The study of possible cytogenetic activity of spironolactone
Research Article
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Abbreviations: chromosomal aberrations, (CA); cyclophosphamide, (CP); micronucleus, (MN); polychromatic erythrocytes, (PCEs); polycystic ovary syndrome, (PCOS); spironolactone, (SPL)

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Summary
Due to its anti-androgen effect, spironolactone (SPL) is used to treat polycystic ovary syndrome (PCOS) and hirsutism. This drug is widely used in medicine, but practically nothing is known about its mutagenic activity, although SPL was found to be carcinogenic in rats. The aim of this study was the evaluation of mutagenic activity of SPL by means of chromosomal aberrations test in lymphocytes obtained from healthy women and PCOS patients, and micronucleus (MN) assay in mouse bone marrow cells. The results of experiments with lymphocytes of healthy women and PCOS patients showed that it was inactive even at highest doses used. The drug did not induce also MN in bone marrow cells of mice at high doses. Our results could suggest that carcinogenic activity of SPL may not be connected with genotoxicity.

I. Introduction
Spironolactone (SPL) is a synthetic 17-lactone steroid which is a renal competitive aldosterone antagonist in a class of pharmaceuticals called potassium-sparing diuretics. Due to its anti-androgen effect, it can also be used to treat polycystic ovary syndrome (PCOS) and hirsutism (Armanini et al, 2007; Koulouri and Conway, 2008). SPL inhibits the effect of aldosterone by competing for intracellular aldosterone receptor in the distal tubule cells. This increases the secretion of water and sodium, while decreasing the excretion of potassium. SPL acts both as a diuretic and as an antihypertensive drug by this mechanism (IARC, 2001).

Although this drug is widely used in medicine, practically nothing is known about its mutagenic activity. Search in scientific databases such as PubMed-Medline, ScienceDirect and Scopus revealed the absence of relevant data. Only the data concerning the absence of its activity in the Ames (Salmonella/microsomes) assay on 5 strains with and without exogenous metabolic activation was found in the website of one of the producers of SPL as pharmacological preparation, Pfizer, Australia (http://www.pbs.gov.au/pf/pfaldat11106.pdf). No data were found in IARC monograph, although this substance was evaluated for its carcinogenicity by the experts (IARC, 2001). It is written in the monograph that “No data were available to the Working Group” and “No data on the genotoxicity of SPL were available”.

This is surprising because there is some evidence that SPL possess carcinogenic activity in rats (thyroid follicular-cell adenomas in males and females and Leydig-cell tumors in males) (IARC, 2001).

The aim of this study was the evaluation of mutagenic activity of SPL by means of chromosomal aberrations (CA) test in human lymphocytes and micronucleus (MN) assay in mouse bone marrow cells. Since this drug is used very frequently for treatment of hirsutism (Armanini et al, 2007; Koulouri and Conway, 2008), lymphocytes of both women with PCOS and healthy women were used. Recently we showed significantly increased level of CA in lymphocytes of women with PCOS (Nersesyan et al, 2006).

II. Materials and methods
A. Subjects
The study comprised 6 females of Armenian nationality newly diagnosed with polycystic ovary syndrome (PCOS) at Institute of Obstetrics and Gynecology, Ministry of Health, Yerevan, Armenia. All these women had expressed hirsutism.

Six healthy female non-smoking volunteers with similar age and physical parameters (25-33 years old, mean age was 27.5±1.7 years) were included in investigations as controls. All biochemical analyses were carried out at Research Center of Maternal and Child Health Protection, in Laboratory of Biochemistry. Hirsutism score was evaluated by a dermatologist using Ferriman-Gallway score.

B. Chromosomal analysis
The CA assay was carried out using conventional techniques (Moorhead et al., 1960) with some modifications used in our laboratory (Batikian et al., 1981; Nersesyan et al., 2006). All reagents and chemicals used in this study, except specially noted, were produced in Russia. Heparinated blood (0.5 ml) was added to 4.5 ml medium, containing 78% RPMI 1640, 20% inactivated foetal bovine serum, antibiotics (penicillin and streptomycin) and stimulated with 2% of phytohaemagglutinin (Difco, USA), and incubated for 72 h at 37 °C. SPL (Sigma, St. Louis, USA) was dissolved in pure warm ethanol (30 mg in 1 ml) (O’Brien et al., 1985). It was added to cell cultures at 46 h of cultivation at concentrations of 300, 150 and 75 µg/ml. Based on the solubility of SPL and the toxicity of ethanol in human lymphocytes (maximal concentration of it in cell media must not be more than 1%, Anupama et al., 2008), the abovementioned concentrations of SPL were studied. As a positive control anatase (TiO2, Merck, Germany) was used at a dose of 40 mg/kg (dissolved in pure ethanol) which was administered either orally or i.p., based on the route of administration of SPL in various experiments. Bone marrow was flushed by means of newborn calf serum (0.2 ml, Sigma, USA) onto slides and smears were prepared. The slides were fixed with cold methanol for 20 min 24 h after the slides preparation. Slides were stained with azure-cosin. After being stained, the slides were coded so that the reader was unaware of the identity of slides being scored. Each slide was assessed for MN in 2,000 polychromatophilic erythrocytes (PCEs). In addition, the per cent content of PCEs was calculated among 1,000 erythrocytes.

D. Statistics
Analysis of human cells data was carried by application of chi-square test calculated by means of web calculator with Yate's correction: http://people.ku.edu/~preacher/chisq/chisq.htm. Non-parametric Mann-Whitney U-test was used to compare the data obtained in animal experiments (GraphPad Prism, version 3.02).

III. Results and discussion
The results of CA test in lymphocytes of healthy women and patients with PCOS treated with SPL are presented in Tables 1 and 2, respectively. Absolutely no changes were noted in lymphocytes of healthy women. In contrast, 1.3-fold increase was observed in lymphocytes of patients when the concentration of SPL was 300 and 150 µg/ml, although the differences with negative and solvent controls were not significant. The types of CA in treated lymphocytes also were not changed compared with the negative control. Mostly chromosomal breaks were registered in lymphocytes of PCOS patients which is in concordance with our previous work where 15 patients were investigated (Nersesyan et al., 2006). The percent of aberrant cells in lymphocytes induced by antitumor drug PTR increased circa 20- and 38-fold compared with negative controls in PCOS patients and healthy women, respectively.

Martirosyan found in 1996 that heterochromatin content in chromosome #1 of women with expressed hirsutism (also due to PCOS) is decreased significantly by 22%. At the same time she found that close female relatives of these patients also have decreased level of heterochromatin (17%). It is noteworthy, that Druzhinin showed in 1990 that heterochromatin content in chromosomes #1 and #9 leads to increased sensitivity to environmental mutagens. Earlier Martirosyan found in 1996 that chromosomes of PCOS patients are more sensitive to some hormonal agents used in therapy of PCOS. In our experiments we did not observe any signs of increased sensitivity of chromosomes obtained from PCOS patients. Furthermore, the effect of PTR was more expressed in healthy women (37.6-fold increase compared with negative control in healthy women vs. 20-fold in patients).

These observations can be interpreted as an complete inactivity of SPL in lymphocytes of healthy women and PCOS patients.

The results of MN experiments on mice are presented in Tables 3 and 4. It can be seen that SPL did not induce significant changes in MN level compared with the negative (solvent) controls administrated by both routes. Only the number of PCEs was significantly decreased in the group of mice treated i. p. with SPL at dose of 500 mg/kg (by 12.4%). It means that mentioned dose is slightly toxic for bone marrow. CP used permanently as positive control in our laboratory, increased the number of MN in PCEs circa 10-fold compared with solvent control. Ethanol (0.5 ml of 40%) was not MN-inducing in the experimental conditions. CP was non-significantly more active when administered i. p. which was also shown in other studies (Nersesyan, 1987).

Table 1. Chromosomal aberrations level in lymphocytes of patients with PCOS treated with spironolactone.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number</th>
<th>Number Aberrant</th>
<th>Number of</th>
<th>Total number of aberrations</th>
</tr>
</thead>
</table>

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Table 2. Chromosomal aberrations level in lymphocytes of healthy women treated with spironolactone.

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Number of studied cells</th>
<th>Number of aberrant cells</th>
<th>Aberrant cells (%)</th>
<th>Number of aberrations per 100 cells</th>
<th>Gaps</th>
<th>Chromatid breaks</th>
<th>Chromosomal breaks</th>
<th>Exchanges</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPL (300)</td>
<td>395</td>
<td>15*</td>
<td>3.80*</td>
<td>4.5*</td>
<td>5*</td>
<td>7*</td>
<td>10*</td>
<td>1</td>
</tr>
<tr>
<td>SPL (150)</td>
<td>359</td>
<td>12*</td>
<td>3.84*</td>
<td>4.7*</td>
<td>6*</td>
<td>5*</td>
<td>11*</td>
<td>0</td>
</tr>
<tr>
<td>SPL (75)</td>
<td>383</td>
<td>13*</td>
<td>2.87*</td>
<td>3.4*</td>
<td>4*</td>
<td>5*</td>
<td>9*</td>
<td>1</td>
</tr>
<tr>
<td>Ethanol (solvent control)</td>
<td>363</td>
<td>10*</td>
<td>2.85*</td>
<td>3.3*</td>
<td>2</td>
<td>12*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PTR (0.25, positive control)</td>
<td>435</td>
<td>201**</td>
<td>46.2**</td>
<td>51.7**</td>
<td>32*</td>
<td>148**</td>
<td>54**</td>
<td>23**</td>
</tr>
<tr>
<td>Saline (negative control)</td>
<td>620</td>
<td>14*</td>
<td>2.3*</td>
<td>2.6*</td>
<td>2</td>
<td>6*</td>
<td>10*</td>
<td>0</td>
</tr>
</tbody>
</table>

* p<0.05; ** p<0.001; each parameter was compared with corresponding one of healthy women; † p<0.001 compared with negative and solvent controls; chi square test with Yate’s correction

Table 3. Micronucleus level in polychromatic erythrocytes of Swiss male mice treated with spironolactone orally.

<table>
<thead>
<tr>
<th>Group of mice</th>
<th>Dose (mg/kg)</th>
<th>PCEs with MN (%; mean ± S.D.)</th>
<th>Total number per 1000 PCEs (mean ± S.D.)</th>
<th>Number of MN among erythrocytes (mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPL</td>
<td>500</td>
<td>3.2±0.6</td>
<td>3.2±0.6</td>
<td>52.2±4.2</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>2.7±1.0</td>
<td>2.7±1.0</td>
<td>52.8±5.1</td>
</tr>
<tr>
<td>Positive control (CP)</td>
<td>40</td>
<td>20.0±3.8*</td>
<td>23.6±4.5*</td>
<td>51.6±6.2</td>
</tr>
<tr>
<td>Solvent control (40% ethanol)</td>
<td>20</td>
<td>2.6±0.9</td>
<td>2.6±0.9</td>
<td>55.2±6.2</td>
</tr>
</tbody>
</table>

* p<0.001 compared solvent control; Mann-Whitney U-test with Gaussian approximation

Table 4. Micronucleus level in polychromatic erythrocytes of Swiss male mice treated with spironolactone intraperitoneally.

<table>
<thead>
<tr>
<th>Group of mice</th>
<th>Dose (mg/kg)</th>
<th>PCEs with MN (%; mean ± S.E.)</th>
<th>Total number of MN per 1000 PCEs (mean ± S.E.)</th>
<th>Number of MN among erythrocytes (mean ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPL</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Positive control (CP)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solvent control (40% ethanol)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

* p<0.001 compared solvent control; Mann-Whitney U-test with Gaussian approximation
Since SPL did not induce significant increase of MN at doses equivalent to ½ and ¾ of LD₅₀, it can be concluded it has no cytogenetic activity in mice.

To our knowledge, this is the first study on cytogenetic activity of SPL in human and animal cells. Since we did not obtain any evidence of activity in human cells in vitro and mouse bone marrow erythrocytes in vivo, and the producers of SPL reported about the absence of its activity in the Ames assay, it may be proposed that SPL is genetically inactive. Carcinogenic activity of this compound in chronic experiments in rats is possibly connected with its influence on metabolism and antiandrogenic activity (IARC, 2001). And, hence, carcinogenic activity of SPL may not be connected with its genotoxicity.

References