**In vitro** assessment of chromosomal aberrations of cultured human peripheral blood lymphocyte following antiviral drug models exposure

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Ribavirin and acyclovir are two wide spectrum antiviral drugs. The present work aimed to draw attentions toward the cytotoxicity and genotoxicity of antiviral drugs. Also, it aimed to evaluate the cytogenetic/genotoxicity relatively to dose concentration (low, therapeutic, and high). Cytotoxicity of test antiviral drugs was carried out using human lymphocytes and macrophages. Ribavirin and Acyclovir were tested (low dose, 5 µg/ml; therapeutic dose, 10 µg/ml and high dose, 20 µg/ml) against macrophage cells and human lymphocytes. Chromosomal aberrations were significantly recorded in human lymphocytes as tetrapolidy, pulverized metaphase and dicentric chromosomal aberrations. These aberrations due to Ribavirin post-treatment are dose dependant.

**Key words:** Ribavirin, acyclovir, lymphocytes, chromosomal aberration.

**INTRODUCTION**

Many reports and retrospective studies have suggested a strong association between particular environmental factors and the occurrence of congenital malformations in humans. Examples of these environmental factors: carbon momoxide, smoking (Wolfe et al., 2006), organic solvents (Savitz, 2003), lead (Landdrigan et al., 2008), anesthetic agents (Polifka and Friedman, 1999), alchol (Coles, 1994), and drugs (Michaelis et al., 1983). Drug utilization may interfere with normal embryonic cells which may cause fetal differentiation and morphogenesis (teratogenicity). The evidence for many drugs is less convincing, but some are highly suspected (Persaud et al., 1985). Antiviral drugs were found to exhibit potent antiviral properties crucial for the clinical treatment of viral diseases, although their uses remain controversial due to side effect (Tatar et al., 2005).

Ribavirin is active against a number of DNA and RNA viruses including avian influenza, hepatitis B, polio, measles and small pox, Ebola virus, Lassa fever, Crimean-Congo hemorrhagic fever and Hantavirus infection (Sidwell et al., 1972). It is a member of the nucleoside anti-metabolite drugs that interfere with duplication of viral genetic material. Ribavirin is concentrated and persists in red blood cells for the life of the erythrocyte (Manns et al., 2001). In the United States the oral (capsule or tablet) form of ribavirin is used in the treatment of hepatitis C trial either as sole agent or in combination with interferon (McHutchison, 1998). The aerosol form is used to treat respiratory syncytial virus related diseases in children. The primary serious adverse effect of ribavirin is hemolytic anemia. It is dose dependant and may sometimes be compensated by decreasing dose (Fried et al., 2002). Ribavirin also can induce point mutations in germ cells yielding abnormal sperms. The genotoxic effects of ribavirin are not exerted...
in a dose-dependent pattern in mouse (Rao and Narayana, 2005). Addition of ribavirin up to 500 μg/ml to peritoneal macrophages freshly isolated from BALB/c J mice had no toxic effects as demonstrated by trypan blue exclusion (> 95% viable) (Ning et al., 1998).

Acyclovir is a guanine analogue extremely selective and of low cytotoxicity, was seen as the start of a new era in antiviral therapy against most species of herpes virus family (O’Brien et al., 1989). All formulations of acyclovir are assigned Food and Drug administration. In general, acyclovir seems to have a low toxic potential. Data from available investigations do not give support for a mutagen, teratogen or carcinogen hazards of acyclovir in patients receiving recommended clinical doses. After termination of pregnancy, the fetus was found as having ventricular septum defect. The presence of the triploid cell lines mocaicism involving chromosome 2 and 19 were confirmed by the analysis of fetal skin tissues. No attributable finding to herpes simplex virus infection and acyclovir treatment was found, and the presence of the triploid cell lines mocaicism were appeared to be purely coincidental (Aktas et al., 2001).

The effect of acyclovir used as 11.2 to 22.5 μg/ml (50 to 100 μM) on human cells was measured. It can inhibit the division of fibroblasts to a variable extent. Inhibition was exerted on T-cell proliferation without apparent effect on the release of lymphokines or on monocyte function. No evidence of adverse effects on the immune system of animal occurred except at excessively high doses in dogs where marked lymphoid hypoplasia occurred (Furman et al., 1980).

Since ribavirin and acyclovir commonly used in developing countries, the use of these drugs during pregnancy may increase the birth defect rates. This raises the idea to evaluate the in vitro genotoxicity of these drugs in cultured human lymphocytes, evaluating the chromosomal aberrations that can be induced post exposure to different concentration of these drugs and the cytotoxicity as well.

MATERIALS AND METHODS

Drugs

Ribavirin was commercially supplied from T3A Company and Acyclovir was commercially obtained as Zovirax from Glaxo Smith Kline -Swiss. Ribavirin and Acyclovir were used as 5, 10 and 20 μg/ml in hanks balanced salt solution (HBSS) representing Low, therapeutic and high doses respectively.

Blood samples

Blood samples were collected from twenty healthy donors (Holding Company for Biological Products and Vaccines) blood bank attendants. Patients were divided into 10 males and 10 females. The mean age was ranged from 24 to 34 years. Patients were elicited according to the designed questionnaire format; no exposure to radiation (for example, X-ray), no smoking and no medication for three months pre submitting to sample collection.

Human lymphocytes were separated using ficoll (Sigma-Aldrich – USA). Cells were aseptically separated and washed twice with sterile NaCl. Chromosomal study was arranged for every donor to make sure that there was no chromosomal aberration either numerical or structural. Treatment doses designs were chosen according to the considered ribavirin and acyclovir plasma levels.

Peripheral blood culture technique

Five ml of venous blood were aspirated in a heparinized vacutainer tubes and 0.5 ml of each sample was placed in a sterile 15 ml Falcon tube (TPP-Swiss) containing 10 ml RPMI 1640 media supplemented with 10% Fetal Calf Serum, L-glutamine, phytohaemagglutinin and Fungizone (Sigma-Aldrich-USA). Low, therapeutic and high dose of Ribavirin and/or Acyclovir was added at the beginning of the cultures. Cultures were incubated for 96 hours at 37°C. Control cultures were processed using saline. 0.1 ml of colcemid was added to the culture to stop mitosis and prevent spindle formation and incubated for 30 min before cell harvesting. Cells were cold centrifuged at approximately 1500 rpm for 10 min (Jouan-France). The supernatant was removed and 10 ml of a 37°C pre-warmed hypotonic solution KCl (0.075 M) were added helping the cells to swell and to separate the chromatids while, leaving the centromeres intact. Cells were resuspended and incubated at 37°C for 45 min. The supernatant was removed by centrifugation, and 10 ml of freshly prepared carnoy’s fixative (methanol: glacial acetic acid, 3:1) were dispensed. The first pipette full of carnoy’s fixative was added drop by drop while shaking the tube after each drop. The fixative was removed and the procedure was repeated twice. The supernatant fluid was decanted and the pellet was suspended in a small amount of the fixative. On a pre-cleaned slide, 2-3 drops of the suspension were placed. The spreading of the cells was done by the use of short, hard breaths and blowing directly perpendicularly on the slide for 4 to 6 times. The slides were air dried for 2 min. Cells were stained using Giemsa stain (Sigma-USA).

Both Microscopic analysis and Photographic analysis were carried out. The analysis included chromosome counts in metaphase and chromosome aberrations.

Cell viability

Cell viability was evaluated using MTT biochemical assay according to (John, 2004). Macrophages were collected from the peritoneal cavity of the mice using cold media then cells were centrifuged at 1200 rpm at 4°C for 5 min. The cells were suspended in media then were counted using trypan blue exclusion assay according to Walum et al. (1990). Macrophages were dispensed as 2 x 105/ml then incubated at 37°C for 24 h. Adhered cells were treated with the drugs. After 48 h cells were microscopically investigated post treatment to monitor drug toxicity. Residual living cells were stained using neutral red dye and OD was measured Using ELISA reader at 450 nm. Macrophage were seeded into 96-well microtiter plates (Nunclon TM, Nunc, Germany) at a density of 10000 cells/well. Twenty four hours later, the culture medium was replaced with serial dilutions of antiviral drugs stock solution in antibiotic-free DMEM. Twenty four hours post incubation, 20 μl MTT 0.5 mg/ml (Sigma, Deisenhofen, Germany) in PBS were dispensed to the replisal wells for 4 h in a dark incubator. Unreacted dye was removed by aspiration and the purple formazan product was dissolved in 200 μl/well Dimethyl Sulfoxide; DMSO (Merk, Darmstadt, Germany) and quantified using plate reader (Titertek plus MS212, ICN, Germany) at 570 nm. The mean optical densities of tested samples and control were recorded and the percentage of cell viability was determined as follow formula:
Figure 1. Percentage of cell viability pre and post treatment of macrophage cells with the antiviral drugs Ribavirin and acyclovir.

Table 1. Assessment of chromosomal aberrations post human lymphocyte treatment with low, therapeutic and high dose of Ribavirin.

<table>
<thead>
<tr>
<th>Ribavirin concentration (µg)</th>
<th>Tetrapoloidy</th>
<th>Pulverized metaphase</th>
<th>Dicentric chromosome</th>
<th>Non-treated cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0</td>
<td>4.60 ± 0.66</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>0.050 ± 0.002</td>
<td>15.70 ± 1.7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>0.60 ± 0.082</td>
<td>29.60 ± 1.56</td>
<td>0.75 ± 0.08</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are means ± SD. Number of tested samples = 20 and P < 0.05.

RESULTS

Cytotoxicity and related viability % of antiviral drug treated cells revealed that both Ribavirin and Acyclovir showed no cytotoxic effect on macrophage cells when they used as 80 µg/ml (Figure 1).

Chromosomal aberrations had been detected in metaphase division of lymphocyte treated with (low, 5 µg/ml; therapeutic, 10 µg/ml; and high dose, 20 µg/ml) of Ribavirin (Table 1). The aberrations appeared in the form of tetraploidy, pulverized metaphase and dicentric chromosomal aberration. The mean value of such abnormalities was statically significant (P < 0.05) as shown in Table 1.

Clear direct correlation was found in 14 cases. Chromosomal aberrations in metaphase relatively to the concentration of ribavirin are shown in Figure 2. The most prevalent aberration was tetrapoloidy, pulverized metaphase, and dicentric chromosome. Results also showed that the high dose (20 µg/ml) of ribavirin gave highest aberration comparing with therapeutic and low doses. No abnormalities were detected in control samples treated with phosphate buffer saline instead of Ribavirin.

No change detected in chromosomes of the lymphocytes examined at the metaphase post Ribavirin treatment with low dose (Figure 2A). While the therapeutic dose show pulverized metaphase (Figure 2B), while the high dose show pulverized metaphase (Figure 2C), tetraploidy (Figure 2D) and dicentric chromosome (Figure 2E). On the other hand, acyclovir had been examined using human lymphocytes and the same concentrations as used in case of Ribavirin. Results in Figure 3, indicated that there is no numerical or structural aberrations detected.

DISCUSSION

The cytotoxic effect of antiviral drugs is almost due to the induction of cell death and suppression of cell division and proliferation (D’Souza and Narayana, 2002). However, it is still controversial whether such drug is genotoxic in humans or not. Some conflicting results
Figure 2. Photomicrographed metaphase chromosomal aberrations of human lymphocyte show (A) normal Metaphase treatment with a low dose of Ribavirin, (B) Pulverized Metaphase after treatment with a therapeutic dose of Ribavirin (C) Pulverized Metaphase after treatment with a high dose of Ribavirin, (D) Tetraploidy Metaphase after treatment with a high dose of Ribavirin and (E) Dicentric chromosome at chromosome 1, 2 after treatment with a high dose of Ribavirin.

Figure 3. Photomicrographs showing normal metaphase chromosomal pattern post low, therapeutic and high dose treatment with Acyclovir (A, B and C) Showed normal metaphase after treatment with Acyclovir.

have been reported. In our study, several chromosomal damages have been found in the human lymphocyte cultures in vitro post ribavirin treatment such as tetraploidy, pulverized metaphase and dicentric chromosome at therapeutic dose (10 µg/ml) and high dose (20 µg/ml). No numerical or structural aberrations were detected in metaphases of human lymphocyte cultures after treatment with a low, therapeutic and a high dose of acyclovir. Neither Ribavirin nor Acyclovir exhibited significant effect on the WISH and macrophage cell viability. The previous studies on the genotoxicity of these drugs depending on whether genotoxicity was assessed using an in vivo or in vitro assay because of the formation of their metabolites. These metabolites are more toxic than either Ribavirin or Acyclovir and are only formed following administration. Therefore, they are not generated in an in vitro assay system (Narayana et al., 2005). In this study, we used two currently used antiviral drugs for in vitro assays and our finding demonstrated that ribavirin was genotoxic coincides with the previously published studies conducted by Joksić et al. (2000). Different doses of Ribavirin (5, 10, and 20 µg/ml) were shown to suppress the human lymphocyte cellular proliferation. Similarly, Joksic et al. (2006) reported that ribavirin increased micronuclear frequency in cultured lymphocytes when it was used at high doses (0.47 and 0.65 µM/ml) for long time. This confirmed the existence of positive relation between genotoxicity of the antiviral drugs and their concentration as well as their time of application. Furthermore, Tater et al. (2009) showed that lymphocytes of peripheral blood samples collected on the 9th to 10th day of therapeutic regime of ribavirin, were
significantly exchanged using sister chromatid and micronuclear assays. Comparative analysis of DNA breakage, chromosomal aberration and apoptosis induced by acyclovir (ACV), ganciclovir (GCV), and penciclovir (PCV) revealed that these drugs were strong inducers of single strand, double strand DNA breakage and abnormal mitosis. This genotoxic effects involved in killing of cells 7-fold for (ACV), 60-fold for (GCV), and 400-fold for (PCV) (Tomicic et al., 2002). In further study carried out by Klug et al. (2004) it was concluded that acyclovir at 25 µM causes minor impact of embryonic development in mammalian culture in vitro while concentrations of 50 µM acyclovir causes additional disturbances of embryonic cultures in vitro. In the limb bud culture (mouse embryos, starting with day 11 of gestation) acyclovir interfered with the differentiation of cartilaginous bone anlagen at concentrations of 200 µM and more in the culture medium. On the contrary, Clive et al. (1991) reported that ACV treatment didn’t increase chromosomal aberration and sister chromatid exchange in human cultured peripheral blood lymphocytes. Another study on Hela cells revealed that ACV had no appreciable changes in cell number and the surviving fraction of cells was reduced to half at 100 µM ACV (Jegetia and Aruna, 1999). These results seemed to be in agreement with our results.

Collectively, our study was carried out in vitro condition to mange safe dose administration. Also, description should not include genotoxic drugs especially at pregnancy and patient should be restricting to the dose designed by physician.

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