Treatment of Pregnant Women with a Betamimetic and Verapamil Increases the Micronuclei Frequency in Umbilical Cord Blood Lymphocytes

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In prevention of preterm labor, betamimetics are used in gynecological practice mostly combined with antiarrhythmic verapamil because of their therapeutic cardiovascular side effects. The aim of this study was to investigate the influence of a betamimetic (ritodrine hydrochloride, fenoterol or hexoprenaline) and verapamil (administered to mothers) on the frequency of micronuclei (MN) in umbilical cord blood lymphocytes of neonates, using cytokinesis-block micronucleus test. The analyzed sample included 23 babies whose mothers received the therapy and 30 control babies whose mothers received no therapy. The average MN frequency was significantly higher in the neonates whose mothers received the therapy (8.13 ± 2.69 MN/1000 BN cells), in comparison with the baseline frequency in untreated controls (3.30 ± 2.63 MN/1000 BN cells), with probability p < 0.05. The highest MN frequency was found in neonates whose mothers received fenoterol and verapamil (2.8-fold i.e. 9.10 ± 3.00 MN/1000 BN cells), while ritodrine hydrochloride and hexoprenaline combined with verapamil induced 2.3-fold and 2.2-fold higher MN values than in controls (7.50 ± 3.33 MN/1000 BN cells and 7.29 ± 0.95 MN/1000 BN cells). Multiple linear regression analysis showed that MN frequency was affected only by the maternal therapeutic treatment, while the neonates’ sex, maternal age, cigarette smoking, and therapeutic doses did not affect the MN frequency in umbilical lymphocytes of neonates. We conclude that the treatment of pregnant women with a betamimetic and verapamil significantly increases the MN frequency in umbilical cord blood lymphocytes of neonates, regardless to therapeutic doses.

Betamimetics (ritodrine hydrochloride, fenoterol or hexoprenaline) are selective β2-adrennergic agonists used as uterine relaxant. They stimulate β2-adrenergic receptors, inhibiting the contractility of uterine smooth muscle that results in the arrest of premature labor (Graves 1996). The most important problem of labor inhibition with β2-adrenergic agonists is associated with cardiovascular side effects such as hypotension and tachycardia (Bieniarz et al. 1972; Benedetti,
as maternal therapy, on the frequency of MN in umbilical cord blood lymphocytes of neonates.

**MATERIAL AND METHODS**

This study was approved by Ethical Committee of Clinic of Kragujevac and consisted of 53 phenotypically healthy neonates observed at the Department of Obstetrics and Gynecology in Kragujevac, Serbia, in 2007. The analyzed sample included 23 healthy neonates (12 males and 11 females) whose mothers received one of the betamimetics (ritodrine hydrochloride, fenoterol or hexoprenaline) combined with verapamil (2 × 40 mg per day) in the second and the third trimester of pregnancy. Six mothers received ritodrine hydrochloride (3 × 40 mg per day), ten mothers received fenoterol (6 × 5 mg per day), while seven received hexoprenaline (6 × 0.5 mg per day). The length of therapy varied from 7 days to 5 months, depending on physiological status of the patients; therefore total doses were different (45 – 14,400 mg for betamimetics and 560 – 12,000 mg for verapamil). All mothers (mean age 25.39 ± 4.99 years) were not occupationally exposed to known mutagenic agents and kept standard dietary habits. Eleven of the 23 mothers were smokers (< 10 cigarettes).

Control sample comprised 30 healthy neonates (15 males and 15 females) whose mothers (mean age 28.70 ± 4.66 years) received no therapy during their pregnancy and without any recent exposure to genotoxic agents or drugs. Twelve of the mothers were smokers (< 10 cigarettes). Further details on the neonate samples are given in Table 1.

The MN frequency in umbilical cord blood lymphocytes of neonates was determined by using cytokinesis-block micronucleus test (CBMN test) described by Fenech (2000). Venous blood samples of babies were collected by umbilical puncture. Whole heparinized blood (0.5 ml) was added in 5 ml complete medium for the cultivation of cells PBMax Karyotyping (Invitrogen, California, USA). All cultures were set up in duplicate and incubated at 37°C for 72 hours. Cytochalasin B (Sigma, St Louis, MO, USA) was added 44 hours after the beginning of incubation in final concentration of 4 μg/ml. The cultures were incubated for another 24 hours. The preparation was standard. Hypotonic solution was 0.56% KCl, the fixation being performed 3 × 15 min with the fixative consisting of glacial acetic acid and methanol (1 : 3). The obtained material was spread onto specially prepared, cold and lamp-dried slides. After 5 - 7 days, the slides were stained with 2% Giemsa.
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The MN frequency was determined by analysing 1,000 binucleated cells (BN) per neonate, according to the criteria described by Fenech (2000). The MN frequency was expressed as average ± s.d. The results were compared by application of non-parameter Mann-Whitney test. Differences with p < 0.05 were considered to be statistically significant. The difference of MN frequencies between the analyzed and control neonates was determined by multiple linear regression analysis in relation to the sex of newborns, age and smoking habits of the mothers as well as to the therapeutic treatment of the mothers and therapeutic doses, as factors important for the increase of MN frequency.

**RESULTS**

The results of MN analysis in the neonates whose mothers received the therapy and controls are presented in Tables 1-3. Binucleated (BN) cell with well preserved membrane of a neonate is shown in Fig. 1. General characteristics of the analyzed samples of neonates and their mothers are presented in Table 1.
Table 2 shows individual variability of MN values in umbilical blood lymphocytes of neonates, type and length of therapy, as well as total doses administered to their mothers during pregnancy.

In control neonates, the baseline MN frequency was 3.30 ± 2.63 MN/1000 BN cells ranging from 0 to 9 MN. Total average MN frequency in lymphocytes of neonates whose mothers received betamimetics and verapamil was 8.13 ± 2.69 MN/1000 BN cells, and ranged from 4 to 14, which was significantly different from the baseline MN frequency in controls (p < 0.05). The highest average MN frequency was found in the neonates whose mothers received fenoterol and verapamil (9.10 ± 3.00 MN/1000 BN cells, ranging from 5 to 14 MN per 1000 BN cells). In the therapy with ritodrine hydrochloride and verapamil, the MN frequency was 7.50 ± 3.33 MN/1000 BN cells, ranging from 4 to 13; in the therapy with hexoprenaline and verapamil the MN frequency was 7.29 ± 0.95 MN/1000 BN cells, ranging from 6 to 9 MN per 1000 BN cells (Table 3). These values were significantly different from the controls (p < 0.05). The same Table shows the results of MN distribution in the analyzed BN cells. Out of 23000 BN cells analyzed in the lymphocytes of neonates whose mothers received the therapy, 180 cells (0.78%) with MN were found, which was a higher value in comparison to controls (98 cells i.e. 0.33%). The greatest number of BN cells had 1 MN in both neonate samples (0.75% and 0.32%, respectively), while the cells with 2 MN were less present (0.03% and 0.003%, respectively).

The application of multiple linear regression analysis regarding factors that may affect MN (i.e. sex of neonates, maternal age and smoking habits as well as therapeutic treatment and therapeutic doses) showed that the difference in lymphocyte MN frequencies between controls and neonates whose mothers received the therapy was affected only by therapeutic treatment of the mothers (P = 0.000). Sex of newborns, age and smoking habits of the mothers as well as therapeutic doses did not affect MN frequency in newborns.

**DISCUSSION**

Numerous studies show that fetus and children are more sensitive to many environmental toxins than adults (Whyatt and Perera 1995; Haley and Talbort 2004; Neri et al. 2006). One of the reasons is that the placenta does not completely protect the fetus from the outside influences (Vähäkangas and Myllynen 2006; Myren et al. 2007). Factors that may increase fetal susceptibility include higher rates of cell proliferation, the greater number of target cells at risk, lower immunologic competence, and decreased capacity to activate and detoxify carcinogens as well as to repair DNA (Anderson et al. 2000; Perera et al. 2004; Whyatt et al. 2004). A great number of authors showed that the frequency of damages in genetic material of neonates and children, such as MN and chromosome aberrations, increased with the concentration of environmental agents (Bocskay et al. 2005; Milošević-Djordjević et al. 2005, 2007b; Neri et al. 2006). In addition to the environmental pollutants, the therapy prescribed to pregnant women as a uterine relaxant attracts...
**Table 2.** Micronuclei frequency in the lymphocytes of neonates depending on the kind, length and total doses of therapy administered to the mothers in the second and third trimester of pregnancy.

<table>
<thead>
<tr>
<th>Mothers age (years)</th>
<th>Mothers Non-smokers (−) Smokers (+)</th>
<th>Neonates sex</th>
<th>Total length of therapy (days)</th>
<th>Total of administered doses of betamimetics (mg)</th>
<th>Total of administered doses of verapamil (mg)</th>
<th>Analyzed BN cells</th>
<th>MN/1000 frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>a) ritodrine hydrochloride + verapamil</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. 27 − M 60</td>
<td>7,200</td>
<td>4,800</td>
<td>1,000</td>
<td>13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. 21 + M 30</td>
<td>3,600</td>
<td>2,400</td>
<td>1,000</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. 26 − M 120</td>
<td>14,400</td>
<td>9,600</td>
<td>1,000</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. 30 + M 30</td>
<td>3,600</td>
<td>2,400</td>
<td>1,000</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. 17 − F 60</td>
<td>7,200</td>
<td>4,800</td>
<td>1,000</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. 25 − F 120</td>
<td>14,400</td>
<td>9,600</td>
<td>1,000</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>b) fenoterol + verapamil</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. 25 + M 30</td>
<td>900</td>
<td>2,400</td>
<td>1,000</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8. 25 − M 150</td>
<td>4,500</td>
<td>12,000</td>
<td>1,000</td>
<td>13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9. 32 − M 60</td>
<td>1,800</td>
<td>4,800</td>
<td>1,000</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10. 18 + M 7</td>
<td>210</td>
<td>560</td>
<td>1,000</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11. 26 − M 45</td>
<td>1,350</td>
<td>3,600</td>
<td>1,000</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12. 26 − F 7</td>
<td>210</td>
<td>560</td>
<td>1,000</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13. 33 − F 30</td>
<td>900</td>
<td>2,400</td>
<td>1,000</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14. 23 + F 120</td>
<td>3,600</td>
<td>9,600</td>
<td>1,000</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15. 23 + F 120</td>
<td>3,600</td>
<td>9,600</td>
<td>1,000</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>16. 25 − F 75</td>
<td>2,250</td>
<td>6,000</td>
<td>1,000</td>
<td>6</td>
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<td></td>
<td></td>
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<tr>
<td><strong>c) hexoprenaline + verapamil</strong></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>17. 23 + M 60</td>
<td>180</td>
<td>4,800</td>
<td>1,000</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18. 24 + M 15</td>
<td>45</td>
<td>1,200</td>
<td>1,000</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19. 35 − M 120</td>
<td>360</td>
<td>9,600</td>
<td>1,000</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20. 34 + F 150</td>
<td>450</td>
<td>12,000</td>
<td>1,000</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21. 28 − F 30</td>
<td>90</td>
<td>2,400</td>
<td>1,000</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22. 18 + F 30</td>
<td>90</td>
<td>2,400</td>
<td>1,000</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23. 20 + F 90</td>
<td>270</td>
<td>7,200</td>
<td>1,000</td>
<td>7</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
great attention as well. The therapy with betamimetics combined with verapamil is considerably administered in gynecological practice; therefore, it is particularly important to identify their undesirable effects especially on growth, development and genetic structure of the fetus.

In the present study, genotoxicity of maternal pharmacotherapy based on the one of betamimetics combined with verapamil was investigated by analyzing MNs in umbilical blood lymphocytes of neonates. Application of the administered therapy produced significant increase in the MN frequency in comparison to the controls ($p < 0.05$).

The fact that verapamil (Gembruch et al. 1988) and betamimetics (Friedman et al. 1994), cross the placenta and that verapamil when combined with other medicaments reveals co-mutagenic effect was pointed out in many papers (Friedman et al. 1990; Scheid et al. 1991, Scheid and Traut 1993; Nesterova et al. 1999; Seredenin et al. 1999). Our results confirm the possibility that in modified intrauterine conditions the fetus reacts at the cell level, directly increasing MN frequencies in umbilical blood lymphocytes.

The analysis of specific effects of administered medicaments showed that each of betamimetics combined with verapamil significantly increased average MN frequency in lymphocytes of neonates ($p < 0.05$). The highest average MN frequency was found in neonates whose mothers received fenoterol and verapamil (2.8-fold i.e. $9.10 \pm 3.00$ MN/1000 BN cells), while ritodrine hydrochloride and hexoprenaline in combinations with verapamil induced 2.3-fold and 2.2-fold higher MN values than in controls ($7.50 \pm 3.33$ MN/1000 BN cells and $7.29 \pm 10.95$ MN/1000 BN cells vs $3.30 \pm 2.63$ MN/1000 BN cells).

Not very long ago, in the in vivo study of genotoxicity of anesthetic substances and combined therapy in post-surgical treatment of cervical cerclage based on ritodrine hydrochloride, erythromycin and verapamil, we concluded that the combined pharmacotherapy treatment significantly increased the MN frequency in peripheral blood lymphocytes of pregnant women who received therapy during six days (Grujičić et al. 2007).

The obtained results of MN distribution show that the administered therapy not only increased number of cells with MN, but also the number of MN in the cells. Thus, our previously published results on the mothers who received partly the same therapy (Grujičić et al. 2007), showed the increase of both number of BN cells containing MN and number of MNs in BN cells.

### Table 3. Distribution of micronuclei in the umbilical blood lymphocytes of neonates.

<table>
<thead>
<tr>
<th>Therapy</th>
<th>No of analyzed BN cells</th>
<th>No of cells with MN</th>
<th>Average frequency of MN/1000 BN cells</th>
<th>Range of variation MN/1000 BN cells</th>
<th>Distribution of MN</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) ritodrine hydrochloride + verapamil</td>
<td>6</td>
<td>6,000</td>
<td>44 (0.73%)</td>
<td>7.50 ± 3.33 $^a$</td>
<td>4-13</td>
</tr>
<tr>
<td>b) fenoterol + verapamil</td>
<td>10</td>
<td>10,000</td>
<td>85 (0.85%)</td>
<td>9.10 ± 3.00 $^b$</td>
<td>5-14</td>
</tr>
<tr>
<td>c) hexoprenaline + verapamil</td>
<td>7</td>
<td>7,000</td>
<td>51 (0.73%)</td>
<td>7.29 ± 10.95 $^c$</td>
<td>6-9</td>
</tr>
<tr>
<td>Total: (Betamimetics+verapamil)</td>
<td>23</td>
<td>23,000</td>
<td>180 (0.78%)</td>
<td>8.13 ± 2.69 $^d$</td>
<td>4-14</td>
</tr>
<tr>
<td>Control sample</td>
<td>30</td>
<td>30,000</td>
<td>98 (0.33%)</td>
<td>3.30 ± 2.63</td>
<td>0-9</td>
</tr>
</tbody>
</table>

$^a, b, c, d$ Statistically significant difference, compared with control sample ($p < 0.05$; Mann-Whitney test)
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The increase was explained by accumulation of DNA damage as a consequence of the applied therapy.

Many authors showed that there was a correlation between the amount of damage of the genetic material and the MN frequency. Thus, Minozzo et al. (2004) point out that the cells with greater number of MN suffered from more serious damage of genomes and that many of them could not survive, being eliminated from the further division by the activation of apoptosis mechanisms. The cells with 1MN have better chances to survive, since they have suffered from lesser genome damages (Hando et al. 1997).

The multiple linear regression analysis showed that only therapeutic treatment of mothers, i.e. transplacental exposure of the fetus to betamimetics and verapamil, significantly influenced the MN frequency in umbilical blood of neonates, while the neonates’ sex, maternal age, smoking habits and therapeutic doses did not affect difference of MN frequency in umbilical blood lymphocytes in the investigated neonates. The neonates whose mothers had longer treatment did not necessarily have higher MN frequencies, although they were transplacentally longer exposed to its effects. This variability of the MN response to the transplacental effect of therapy can be explained by individual sensitivity of umbilical blood lymphocytes in the neonates to the mutagen effect. Our results show that there was no significant difference in MN frequencies between the neonates whose mothers were 17 – 30 and 31 – 40 years old ($p > 0.05$).

The results of both genders did not show a significant difference in the baseline and induced MN frequency relating to gender of neonates. A number of published studies showed the same frequency of MN among males and females aged ≤ 19 years (Barale et al. 1998; Shi et al. 2000). Analyzing a pooled database of nearly 7000 subjects, Bonassi et al. (2001) showed an effect of sexual differences of MN only in adults.

Maternal cigarette smoking during pregnancy and a possible transplacental effect on the fetal development were the subject matter of numerous investigations (Pluth et al. 2000; Neri et al. 2003). Our results show that there was no significant difference in the MN frequency between the neonates whose mothers were smokers and non-smokers. These findings are in agreement with the results by other authors who concluded that smoking of less than 20 cigarettes per day had no effect on the change of MN frequency, and that only a few cigarettes per day stimulated protective cell response (Gourabi and Mozdarani 1998; Rothfuss et al. 1998). In our study, mothers smoked 5-10 cigarettes per day.

The results obtained by application of CBMN test reveal that the investigated betamimetics (ritodrine hydrochloride, fenoterol and hexoprenaline) combined with verapamil, administered to pregnant women in total therapeutic doses (45 – 14,400 mg for betamimetics and 560 – 12,000 mg for verapamil) during 7 days to 5 months in the second and third trimester, had an important genotoxic effect on the body cells of neonates as judged by the significant increase in MN frequency in umbilical cord blood lymphocytes, without regard to therapeutic doses.

Acknowledgments

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References


Bonassi, S., Fenech, M., Lando, C., Lin, Y.P., Ceppi, M., Chang, W.P., Holland, N., Kirsch-Volders, M., Zeiger, E., Ban, S.,


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