Original Article

Assessment of Genotoxic Potential of *Hridayarnava Rasa* (A Herbo-Mineralo-Metallic Ayurvedic Formulation) Using Chromosomal Aberration and Sperm Abnormality Assays

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ABSTRACT

Objectives: Herbo-mineral formulations are being successfully used in therapeutics since centuries. But recently, they came under the scanner for their metallic contents especially the presence of heavy metals. Hence it is the need of the hour to assess and establish the safety of these formulations through toxicity studies. In line with the various toxicity studies that are being carried out, Government of India expressed the need for conducting genotoxicity studies of different metal- or mineral-based drugs. Till date very few Ayurvedic herbo-mineral formulations have been studied for their genotoxic potential. The present study is aimed to evaluate the genotoxic potential of *Hridayarnava Rasa*. **Materials and Methods:** It was prepared as per classical guidelines and administered to Swiss albino mice for 14 consecutive days. Chromosomal aberration and sperm abnormality assay were done to evaluate the genotoxic potential of the test drugs. Cyclophosphamide (CP) was taken as positive group and results were compared. **Results:** All treated groups exhibited significant body weight gain in comparison to CP group. Results revealed no structural deformity in the above parameters in comparison to the CP-treated group. **Conclusion:** Reported data showed that both tested samples of *Hridayarnava Rasa* does not possess genotoxic potential under the experimental conditions and can be safely used.

Key words: Colchicine, cyclophosphamide, genotoxicity, herbo-mineral formulations, *Tamra bhasma*

INTRODUCTION

Rasashastra is one of the inseparable parts of Indian Traditional Medicine, which comprehensively deals with different metals, minerals, gems, herbs, poisonous plants, their properties, different processing techniques and therapeutic uses. This branch not only deals with the clinical uses of these materials but also their possible adverse effects and their management. Most of the preparations of Rasashastra are 'herbo-mineralo-metallic' in nature, that is, they contain minerals and metals as an integral part of their formulations along with the herbs. Use of metals in medicine is often associated with toxicity,[1] but they are made biocompatible in a particular chemical form by a detoxification process, which removes the toxic potential from metals and imparts them with higher level of therapeutic efficacy.[2] Mainly two facts encouraged the use of these formulations in therapeutics; first, they are being routinely used as effective drugs for centuries and secondly, these drugs do not show any noticeable side effects with

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the recommended doses. Some recent studies have also postulated that the herbo-mineralo-metallic formulations, if prepared and administered properly, are safe and efficacious.[3] But in the current scenario, some researchers are frequently raising doubts regarding the safety of these herbo-mineral preparations especially over their heavy metal content. To answer their allegations, many studies have been carried out in the recent past that evaluated the safety of these preparations, especially metallic Bhasmas. To name a few, toxicity studies of *Abhraka Bhasma*, *Tamra Bhasma*, *Trivanga Bhasma*, *Yashada Bhasma*, *Shilasindura*, etc., and many more have concluded their safety at therapeutic dose levels.[4-8]

Furthermore in addition to the toxicity studies going on, Government of India expressed the need for conducting genotoxicity studies of different metal- or mineral-based drugs.[9] Till date, very few studies have been carried out to evaluate the genotoxic potential of Ayurvedic herbo–mineral formulations. *Swarna makshika Bhasma*, *Rasamanikya, Lauha Bhasma, Tamra Bhasma, Kajjali, Abhraka Bhasma*, *Mandura Bhasma*, *Lauha Bhasma*, *Tamra Bhasma*, *Kajjali*, *Shwasakuthara Rasa* and *Smritisagar Rasa* are tested for their genotoxic potential by maximum single dose 2000 mg assessed through micronucleus (MN) assay and Comet assay.[10-12]

In the present study, *Hridayarnava Rasa*, a herbo-mineral formulation, which is prepared by triturating *Kajjali* (black sulfide of mercury-HgS) and *Tamra Bhasma* (incinerated copper) with the decoction of three myrobalans and juice of *Solanum nigrum* Linn. is evaluated for its genotoxic potential by chromosomal aberration (CA) and sperm abnormality assays. Both the tests are commonly used to assess the genotoxic potential of drugs.

MATERIALS AND METHODS

Test drugs

Test Drug 1: *Hridayarnava Rasa* prepared by using *Shodhita Tamra Bhasma* [STBHR].

Test Drug 2: *Hridayarnava Rasa* prepared by using *Ashodhita Tamra Bhasma* [ATBHR].

Both the test drugs were prepared in the laboratory of *Rasashastra* and *Bhaishajya Kalpana*, Institute for Post Graduate Teaching and Research in Ayurveda (I. P. G. T. and R. A), Gujarat Ayurved University, Jamnagar by following standard guidelines as prescribed in classical Ayurvedic literature.[13,14]

For the preparation of *Shodhita Tamra Bhasma,* copper scraps with 99.89% pure copper was subjected to general and specific purification procedure. For general purification, copper scrap was heated in iron pan to red hot state and then quenched seven times in each of these media – sesame oil, buttermilk, cow urine, sour gruel and decoction of *Dolichos biflorus* Linn.[15] Specific purification of thus obtained copper was carried out by boiling in cow urine for three hours.[16] After that, it was washed with hot water and dried. This *Shodhita* copper was then subjected to incineration by mixing equal amount of purified sulfur, *Kajjali* (black sulfide of mercury) and juice of *Citrus medica* Watt.[17] *Ashodhita Tamra Bhasma* was prepared by the same manner but the procedures of general and specific purification were excluded in it. The process of *Amritikarana* (nectarization) was carried out in both the *Bhasmas*.[18]

Chemicals

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

Animals

Adult Swiss albino mice of either sex, weighing 35 ± 5 gm were used in the study. Animals were obtained from animal house attached to the Pharmacology laboratory, S. S. R College of Pharmacy, Silvassa, and were exposed to natural day and night cycles, with ideal laboratory conditions in terms of ambient temperature and humidity. Temperature during the time of carrying out the experiment was between 24 ± 2°C and humidity 50–60%. Animals were fed *ad libitum* with Amrut brand mice feed supplied by Pranav Agro Industries and RO (reverse osmosis) purified water. The experiment was carried out after obtaining the permission from Institutional Animal Ethics Committee (Approval number: IAEC/2013/05) and care of animals was taken as per the CPCSEA guidelines.

Dose fixation

Clinical dose of *Hridayarnava Rasa* is 125 mg twice a day.[14] The suitable dose for mice was calculated by referring to the table of Paget and Barnes and was found to be 32.5 mg/kg.[19] The test drugs were administered in the form of suspension made in honey orally with the help of rubber catheter attached to a disposable syringe. For the preparation of stock solution, both the test drug samples were taken in requisite quantity in small porcelain mortar and honey 10 ml/kg body weight of mice was added, the formed mixture was further grounded for 5 minutes to make it homogenoius.

Experimental design

The animals were randomized into five groups consisting of five animals in each group for evaluating the influence of STBHR and ATBHR on chromosoms

and sperm morphology. Group I served as normal control (NC) receiving tap water and normal food. Group II served as positive control and treated with CP single dose 25 mg/kg intra-peritoneally 24 hour prior to termination.[10,20] Group III served as vehicle control (VC) and treated with honey 10 ml/kg body weight. Group IV and V were treated with test drugs [stock solution 10 ml/kg body weight (containing test drug 32.5 mg/kg body weight and honey 10 ml/kg body weight)] for 14 consecutive days and sacrificed on 15th day [Table 1].

Body weight

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

Chromosomal aberration assay

Animals were injected colchicine intra-peritoneally at the dose of 4 mg/kg body weight, on the 15th day in order to arrest dividing cells in metaphase[12] and sacrificed by cervical dislocation, 90 minutes after the colchicine treatment. Bone marrow cells from both femurs were extracted, subjected to hypotonic shock treatment (KCL 0.075M) for about 30 minutes at room temperature and then centrifuged at 1000 rpm for 10 minutes. 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 hot plate at 40°C. One more drop of fixative was added on slides to see more reliable pictures of chromosomes and then the slides were air dried at room temperature and finally stained with 5% dilution of Giemsa reagent in phosphate buffer (pH 6.8) for 15 minutes.[13] The chromosomes of 100 metaphase cells per group and 20 metaphases per animal were analyzed with × 100 oil immersion objective, using a Trinocular Research Carl Zeiss Microscope (Germany). Metaphases with chromosomes and chromatid breaks, gaps, rings, stickiness, dicentrics, centric fusion and deletion (if any) were recorded.[21]

Sperm abnormality assay

The method of Wyrobek and Bruce was used for investigating sperm morphology abnormality assay.[22] 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 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 minutes. 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 to assess the change in body weight by applying paired *t* test. The results were presented as Mean ± SEM for five mice in each group by using Sigmastat software (version 3.1) for all the treated groups with the level of significance set at $P < 0.05$.

OBSERVATIONS AND RESULTS

The effect of STBHR and ATBHR in body weight, chromosomal aberration and sperm abnormality assays are shown in Tables 2-4.

DISCUSSION

To assess the chemicals' potential to cause DNA damage that may lead to cancer, many sophisticated techniques including Ames assay, *In vitro* and *in vivo* Toxicology Tests, and comet assay have been developed. Since CA assay and sperm abnormality assay are not employed till date to any herbo-mineral formulation to test their DNA damage potential, in the present study, a 14-day genotoxic profile

Table 1: Treatment schedule

Group	No of animals	Drug	Dose	Duration
I	5	NC	-	14 days
II	5	CP	25 mg/kg	
III	5	VC	10 ml/kg	
IV	5	STBHR	32.5 mg/kg	
V	5	ATBHR	32.5 mg/kg	

NC = Normal control, CP = Cyclophosphamide control, VC = Vehicle control, STBHR = *Hridayarnava Rasa* prepared using *Shodhita Tamra Bhasma*, ATBHR = *Hridayarnava Rasa* prepared using *Ashodhita Tamra Bhasma*

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

Group	Treatment	Body weight (g)		Actual % change
		Before treatment	On 15th day	
I	NC	34.80±1.02	45.20±1.02	10.40±0.98↑
II	CP	39.67±1.17	44.40±2.04	4.80±1.49↑
III	VC	36.80±1.20	43.60±2.14	18.07±2.82**↑
IV	STBHR	39.67±1.41	44.67±1.52	12.68±1.25*↑
V	ATBHR	38.67±2.04	44.00±1.46	14.54±3.31*↑

Data: Mean±SEM, ↑ = Increase, ↓ = Decrease, *P<0.05, **P<0.01 (Unpaired *t* test in comparison to CP group). NC = Normal control, CP = Cyclophosphamide control, VC = Vehicle control, STBHR = *Hridayarnava Rasa* prepared using *Shodhita Tamra Bhasma*, ATBHR = *Hridayarnava Rasa* prepared using *Ashodhita Tamra Bhasma*

of STBHR and ATBHR was evaluated by employing these two tests.

Body weight of mice was recorded after 14 days of drug administration and compared with CP group. It showed significant normal progressive weight gain in all treated groups. This indicates toward the absence of any toxic degenerative potential in test drugs [Table 2].

Table 3: Results of chromosomal aberration assay

Groups	Chromosomal aberration									
	Chromatid		Chromosomal		De	Ex	Fg	PS	R	Dc
	Gap	Break	Gap	Break						
NC	–	–	–	–	–	–	–	–	–	–
CP	+	+	+	+	+	+	+			+
VC	–	–	–	–	–	–	–	–	–	–
STBHR	–	–	–	–	–	–	–	–	–	–
ATBHR	–	–	–	–	–	–	–	–	–	–

De = Deletion, Ex = Exchange, Fg = Fragments, PS = Pulverization and stickiness, R = Ring, Dc = Dicentric, + = Present, – = Absent, NC = Normal control, CP = Cyclophosphamide control, VC = Vehicle control, STBHR = *Hridayarnava Rasa* prepared using *Shodhita Tamra Bhasma*, ATBHR = *Hridayarnava Rasa* prepared using *Ashodhita Tamra Bhasma*

Table 4: Results of sperm abnormality assay

Groups	Sperm abnormality assay					
	Head abnormalities				Tail abnormalities	
	Amorphous shape	Hook less	Banana shaped	Folded	Double tailed	Coiled
NC	–	–	–	–	–	–
CP	+	+	+	+	–	+
VC	–	–	–	–	–	–
STBHR	–	–	–	–	–	–
ATBHR	–	–	–	–	–	–

+ = Present, – = Absent, NC = Normal control, CP = Cyclophosphamide control, VC = Vehicle control, STBHR = *Hridayarnava Rasa* prepared using *Shodhita Tamra Bhasma*, ATBHR = *Hridayarnava Rasa* prepared using *Ashodhita Tamra Bhasma*

In vivo, chromosome aberration assay has been recommended by regulatory authorities for the assessment of genotoxicity and mutagenicity of many chemicals and natural compounds.[23] The assay is sensitive and the data obtained from it is considered highly relevant in human context.[24] Here the deleterious effects (aberrations) on whole chromosomes or single chromatid and types of morphological alterations like breaking or rearrangements can be directly visualized under microscope.[25] Colchicine is effective in causing metaphase stasis in matrix, thus used to arrest metaphase, when chromosome structure seen noticeably.[26] 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) is used to facilitate visual analysis because it causes the cells to swell and enhances eventual separation of the chromosomes. CP is a covalent DNA-binding agent.[27] Its cytogenotoxicity has been reviewed and updated by Anderson, and its use as a positive control chemical in genotoxicity tests has been recommended.[28,29]

In the present study, different chromosomal abnormalities like gap, ring formation, stickiness etc., were observed in CP treated group along with more number of aberrations in the form of chromosome breaks and centric fusion. [Figure 1a–f] Centric fusion was observed more frequently because almost all mouse chromosomes

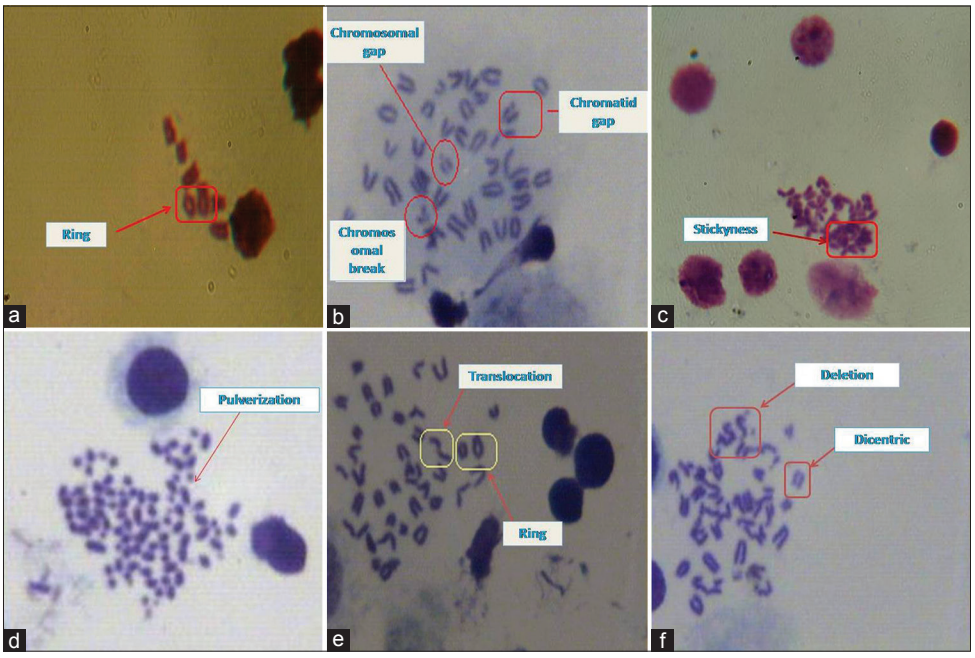

Figure 1: (a) CP- Ring; (b) CP- Chromosomal gap, chromatid gap, chromosomal break; (c) CP- Stickiness, (d) CP- Pulverization; (e) CP- Translocation and Ring; (f) CP- Deletion and dicentric (×100 magnification)

are acrocentric and thus they exceptionally merge with each other. Both the test drugs are devoid of any of the above aberrations. Observations regarding various structural aberrations in test groups are given in Table 3 [Figure 2a–d].

Sperm morphology test provides a direct measure of the quality of sperm produced in chemically treated animals such as in the use of formaldehyde.[30] In this, assessment of chemical effects on exposed mice is based on visual scoring of the percentage of sperms with abnormal head forms and shapes in smear.[31,32] It is known that during spermatogenesis, DNA synthesis occurs before the pre-meiotic phase and no further synthesis occurs throughout the duration of spermatogenesis in the cell cycle. Once sperm head develops its shape, it becomes extremely stable. Thus, according to the work of Bruce and Heddle, sperm-head morphological abnormalities may occur as a consequence of natural mistakes in the spermatozoon differentiating process and a chemical mutagen might increase the frequency of these mistakes.[33] Thus much importance is given to the morphological changes in head of the sperms. Different abnormalities in head are – amorphous shape, without hook, banana shaped and folded head. Abnormalities in the tails of sperms like coiled tail, double tail etc., are also assessed.

Maximum numbers of sperm abnormalities in head and tail were observed in CP treated group. The abnormalities like amorphous-shaped head, banana-shaped, hook-less head and coiled tail were more frequent than other abnormalities of head and tail in CP treated group. [Figure 3a–d] The test preparations were devoid of any such sperm abnormalities showing their non toxicity to sperms. Results are shown in Table 4 [Figure 4a–d].

The results of the current study indicate that therapeutic use of *Hridayarnava Rasa* is safe from the genotoxic point of view since its 14-day administration does not produce any chromosomal aberration and sperm morphological abnormality. Even the *Hridayarnava Rasa* prepared by using *Ashodhita Tamra Bhasma* was found to be safe in both the tests. However, to support this observation single cell alkaline gel electrophoresis coupled with micronuclei induction test can also be carried out along with other toxicity studies.

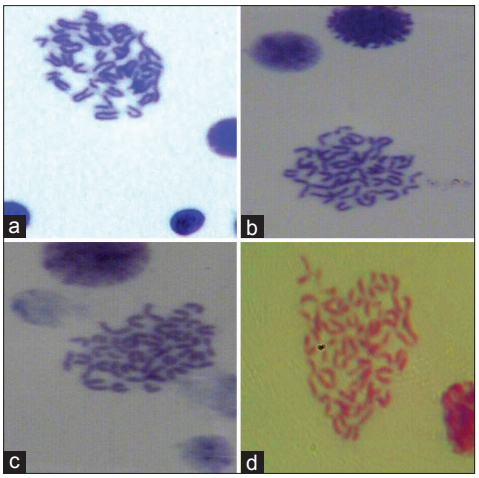

Figure 2: (a) NC - Normal chromosome; (b) VC - Normal chromosomes; (c) STBHR - Normal chromosome; (d) ATBHR - Normal chromosome (×100 magnification)

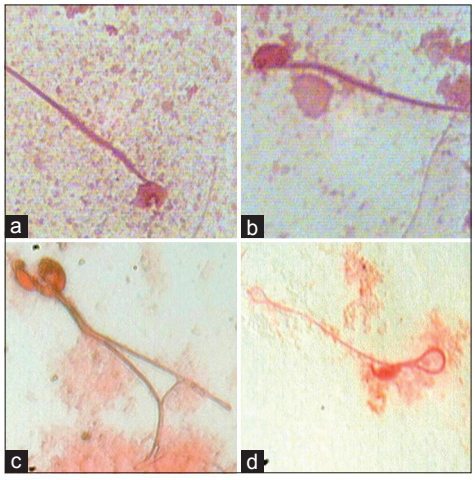

Figure 3: (a) CP- Amorphous head; (b) CP- Hook-less head; (c) CP- Amorphous, Banana shaped and hook-less head; (d) CP- Coiled tail (×400 magnifications)

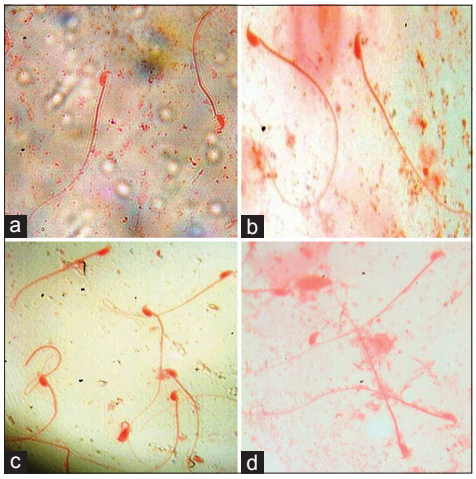

Figure 4: (a) NC - Normal sperm; (b) VC - Normal sperm; (c) STBHR - Normal sperm; (d) ATBHR - Normal sperm (×400 magnification)

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