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Evaluation of Ribavirin Genotoxicity with Sister Chromatid Exchange and Micronuclei Assays in Humans

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Aim: The main objective of this study was to assess the genotoxicity of ribavirin in 15 patients who were suffering from Crimean–Congo haemorrhagic fever and treated with the therapy dose of the antiviral agent.

Materials and Methods: Genotoxicity was evaluated using the cytokinesis-blocked micronucleus (MN) and sister chromatid exchange (SCE) assays in lymphocyte cultures that were prepared from blood samples collected from the 15 patients. The blood samples were taken on day 9 of a 10-day therapeutic regimen of ribavirin and 1 month after the cessation of therapy.

Results: In all patients, the frequency of sister chromatid exchanges and the formation of micronuclei were significantly higher in the lymphocytes of blood samples that were taken in day 9 of the therapy compared to those that were collected 1 month after the cessation of therapy. In addition, on day 9, SCE and MN values were statistically different from the values of no-ribavirin control group.

Conclusions: These results showed that ribavirin has a reversible genotoxic effect in humans and this effect could be due to toxic metabolites of ribavirin.

Key Words: Ribavirin, genotoxic effect, sister chromatid exchange, micronuclei formation

İnsanlarda Ribavirin Genotoksisitesinin Kardeş Kromatid Değişimi ve Mikronükleus Yöntemleri ile Değerlendirilmesi

Amaç: Crimean Congo Hemorrhagic Fever nedeniyle tedavi dozunda ribavirin alan 15 hastada ribavirin genotoksisitesinin değerlendirilmesi amaçlanmıştır.

Yöntem ve Gereç: Onbeş hastadan alınan kan örneklerinden yapılan lenfosit kültürlerinde kardeş kromatid değişimi (KKD) ve mikronükleus (MN) yöntemleri kullanılarak genotoksik etki değerlendirildi. On günlük ribavirin tedavisinin dokuzuncu gününde ve tedavi kesildikten 1 ay sonra kan örnekleri alındı.

Bulgular: Tüm hastalarda, kardeş kromatid değişimi sıklığı ve mikronükleus oluşumu, tedavinin dokuzuncu gününde alınan kan örneklerinde tedavi kesildikten 1 ay sonra alınan örneklere göre belirgin bir biçimde yüksekti. Ayrıca, dokuzuncu gün KKD ve MN değerleri ribavirin almamış kontrol grubu değerlerinden istatistiksel anlamda farklıydı.

Sonuç: Sonuçlarımız göstermiştir ki, ribavirin insanlar üzerinde geri dönüşümlü bir genotoksik etkiye sahiptir ve bu etki ribavirinin toksik metabolitlerinden kaynaklanmaktadır.

Anahtar Sözcükler: Ribavirin, genotoksik etki, kardeş kromatid değişimi, mikronükleus oluşumu

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Introduction

Ribavirin (RBV) (1–β–D–ribofuranosyl–1,2,4– triazole–3–carboxamide; $C_8H_{12}N_4O_5$) is a synthetic purine nucleoside analogue and is used therapeutically as a broad spectrum antiviral agent (1). It exerts its antiviral action by inhibiting inosine monophosphate dehydrogenase, thereby preventing replication of some DNA and RNA viruses (2). RBV has been reported to be beneficial in the treatment of measles, Lassa fever, influenza, parainfluenza, hepatitis C, and in infections due to herpes virus, respiratory syncytial virus, and human immunodeficiency virus (HIV), but its therapeutic use is limited because of its toxicity (3,4). Its adverse effects include hemolytic anemia, hepatotoxicity, mitochondrial toxicity, and teratogenicity (5-8). It has also been demonstrated that RBV inhibits osteoblast proliferation in vitro (9). Because of its teratogenic potential, women

are recommended to use contraceptives for 6 months after RBV treatment. It has been reported that RBV is not genotoxic in some studies (10,11). Some other reports describe it as a potential genotoxin in human in vitro and experimental animals (12). Recently, Narayana et al. (2002) demonstrated that RBV is mutagenic to the germ cells of Wistar rats (13).

The sister chromatid exchange (SCE) and micronucleus (MN) tests are used to establish the genotoxic effects of physical and chemical agents (14,15). SCEs are reciprocal interchanges of DNA replication products at homologous sites between 2 chromatid arms within a single chromosome in dividing cells (14,16). These interchanges occur spontaneously in all cells at a low rate, and the rate of their formation increases when DNA is damaged and/or improperly repaired. SCEs can be detected after incorporating bromodeoxyuridine (BrdU) into chromosomal DNA for 2 cell cycles.

The MN assay is a quantitative test that detects structural damage to chromosomes and is now widely used in genotoxicity studies (15,17). Micronuclei can be formed when either the intact chromosome or acentric chromosomal fragments become separated from the nucleus during mitosis or meiosis, and are seen within a binucleotide cell after blocking cytokinesis (17,18). The method is now applied to various cell types to disclose genetic damage that can be caused by ionizing radiation or potential genotoxins (19).

In view of the uncertainty of the genotoxic effects of RBV in humans, we evaluated its genotoxicity using SCE and MN assays in lymphocytes taken from patients who were suffering from Crimean–Congo hemorrhagic fever (CCHF) and treated with RBV.

Materials and Methods

Patients and RBV Dosages

The study involved an experimental group composed of 15 patients and a control group with 12 healthy volunteers. All individuals in the study lived in a rural area, were not habitual smokers or drinkers, and had not been exposed to any known toxic agents. The patients were diagnosed as suffering from Crimean–Congo haemorrhagic fever (CCHF) after a clinical examination and based on the results of microbiological and routine clinical laboratory tests. The diagnosis of CCHF for each patient was confirmed by the detection of immunoglobulin M seropositivity using an enzyme-linked immunosorbent assay (ELISA) and/or demonstration of RNA of the virus using reverse transcriptase polymerase chain reaction (RT-PCR).

Oral RBV treatment at the dosage recommended by the World Health Organization was started in all patients immediately after making a provisional diagnosis of CCHF, and was continued for 10 days (20). The starting dose of RBV was 2000 mg, and then followed by 1000 mg RBV every 6 h for the first 4 days and 500 mg RBV every 6 h during the following 6 days. This dosing regimen has been used successfully to treat CCHF (21,22). If necessary, intensive supportive therapy was added to the RBV treatment and no other antimicrobial agents were administered to any of the patients. On day 9 of therapy and 1 month after completion of therapy, 2–5 ml samples of peripheral blood were taken from each individual in the experimental and control groups for use in the SCE and MN assays.

Control individuals had not taken any drugs in the previous 6 months.

Sister chromatid exchange (SCE) assay

To determine the frequency of SCE in the lymphocytes, a 1–2.5 ml sample of peripheral blood from each individual was added to 5 ml of chromosome medium B (Biochrom, Berlin, Germany) supplemented with 5 µg/ml of phytohaemagglutinin (Biochrom). BrdU (5–bromo–2–deoxyuridine, Sigma, MO, USA) was then added to each culture (10^{-4} M final concentration) and the cultures were incubated in complete darkness for 72 h at 37 °C. Colchicine (final concentration 0.5 µg/ml, Sigma) was added to each culture during the last 2 h of the incubation to block the cells in the metaphase stage of mitosis. Upon completion of the incubation, each cell culture was subjected to hypotonic treatment by adding 8 ml of 0.075 M KCl and was maintained for 30 min at 37 °C. Three repetitive fixations of each cell suspension were then performed with cold methanol/acetic acid (3:1, v/v). An aliquot of each cell suspension was then dropped onto a cold slide. The slides were dried at room temperature, and were then kept in the dark for 3 days after which they were stained using the fluorescence-Giemsa method

(23). Initially, slides were treated with 0.5 µg/ml bisBenzimide (Hoechst 33258, Sigma, USA) for 20 min and then exposed to UV-A (366 nm wavelength) for 1 h. The slides were incubated at 65 °C in 2XSSC (1:1, v/v, 0.03M sodium citrate/0.03M NaCl) solution for an hour. After that they were stained with 5% Giemsa for 10 min. The slides were coded before analysis (23). The number of SCEs was counted in at least 30 metaphases in each sample from each individual. The number of SCEs per metaphase was scored for each individual.

Micronucleus (MN) assay

In order to determine the number of micronucleated lymphocytes, a 1–2.5 ml sample of peripheral blood from each individual was added to 5 ml of chromosome medium B (Biochrom, Berlin, Germany) supplemented with 5 µg/ml of phytohaemagglutinin (Biochrom). The cultures were incubated for 72 h at 37 °C. After 44 h, 4.5 µg/ml of cytochalasin–B (Sigma, MO, USA) was added to each culture to block cytokinesis (19). Upon completion of the incubation, each cell culture was then subjected to hypotonic treatment by adding 8 ml of 0.075 M KCl and was maintained for 7 min at 37 °C. Three repetitive fixations of each cell suspension were then performed with methanol/acetic acid (3:1, v/v). An aliquot of each cell suspension was then dropped onto a cold slide. The slides were air–dried and kept at room temperature for 1 day after which they were stained with 5% Giemsa. The frequency of cells with 2 or more micronuclei was determined after assessing between 800 and 1900 binucleated cells from each cell culture. The slides were scored using the following scoring criteria described by Fenech et al. (24); the diameter of the MN should be less than 1/3 of the main nucleus, MN should be separated from or marginally overlap with main nucleus as long as there is clear identification of the nuclear boundary and MN should have similar staining as the main nucleus.

Statistical analyses

The mean and standard deviation (SD) were calculated from the individual results of each assay during therapy and after the cessation of therapy and of the control group. The data were analyzed using the Wilcoxon signed rank sum test. The level of significance was set at 5%.

Results

The frequency of SCEs in the lymphocytes after 9 days of RBV therapy was significantly higher than that determined 1 month after the cessation of therapy in 14 out of 15 patients ($Z = -3.408$, $P = 0.001$) (Figure 1). The mean frequency of SCEs on day 9 of therapy was 10.37 ± 2.61 (range: 6.42–15.49). The mean SCE frequency was 6.02 ± 0.65 (range: 4.78–7.23) 1 month after the cessation of therapy.

The MN scores in the lymphocytes of the 15 patients during therapy were significantly higher than those determined 1 month after the cessation of therapy ($Z = -3.408$, $P = 0.001$). The mean MN scores on day 9 of therapy was 22.1 ± 0.98 (range: 10.45–38.46) (Figure 2). Furthermore, we found that there was a positive correlation between the frequency of SCEs and the number of binucleated cells with micronuclei after day 9 of RBV therapy in the 15 patients. This correlation was not observed 1 month after the cessation of therapy. Likewise, there was no correlation between SCE numbers and prognosis of the patients.

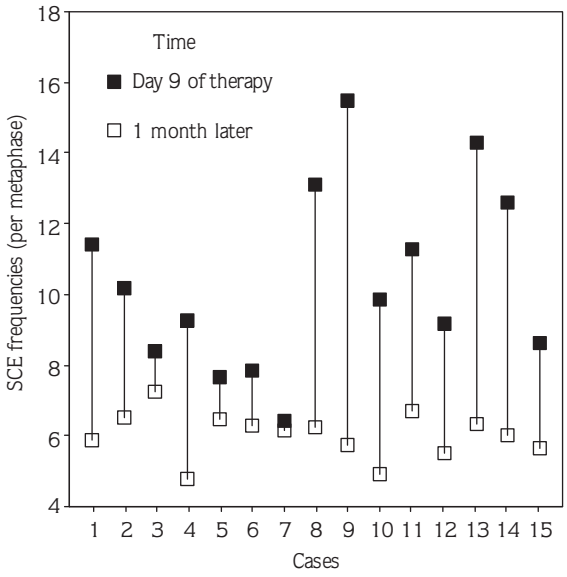

Figure 1. Individual variation in the frequency of SCEs during therapy and 1 month after the cessation of therapy. Variations among the cases with respect to differences in the values on day 9 of therapy and 1 month after the cessation of therapy are shown.

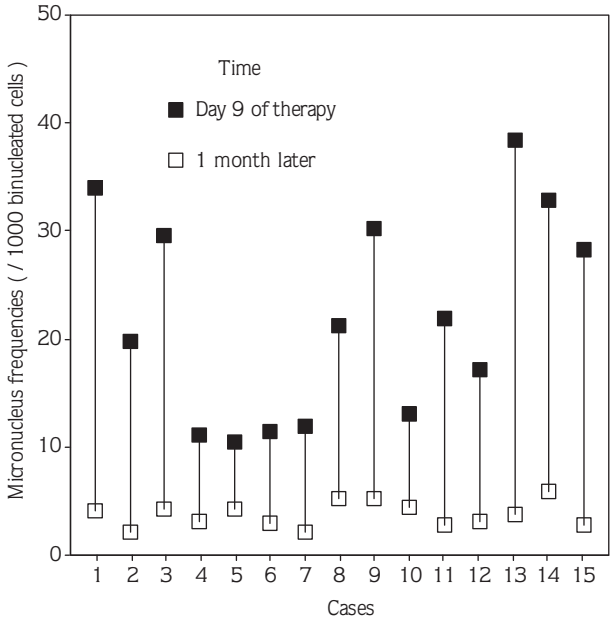

Figure 2. Individual variation in the formation of micronuclei during therapy and 1 month after the cessation of therapy.

In the control group, the SCE frequency and MN scores were 5.71 ± 1.15 and 3.65 ± 0.97, respectively (Tables 1 and 2). Day 9 group showed significant difference compared to the control group regarding both SCE and MN tests ($P < 0.0001$). However, there were no statistical differences between the control group and the experimental group after 1 month of therapy ($P > 0.005$).

Discussion

The results of previous studies on the toxicity of RBV in humans have demonstrated that RBV is cytotoxic to numerous cell types, especially erythrocytes, lymphocytes, and germ cells, when given at doses greater than 10 mg/kg (11,13,25). The cytotoxic effect of this drug is almost certainly due to the induction of cell death and the suppression of cell division and proliferation (25). However, it is still controversial whether RBV is genotoxic in humans, and conflicting results have been reported. The results of our study have shown that RBV has a reversible genotoxic effect in humans.

The results of previous studies on the genotoxicity of RBV are controversial because different methods have been used to assess its genotoxicity. The measured toxicity of RBV may depend on whether genotoxicity was assessed using an in vivo or an in vitro assay because of the formation of its metabolites, ribavirin–5′–triphosphate, and ribavirin–5′–monophosphate. These 2 metabolites are more toxic than RBV and are only formed following in vivo administration of RBV. Therefore, they are not generated in an in vitro assay system to which RBV is added (26,27). The type of assay used to assess RBV toxicity therefore needs to be considered comparing the results of toxicity studies of RBV. In this investigation, we used SCE and MN assays to assess the genotoxicity of RBV in vivo. Therefore, our results encompass the toxicity of the metabolites as well as that of intact RBV because we collected blood samples from patients who were treated with RBV. Our finding that RBV is genotoxic in humans coincides with previously published in vivo animal studies (12,28,29).

Hoffmann et al. reported that RBV had no mutagenic potential in male rats, following the use of the dominant lethal assay (10). However, this method may not be sufficiently sensitive to assess the mutagenic potential of RBV. The dominant lethal effect is the result of chromosomal damage and it may not be possible to demonstrate these genomic alterations experimentally, as they are not fatal to the fertilized egg or the developing embryo. On the other hand, Narayana et al. showed a mutagenic effect of RBV on rat germ cells using identical

Table 1. Statistical analyses of SCE frequencies on day 9 of therapy group, the experimental group 1 month after the cessation, and the control group.

	n	Mean ± SD	P
Day 9 of therapy	15	10.37 ± 2.61	P < 0.00011
month after therapy	15	6.02 ± 0.65	P > 0.005
Control	12	5.71 ± 1.15	-

Table 2. Statistical analyses of MN scores on day 9 of therapy group, the experimental group 1 month after the cessation, and the control group.

	n	Mean ± SD	P
Day 9 of therapy	15	22.1 ± 0.98	P < 0.00011
month after therapy	15	3.74 ± 1.16	P > 0.005
Control	12	3.65 ± 0.97	-

doses that were used by Hoffmann et al. (13). In an in vitro study conducted by Joksic et al., RBV was shown to suppress cellular proliferation at all the doses used, but it did not increase micronuclear formation after blocking cytokinesis in cultured human lymphocytes (11). On the contrary, the results of another study showed that RBV increased MN frequency in cultured lymphocytes when RBV was used at high doses and the cells were exposed for a long time (30). Using 2 methods to assess its genotoxicity, we have shown that RBV is a genotoxic chemical. We are convinced that this conclusion is valid because different researchers performed each of the 2 genotoxicity assays in order to prevent researcher bias in the collection and interpretation of the results.

Several investigators have used the MN assay to evaluate RBV toxicity and have shown that RBV has mutagenic activity in the bone marrow of mice and rats when administered at doses greater than 10 mg/kg (12,28,29). They have also demonstrated that this effect was not dose-dependent. Narayana et al. did not find a linear correlation between the administered dose and toxicity when using the in vivo sperm morphology assay to assess RBV toxicity, although they found a weak concentration-dependent effect when they used an in vitro assay to assess its mutagenicity (13). Joksic et al., however, reported the existence of a positive correlation between the genotoxicity of RBV and its exposure time, and between its genotoxicity and concentration, using the MN assay (30). In our study, the combination of time (9 days) and dose (30–50 mg/kg/day) was sufficient to produce a toxic effect of the drug. We were not able to investigate the correlation between the dose of RBV and its genotoxicity because RBV was administered to the patients only at the recommended dose. Nevertheless, we found that there were individual differences in the severity of genotoxicity at identical doses of administration and we believe that the individual's ability to metabolize the drug accounts for this difference. The frequency of SCEs and the formation of micronuclei during treatment were much greater than those found 1 month after the cessation of therapy. There were no statistical difference between the control group and the experimental group 1 month after cessation for both SCE and MN tests (Tables 1 and 2). The frequency of SCEs during therapy ranged widely, whereas it was within a very narrow range after therapy. We thus suggest that individual sensitivity of subjects to RBV can affect the severity of its genotoxicity.

It is widely accepted that RBV is a cytotoxic and genotoxic agent (12,31). In our preliminary study, which included only 3 patients, we proved that RBV may be a potent genotoxic agent in humans (32). This present study including a larger number of adult subjects further supports that RBV is a drug having genotoxic effect. The results of this study provide further support that RBV is a drug that has reversible genotoxic effects. There are insufficient studies on RBV toxicity in humans. To the best of our knowledge, this is one of the early studies reporting RBV-induced genotoxicity in humans in vivo. Because the use of RBV to treat CCHF in all likelihood will increase (14,33,34), physicians should keep the genotoxicity of RBV in mind when administering it to patients, despite the fact that its genotoxic effect appears to be transient.

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