

ANALYSIS OF SISTER-CHROMATID EXCHANGES IN CULTURED HUMAN LYMPHOCYTES TREATED WITH CYMIAZOLE HYDROCHLORIDE

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Cymiazole hydrochloride is the active component of the acaricides Apitol[®] and Apichem[®], which are used as varroacides in beekeeping. Considering that residues of cymiazole hydrochloride were detected in all bee products being used for human nutrition and as alternative medications in human and veterinary medicine, the aim of this study was to evaluate the ability of this substance to produce genotoxic effects in human peripheral blood lymphocytes. The genotoxic effects were studied by sister chromatid exchange (SCE) test in vitro.

The results obtained for all doses applied to the cultures (0.01; 0.10 and 1 mg/ml) showed very significant increases in frequencies ($p < 0.001$) of SCEs in comparison to the negative control, i.e. cymiazole hydrochloride exhibited genotoxic properties.

Key words: cymiazole hydrochloride, acaricides, varroacides, pesticides, genotoxicity, SCE-test, chromosomal aberrations, beekeeping

INTRODUCTION

Cymiazole hydrochloride is the active ingredient of Apitol[®] and Apichem[®], which are synthetic, systematically acting acaricides. The treatment of honey bee colonies with acaricides influences the quality of bee products. Cymiazole hydrochloride especially endangers the quality of honey, as it is water-soluble and hence, easily diluted in honey. Use of this substance during the nectar flow always results in considerable amounts of residues. Residues of cymiazole hydrochloride can be detected not only in honey and honey bees, but in beeswax and other bee products too (Eyrich and Ritter, 1986; Eischen *et al.*, 1988; Patetta and Manino, 1988; Eyrich and Ritter, 1990; Cabras *et al.*, 1993; Cabras *et al.*, 1994; Bogdanov *et al.*, 1988; Volante *et al.*, 2001; Korta *et al.*, 2001; Korta *et al.*, 2002). With respect of the presence of this substance in bee products used in human and veterinary medicine as alternative drugs and in human nutrition, the aim of this study was to evaluate the eventual genotoxic properties of cymiazole hydrochloride. In some previous investigations of the genotoxic effects of cymiazole hydrochloride in cultured human lymphocytes (Pejović *et al.*, 1999, 2000, Stanimirović *et al.*, 2001), all applied doses of this drug induced a significant frequency of structural chromosomal changes of the lesion and break types. Stanimirović *et al.* (2003) found that all

experimental concentrations of cymiazole hydrochloride increased the mitotic and proliferation indices. However, all possible genotoxic effects have not been thoroughly investigated. The sister chromatid exchange (SCE) test is considered to be more sensitive than analysis of chromosome aberrations. Although the molecular mechanisms of SCE induction remain unclear, it seems that SCEs reflect DNA breakage and reunion at homologous loci of sister chromatids (Iannuzzi *et al.*, 1991), so that analysis of the frequency of SCE, might be a useful parameter to indicate possible genotoxic properties of small amounts of cymiazole hydrochloride.

MATERIAL AND METHODS

Test substance and controls. Cymiazole hydrochloride (Apitol® JKL: 03 6, 6/005-011/005; Evrotom, Ruma, Yugoslavia) was used as the test substance. The positive control was cyclophosphamide (Sygma Chemical Co., St. Louis, MO) at the final concentration of 40 µg/ml. The negative control was 0.9 % NaCl.

Lymphocyte culture. Human peripheral blood lymphocyte cultures were prepared according to a slight modification of the protocol described by Evans and O'Riordan (1975). Heparinised whole blood samples (0.8 ml) obtained from three healthy men under 35 years of age were added to vials with 9.2 ml of pre-warmed Parker 199 medium (Torlak, Belgrade, Yugoslavia) containing 30% of inactivated calf serum (Serva, Heidelberg, Germany) and 0.04 mg/ml of phytohaemagglutinin (Murex Diagnostics Ltd., Dartford, England). At the beginning of incubation 5-bromo-2'-deoxyuridine (BrdUrd, Sigma Chemical Co., St. Louis, MO) was added to each culture to obtain a final concentration of 25 µM. Cultures were incubated in the dark for 72 h at 37°C.

Treatment. Exactly 47 h and 30 min after the beginning of incubation cymiazole hydrochloride (Apitol®) was added to cultivation vials in such amounts to obtain final experimental concentrations of: 0.01, 0.1, and 1 mg/ml. Positive and negative control substances were added to separate cultivation vials (40 µg/ml cyclophosphamide and 0.9% NaCl).

Sister-Chromatid Exchange (SCE) test. In order to obtain visible SCEs, 5-bromo-2'-deoxyuridine (BrdUrd, Sigma Chemical Co., St. Louis, MO, final concentration 25 µM) was added to each culture one hour after the beginning of incubation. Two hours before harvesting, colchicine (Sigma Chemical Co., St. Louis, MO) was added to the cultures to achieve a final concentration of 0.5 µg/ml. After hypotonic treatment (0.075 M KCl) followed by three repetitive cycles of fixation in methanol/ acetic acid solution (3:1, v/v), centrifugation and resuspension, the cell suspension was dropped on chilled, grease-free microscopic slides, air-dried and aged for at least four days. Differential staining for the inspection of the SCE rate was performed according to the fluorescence-plus-Giemsa (FPG) procedure (Perry and Wolff, 1974). A total of 60 well-spread mitoses per donor were scored and the values obtained were calculated as SCEs per cell.

Statistical analysis. Statistical analysis of experimental values in the SCE test was performed using Student's t-test.

RESULTS

The result of the SCE test *in vitro* are shown in Table 1 and Figure 1.

Table 1. Influence of cymiazole hydrochloride on SCE frequency in cultures of human peripheral blood lymphocytes

| Cymiazole hydrochloride concentrations | SCE range | Mean value SCE | Standard deviation SD | Standard error SE | Percent of mean value of SCE in respect to control Xk |
|--|-----------|----------------|-----------------------|-------------------|---|
| Negative control | 3 – 9 | 5.93 | 1.901 | 0.134 | 100.0 |
| 0.01 mg/ml | 3 – 10 | 8.19 | 1.812 | 0.128 | 138.2 |
| 0.10 mg/ml | 5 – 12 | 8.78 | 1.802 | 0.127 | 148.1 |
| 1.00 mg/ml | 7 – 16 | 13.46 | 1.883 | 0.133 | 227.1 |
| Positive control | 9 – 29 | 23.69 | 4.460 | 0.315 | 399.6 |

The average number of SCEs per cell was 5.93 ± 0.13 in the negative control.

A greatly elevated SCE frequency per-cell ($p < 0.001$) was observed after treatment with the positive control, and the average value was 23.69 ± 0.31 .

After treatment with cymiazole hydrochloride the rate of SCEs per-cell was increased significantly ($p < 0.001$), to average values of 13.46 ± 1.88 for 0.01 mg/ml; 8.78 ± 1.80 for 0.10 mg/ml, and 8.19 ± 1.81 for 1 mg/ml,.

These data demonstrate significantly elevated values of SCE frequencies in each treated culture and indicate the genotoxic properties of cymiazole hydrochloride.

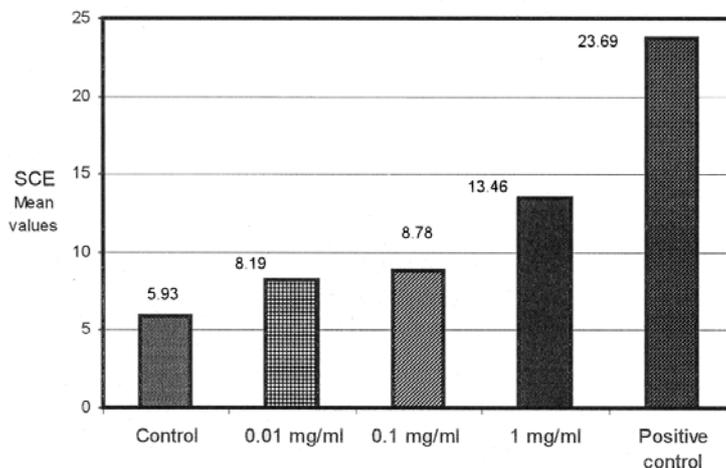


Figure 1. Influence of cymiazole hydrochloride on SCE frequency in cultures of human peripheral blood lymphocytes

DISCUSSION

In general, the use of varroacides in bee colonies leaves residues in various bee products. Hence, all around the world, maximum allowed residue levels – (MRL) for cymiazole hydrochloride in honey are set for the purpose of consumer safety. They range from 0.01ppm in Italy and Germany to 1 ppm in the whole EU. The US does not have an MRL for cymiazole hydrochloride in honey. No MRL has been established for beeswax (Wallner, 1999).

Cymiazole hydrochloride was found to be moderately toxic to honey bees when ingested at a rate of 3500ppm (Patetta and Manino, 1988). Cymiazole hydrochloride fed to bees reduced the development of the hypopharyngeal glands and increased the amount and acidity of rectal contents (Omar and Shoriete, 1992).

Cabras *et al.* (1994) measured residues of cymiazole hydrochloride in honey bees and in honey. Cymiazole hydrochloride residues in unsealed honey decreased from an average 2.45 ppm (after one-day treatment) to 0.14 ppm (112 days after treatment), but in honey bees residues were 84.12 ppm after one-day treatment but decreased to 0.07 ppm after 15 days. From these results, the authors concluded that honey bees can rapidly degrade cymiazole hydrochloride, but that levels higher than the permitted 0.01 ppm (in Italy) can easily occur in honey from treated colonies.

Kezic *et al.* (1992) investigated the influence of cymiazole hydrochloride on honey bee enzymes and affirmed that this drug, in the dose recommended by the producer, increase benzo-(a)-pyrene monooxidase activity by 300%.

The results of this study suggest caution in using cymiazole hydrochloride by strictly respecting withdrawal time, having in mind the data on cymiazole hydrochloride residues in the range of 0.01 to 1.1 mg/kg in honey, 0.2 to 9.3 mg/kg in honeycomb and 0.37 to 1.25 mg/kg in beeswax following drip-on treatment at the recommended dose. Cymiazole hydrochloride concentrations in the range of 0.01 to 0.34 mg/kg in honey, 0.33 to 2.4 mg/kg in honeycomb and 0.3 to 1.02 mg/kg in beeswax were found after administration by the feeding method (*Committee for Veterinary Medicinal Products*).

Strict respect for the withdrawal time while using cymiazole hydrochloride is of great importance because, levels higher than the permitted one can easily occur in honey from treated colonies even though honey bees can rapidly degrade cymiazole hydrochloride (Cabras *et al.* 1994). Disrespect for the recommended doses of cymiazole hydrochloride and application rules can lead to disturbances in functioning of the honey bee colony by reducing development of the hypopharyngeal glands and increasing the amount and acidity of rectal contents (Omar and Shoriete, 1992) as well as increasing benzo-(a)-pyrene monooxidase activity (Kezic *et al.*, 1992). Also, ignoring the directions for use could cause disarrangements in spermatogenesis and oogenesis in honey bees, as well as in consumers (Stanimirović *et al.*, 2001).

The fact that concentrations of 0.01, 0.1 and 1 mg/ml of cymiazole hydrochloride significantly increased mitotic and proliferative activities of cultured human lymphocytes (Table 1, Figure 1) suggests caution in consuming bee prod-

ucts (especially honey) from honeybee colonies treated with Apitol® or Apichem®, particularly in those predisposed to malignant diseases.

It is well known that many pesticides – insecticides contain active substances which could express genotoxic properties. There are many different tests for the detection of genotoxic effects (Zimonjić *et al.*, 1990). The sensitivity of tests applied in cytogenetic studies could be different in the various systems that have been used. Detection of frequencies of the SCEs in human blood lymphocyte cultures is considered to be one of the more sensitive methods to detect genotoxic activities. However, the use of several *in vitro* and *in vivo* test systems has been recommended. There have been some indications that cymiazole hydrochloride could express genotoxic properties. Thus, in earlier investigations (Pejović *et al.*, 1999, 2000; Stanimirović *et al.*, 2001) all tested doses of this drug induced very significant increases in frequencies of structural chromosomal changes of the lesion and break types in cultures of human blood lymphocytes. Mitotic index values and proliferation activities showed very significant increases ($p < 0.001$), in comparison to the negative control (Stanimirović *et al.*, 2003). Elevated mitotic index values accompanied by a great number of polyploid cells reveal a possible cancerogenic effect. It could be useful to apply some more *in vivo* tests, regardless of the described tests which have already demonstrated some genotoxic properties of cymiazole hydrochloride.

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**ANALIZA RAZMENE SESTRINSKIH HROMATIDA U KULTURAMA LIMFOCITA
ČOVEKA TRETIRANIH CIMIAZOL HIDROHLORIDOM**

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SADRŽAJ

Cimiazol hidrohlorid je aktivna komponenta Apitol[®]-a i Apichem[®]-a koji se koriste kao varoacidi u gajenju pčela. Kako su rezidue cimiazol hidrohlorida utvrđene u svim pčelinjim proizvodima - kako onima koji se koriste u ljudskoj ishrani, tako i onima koji se koriste kao alternativni medikamenti u ljudskoj i veterinarskoj medicini, cilj ovog proučavanja je bio da se procene mogućnosti ove substance da izazove genotoksična dejstva na limfocite periferne krvi čoveka. Efekti genotoksičnosti ispitivani su u *in vitro* SCE testu. Dobijeni rezultati, za sve doze koje su primenjene u kulturama (0.01; 0.10 and 1 mg/ml), pokazali su statistički vrlo značajno povećanje ($p < 0.001$) učestalosti SCEa u poređenju sa negativnom kontrolom; tj. cimiazol hidrohlorid je, pri korišćenim uslovima eksperimenta, iskazao genotoksična svojstva u kulturi humanih limfocita.