Research Paper

In vivo chromosome damaging effects of an inosine monophosphate dehydrogenase inhibitor: Ribavirin in mice

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ABSTACT

Objective: To investigate the *in vivo* mutagenic effects of ribavirin in mice.

Methods: Mice were injected (i.p.) 20, 100, or 200 mg/kg ribavirin (single exposure) for bone marrow micronucleus, peripheral blood micronucleus and bone marrow chromosome aberration tests. Five treatments of 200 mg/kg ribavirin was given (i.p.) for sperm morphology test. The tests were performed as per the standard procedures.

Results: Ribavirin induced significant number of micronucleated polychromatic erythrocytes (MNPCEs) at 24, 48 and 72 h following the exposure with more effects at 24 h ($p<0.05$-0.001). Micronucleated PCEs were more at 48 h in lower dose-levels and at 72 h in highest dose-level in the peripheral blood ($p<0.05$-0.001). Ribavirin induced structural chromosomal damage hence producing the fragments for the micronucleus formation. Ribavirin decreased the PCE%, P/N ratio and the mitotic index indicating that it prevents cell division in mouse bone marrow. Ribavirin also decreased the testis weight and induced the formation of abnormal sperms.

Conclusion: Ribavirin is a potent mutagen and cytotoxic agent in mice *in vivo*. Further, it also induces point mutations in germ cells yielding abnormal sperms. The genotoxic effects of ribavirin are not exerted in a dose-dependent pattern in mouse.

KEY WORDS: Acentrics, bone marrow, genotoxicity, mutagenesis

Introduction

Ribavirin (Tribavirin) is a synthetic nucleoside analog used in the treatment of various viral infections.[1] It inhibits viral replication *via* a variety of mechanisms; however, its antiviral properties appear to be by inhibition of the cellular inosine 5' monophosphate dehydrogenase activity that consequently depletes the intracellular GTP pool.[2] Ribavirin treatment induces reversible anemia,[3] and other hematological disorders.[4-6] Although there are no comparable data for humans,[2] ribavirin is a teratogen in all animals tested affecting the limb developments, eye formation and development of the central nervous system.[7-10]

Ribavirin increased the incidence of mutations *in vitro* in mouse Balb/c3T3 (fibroblasts), and lymphoma cells, although its carcinogenic potential is not yet clear.[11] It induced the formation of micronuclei in mouse bone marrow erythrocytes at the dose-levels of 20, 100 or 200 mg/kg, and chromosomal damages at 200 mg/kg after 2-5 exposures,[12] and following a single acute exposure of very high doses.[13] Ribavirin was found to be non-mutagenic in rats when investigated by dominant lethal test, although the results were not unequivocal.[14] However, it induced the generation of a significant number of abnormal spermatozoa[15] and decreased the sperm counts[16] with some relation to the doses and sampling points tested in rats. In rat bone marrow cells, a single exposure of 10-200 mg/kg ribavirin induced the formation of micronuclei except at 10 mg/kg, almost in a dose-dependent pattern but without any well-defined sex-differences.[17] Even though its mutagenicity has been reported based on the results of bone marrow micronucleus test and sperm morphology test in the rat, the results of *in vitro* [18] studies revealed otherwise. Besides, the previous studies in mice have employed multiple treatments[12] or the exposure of very high doses[13] and the toxicity was evaluated by the micronucleus test. To establish the mutagenicity of any chemical, the chromosome-damaging effects should be evaluated by multiple tests. Although a few reports exist on the toxic effects of ribavirin, there is still a controversy regarding whether or not ribavirin induces any chromosomal damage. Moreover, the micronucleus or sperm morphology tests have limitations as they are unable to identify the type of chromosomal damage induced. Hence, the present study was planned to investigate the genotoxicity of ribavirin in mice *in vivo*.

Materials and Methods

Animals

Swiss albino inbred mice (Mus musculus; 8-10 weeks old; 26-30 g, body weight) were housed in plastic cages with paddy husk bedding. Animal house temperature was maintained at 28±1 ^{0}C. Animals were given food and water *ad libitum*. Mice were segregated into groups of 5 each. All experiments were conducted as per the guidelines of the institutional ethical committee and those of Government of India. In all experiments, the animals were anesthetized (Pentabarbitone sodium 45 mg/kg, i. p.) and then sacrificed by overdose of the same.

Chemicals

Ribavirin (Virazole; ICN pharmaceuticals, Inc., CA; Lot No. 94J02) was dissolved in water just before use and injected intraperitoneally. The dose selection was based on the previous studies.[12,16] Briefly, 20 mg/kg is an antiviral dose, whereas the other two are the higher doses. The basis for their selection was that they were used in earlier studies which revealed the controversial results.

Bone marrow micronucleus assay

One group which received water served as control. Three groups were treated with 20 mg/kg, another 3 groups with 100 mg/kg and remaining 3 groups with 200 mg/kg. From the ribavirin-treated category one group each from different dose-levels was selected either at 24, 48 or 72 h (the same animals were used for peripheral blood micronucleus test and chromosome aberration test; vide infra). The animals were anesthetized and the bone marrow was aspirated from femurs into 1 ml of 5% bovine albumin in phosphate buffered saline (pH 7.2) as previously described.[12, 17, 19] The cell suspension was centrifuged (1000 rpm for 5 min) and the smears were prepared from the pellet on chemically cleaned glass slides and stained with May-Gruenwald–Giemsa.[12] The smears were analyzed under the oil immersion objective and 2000 polychromatic erythrocytes/animal were counted. Concurrently encountered normochromatic erythrocytes (NCEs), micronucleated PCEs (MNPCEs) and MNNCEs were also counted.

Peripheral blood micronucleus assay

This test was performed to investigate the mutagenicity in circulating erythrocytes. If this test is conducted along with bone marrow micronucleus test and chromosome aberration test, the accuracy of genotoxicity evaluation is 100%. This assay was conducted following a single exposure to ribavirin 20, 100 or 200 mg/kg at 0, 24, 48 or 72 h in the same animals used for bone marrow micronucleus assay (5 animals/group/sample time). The blood was collected from the ventral tail vein and the blood sample before treatment served as control. The smears were prepared on clean glass slides and stained with the May-Gruenwald-Giemsa.

Two thousand PCEs were scored and concurrently met MNPCEs were recorded and expressed as percentage incidence. Another 100 PCEs were separately recorded and simultaneously met NCEs were counted to express NCE% and MNNCE%. Number of PCEs scored was reduced when it was not possible to score 100 PCEs in cases of severe inhibition of erythropoeisis.

Bone marrow mitotic chromosome analysis

This test was also conducted in the same animals used for the micronucleus tests. Single treatment of 20, 100 or 200 mg/kg of ribavirin was given (5 animals/sample time/dose) to study the time- and dose-responses. The sampling was done at 48 and 72 h for 20 and 100 mg/kg and at 24, 48 or 72 h for 200 mg/kg. The procedure of the chromosome preparation adopted was that of Adler[20] and Savage.[21] Briefly, 0.2 ml of 0.02% colchicine (Sigma Chemicals) was injected intraperitoneally to arrest the cell division and after 2 h the animals were sacrificed by the lethal dose of anesthesia. The marrow was aspirated from the femurs into a syringe containing 0.075KCl (560 mg/100 ml distilled water). The marrow suspension was incubated at 37 ^{0}C for 15-20 min and centrifuged at 1000 rpm for 10 min. The supernatant was discarded. The pellet was mixed with the fixative (3:1, methanol: acetic acid) and the suspension was allowed to stand for 30 min and then centrifuged. The preparation was given two changes of fixative. The pellet was mixed thoroughly in 1 ml of fresh fixative and 2-3 drops of the suspension was placed on a clean glass slide from a height. The slides were flame-dried and stained with 10% Giemsa at pH 6.8 for 15-20 min. Slides were screened for chromosome abnormalities as per the standard method.[20, 21]

Sperm morphology assay

One group of mice was treated (i.p.) with ribavirin 200 mg/kg/day for 5 consecutive days at intervals of 24 h. Another group of mice, which received water served as control (5 animals/group). All animals were sacrificed on Day 35 following the last exposure. Laparotomy was conducted and the reproductive tract was exposed. The testes and the epididymes were removed. The tunica albugenia was removed and the testes were weighed (paired weight). The epididymis was minced in 1ml phosphate buffered saline into a suspension and filtered through a nylon cloth. To the filtrate, 1 drop of eosin Y was added and smears were prepared. Two thousand spermatozoa/rat were examined for shape abnormalities as per the standard procedure,[22, 23] and expressed as percentage incidence of abnormal sperms.

Statistical analysis

Data were expressed as mean±SEM for each group and subjected to the statistical analysis by ANOVA (one-way) and Bonferroni post hoc test. $P<0.05$ was considered as the level of significance.

Results

Bone marrow micronucleus assay

All three doses of ribavirin induced the formation of MNPCEs ($P<0.05$-0.001; Figure 1a) following a single exposure except at 72 h in 20 mg/kg. At 24 and 48 h, there were no differences in MNPCE% at 20 and 100 mg/kg but the effects of 200 mg/kg significantly differed from that of the other two doses. The two higher doses significantly increased the

MNPCE% at 72 h, but 20 mg/kg dose did not. The PCE% was decreased at the two higher dose-levels at 24 and 48 h, and also at 72 h in 200 mg/kg and similar was the case with the P/N ratio (Table 1). Micronucleated NCEs (Figure 1b) did not respond significantly to the toxicity of the drug.

Peripheral blood micronucleus assay

At 24 h, a significant elevation in MNPCEs (Figure 1c) was observed in 20 mg/kg group, but not in the other two treated groups. At 48 h, there was a dose-dependent increase in MNPCE% with more than two-fold increase over the 20 mg/kg group and similar was the case at 72 h. At 24 and 72 h, MNNCE% (Figure 1d) did not show any significant increase, but at 48 h, it was significant in the 20 and 100 mg/kg groups. The two cytotoxic parameters (PCE% and P/N) did not show any change at 20 mg/kg, but at 100 mg/kg, and as time advanced the cytotoxicity also increased. The higher dose, 200 mg/kg also showed cytotoxicity, however, the 100 mg/kg dose-group was the most affected (Table 2).

Figure 1. Photomicrograph showing micronucleated erythrocytes. A) MNPCE in bone marrow (a), B) MNNCE in bone marrow (b), C) MNPCE in peripheral blood (b), and D) MNNCE in peripheral blood (b). An NCE is indicated (a). May Gruenwald-Giemsa, 1000X.

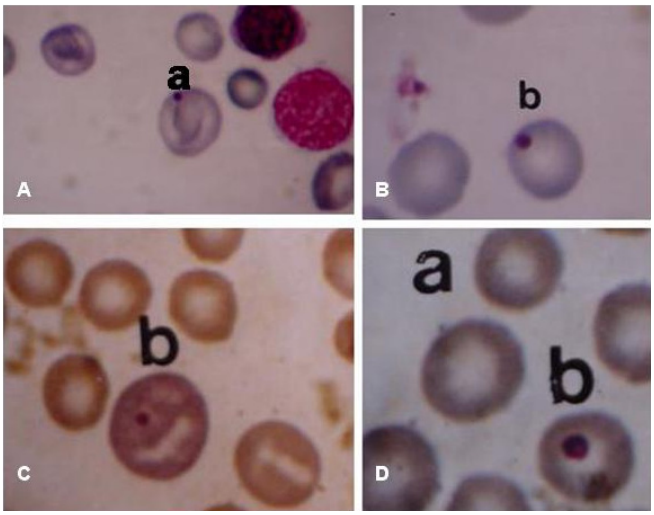

Table 1

Bone marrow micronucleus test in ribavirin treated mice

Drug (mg/kg)	*Sample time (h)*	*MNPCE%*	*MNNCE%*	*PCE%*	*P/N*
0	0	0.18±0.04	0.21±0.06	56.67±2.92	1.34±0.15
	24	0.72±0.09***	ND	62.34±7.49	1.94±0.47
20	48	0.48±0.01**	0.13±0.01	56.74±2.30	1.33±0.13
	72	0.25±0.04	0.11±0.04	61.36±0.49	1.59±0.03
	24	0.73±0.14**	0.09±0.02	40.68±3.59**	0.70±0.10**
100	48	0.47±0.09**	0.26±0.05	36.94±4.77**	0.66±0.16**
	72	0.37±0.05*	0.29±0.02	53.60±3.81	1.24±0.18
	24	0.96±0.18***	0.15±0.02	25.54±3.16***	0.36±0.07***
200	48	0.55±0.08**	0.18±0.02	14.12±2.12***	0.17±0.03***
	72	0.92±0.16***	0.40±0.18	39.05±8.83**	0.77±0.31**

Values are mean±SEM from 5 animals/group. Bone marrow was collected from 2 rats at 24 h, 2 rats at 48 h and 1 rat at 72 h in control group. *P<0.05, **P<0.01 and ***P<0.001, versus control (multiple comparisons by Bonferroni test). Intergroup differences were significant (P<0.05-0.001) except for MNNCE% (One-way ANOVA). ND= not done.

Table 2

Peripheral blood micronucleus test in ribavirin-treated mice

Drug (mg/kg)	*Sample time (h)*	*MNPCE%*	*MNNCE%*	*PCE%*	*P/N*
	0	0.14±0.02	0.09±0.01	1.51±0.32	0.01±0.00
20	24	0.27±0.04**	0.10±0.01	1.57±0.14	0.01±0.00
	48	0.28±0.05**	0.17±0.02**	1.59±0.11	0.02±0.00
	72	0.18±0.05	0.11±0.01	1.27±0.06	0.01±0.00
	0	0.20±0.04	0.11±0.01	1.94±0.15	0.02±0.00
100	24	0.23±0.04	0.12±0.02	1.22±0.10***	0.01±0.00***
	48	0.54±0.06***	0.16±0.02	0.81±0.07***	0.01±0.00***
	72	0.35±0.05***	0.16±0.02*	0.70±0.05***	0.01±0.00***
	0	016±0.04	0.15±0.02	2.73±1.09	0.03±0.01
200	24	0.18±0.05	0.15±0.04	2.39±1.19	0.02±0.01
	48	0.65±1.15***	0.12±0.02	0.93±0.46**	0.01±0.01**
	72	0.80±0.55***	0.18±0.07	1.77±1.02*	0.02±1.02*

Values are mean±SEM from 5 animals/group. *P<0.05, **P<0.01 and ***P<0.001, versus respective control (multiple comparisons by Bonferroni test). Inter-group differences were significant for MNPCE% (P<0.05-0.001); for MNNCE% at 48 h (P<0.01) in 20 mg/kg, and at 48 h and 72 h in 100 mg/kg (P<0.01). For PCE% and P/N, at 48 h in 20 mg/kg, at 48 h and 72 h in 100 mg/kg (P<0.001), and in all groups in 200mg/kg (P<0.01) (One-way ANOVA).

Figure 2. Photomicrograph showing metaphase chromosome preparation from bone marrow. a) Chromatid break (arrow), b) chromosome break (arrow), c) ring chromosome (arrow), d) exchanges (arrows), e) deletion (arrow), f) minute (arrow), g) multiple breaks, h) pulverization, and i) fragmentation. Giemsa stain, 1000X.

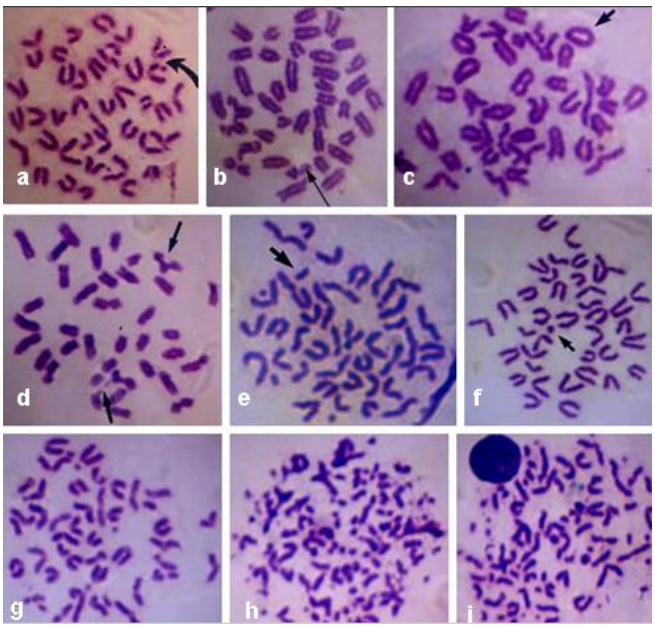

Figure 3. Photomicrograph showing different sperm abnormalities. a) Normal sperm, b) hook-less, c) amorphous, d) banana-shaped, e) folded, and f) two-tailed sperms. Eosin Y, 1000X.

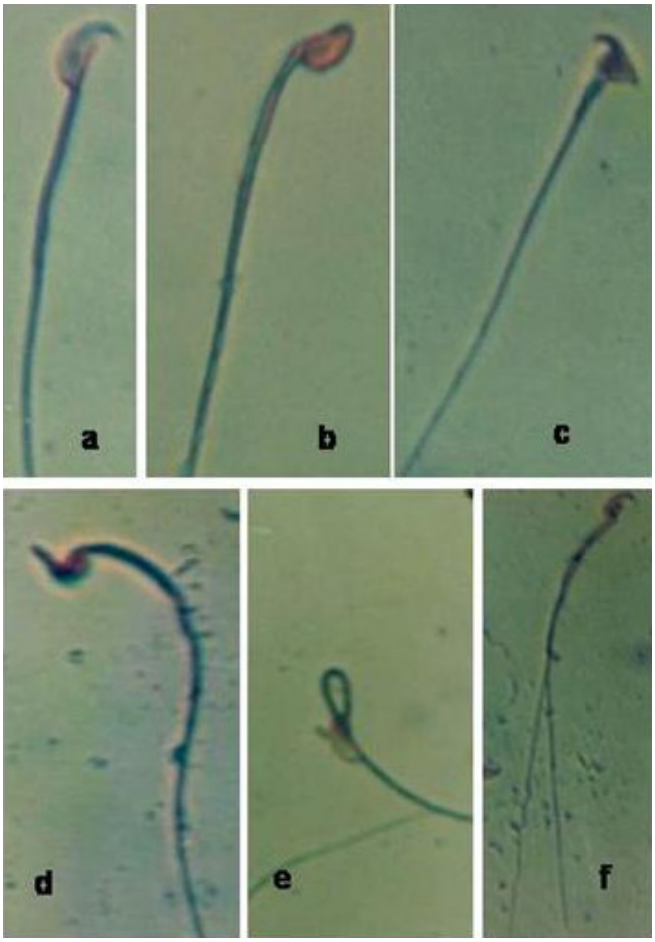

Table 3

Bone marrow mitotic chromosome test in ribavirin-treated mice

Drug (mg/kg)	*Sample time (h)*	*Total cells*	*1*	*2*
0	24	300	0.67±0.54	0
	48	300	18.33±2.84**	16.33±2.84**
20	72	300	7.00±0.82*	6.67±0.98*
	48	600	8.50±1.22*	7.17±1.30*
100	72	600	7.67±1.02*	6.67±0.65*
	24	300	9.00±1.70**	6.67±0.98*
200	48	300	11.33±2.37**	10.00±1.89*
	72	300	8.67±1.09*	6.67±1.19*

Values are mean±SEM from 5 animals/group. 1= including cells with gaps, and 2= excluding cells with gaps. *P<0.01 and **P<0.001, control versus treated (Bonferroni test). Intergroup differences are significant for 20 mg/kg at both sample times and for 200 mg/kg at 48 h (P<0.01-0.001; One-way ANOVA).

Table 4

Mitotic index in ribavirin-treated mice

Drug (mg/kg)	*24 h*	*48 h*	*72 h*
0	6.38±0.33	ND	ND
20	1.90±0.20*	2.02±0.07*	ND
100	1.53±0.16*	2.12±0.10*	ND
200	2.27±0.03*	2.15±0.65*	3.22±0.57*

Values are represented as mean±SEM from 5 animals/group. *P<0.01 against control (Bonferroni test). ND= not done. The 24 h values served as control for 48 h and 72 h groups.

Bone marrow mitotic chromosome analysis

Ribavirin induced the formation of cells with structurally damaged chromosomes (Figure 2). Chromatid gaps and breaks, chromosome breaks, deletions and exchange figures were the most common structural damages. There was no dose-dependent increase in chromosomal damage and the effects of 20 mg/kg were more at 24 h (Table 3). All doses of ribavirin decreased the mitotic index, but without any particular relation to the doses or time points employed (Table 4).

Sperm morphology assay

Ribavirin decreased the testes weight (control 257.28 ±11.54 and treated- 200.00±10.90, P<0.01) and increased the number of sperms with abnormal morphology (control-1.59±0.26 and treated-9.14±1.16, P<0.001) (Figure 3).

Discussion

The bone marrow micronucleus test has been a most suitable genotoxicity test[24] along with simultaneously used tests like chromosome aberration, peripheral blood micronucleus and sperm morphology tests. This battery of tests would ensure a 100% accurate genotoxicity testing.[25]

Previous studies on genotoxicity of ribavirin have employed any one of these tests except a study by Rao and Rahiman[12] which also included chromosome aberration test, but following multiple treatments with 200 mg/kg, and another study by Phillips *et al* [13] who also conducted the peripheral blood micronucleus test after multiple exposures to very high doses. The doses of 500-2000 mg/kg used in the latter study[13] were so high so that they might have been highly toxic and arrested cell proliferation, and consequently micronucleus formation. However, they were able to observe the micronucleated cells in the venous blood.

A significant increase in MNPCE% indicated that ribavirin induced chromosomal damage in mouse bone marrow cells. In mice exposed to two treatments of 200 mg/kg[12] or three treatments of 500-2000 mg/kg,[13] the frequency of micronuclei did not show any dose-dependent responses. The results indicated that dose-response was not well defined as regards the MNPCE% in mice, except at 72 h (Table 1). The study by Phillips *et al* [13] did not however examine the incidences of micronuclei at that sample time. On the other hand, dose-dependent increase in MNPCE% was observed in Wistar rats following a single treatment of 10-200 mg/kg; at 10 mg/kg the drug did not induce any mutagenicity and it was concluded that the 10 mg/kg was a 'no observed adverse effect dose-level[17]. In that study even the MNNCEs significantly increased due to conversion of the MNPCEs by 24 h itself, however, no such effect was observed in the present study. This was due to the higher degree of variations among the animals. Why do these differences exist between different species? It is known that the metabolites – ribavirin 5' monophosphate (RMP) and ribavirin 5' triphosphate (RTP) are more toxic than the parent compound.[2] The availability of these metabolites to the target tissue (in this case, the bone marrow) therefore would depend on the extent of metabolism of ribavirin in the liver which might vary from species to species. Only at 200 mg/kg, the MNPCEs were less in number at 48 h than the other 2 samples and that was due to increased toxicity of higher dose, which did not allow the cells to enter into mitosis. This quality of the drug was not observed in the rat following a single treatment and there was an increase in MNPCE% at 48 h compared to 2 other samples.[17] Thus, the present study differs from that of Narayana *et al*,[17] and the differences are probably due to, 1) lack of well-defined dose-response, 2) time-response, and 3) negligible number of MNNCEs in the mouse bone marrow.

The results from the peripheral blood micronucleus test further indicated that the responses of MNPCEs were totally different from that of the bone marrow. In contrast to the bone marrow, the MNPCEs were more at 48 h, and by 72 h they decreased except in 200 mg/kg. Relatively more MNPCEs observed at 24 h in the bone marrow reached the blood by 48 h, which may be responsible for such an observation in the blood. Increased incidence of micronucleated cells at 72 h in the 200 mg/kg group was an aftermath of extended toxicity of this higher dose, which kept on generating the micronuclei in the bone marrow and consequently in the blood. Lack of dose-dependent increase of MNPCEs at 24 h was probably due to the drug toxicity, which prevented the cells from leaving the bone marrow to reach the blood by 24 h, which otherwise would have reached approximately at that time.[26] Compared to the study of Phillips *et al* [12] the MNPCEs were more in this case. Since the doses employed here were comparatively lower, that probably exerted less cytotoxicity providing more dividing cells in the marrow to be acted upon.

Following the exposure to ribavirin, structural damage took place in affected cells that resulted in the formation of acentrics (chromosome fragments). When the enucleation of erythrocytes occurs, the acentrics are not extruded from the cells; instead they appear as small bodies called micronuclei. The whole chromosome left in the cell due to non-disjunction could also form the micronuclei, but in that case the latter would be larger in size. Thus, the small-sized micronuclei which appeared in the cells in the present study indicate that they are formed by the acentrics and that this finding is in consensus with the type of chromosomal damage observed. However, the formation of acentrics did not show any dose-dependent response. Thus formed acentrics transformed into micronuclei by 24 h resulting in a significant increase in their number in the bone marrow by that time (Table 1). Inhibition of inosine monophosphate dehydrogenase would result in decreased xanthosine monophosphate formation required for DNA synthesis,[27] and ribavirin 5' triphosphate also could inhibit RNA polymerase by competing with GTP or ATP for substrate sites.[28] These actions of the drug therefore could affect the DNA structure leading to chromosomal damage in cells. Primarily, ribavirin induced the chromatid type of aberration (chromosome type was also seen at higher dose levels), which indicated that the drug possibly acted on cell-cycle stages late S and G_2 similar to other drugs like Ara C and 5-Fluorouracil.[29] Compared to the total chromosome damage that led to the formation of acentric fragments, the frequencies of micronuclei were less in the bone marrow and consequently in the blood. This might account for the fact that all acentrics are not converted into micronuclei,[26] or not all of them are observed under the light microscope.[25]

Evaluation of mitotic index (MI) revealed that ribavirin affected the cell division adversely in the bone marrow supporting the previous findings on cytotoxicity that induced the cell death in rat bone marrow and testis.[30] The decrease in testis weight was due to decreased spermatogenesis owing to the drug-induced cytotoxicity.[23] In previous studies, this drug was found to induce the formation of abnormal sperms and decrease the sperm count in the rat.[15,16] Nevertheless, in this study hook-less sperms were more and also the percentage abnormality at 200 mg/kg was less than that in the rat. Sperm abnormalities are induced by exogenous compounds *via* point mutations[15] or due to the inimical nature of chemicals on spermato-genesis.[23]

In conclusion, the results of this study infer that ribavirin is a potent mutagen that causes structural damages in chromosomes and acts as a cytotoxic agent in mice. Further, it also induces point mutations in the germ cells thus inducing the formation of abnormal sperms. The genotoxicity of this drug is not exerted in a dose-dependent pattern.

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PHARMA CME – 2005

Date : July 9th – 10th, 2005

Place : Rangaraya Medical College, Kakinada, Andhra Pradesh

Theme : Modern Trends in Pharmacotherapeutics

Workshop : Non-invasive Techniques in Clinical Pharmacology

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