer for 30 minutes at 0.7 V/cm (electrode to electrode) and 300 mA current. After electrophoresis, slides were immersed in an excess amount of 0.4 M Trizma base to neutralize the alkali and then fixed in 100% ethanol [10].

Following fixation, the slides were air dried and stored at

room temperature in desiccators until scoring. Prior to scoring slides, the DNA was 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 200X magnifications using imaging system with comet assay software. The extent of DNA migration for all samples was evaluated according to the following endpoint measurements.

% Tail DNA: Intensity of all tail pixels divided by the total intensity of all pixels in the comet, expressed as a percentage.

Tail Length: Horizontal distance from the center of the head to the end of the tail.

Olive Tail Moment: The distance between the center of gravity of the DNA distribution in the tail and the center of gravity of the DNA distribution in the head multiplied by the fraction of DNA in the tail.

2. Micronucleus Assay Test

It was carried out in bone marrow cells by the method suggested by Witt KL et al., [12]. The bone marrow was aspirated from the shaft of femurs into 5% Bovine Serum Albumin (BSA) using 25 guage needle. The marrow was made homogeneous suspension and centrifuged at 1000 rpm for 5 minutes. The supernatant was removed. The sediment was mixed thoroughly with 0.5 ml of 5% BSA and smeared on a clean slide. Prepared slides were air-dried, fixed for 5 minutes in absolute methanol. The air dried smears were first stained with 1:1 diluted May-Grunwald stain using phosphate buffer of pH 6.8 for 15 minutes. Then, the slides were transferred to Giemsa stain diluted with phosphate buffer for 10 minutes. And in buffer solution slides were washed for 5 minutes. Air dried slides were observed under 100X oil immersion objective to score the Micronucleated Polychromatic Erythrocytes (MnPCEs) and Micronucleated Normochromatic Erythrocytes (MnNCEs). About 1000 PCE and corresponding number of NCE scored for the presence of micronuclei and for the determination of PCE/NCE ratio. These ratios were used as a measure of toxicity of test materials. Statistical analysis was done to find out the significance of micronucleus induction.

3. Chromosomal Aberration Test

It was carried out in bone marrow cells by the method suggested by Hayashi et al., [13]. 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 minutes. The cells were fixed with freshly prepared methanol-acetic acid (3:1) fixative for 45 minutes at room temperature and centrifuged at 1000 rpm for 8 minutes. Again the cells were re-suspended in fresh fixative for 10 min and centrifuged for 8 min at1000 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 was dropped from a distance using Pasteur's pipette on a clean pre-chilled glass slides and were dried on 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 minutes. The chromosomes of 1000 cells in metaphase were analysed with a 100X 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 was expressed in mean±SEM and analysed one-way-ANOVA followed by Dunnet's multiple 't' test using GraphPad instant version 3.5 for determining the level of significance of the observed effects. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Alkaline (pH>13) comet assay: The following parameters in comet assay was observed at the end of the experiment by using comet score software such as comet length, comet height, comet area, comet diameter, comet tail length, % DNA (delete word comet), tail movement and olive movement. Among the above mentioned parameters Tamra Bhasma administered at both therapeutic and five times of therapeutic dose groups showed slightly or moderate increase in comet height, comet tail length, % DNA and Tail movement, however the increase was not statistically significant as compared to normal control. And parameters such as comet length, comet area, comet

Group	Comet Length	Comet Hieght	Comet Area	Comet Diameter	Comet Tail Length	% DNA	Tail Movement	Oilive Movement
Normal Control	132±12.98	113± 10.94	10352.38± 1305.5	114.69± 10.85	1.71±0.42	0.82±0.31	0.00228± 0.0016	7.30±2.18
Positive Control	233.08± 12.97*	180.08± 9.39**	28484.46± 3614.9*	232.62± 13.02**	18.75±5.84**	22.30±4.64**	6.36±2.15**	19.31±2.04*
Tamra Bhasma (TED)	112.15±5.25	102.62± 4.22	7511.85± 664.36	101.08± 6.37	11.07±4.23	10.53±4.22	2.87±1.96	9.02±2.48
Tamra Bhasma (TED x5)	151.23± 11.38	127.92± 8.46	13408± 1697.9	132.15± 9.90	12.16±5.40	8.99±3.73	2.49±1.07	15.86±5.48

[Table/Fig-1]: Effect of Tamra bhasma on DNA damage in comet assay.
Data expressed in mean±SEM, *p<0.05, **p<0.01 in comparison to normal control

diameter and olive movement revealed almost nearly normal profile when compared to normal control [Table/Fig-1].

Micronucleus assay: Cyclophosphamide group has showed significant increase in Micronuclear Polychromatic Erythrocytes (MNPCE) in comparison to normal control. Whereas, repeated administration of test drug Tamra Bhasma of both the dose groups produced remarkable reduction in the MNPCE and it is almost similar as compared to normal control [Table/Fig-2].

Group	MNPCEs/ 2000 PCEs
Normal control	1.16±0.65
Cyclophosphamide control	60.66±6.86**
Tamra Bhasma (TED)	0.83±0.30
Tamra Bhasma (TED X5)	0.66±0.33

[Table/Fig-2]: Effect of Tamra Bhasma on bone marrow cells in micronuclear test.
*Mean±SEM, **p<0.01 in comparison to normal control group

Chromosomal aberration assay: Microscopically, examined the cells arrested in the metaphase for both numerical and structural chromosomal aberrations such as chromosomal gap, chromatid gap, pulverization and stickiness, ring, deletion, fragments and translocation. Repeated administration of test drug Tamra Bhasma at both the dose groups didn't produce chromosomal abnormalities in both structurally as well as numerically [Table/Fig-3].

Group	CtG	CG	Ex Gap	D Gap	Fg	PV	R	DL
Normal control	-	-	-	-	-	-	-	-
Cyclophosphamide control	+	+	+	+	-	+	+	+
Tamra Bhasma (TED)	-	-	-	-	-	-	-	-
Tamra Bhasma (TED X 5)	-	-	-	-	-	-	-	-

[Table/Fig-3]: Effect of Tamra Bhasma on chromosomal abberation assay.
Chromosomal gap-CG, Chromatid gap-CtG, Exchange-Ex, Dientric-D, Fragments-Fg, Pulverization-PV, Ring-R, Deletion-DL

DISCUSSION

Genotoxicity profile was evaluated on the effect of Tamra Bhasma on following parameters such as comet assay, micronuclei assay and chromosomal aberration assay. The cytotoxic effects of chemotherapeutic drugs like Cyclophosphamide often results in defective DNA, abnormal cell function and or cell death. This has been evident in the form of gene mutations, chromosomal aberrations and presence of micronuclei in animal models [14]. Antibacterial activity against enteric pathogens and anti-hyperlipidemic activity of Tamra Bhasma was reported by Tambekar DH et al., [15] and Jagtap CY et al., [16].

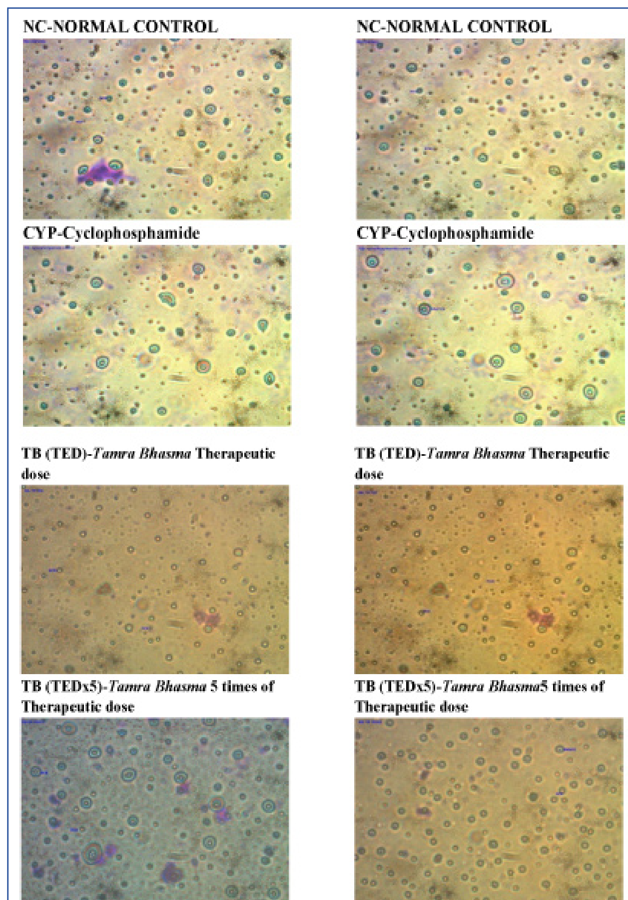
Comet Assay: The comet assay is increasingly being used in assessing genotoxic potential of pharmaceutical products, industrial chemicals, environmental toxins etc. The comet assay is rapid, simple to perform and can be performed on any eukaryotic cells [17]. Thus, in early drug development comet assay serves as an important tool as a mechanistic and genotoxic predictor [18]. Increase in the DNA fragments represents the DNA damage that has migrated out of the cells nucleolus in the form of a characteristic streak similar to the tail of a comet. Tamra Bhasma administered at both therapeutic and five times of therapeutic dose groups showed slight or

[Table/Fig-4]: Photomicrograph of comet assay- Tamra bhasma.

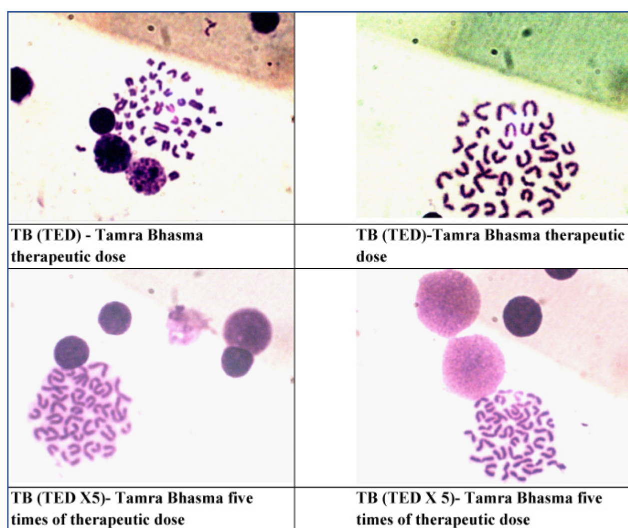
moderate increase in comet height, comet tail length, % DNA and Tail movement, however the increase was not significant as compared to normal control [Table/Fig-4].

Micronucleus Assay: Micronuclear assay is a genotoxicity test for detecting micronuclei in the cytoplasm of interphase cells. MN may originate from acentric chromosomal fragments or whole chromosome unable to migrate to the poles during the anaphase stage of cell division [19,20]. Repeated administration of test drug Tamra bhasma at both the dose level groups produced remarkable reduction in the MNPCE and it is almost similar as compared to normal control. The decreased micronucleus cells in micronuclei assay test indicates that

the administered test drug Tamra Bhasma did not induce chromosomal breakage or chromosomal gain or loss in bone marrow cells. Hence, the test substances administered at both dose levels were found to be negative and indicates the test substances do not induce chromosomal breaks or gain and loss in bone marrow cells of mice [Table/Fig-5]. This is clear indication of absence of mutagenicity potential in the test drug even on repeated administration for 28 days and at five times higher dose than the one used in therapeutic settings.



[Table/Fig-5]: Photomicrograph of Micronucleus assay test in bone marrow cell smear Tamra bhasma.



[Table/Fig-6]: Photomicrograph of chromosomal aberration test.

Chromosomal Aberration Assay: The chromosomal aberration test in bone marrow cells in vivo cytogenetic analysis provides a valuable technique for evaluating damage to chromosomes on the basis of direct observation and classification of chromosomal aberrations [21]. On microscopic examination, the cells arrested in the metaphase for both numerical and structural chromosomal aberrations. Repeated administration of test drug Tamra Bhasma at both the dose level groups did not produce chromosomal abnormalities both structurally as well as numerically [Table/Fig-6]. Chaudhari CY et al., suggests the use of therapeutic drugs prepared in Tamra Bhasma (TB) and in Amritikirana is safe at the genetic level as demonstrated to be lack of deformity in chromosomal aberration assay [22].

LIMITATION

The limitations of the study were the sample size and duration of the experimental dosage. Parameters used are preliminary to evaluate the chromosomal aberrations hence further techniques like karyotyping etc., may be recommend to analyse the chromosomal aberrations.

CONCLUSION

The premise is that the metal, mineral or herbo-mineral preparations used in the Indian system of medicine may cause toxic effect on mammalian tissues. Hence, the present study was designed to undertake genotoxicity effect of test drug (Tamra Bhasma) in Swiss albino mice. The test substances did not cause changes in the molecular (DNA) level, which are expressed at the level of cellular and organ toxicity.

From the repeated dose genotoxicity study we can conclude that Tamra Bhasma was relatively safer and did not produce any marked toxic effect or abnormalities in genetic material. However, the higher dose used in the present study was much higher than routinely practiced clinical doses. This work may inspire more efficacious and safer use in clinical set up and global acceptance of the test formulations.

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