ORIGINAL ARTICLE

Genotoxicity studies of Rheumavedic capsule

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Rheumatoid arthritis is a chronic systemic autoimmune disorder and the drugs used to treat are taken for a long period of time, so it is necessary to ensure that Rheumavedic (RV) capsule, used to treat rheumatoid arthritis, is free from any toxicity. The present study was taken up to evaluate the genotoxic potential of RV capsule. Swiss albino mice (25–30 g) were divided into eight groups (n=6). Control mice received vehicle (2 ml distilled water, p.o.), Group II, III, IV mice received cyclophosphamide (CPA) dissolved in vehicle, Group V animals received RV (780 mg/kg,p.o.×7 days), Group VI, VII, VIII animals received RV for seven days followed by CPA on the 7th day. Animals were sacrificed after 24 (Group II and VI), 48 (Group III and VII) and 72 h (Group IV and VIII) of CPA challenge. Bone marrow micronucleus test (MNT) and chromosomal aberration test was carried out against CPA-induced clastogenicity (100 mg/kg, IP) in mouse. One-way ANOVA, Post-hock analysis by Tukey's multiple comparison tests was done using GraphPad Prism 5. There was no significant decrease in micronucleus formation and chromosomal aberrations in RV-treated mice against CPA-induced clastogenicity. RV is free of genotoxicity, but do not have antigenotoxic effect.

Key words: Bone marrow, chromosomal aberrations, cyclophosphamide, Rheumavedic, micronucleus

INTRODUCTION

Genotoxicity testing is an important part of preclinical safety assessment of any drug. It is designed to detect genetic damage such as gene mutations and chromosomal aberration, which may be reflected in tumorigenic or heritable mutation potential of the drug. As the mechanisms of micronucleus formation are related to those inducing chromosomalaberrations, both micronuclei and chromosomal aberrations can be accepted as assay systems to screen for clastogenicity induced by test compounds.[1] Micronucleus test (MNT) and chromosomal aberration test (CAT) using mouse bone marrow system are proven test to detect anticlastogenic or antigenotoxic properties of a drug or chemical comp ound.

"Rheumavedic capsule", a polyherbal formulation of Vedic Bio-labs Pvt. Ltd., Bangalore, contains the extracts of 15 different medicinal plants *viz.*, Rasna extract (*Alpinia galanga*), Shallakiniryasa extract (*Boswellia serrate*), Shuddhashilajith extract (*Asphlatum* (purified), Shuddha Guggulu extract (*Commiphora mukul* (purified)), Bala extract Nirgundi extract, *Sida cordifolia), Vitex negundo*), Ashwagandha extract (*Withania somnifera*), Vishwabheshaja extract (*Zingiber officinale*), Nagakesara extract (*Mesua ferrea*), Erandamoola extract (*Ricinus communis*), Devadaru extract (*Cedrus deodara*), Punarnava extract (*Boerhavia diffusa*), Gokshura extract (*Tribulu sterrestris*), Shuddhavatsnabha powder (*Aconitum heterophyllum*), Shuddhaarkamula powder (*Calotropis procera* (purified) and is used for the treatment of inflammation and pain associated with arthritis.[2] As the drug to be taken lifelong by the patient, it is necessary to ensure that it is free of genotoxicity. The present study is taken up to evaluate genotoxicity of RV capsule.

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MATERIALS AND METHODS

Chemicals

RVcapsules were purchased from Lakshmi Ayurvedic Centre, Jayanagar, Bangalore, India. Colchicine, bovine albumin fraction from Hi Media Laboratories Pvt. Ltd., Mumbai, India, cyclophosphamide (Endoxan-N) from Zydus Biogen, Ahmadabad, India. May-Grünwald's from S.D. Fine Chem Limited, Mumbai, India. Giemsa stain from Merk Pvt. Ltd., Mumbai, India. Glycerin I.P from Multi-Labs Pvt. Ltd., Bangalore, India and methanol was purchased from Spectrum Reagents and Chemicals Pvt. Ltd., Mumbai, India.

Animals

Healthy 7–8 weeks old male Swiss albino mice weighing

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25–30 g were obtained from *In Vivo* Biosciences, Bangalore, and maintained under standard lab conditions of 25±2°C, relative humidity 65±10%. Animals received a standard pellet diet (Mysore Feeds Pvt. Ltd., Bangalore, India) and water *ad libitum*. The experimental protocols for genotoxicity studies are done in accordance with the Organisation for Economic Co-operation and Development (OECD) guidelines (No.474,475)[3] for mutagenicity studies in animals. It was approved by the Institutional Animal Ethics Committee, Visveswarapura Institute of Pharmaceutical Sciences, Bangalore [Reg. No.: 152/1999/CPCSEA].

Study Design

After acclimatization for seven days, the animals were divided into eight groups of six animals each.

Group I : Mice treated with vehicle (10 ml/kg) orally for seven consecutive days

Group II : Mice treated with CPA (100 mg/kg;i.p.). Bone marrow extract (BME), 24 h after CPA inj.

Group III : Mice treated with CPA (100 mg/kg;i.p.). BME,48 h after CPA inj.

Group IV : Mice treated with CPA (100 mg/kg;i.p.). BME, 72 h after CPA inj.

Group V : Mice treated with RV (780 mg/kg) orally for seven consecutive days.

Group VI : Mice treated with RV(780 mg/kg) orally for seven consecutive days,followed by CPA (100 mg/kg;i.p.) as a challenge on the seventh day. After 24 h of CPA inj., BME was performed.

Group VII : Mice treated with RV(780 mg/kg) orally for seven consecutive days followed by CPA (100 mg/kg;i.p.) as a challenge on the seventh day. After 48 h of CPA inj., BME was performed.

Group VIII : Mice treated with RV(780 mg/kg) orally for seven consecutive days followed by CPA (100 mg/kg;i.p.) as a challenge on the seventh day. After 72 h of CPA inj. BME was performed.

Bone Marrow Micronucleus Assay

On the seventh day, experimental animals were sacrificed by cervical dislocation and bone marrow was aspirated from femur and tibia into one ml of 5% bovine albumin in phosphate-buffered saline (pH 7.2), centrifuged (1000 rpm for 5 min.) and the smears were prepared from the pellet on chemically cleaned glass slides and stained with May–Grünwald's, followed by Giemsa stain. The smears were analyzed under oil immersion using Labomed-Model Digi 2 microscope (90–260 V) for the presence of micronuclei (MN) in polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE). Polychromatic erythrocyte/Normochromatic erythrocyte (P/N) ratio was determined by counting a total of about 500 erythrocytes per animal and 2000 erythrocytes were examined for the presence of MN.[4]

Chromosomal Aberrations Test

On the seventh day, animal was injected with 0.04% colchicine, 4 mg/kg, i.p., 90 min prior to death, for mitotic arrest. Experimental animals were sacrificed by cervical dislocation; the bone marrow was aspirated from femur and tibia into suspending medium 0.075 M KCl, centrifuged and supernatant discarded and the pellet was mixed with fixative (3 : 1, methanol : acetic acid) and centrifuged. The preparation was given two changes of fixative and smears were prepared. The slides were flame-dried and stained with 10% Giemsa at pH 6.8 for 15–20 min. Smears were screened for different types of chromosomal abnormalities – rings, breaks, exchanges and minute.[4]

Statistical Analysis

The results were expressed as mean±SEM and analysis was carried out by one-way ANOVA. Post-hock analysis was done by Tukey's multiple comparison test to estimate the significance of difference between various individual groups. $P<0.001$ was considered significant.

RESULTS

There was no significant decrease in micronucleus formation [Table 1] and chromosomal aberrations [Figure 1] in RV-treated mice against CPA-induced clastogenicity. There was no variation in %MNPCE, %MNNCE, P/N ratio and total no. of chromosomal aberrations in Group V mice.

DISCUSSION

Rheumatoid arthritis is a chronic systemic autoimmune

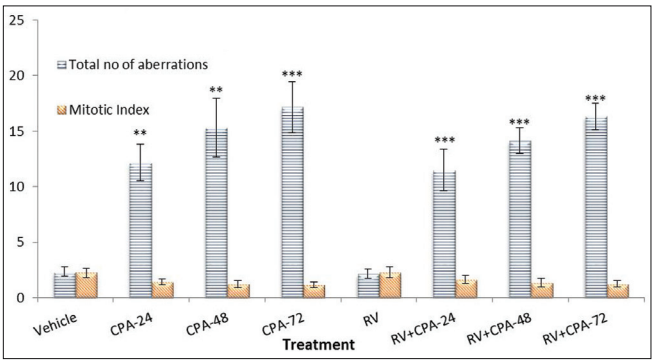

Figure 1: Effect of Rheumavedic capsule on chromosomal aberration in mice using cyclophosphamide as clastogen

n=6, Values are expressed in Mean±SEM, One way ANOVA followed by Tukey's multiple comparison test. ***P*<0.01, ****P*<0.001 Vs Vehicle Control, CPA 24, 48, 72: Bone marrow was extracted after 24, 48 and 72 hrs of cyclophosphamide injection in mice RV – Rheumavedic alone orally for 7 consecutive days. RVCPA 24, 48, 72: Bonemarrow extraction was performed after 24, 48 and 72 hrs of cyclophosphamide injection in mice, pretreated with RV orally for 7 consecutive days

Table 1: Effect of Rheumavedic capsule on micronuclei in mice using cyclophosphamide as clastogen

	Vehicle	CPA-24	CPA-48	CPA-72	RV	RV+CPA-24	RV+CPA-48	RV+CPA-72
% Mn PCE	0.35±0.043	1.883±0.145^{+}	2.45±0.148^{+}	2.967±0.187^{+}	0.317±0.048	1.633±0.133^{ns}	1.85±0.165^{ns}	2.183±0.114*
% Mn NCE	0.217±0.065	0.65±0.076	0.967±0.161^{+}	1.433±0.173^{+}	0.233±0.042	0.617±0.091	0.833±0.117^{ns}	0.983±0.095*
P/N ratio	0.878±0.08	0.804±0.058	0.73±0.047	0.673±0.033	0.898±0.082	0.815±0.07	0.793±0.057	0.746±0.054

n=6, Values are expressed in Mean±SEM, One way ANOVA followed by Tukey's multiple comparison test. ^{+}P<0.001 Vs Vehicle Control, $^{*}P$<0.01 Vs CPA Control, ns – Not Significant; CPA 24, 48, 72: Bone marrow was extracted after 24, 48 and 72 hrs of cyclophosphamide injection in mice; RV – Rheumavedic alone orally for 7 consecutive days. RVCPA 24, 48, 72: Bonemarrow extraction was performed after 24, 48 and 72 hrs of cyclophosphamide injection in mice, pretreated with RV orally for 7 consecutive days.

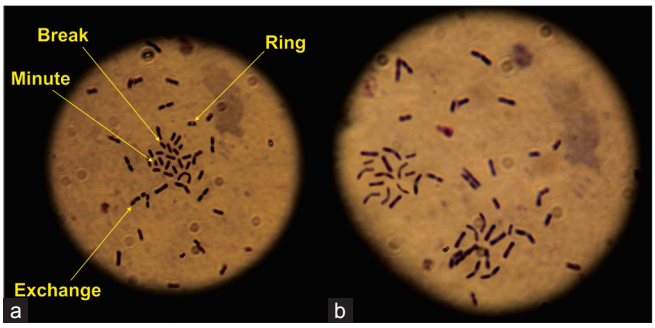

Figure 2: Effect of Rheumavedic on chromosomes a) Aberrated chromosomes in erythrocytes of CPA treated mice: Breaks, Rings, Minutes, Exchanges due to cyclophosphamide are observed (M. G. & G, x100) b) Normal chromosomes in erythrocytes of Rheumavedic treated mice: Breaks, Rings, Minutes, Exchanges are absent (M.G.& G, x100)

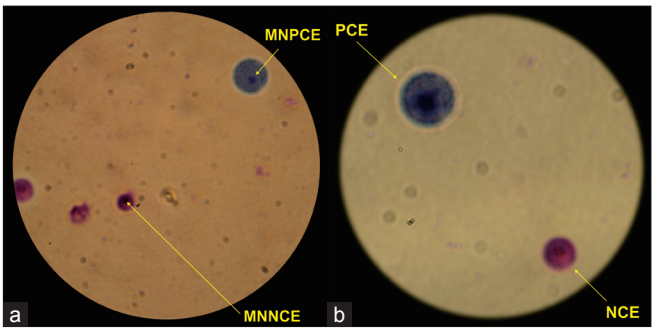

Figure 3: Effect of Rheumavedic on Micronucleous formation a) Micronucleous formation in cyclophosphamide treated mice: MNPCE - micronucleated polychromatic erythrocytes MNNCE – micronucleated normochromatic erythrocytes (M.G.& G, x100) b) Absence of Micronucleousinrheumavedic treated mice: PCE - polychromatic erythrocytes, NCE - normochromatic erythrocytes (M. G. & G, x100)

disorder and the drugs used to treat are taken for a long period of time, so it is necessary to ensure that RVcapsule, used to treat rheumatoid arthritis, is free from any toxicity. The bone marrow micronucleus test (MNT) and chromosomal aberration test (CAT) are the most suitable genotoxicity tests. Other tests include peripheral blood micronucleus and sperm morphology tests.[4] The antimutagenic activity of RV was evaluated by measuring their inhibitory effect on CPA-induced mutagenesis.[4]

CPA has been shown to produce gene mutations, chromosome aberrations, MN and sister chromatid exchanges in rats, mice and Chinese hamsters, and gene mutations in the mouse spot test and in the transgenic 1acZ construct of Muta™ Mouse.[5] CPA gets metabolized to phosphoramide mustard and acrolein before it can act as a mutagenic agent to promote chromosomal aberrations; these are due to lesions in Deoxyribo nuclic acid (DNA) caused by phosphoramide mustard which lead to discontinuities of the DNA helix.[5] CPA produced a significant decrease in the P/N ratio, which is due to increase in NCEs and also a significant rise in the total number of chromosomal aberrations – rings, breaks, exchanges and minute [Figure 2] – of CPA-treated animals, when compared with normal control animals, which signals a cytotoxic effect. RVslightly (statistically not significant) inhibits the CPA-induced chromosomal aberrations and a formation of MN in PCE and NCE [Figure 3]. RV could not bring P/N ratio to normal level. RVwhen *administered* alone didnot produce any significant variation in %MNPCE, %MNNCE, P/N ratio and total no. of chromosomal aberrations in group V mice, indicating that it is devoid of any genotoxicity. In our study, we found a significant decrease in mitotic index of CPA-treated animals, which can be due to the affected cell division in the bone marrow. Mitotic index was not altered in RV-treated mice.

RV is free of genotoxicity, but do not have antigenotoxic effect.

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