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Short Communication

Genotoxic Effect of Ribavirin in Patients with Crimean-Congo Hemorrhagic Fever

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SUMMARY: In this study, we investigated the in vivo genotoxicity of ribavirin in humans, studying 3 patients with Crimean-Congo hemorrhagic fever who were treated with high-dose ribavirin. In order to evaluate genotoxicity, both the micronucleus (MN) test and the sister chromatid exchange (SCE) test were used. In all patients, blood samples were taken during and after therapy. Whole blood cultures were performed for 72 h and the MN assay and SCE test were then carried out to demonstrate the genotoxicity. In all patients, both SCE and MN amounts were found to be higher in the samples which were taken during therapy than in those at 1 month after therapy. The results of our study reveal that ribavirin has a reversible in vivo genotoxic effect on humans.

Ribavirin (RBV) (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide; $C_8H_{12}N_4O_5$) is a synthetic purine nucleoside analogue with a broad spectrum of antiviral activity which blocks viral replication by inhibiting inosine monophosphate dehydrogenase (1). RBV has many adverse effects including hematologic toxicity, liver toxicity and teratogenicity (2). Nevertheless, few studies have been conducted on RBV, and in vivo genotoxicity has not yet been definitively determined.

The genotoxic effects of physical and chemical agents can be detected by micronucleus (MN) test prior to the cytokinesis stage, or by sister chromatid exchange (SCE) test at the chromosome level (3,4). The MN test can be carried out in all cells which multiply by mitosis. In the case of exposure to a genotoxic agent, an MN is formed in divided cells during the metaphase/anaphase transition and appears within binucleated cells as a small nucleus (5). This MN originates from acentric fragments, which are excluded from the two daughter nuclei at the late stages of mitosis (3). On the other hand, the SCE method shows the exchange of parts between two sister chromatids of a single chromosome. SCEs are visualized by a combination of fluorescent and Giemsa techniques. The rate of SCE increases in cells which have been exposed to genotoxic agents. Both SCE and MN are reliable markers of genotoxicity (6,7).

Our purpose in the present study was to determine the in vivo genotoxicity of RBV by using both methods in humans. Our subjects were three patients with Crimean-Congo hemorrhagic fever (CCHF) which had been diagnosed clinically, microbiologically and by laboratory tests. All diagnoses were confirmed by ELISA. None of the patients used any antimicrobial agent other than RBV.

Case 1: A 21-year-old female was admitted to our clinic with f a 2-day fever and a 5-day malaise, myalgia, fatigue, headache, nausea, vomiting, hematemesis, melena and vaginal bleeding. On physical examination, abdominal tenderness, cervical lymphadenopathy, petechiae and ecchymoses on the body were detected. Laboratory results revealed the following: hemoglobin (Hb), 12.6 g/dL; white blood cells (WBCs), 2.2×10^9/L; platelets (PLTs), 7×10^9/L; alanine aminotransferase (ALT), 1,393 U/L; aspartate aminotransferase (AST), 5,218 U/L; lactate dehydrogenase (LDH), 7,650 U/L; creatin phosphokinase (CK), 3,888 U/L; prothrombin time (PT), 60 sec. Anti CCHFV-IgM antibody was positive. CCHF was considered with these results and oral RBV therapy at a high dose (2,000 mg as an initial dose followed by 1,000 mg per 6 h in the first 4 days and 500 mg per 6 h in the following 6 days) was given with intensive supportive therapy. No antibiotics were not added to the therapy. After a 10-day treatment, the patient had improved.

Case 2: A 45-year-old male was admitted our clinic complaining of a 3-day fever and a 7-day malaise, myalgia, fatigue, headache, nausea, vomiting, hematemesis and epistaxis. On physical examination hepatomegaly and abdominal tenderness were recorded. Laboratory results revealed Hb, 11.5 g/dL; WBCs, 3.4×10^9/L; PLTs, 25×10^9/L; ALT, 3,435 U/L; AST, 1,260 U/L; LDH, 6,014 U/L; CK, 1,870 U/L; PT, 19 s. Anti CCHFV-IgM antibody was positive. Oral RBV therapy at the same doses as in Case 1 and intensive supportive therapy were started. After a 13-day therapy, the patient had improved.

Case 3: A 54-year-old male was admitted our clinic with a 3-day fever and a 6-day malaise, myalgia, fatigue, headache, nausea, vomiting, hematemesis and epistaxis. On physical examination hepatomegaly and regional lymphadenopathy were recorded. Laboratory results revealed Hb, 13.9 g/dL; WBCs, 1.3×10^9/L; PLTs, 35×10^9/L; ALT, 415 U/L; AST, 721 U/L; LDH, 1,651 U/L; CK, 739 U/L; PT, 32 s. Anti CCHFV-IgM and IgG antibody were positive. Oral RBV therapy and intensive supportive therapy were given for 10 days. The patient was improved on the 11th day of hospitalization.

We studied the genotoxic effects of RBV in these patients by both MN and SCE tests. Before the initiation of therapy, no blood samples were taken as a precaution against virus transmission to laboratorians. Two milliliter of peripheral whole blood sample from each patient were taken for culturing on the 9th day of treatment and at 1 month after the completion of therapy. Two distinct blood cultures were performed on each patient for the SCE and MN tests. Periph-

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eral blood lymphocyte cultures were performed according to a slightly modified version of the protocol described by Evans and O'Riordan (8). Briefly, 1 mL of whole blood was cultured in 5 mL of culture medium (Chromosome Medium B; Biochrom, Berlin, Germany) with 5 μg/mL of phytohemagglutinin (Biochrom). For SCE tests, 5-bromo-2'-deoxyuridine (Sigma Chemical Co., St. Louis, Mo., USA; final concentration 10^{-4} M) was added to the cultures (9). The cultures were incubated in complete darkness for 72 h at 37°C. After 70 h and 30 min of incubation, colcemid (Sigma) was added to the cultures to achieve a final concentration of 0.5 μg/L. After hypotonic treatment (0.075 M KCl) followed by three repetitive cycles of fixation in methanol/acetic acid solution (3:1, v/v), the cell suspension was dropped onto glass slides. The slides were dried at room temperature for 3 days and then differentially stained for inspection of the SCE amount according to the fluorescence plus Giemsa (FPG) procedure (9,10). Twenty metaphases containing 46 chromosomes were examined by Olympus BH-2 microscope (Olympus Corp., Tokyo, Japan), and the scores were calculated as SCEs per cell.

For MN studies, 1 mL of peripheral blood samples was added to 15-mL Falcon tubes each containing 5 mL of culture medium. Cytochalasin-B (4.5 μg to each mL of culture) was added to each culture tube at 44 h (6). At 72 h, the cultures were harvested and slides were prepared. The slides were dried at room temperature for 3 days, and were then stained by 5% Giemsa. For each patient, 1,000 binucleated cells were examined to determine the frequency of micronuclei, and the number of cells with MN was determined according to the scoring method described by Fenech et al. (11).

Interpretation of the results revealed that SCE rates at the 9th day of therapy were higher than those at 1 month after the completion of therapy. Similarly, MN amounts were found to be increased in the samples which were taken on the 9th day of therapy but normal in those taken at 1 month after therapy. Although both SCE and MN rates were similar in Cases 2 and 3, these rates were markedly high in Case 1 (Table 1).

It has been shown that RBV is a cytotoxic agent above a dose level of 10 mg/kg on different cell plates, especially in erythrocytes, lymphocytes and sperm (12-14). The cytotoxic effect of this drug is due to the induction of cell death and the suppression of cell division and proliferation. Despite the widespread use of RBV, its genotoxicity is presently under debate and it has also been reported to be a potential mutagenic agent (15).

In one study (16), it was reported that RBV does not cause chromosomal aberrations in human leukocyte cultures. In another in vitro study (13), the micronuclei-inducing ability of RBV was found to be relatively low. These studies were performed under in vitro conditions in which no products of RBV metabolism were generated. Nevertheless, the metabolites of this drug, especially RBV 5'-triphosphate (RTP), are more toxic than unmetabolized RBV (17).

Table 1. Sister chromatid exchange (SCE) and micronucleus (MN) amounts of the cases

Case	9th day therapy		1 month following therapy	
	SCE amount (per cell)	MN amount (/1,000 cells)	SCE amount (per cell)	MN amount (/1,000 cells)
1	12	28	5.8	1
2	7.8	17	4.2	0
3	7.3	12	4.2	1

In vivo animal studies by MN assay have revealed that RBV at high doses is genotoxic in bone marrow cells (18,19). Additionally, Rao and Rahiman (18) have pointed out the cytogenetic effect of this drug on mitotic chromosomes in mice. Phillips et al. (20) have reported that seven nucleoside analogues, including RBV, show genotoxic activity at different levels in mouse bone marrow. To the best of our knowledge, there has been no previous in vivo study on the genotoxicity of RBV in humans.

We used two different methods to determine the in vivo genotoxicity of RBV in humans treated with this drug alone. At the 9th day of therapy, SCE rates and MN numbers were significantly higher than those at 1 month after therapy. The results of both tests therefore clearly demonstrate the in vivo genotoxicity of RBV. Although the number of patients was inadequate, the concordance of the results of both the MN and SCE methods supports the existence of an in vivo genotoxicity. Joksic et al. (13) report that RBV does not induce the formation of MN in cultured human lymphocytes, but that study may be insufficient to estimate in vivo genotoxicity because the metabolites of this drug have a toxic effect. In the literature, RBV is generally reported to be non-toxic in in vitro studies, while its toxic effects have been documented in in vivo studies (18-20). These results are in good agreement with the present results.

In Case 1, both SCE and MN rates were higher than in the other two cases (Table 1). This difference was probably due to the high individual sensitivity of this patient to RBV or to the presence of an unknown external genotoxic agent or agents.

The data obtained in the present study indicate that RBV has a reversible in vivo genotoxic effect in humans.

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