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# EVALUATION OF GENOTOXIC EFFECTS OF FUMAGILLIN BY SISTER CHROMATIDE EXCHANGE AND CHROMOSOMAL ABERRATION TESTS IN HUMAN CELL CULTURES

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*Fumagillin is a naturally secreted antibiotic of the fungus Aspergillus fumigatus. In veterinary medicine fumagillin is used against nosemosis in bees and microsporidiosis in fish. Since fumagillin is stable in honey in a honey bee hive there is a possible genotoxic risk to beekeepers and consumers of honey contaminated with this substance. The genotoxic effect of fumagillin was evaluated in sister-chromatid exchange (SCE) and chromosome aberration tests in cultured human peripheral blood lymphocytes at three concentrations (1.02, 3.07 and 9.20 μg/mL), with 1:1 water-sugar syrup as the negative control and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) as the positive control. The mitotic and proliferative indices were calculated to detect the cytostatic effect of fumagillin.*

*The results revealed that all tested concentrations of fumagillin (1.02, 3.07 and 9.20 μg/mL) significantly ($p<0.001$) increased the SCE frequency per cell and decreased the proliferative activity of human cultured lymphocytes which was manifested in the decrease in mitotic and proliferative indices. Significant increase ($p<0.001$) in aneuploidy and polyploidy was induced by medium and maximum concentrations of fumagillin. Besides, maximum concentrations of fumagillin significantly ($p<0.001$) increased the frequency of monitored structural aberrations of gap, break and insertion type. The results of this study demonstrate the genotoxic potential of fumagillin in vitro.*

*Key words: Antiproliferative effect, Chromosome aberration (CA), Fumagillin, Genotoxicity, Sister chromatid exchange (SCE)*

## INTRODUCTION

Fumagillin (IUPAC Name: 10-[[5-methoxy-4-[2-methyl-3-(3-methylbut-2-enyl)oxiran-2-yl]-2-oxaspiro[2.5]oct-6-yl]oxy]-10-oxo-deca-2,4,6,8-tetraenoic acid: Chemical Formula: $C_{26}H_{34}O_7$) is a natural antibiotic produced by some strains of the fungi *Aspergillus fumigatus*. This fungal metabolite is especially active against microsporidia and various amoeba species (McCowen *et al*., 1951; Killough *et al*., 1952; Katznelson and Jamieson, 1952; Bailey, 1953; Shadduck,

1980; Didier, 2005; Didier *et al.*, 2005). In the early 1950s it was first reported to be active against microsporidian pathogen *Nosema apis* (Microsporidia: Nosematidae) in *Apis mellifera* (Katznelson and Jamieson, 1952; Bailey, 1953), when used dissolved in sugar syrup. This approach has also been adapted for other insect species (Whittington *et al.*, 2003). For the treatment of *Nosema* infections in honey bees fumagillin is the only chemical registered.

It was proved that fumagillin is very stable in honey at elevated temperatures (stable for at least 35 days at 80°C) (Assil and Sporns, 1991) and that it is quite stable in honey bee hives (Furgala, 1962). Agner *et al*. (2003) suggest that fumagillin should be stored below -60°C since significant degradation took place even in samples stored in freezer conditions at -20°C. Light also induced a degradation process in fumagillin, thus it is proposed to be stored and transported in brown glassware. Kochansky and Nasr (2004) emphasized that long exposures of fumagillin to sunlight should be avoided, while brief exposure causes no obvious loss of activity. In the same work, samples of fumagillin in syrup, irradiated for 0, 0.5, 5, 30, or 360 minutes were all effective in protecting caged bees from nosemosis suggesting its stability after short exposures to sunlight (max. up to 360 minutes).

The use of fumagillin is permitted in the EU and the USA (EMEA, 2000; EMEA, 2003; FDA, 2005), but the maximum residue level (MRL) is not ascertained neither in the EU, nor in the USA.

There is lack of data regarding fumagillin intake levels in humans as a result of the consumption of fumagillin contaminated honey. Mladjan and Jovic (2000) and Kulic (2006) detected fumagillin residue levels that ranged from 8.5 to 12.3 mg/kg in honey harvested from bee colonies irregularly treated with fumagillin during the intensive honey flow season.

Fumagillin is effective in suppressing nosema in overwinter honey bee colonies and package bees (populations of 0.9 - 2.3 kg of honey bees with a queen), as it attacks the actively multiplying disease-producing parasites in the gut of the bee. Since fumagillin is not effective against dormant *N. apis* spores, treatment with this drug will not completely eliminate the disease from the colony. The infection will continue after all the medicated syrup has been consumed (Mladjan *et al.*, 2000a; Mladjan *et al*., 2000b). Adverse effects on bees after treatment with fumagillin were described (Liu, 1990a; Liu, 1990b). Fumagillin significantly increases the mortality of bees as well as the number of yeasts in comparison with the control (Rada *et al.*, 1997).

Fumagillin was also proposed for the suppression of other microsporidian parasites in invertebrates (Zbinden *et al.*, 2005). Fumagillin, administered in the diet, was used to treat microsporidiosis in fish (El-Matbouli and Hoffmann, 1991; Karagouni *et al.*, 2005; Hedrick *et al.*, 1988; Kent and Dawe, 1994 Le Gouvello *et al*., 1999; Morris *et al.*, 2003). However, in more rigorous tests required for the U.S. Food and Drug Administration approval, fumagillin was not found to be effective against microsporidiosis in fish (Gilbert and Granath Jr, 2003).

In humans, fumagillin was first used more than 40 years ago for the treatment of intestinal amebiosis (McCowen *et al.*, 1951; Killough *et al.*, 1952), it was effective when used topically in the treatment of microsporidial

keratoconjunctivitis (Roserger *et al.*, 1993; Wilkins *et al.*, 1994) and when used orally in the treatment of chronic *Enterocytozoon bieneusi* infection in patients with AIDS and other types of immunodeficiency (Molina *et al.*, 2000; Molina *et al.*, 2002). Moreover, it was revealed that fumagillin has the ability to inhibit endothelial proliferation and block angiogenesis *in vitro* and *in vivo*, although the administration of fumagillin is limited because of its toxic side-effects (Ingber *et al.*, 1990), thus analogues with fewer side-effects have been synthesized (Fardis *et al.*, 2003).

According to the European Agency for the Evaluation of Medicinal Products (EMEA) (EMEA, 2000), indications for the use of fumagillin are only nosemosis of honey bees and proliferative kidney disease of trouts. However, in the EMEA report (2003) fumagillin is registered as "orphan medical product" for the treatment of diarrhoea associated with intestinal microsporidial infection.

In order to determine an effective commercial treatment for fish infected with myxosporeans, Athanassopoulou *et al.* (2004) tested 6 different doses of fumagillin and observed lesions in fish treated with fumagillin. However, pathology due to treatment with fumagillin was observed only at doses >6 mg/kg bw for 6 weeks in the interstitial renal tissue, where slight inflammation arose. The highest dose tested (25 mg/kg b.w.) also produced necrosis of the kidney interstitial tissue, degeneration of the epithelial cells of the tubules and a reduction in melanomacrophage centre numbers. According to Ingber *et al.* (1990) prolonged administration of fumagillin was limited because it caused severe weight loss in mice. *A. fumigatus* produced a number of biologically active substances which slowed ciliary movements and damaged the epithelium, which may have influenced the colonization of the airways. Thus, fumagillin like other *Aspergillus* toxins exerted cilioinhibitory effects (Amitani, 1995). Moreover, fumagillin is toxic when administered systemically to mammals (Didier, 2005).

Genotoxic effects of fumagillin and its compound dicyclohexilamine were demonstrated *in vitro*. Thus, in the study of Stanimirovic *et al.* (1999) all tested concentrations of fumagillin (0.8 mg/mL, 0.4 mg/mL and 0.08 mg/mL) induced numerical (aneuploidy) and structural chromosomal changes (gaps and breaks) in cultured human lymphocytes. Moreover, aneuploid cells among cultured human lymphocytes treated with fumagillin were detected in the study of Stevanovic *et al.* (2000), but only at the highest tested concentration (0.8 mg/mL). In addition, the antiproliferative effect of fumagillin *in vitro* at all applied concentrations (0.8 mg/mL, 0.4 mg/mL and 0.08 mg/mL) was reported in both studies (Stanimirovic *et al.*, 1999; Stevanovic *et al.*, 2000).

As regarding dicyclohexilamine, which is similar to fumagillin, in a cytogenetic study conducted by Stoltz *et al.* (1970), lymphocytes from human blood samples incubated with dicyclohexilamine sulfate expressed a concentration-depended increase in aberration rate from approximately 6% in the controls to nearly 16% in the experimental groups.

Kulic (2006) investigated the genotoxic potential of dicyclohexilamine *in vitro* in concentrations of 0.08, 0.4 and 0.8 mg/mL and *in vivo* in doses of 5, 10 and 20 mg/kg b.w. The highest doses in both experiments increased the frequency of numerical and structural chromosome aberrations. Furthermore, all tested doses

in both experiments exerted antiproliferative effects which were manifested in a decrease in mitotic and proliferation indices.

However, *in vitro* investigations of dicyclohexylamine performed by Purchase *et al.* (1978) and Mortelmans *et al.* (1986) indicated no genotoxic potential in the *Salmonella*/microsome assay. Moreover, investigations on the DNA-damaging effect of dicyclohexylamine in the UMU test and in the DNA synthesis inhibition test in HeLa S3 cells gave no indication that dicyclohexylamine had a damaging effect on DNA (Heil *et al.*, 1996).

In investigations of secondary metabolites of *Aspergillus fumigatus,* the fungus producing fumagillin, gliotoxin induced DNA adduct formation (Golden *et al.*, 1998) and proved to be genotoxic in various *in vitro* test systems (Nieminen *et al.*, 2002) whilst verruculogen was genotoxic in the *Salmonella*/mammalian microsome assay (Sabater-Vilar *et al.*, 2003).

Since CAs, SCEs and micronuclei have been considered essential markers of genotoxicity in *in vitro* studies (Fucic *et al.*, 1998; Maluf and Erdtmann, 2000), the objective of this study was to evaluate possible genotoxic effects of fumagillin *in vitro* in the chromosome aberration (CA) and sister-chromatid exchange (SCE) assays. The endpoints used for genotoxic analysis were the frequencies of CAs and SCEs, as well as the mitotic (MI) and proliferation (PI) indices in human peripheral blood lymphocyte cultures.

## MATERIAL AND METHODS

The investigated substance - Fumagilin-B (MEDIVET Pharmaceuticals LTD, Canada; Purity: ≥90% by HPLC, CAS 23110-15-8) was tested in three concentrations i.e. 1.02, 3.07 and 9.20 μg/mL. The experimental concentrations were obtained by dissolving fumagilin in 1:1 water-sugar syrup, as in the formulation usually used for application in beekeeping. Water-sugar syrup 1:1 was used as the negative control, whereas $10^{-6}$ M N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, Sigma) was the positive control.

*In vitro* investigations of effects of poorly soluble agents (as is fumagillin) require that the maximum concentration be the one being the result of maximum solubility of the agent at a certain cultivation temperature (Zimonjic *et al.*, 1990).

The maximum concentration of fumagillin in this study of 9.20 μg/mL corresponds to the normal therapeutic dose in beekeeping, in accordance with the recommendation that for optimal nosema control each one-chamber honey bee colony should receive 2 L of medicated syrup containing 50 mg of fumagillin during the treatment period of 15 days in the spring. Thus, the maximum concentration of 9.20 μg/mL was obtained by dividing 50 mg by 5460 mL (5460 mL is the average blood volume in adult weighing 78 kg, i.e. 7 % of body weight). Between maximum concentration and further, lower concentrations, semi-log difference is recommended (Zimonjic *et al.*, 1990).

Human peripheral blood lymphocyte cultures for CA and SCE analysis were set up according to a slightly modificated protocol of Evans and O'Riordan (1975). Heparinized whole blood samples (0.8 mL) obtained from five healthy men <35 years of age were added to vials with 9.20 mL of Parker 199 medium containing

30% of inactivated calf serum (Serva, Heidelberg, Germany) and 0.04 mg/mL of phytohemagglutinin (Murex Diagnostics, Ltd., Dartford, UK). Two cultures per donor were incubated in complete darkness at 37°C 72 h. Exactly 47 h and 30 min after the beginning of incubation, fumagillin was added to the cultivation vials in amounts to obtain final experimental concentrations of 1.02, 3.07 and 9.20 μg/mL.

For SCE analysis, 5-bromo-2'-deoxyuridine (BrdUrd, Sigma Chemical Co., St. Louis, MO, USA, final concentration 25 μM) was added 24 h after culture initiation.

Two hours prior to harvesting, colcemid (Ciba, Basel, Switzerland) was added to the cultures up to 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 onto chilled, grease-free microscopic slides, air-dried, aged, and stained.

For detection and identification of CA, slides were stained with 5% Giemsa (Sigma) 10 min. For each donor and for each experimental concentration, as well as for the controls, 60 well-spread metaphases with 46 chromosomes (total number of chromosomes in human cells) were scored on blindly coded slides for CAs. Identification of the aberration type was performed according to the International System for Human Cytogenetic Nomenclature (I.P.S.C, 1985).

G-banding of chromosomes was done by the tripsin methods of Seabright (1971) and Ronne (1991) on blindly selected slides for the purpose of CA scoring.

For the inspection of the SCE frequency, differential staining was performed according to the Fluorescence-plus-Giemsa (FPG) procedure (Perry and Wolff, 1974). For each experimental concentration, as well as for the controls, 60 well-spread mitoses with 46 chromosomes (per donor) were scored on blindly coded slides, and the values obtained were calculated as SCEs per cell (Lioi *et al.*, 1998; Lioi *et al.*, 2004).

The mitotic index was evaluated counting at least 1000 cells per treatment: the number of dividing cells (prophases and metaphases) was divided by the total number of cells (Lioi *et al.*, 1998; Lioi *et al.*, 2004). Cell cycle kinetics was estimated from the proliferation index scored on at least 200 metaphases per donor. The proliferation index was calculated according to the formula: PI = (M1 + 2M2 + 3M3) /100, where M1, M2 and M3 represent the proportion of the first, second and third metaphases, respectively (Lamberti *et al.*, 1983; Stanimirovic *et al.*, 2005).

Experimental data were analyzed using one-way analysis of variance (ANOVA) to determine whether any treatment significantly differed from the controls.

## RESULTS

The effects of fumagillin in the cultures of human peripheral blood lymphocytes were evaluated by determining mitotic and proliferative indices, sister chromatide exchange frequency and structural and numerical chromosomal aberrations frequencies. The results are presented in Tables 1 and 2 and Figure 1.

Table 1. The effect of fumagillin on SCE per-cell frequency and mitotic and proliferative indices in human peripheral blood lymphocyte cultures[b]

| Treatment | MI | | PI | | SCE | | |
|---|---|---|---|---|---|---|---|
| | range | mean±SD | range | mean±SD | range | mean±SD | Xk |
| Negative control 1:1 water-sugar syrup | 5.15-5.53 | 5.36±0.14 | 1.71-1.74 | 1.72±0.01 | 3-9 | 5.78±0.35 | 100.00 |
| Positive control MNNG $10^{-6}$ M | 3.15-3.79 | 3.57[a]±0.15 | 1.51-1.65 | 1.53[a]±0.01 | 7-30 | 13.77[a]±0.39 | 238.24 |
| Fumagillin, 1.02 µg/mL | 3.19-3.55 | 3.37[a]±0.10 | 1.64-1.69 | 1.66[a]±0.02 | 4-11 | 7.64[a]±0.10 | 132.18 |
| Fumagillin, 3.07 µg/mL | 3.11-3.20 | 3.17[a]±0.04 | 1.63-1.67 | 1.64[a]±0.01 | 5-12 | 8.85[a]±0.21 | 153.11 |
| Fumagillin, 9.2 µg/mL | 2.16-2.34 | 2.27[a]±0.07 | 1.61-1.65 | 1.63[a]±0.01 | 7-18 | 13.65[a]±0.54 | 236.16 |

MI, mitotic index; PI, proliferative index; SCE, sister chromatid exchange; [a]$p<0.001$ (ANOVA, Student's *t* test); SD, standard deviation; Xk, percentage of mean value of SCE of the negative control. [b]A total of 600 cells were recorded for the SCE assay and 1000 cells were recorded for the MI and PI

Table 2. Different types of chromosomal aberrations in cultures of human peripheral blood lymphocytes in controls and groups treated with increasing doses of fumagillin[a]

| | Negative control WSS | | Positive control MNNG | | Fumagillin 1.02 μg/mL | | Fumagillin 3.07 μg/mL | | Fumagillin 9.20 μg/mL | |
|---|---|---|---|---|---|---|---|---|---|---|
| Chromosome aberration | CA/cell Mean±SD | % | CA/cell Mean±SD | % | CA/cell Mean±SD | % | CA/cell Mean±SD | % | CA/cell Mean±SD | % |
| Polyploidy | 0.12±0.04 | 0.02 | 16.00±1.06[b] | 2.67 | 0.37±0.16 | 0.06 | 0.87±0.34[b] | 0.14 | 4.75±0.71[b] | 0.79 |
| Aneuploidy | 4.50±0.69 | 0.75 | 105.62±1.07[b] | 17.60 | 4.75±0.71 | 0.79 | 6.12±0.83[b] | 1.02 | 29.87±1.46[b] | 4.98 |
| Gaps | 2.00±0.69 | 0.33 | 16.75±1.03[b] | 2.79 | 2.25±0.71 | 0.37 | 2.50±0.35 | 0.42 | 5.25±0.71[b] | 0.88 |
| Breaks | 0.75±0.21 | 0.12 | 6.50±0.59[b] | 1.08 | 0.87±0.34 | 0.14 | 1.12±0.64 | 0.19 | 4.12±0.64[b] | 0.69 |
| Insertions | 0.00±0.00 | 0.00 | 6.37±0.60[b] | 1.06 | 0.00±0.00 | 0.00 | 0.37±0.16[b] | 0.06 | 2.87±0.41[b] | 0.48 |
| Total cytogenetic changes[c] | 2.75±0.21 | 0.45 | 29.62±0.58[b] | 4.93 | 3.12±0.69[b] | 0.51 | 3.99±0.33[b] | 0.67 | 12.24±0.46[b] | 2.05 |

[a]A total of 600 cells were recorded for analysis of chromosome aberrations (CA); [b]$p<0.001$ (ANOVA, Student's *t* test); [c]Total cytogenetic changes excluding polyploidies and aneuploidies; WWS: 1:1 water-sugar syrup; MNNG: $10^{-6}$ M N-methyl-N'-nitro-N-nitrosoguanidine; SD, standard deviation

Figure 1. Metaphase spread with differentialy stained sister-chromatids (FPG technique) from lymphocytes treated with the higest concentration of fumagillin (9.2 μg/mL)

All experimental concentrations of fumagillin (1.02, 3.07 and 9.20 μg/mL) induced a significant decrease ($p<0.001$) in MI (MI=3.37±0.10, MI=3.17±0.04, MI=2.27±0.07, respectively) in comparison with the negative control (MI=5.36±0.14). The positive control (MNNG) also significantly ($p<0.001$) decreased the MI of human lymphocytes (MI=3.57±0.15). The same experimental concentrations of fumagillin induced a significant decrease ($p<0.001$) in PI (PI=1.66±0.02, PI=1.64±0.01, PI=1.63±0.01, respectively) compared with the negative control (PI=1.72±0.01). In addition to this, MNNG significantly decreased the PI (PI=1.53±0.01) compared with the negative control (PI=1.72±0.01) (Table 1). These results show that all the tested concentrations of fumagillin exerted an antiproliferative effect.

With the aim of ascertaining genotoxic effects of fumagillin, SCE frequency in lymphocyte cultures treated with fumagillin was monitored (Figure 1). The induction of SCE in all treated groups (1.02, 3.07 and 9.20 μg/mL) was significantly ($p<0.001$) increased (SCE=7.64±0.10, 8.85±0.2, 13.65±0.54, respectively) in comparision with the negative control (SCE=5.88±0.35). The highest increase in SCE frequency was observed in the positive control (SCE=13.77±0.39) compared with the negative control (SCE=5.88±0.35).

Considering the mean value of SCE frequency in the negative control 100.00 %, the following increases in SCE frequency were obtained: 32.18 % for minimum, 53.11 % for medium and 136.16% for maximum tested concentrations of fumagillin. However, having compared the mean values of SCE frequency between the negative and positive control groups, an increase at a level of 138.24% in the positive control was revealed.

Cytogenetic analysis showed an increase in frequency of numerical and structural chromosome aberrations in the cultures treated with three experimental concentrations of fumaglillin (1.02, 3.07 and 9.20 μg/mL) in comparison with the negative control.

An increased frequency of aneuploidy and polyploidy was detected in human lymphocyte cultures treated in medium and maximum concentrations of fumagillin. Thus, the concentration of 3.07 μg/mL induced aneuploidies at a level of 1.02 % and polyploidies at 0.14 %; while 9.20 μg/mL led to 4.98 % aneuploidies and 0.79 % polyploidies, compared with 0.75 % of aneuploidies and 0.02 % of polyploidies in the negative control. Moreover, the positive control increased the frequency of aneuploidies and polyploidies to the level of 17.60 % and 2.67 %, respectively, compared to the negative control (0.75 % and 0.02 %, respectively). The statistical analysis indicated that the differences in the frequency of chromosomal damages of aneuploidy and polyploidy induced by medium and maximum concentrations of fumagillin (3.07 and 9.20 μg/mL) and by the positive control were significant ($p<0.001$) in comparison with the negative control group (Table 2).

Fumagillin expressed the ability to induce the following structural chromosome aberrations: gaps, breaks and insertions (Table 2) but only the maximum concentration of fumagillin (9.20 μg/mL) significantly ($p<0.001$) increased the frequency of all monitored structural aberrations, at the level of 0.88 % for gaps, 0.69 % for breaks and 0.48 % for insertions, compared to the negative control group (0.33 % for gaps, 0.12 % for breaks and 0.00 % for insertions). The medium and minimal experimental concentrations (3.07 and 1.02 μg/mL) did not induce a significant increase in the evaluated structural changes. However, the positive control significantly ($p<0.001$) increased the frequencies of gaps, breaks and insertions (2.79 %, 1.08 % and 1.06 %, respectively) in comparison to the negative control (0.33 %, 0.12 % and 0.00 %, respectively). To summarize, these results point to significant genotoxic effects of fumagillin on human lymphocyte cultures.

## DISCUSSION

In the EC fumagillin is registered as "orphan medical product" for the treatment of diarrhoea associated with intestinal microsporidial infection (EMEA, 2000; EMEA, 2003) and it is the only chemical registered for the treatment of *Nosema* infections in honey bees (EMEA, 2000). The residues of fumagillin from honey, due to its high stability in honey (Furgala, 1962) and other food based on honey bee products, can easily reach consumers (including children,

adolescents, convalescents, chronic patients and the elderly) (Stanimirovic *et al.*, 1999; Stevanovic *et al.*, 2000). Furthermore, topical fumagillin is suggested for the treatment of ocular infection caused by microsporidia (Roserger *et al*., 1993; Wilkins *et al.*, 1994). Fumagillin is currently being tested for the treatment of diarrhoea associated with intestinal microsporidial (*Enterocytozoon bieneusi*) infection in severely immunocompromised patients (EMEA, 2003; Molina *et al.*, 2000; Molina *et al.*, 2002). The abovementioned indicated the necessity of evaluation of genotoxic properties of fumagillin, even more so as available data on positive genotoxic effects of fumagillin obtained *in vitro* (Stanimirovic *et al.*, 1999; Stevanovic *et al.*, 2000) are insufficient, and there are no reports based on *in vivo* genotoxicological testing of fumagillin (Bünger *et al.*, 2004). However, there are references about genotoxic effects of gliotoxin and verruculogen, secondary metabolites of *Aspergillus fumigatus* (Golden *et al.*, 1998; Nieminen, 2002; Sabater-Vilar, 2003). In addition, there are data regarding genotoxic effects of dicyclohexilamine, a compound similar to fumagillin, obtained either in *in vitro* mammalian tests (Stoltz *et al.*, 1970; Kulić, 2006) or in bacterial mutagenicity tests (Purchase *et al.*, 1978; Mortelmans *et al.*, 1986; Heil, 1996). The abovementioned require further investigations in both *in vitro* as well as in *in vivo* mammalian tests systems, even more so as fumagillin, contrary to dicyclohexilamine, has primarily two epoxide structures capable of alkylating proteins involved in the packaging of DNA (Birch and Hussain, 1969) thereby establishing conditions for damaging DNA.

In our *in vitro* study all experimental concentrations of fumagillin (1.02, 3.07 and 9.20 μg/mL) induced a significant decrease ($p<0.001$) in MI and PI in comparison with the negative control which is in accordance with the findings of many authors considering the antiproliferative effects (antiangiogenic effects) of fumagillin (Molina *et al.*, 2002; Wang *et al.*, 2000; Mazzanti *et al.*, 2004; Stanimirovic *et al.*, 1999; Stevanovic *et al.*, 2000; Datta *et al.*, 2004). It can be assumed that the decrease in MI and PI in our study is the consequence of fumagillin binding on methionine aminopeptidase-2 (MetAP-2), the molecular target of fumagillin and its analogue TNP-470 (Griffith *et al.*, 1998; Sin *et al.*, 1997; Liu *et al.*, 1998). Fumagillin binds MetAP-2 on His-231, inactivating the enzyme. MetAP-2 removes the N-terminal methionine from most proteins involved in cell cycle regulation as a part of the translocation process, so its inhibition results in cell cycle arrest and apoptosis (Fardis *et al.*, 2003). This mechanism probably underlies the antiproliferative effect of fumagillin which was manifested in the decrease in MI and PI in our study. Moreover, the results of Mazzanti *et al.* (2004) supported the notion that genes DOC1, KLF4, and TC1 are specific for the endothelial cells response to endostatin and fumagillin. Nevertheless, these authors suggested further studies be necessary to clarify these early mechanisms and to better understand the function of these genes (Mazzanti *et al.*, 2004).

The results of investigations of genotoxic effects of fumagillin in this study, using the CA and SCE tests, are in accordance with previous findings of Stanimirovic *et al.* (1999) and Stevanovic *et al.* (2000). However, fumagillin concentrations investigated by these authors (0.8 mg/mL, 0.4 mg/mL and 0.08 mg/mL) were much higher than those investigated in this study (1.02, 3.07

and 9.20 μg/mL). Thus, our results point to the necessity for precautions in the use of fumagillin, since the selected doses are in the therapeutic dose range. A significant increase ($p<0.001$) in the frequency of polyploidies induced by medium and maximum concentrations of fumagillin is probably the consequence of the effects of fumagillin on the cytoskeleton through modifications of the phosphorylation state and subcellular localization of cofilin and hsp27, two proteins involved in actin cytoskeleton. This concept is in accordance with Keezer *et al*. (2003) who used a proteomic approach to identify common targets of a panel of angiogenesis inhibitors (endostatin, thrombospondin-1, fumagillin, and its synthetic derivative, TNP-470). In addition, a significant increase ($p<0.001$) in the frequency of aneuploidies in our study was induced by medium and maximum concentrations of fumagillin. The aneuploidies observed were mostly trisomies of chromosome 3 from group A in the human karyogram. There are a number of possible mechanisms by which chemicals might induce aneuploidy, including effects on microtubules, damage to essential elements for chromosome function (i.e. centromeres, origins of replication, and telomeres), reduction in chromosome condensation or pairing, induction of chromosome interchanges, unresolved recombination structures, increased chromosome stickiness, damage to centrioles, impairment of chromosome alignment, ionic alterations during mitosis, damage to the nuclear membrane, and a physical disruption of chromosome segregation (Oshimura and Barrett, 1986). Nevertheless, in our opinion, further studies are necessary to clarify the mechanisms underlying aneuploidogenic properties of fumagillin. Additionally, in cell culture model systems in which cells are exposed to different carcinogens, chromosomal aneuploidy is the earliest detectable genomic aberration ((Oshimura and Barrett, 1986; Barrett *et al*., 1985). Thus, our finding of aneuploidies (of trisomy type) suggests for the necessity for caution when using fumagillin.

If the total number of gaps, breaks and insertions in the negative control was considered to be 100%, the following increases in frequency of named CAs are obtained: 13.45 % for minimum, 45.09 % for medium and 345.09 % for the maximum tested concentration of fumagillin. These results point to the great clastogenic effect of the evaluated agent. The increase in the SCE frequency observed at all applied concentrations (1.02, 3.07 and 9.20 μg/mL) is probably a consequence of limited capacity of repair enzyme systems of non-homologous recombinations to achieve corrections of such a great number of breaks induced by the investigated agent, which is in accordance with the statement of Miller *et al.* (2001) that nucleotide excision repair enzymes are responsible for removing "bulky" DNA damage that distorts the DNA helix. Besides, Mohrenweiser and Jones (1998) emphasized that recombinational repair involves enzymes that correct double strand breaks and interstrand cross-links and operates primarily via non-homologous recombination in mammals.

However, the SCE frequency could be influenced not only by the genotoxic agent tested, but also by other factors, such as endogenous agents that are capable of interfering with DNA replication (Tucker *et al.*, 1993; Tucker and Preston, 1996; Djelic and Djelic, 2002), oxidative stress (Karbownik *et al.*, 2001; Hu *et al.*, 2004) and interindividual variability (Albertini *et al.*, 2000; Norppa, 2003;

Ingelman-Sundberg, 2001), although the factors mentioned were not the subject of this investigation. Nevertheless, our finding of a significant ($p<0.001$) increase in SCE in all treated groups in comparision with the negative control, enables us to assume that the effects of all the abovementioned factors (endogenous agents, oxidative stress and interindividual variability) were minimised.

In spite of the lack of confident evidence regarding fumagillin residue levels in food, with exception of those of Mladjan and Jovic (2000) and Kulic (2006), our results considering the decrease in MI and PI, and the increases in SCE and CA frequencies induced by fumagillin lead to the conclusion that fumagillin residues in honey, even at doses as tested in our study, if they are consumed by the elderly, chronic patients and convalescents, could have an additional harmful influence on their health condition. In addition, there could be an effect on the absorption of the drugs already consumed because ageing and various xenobiotics reduce the capacity for drug-metabolising enzymes (Ingelman-Sundberg, 2001; Bajic *et al.*, 2004; Stanimirovic *et al.*, 2005). Beekeepers being occupationally exposed to fumagillin may also be at genotoxic risk. Finally, for the purpose of consumer safety, it is necessary to educate beekeepers with regard to the use of fumagillin. A similar caution should be taken with for patients treated with fumagillin against microsporidia. Thus, additional research on the adverse effects of fumagillin should be undertaken in order to provide all the necessary data to define an MRL for this substance, while our results should not be disregarded in any case.

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## EVALUACIJA GENOTOKSIČNOG EFEKTA FUMAGILINA U KULTURI HUMANIH ĆELIJA PRAĆENJEM RAZMENE SESTRINSKIH HROMATIDA I HROMOZOMSKIH ABERACIJA

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SADRŽAJ

Fumagilin je prirodni antibiotik *Aspergillus fumigatus-a*. U veterinarskoj medicini fumagilin se koristi protiv nozemoza kod pčela i mikrosporidioza kod riba. Kako je fumagilin stabilan u medu i pčelinjim košnicama, postoji genotoksični rizik za pčelare i konzumere meda kontaminiranog ovom supstancom. Genotoksični efekat fumagilina je procenjivan testovima razmene sestrinskih hromatida i hromozomskih aberacija u kulturi limfocita periferne krvi ljudi u tri koncentracije (1,02, 3,07 and 9,20 μg/mL), gde je kao negativna kontrola korišćen vodeni rastvor šećernog sirupa, a kao pozitivna kontrola N-metil-N'-nitro-N-nitrozoguanidin (MNNG). Citostatični efekat fumagilina je utvrđivan mitotskim i proliferativnim indeksima.

Rezultati ukazuju da sve testirane koncentracije fumagilina (1,02, 3,07 and 9,20 μg/mL) signifikantno ($p<0,001$) povećavaju frekvenciju SCE po ćeliji i smanjuju proliferativnu aktivnost kultivisanih ćelija koja je manifestovana smanjenjem mitotskih i proliferativnih indeksa. Signifikantno povećanje ($p<0,001$) aneuploidija i poliploidija je indukovano srednjom i maksimalnom koncentracijom fumagilina. Osim toga, maksimalna koncentracija fumagilina signifikantno ($p<0,001$) povećava frekvenciju praćenih strukturnih aberacija tipa gapova, prekida i insercija. Rezultati ove studije pokazuju genotoksični potencijal fumagilina *in vitro*.