Fumagillin (dicyclohexylamine) is a natural antibiotic, secreted by Aspergillus fumigatus. It is used in veterinary medicine against microsporidiosis in bees and fish, as well as in human medicine for the treatment of intestinal amebiasis, microsporidial keratoconjunctivitis and intestinal microsporidiosis due to Enterocytozoon bieneusi in patients with AIDS and other types of immunodeficiency. In this study, the genotoxicity of fumagillin was evaluated in mouse bone marrow cells using the mitotic index (MI) and micronucleus (MN) assay. Fumagillin was administered to BALB/c mice by gavage in doses of 25, 50, 75 mg/kg b.w., repeated for 7 days at 24h intervals, with water-sugar syrup as the negative control and cyclophosphamide as the positive control (40 mg/kg b.w.). All experimental doses of fumagillin induced a significant decrease (p<0.001) in MI (3.47 ± 0.04%, 3.17 ± 0.01% and 2.27 ± 0.02%, respectively) in comparison with the negative control (6.00 ± 0.01%) and with the positive control (14.78 ± 0.09). Fumagillin significantly (p<0.001) increased the frequency of MN (4.98 ± 0.35, 8.45 ± 0.57 and 12.02 ± 0.37, respectively) over the negative control (1.04 ± 0.28). These results suggest that fumagillin (dicyclohexylamine) has an antiproliferative and genotoxic potential in mammal in vivo test.

Key words: Fumagillin (dicyclohexylamine), Honey bee, Nosema-disease, Genotoxicity, Mitotic index (MI), Micronuclei (MN)

INTRODUCTION

Fumagillin, an antibiotic derived from the fungus Aspergillus fumigatus, has particular activity against microsporidia and various amoeba species (McCowen et al., 1951; Killough et al., 1952; Katznelson and Jamieson, 1952; Bailey, 1953; Shadduck, 1980; Didier, 2005). It was first reported to be active against Nosema apis in honey bees (Apis mellifera L.) in the early 1950's (Katznelson and Jamieson, 1952; Bailey, 1953).

Fumagillin is quite stable in honey bee hives (Furgala, 1962). Furthermore, investigations of Assil and Sporns (1991) showed that fumagillin was very stable in honey at elevated temperatures (stable for at least 35 days at 80°C). The use of
fumagillin is permitted in the EU and the USA (EMEA, 2000; FDA, 2005), but the maximum residue level (MRL) is not affirmed neither in the EU, nor in the USA. Fumagillin has also been proposed for suppression of other microsporidian parasites in both invertebrates and vertebrates. Thus, it is revealed that fumagillin is effective against *Octosporea bayeri* in the freshwater crustacean *Daphnia magna* (Zbinden et al., 2005) and *Myxobolus cerebralis* (Myxosporea), which is the causative agent of whirling disease in many fish species (El-Matbouli and Hoffmann, 1991; Karagouni et al., 2005) and *Tetracapsuloides bryosalmonae* (Malacosporea), which is the causative agent of proliferative kidney disease of chinook salmon *Oncorhynchus tshawytscha* (Hedrick et al., 1988; Kent and Dawe, 1994) as well as rainbow trout *Oncorhynchus mykiss* (Le Gouvello et al., 1999; Morris et al., 2003). However, in more rigorous tests required for the U.S. Food and Drug Administration approval, it was found to be ineffective (Gilbert and Granath, 2003).

In humans, fumagillin is used for the treatment of intestinal amebiasis (McCowen et al., 1951; Killough et al., 1952). It is effective when used topically in the treatment of microsporidial keratoconjunctivitis (Roseger et al., 1993; Wilkins et al., 1994) and orally in the treatment of intestinal microsporidiosis due to *Enterocytozoon bieneusi* in patients with AIDS and other types of immunodeficiency (Molina et al., 2000, 2002; Conteas et al., 2000).

It was revealed that fumagillin has the ability to inhibit endothelial proliferation and block angiogenesis *in vitro* and *in vivo*. However, administration of fumagillin is limited because of its toxic side-effects (Ingber et al., 1990, Didier, 2005). Analogues with fewer side-effects have been synthesized, and one of these, TNP-470 (AGM-1470), was shown to inhibit tumor growth in a number of xenograft models (Yamaoka et al., 1993; Yanase et al., 1993; Kurebayashi et al., 1994; Tanaka et al., 1995). However, TNP-470 was found to possess immunosuppressive activity (Turk et al., 1998) and produces excessive cytotoxicity (Ingber et al., 1990; Ruddy et al., 2000). Moreover, in studies of Yoshida et al. (1998) TNP-470 inhibited the production of leukemia inhibitory factor, which may be related to the amelioration of cancer cachexia. TNP470 induced apoptosis and enhanced the expression of P-galactosidase, a biomarker of senescence (Yoshida et al., 1998).

Recent investigations of Huang et al. (2004) have indicated that the antitumor effects of TNP-470 cannot be attributed to the prevention of neoangiogenesis, but instead to its direct action on tumor cells.

Data referring to genotoxic effects of fumagillin were obtained by *in vitro* investigations and the results were either positive (Stoltz et al., 1970; Stanimirovic et al., 1999; Stevanovic et al., 2000) or negative (Purchase et al., 1978; Mortelmans et al., 1986; Heil et al., 1996). In addition, there are no references regarding genotoxic effects of fumagillin *in vivo* (Toxicological Evaluation, 2000).

All listed information point to a necessity of investigations of genotoxic effects of fumagillin *in vivo*. The objective of this study was to evaluate and characterize by the micronucleus assay the possible genotoxic effects of fumagillin *in vivo*, by using different doses which are in the therapeutic range in
beekeeping. The endpoints used for genotoxic analysis were micronuclei (MN) and mitotic index (MI) in mouse bone-marrow cells.

MATERIAL AND METHODS

The genotoxic effects of fumagillin (Fumagillin-ET, 3/0-05-009/001- JKL 022; Evrotom, Ruma, SCG) were investigated in bone marrow cells of BALB/c strain mice, using the micronucleus (MN) test system. The mitotic index (MI) was also determined to detect the cytotoxic effect of fumagillin.

Three experimental doses of fumagillin were tested: 25, 50 and 75 mg/kg b.w. The median experimental dose of 50 mg/kg corresponds to the therapeutic dose-range of fumagillin in beekeeping, since the recommended fumagillin dose for honey bees is 26 mg fumagillin/L (Webster, 1994).

Fumagillin does not dissolve readily in water. To prepare the medicated sugar syrup, it is recommended to mix fumagillin in small amounts of warm water (not above 32-34°C) and stir into a paste, then add the prepared water-sugar syrup gradually and shake the container occasionally. The antibiotic mixture should be admixed with water-sugar syrup shortly before use.

The experimental design for micronucleus (MN) assay included three groups: positive control, negative control, and the treated group. The treated groups were divided into three subgroups based on selected doses of fumagillin. All groups had eight animals per dose/group (both sexes). Our experimental design was carried out in two cycles. We used BALB/c mice of 6 weeks of age with an average weight of 19±2 g. Animals were kept under uniform conditions and were housed under 12/12-h photoperiod at constant temperature (21°C) with free access to standard laboratory chow and water.

Experimental doses were obtained by dissolving fumagillin in 1:1 water-sugar syrup orally administered to mice, as is the case with the formulation usually used in beekeeping.

The negative control group was treated per os with water-sugar syrup. A known mutagen, cyclophosphamide at a dose of 40 mg/kg b.w. was used as the positive control group due to its known clastogenic and mutagenic features (Anderson et al., 1995). Cyclophosphamide was intraperitoneally (i.p.) administered, and the injected volume was 0.01 mL/g b.w. All animals received daily i.p. treatments for a 7-day duration.

Schmid method (Schmid et al., 1975) was used for the analysis of micronuclei (MN) in polychromatic erythrocytes (PCE) of mouse bone marrow. At least four slides were made for each animal and allowed to dry overnight and later stained with May–Gruenwald–Giemsa (MOL d.d., Beograd, SCG) solutions according to the standard technique (Adler, 1984) for conventional assessment of MN frequencies. All slides were coded for microscopic analysis at 1000x magnification. Per animal, 1000 polychromatic erythrocytes (PCE) from each of four slides were scored for the presence of MN. The mitotic index (MI) was determined on 1000 or more cells in accordance with the guidelines of Zimonjic et al. (1990).
Statistical analyses were carried out with the software programme Statistica 6.0 using the Analysis of Variance (ANOVA) and the Student’s t-test and LSD-test.

RESULTS

The effects of fumagillin were evaluated in bone marrow cells of BALB/c strain mice by monitoring the mitotic index (MI) and micronuclei (MN).

The results show (Table 1) that all experimental doses of fumagillin (25, 50 and 75 mg/kg b.w.) induced a significant decrease \( (p<0.001) \) in mitotic index \( (3.47 \pm 0.04\%, 3.17 \pm 0.01\% \text{ and } 2.27 \pm 0.02\%, \text{ respectively}) \) in comparison with the negative control \( (6.00 \pm 0.01\%) \). Moreover, a significant decrease \( (p<0.001) \) in MI was also observed in all experimental doses in comparison with the positive control \( (14.78 \pm 0.09) \).

Table 1. Mitotic index in bone marrow cells treated with fumagillin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Doses</th>
<th>Mitotic index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Min - Max</td>
<td>Mean ± SE</td>
</tr>
<tr>
<td>Water-sugar syrup</td>
<td>1 water : 1 sugar</td>
<td>5.95 – 6.07</td>
</tr>
<tr>
<td>Fumagillin 25 mg/kg b.w.</td>
<td>3.33 – 3.66</td>
<td>3.47 ± 0.04***</td>
</tr>
<tr>
<td>Fumagillin 50 mg/kg b.w.</td>
<td>3.13 – 3.19</td>
<td>3.17 ± 0.01***</td>
</tr>
<tr>
<td>Fumagillin 75 mg/kg b.w.</td>
<td>2.19 – 2.34</td>
<td>2.27 ± 0.02***</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>40 mg/kg b.w.</td>
<td>14.28 – 15.06</td>
</tr>
</tbody>
</table>

SE, standard error; SD, standard deviation;
\***p<0.001, significantly different from the negative control (LSD-test)

Figure 1. Microphotograph shows one polychromatic erythrocyte with two micronuclei of BALB/c strain mice induced by fumagillin in a dose of 25 mg/kg b.w.
Administration of fumagillin showed an ability to induce micronuclei (MN) (Figure 1) in polychromatic erythrocytes (PCE) of bone marrow of BALB/c mice (Table 2). Statistical analysis demonstrated a significant increase \((p<0.001)\) in micronuclei induction \((4.98 \pm 0.35, 8.45 \pm 0.57 \text{ and } 12.02 \pm 0.37)\) at all three doses of fumagillin \((25, 50 \text{ and } 75 \text{ mg/kg b.w., respectively})\) over the negative control \((1.04 \pm 0.28)\). In addition, the results demonstrated a dose-response relationship between exposure to fumagillin and frequency of MN in PCE.

Table 2. Frequency of micronucleated erythrocytes in bone marrow cells of control groups and experimental groups of BALB/c strain mice treated with increasing doses of fumagillin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>Mean value No. of MN</th>
<th>SD</th>
<th>Xk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>water:sugar – 1:1</td>
<td>1.04</td>
<td>0.28</td>
<td>4.76</td>
</tr>
<tr>
<td>Positive control</td>
<td>40 mg/kg b.w.</td>
<td>21.86***</td>
<td>0.71</td>
<td>100</td>
</tr>
<tr>
<td>Fumagillin</td>
<td>25 mg/kg b.w.</td>
<td>4.98***</td>
<td>0.35</td>
<td>22.78</td>
</tr>
<tr>
<td>Fumagillin</td>
<td>50 mg/kg b.w.</td>
<td>8.45***</td>
<td>0.57</td>
<td>38.65</td>
</tr>
<tr>
<td>Fumagillin</td>
<td>75 mg/kg b.w.</td>
<td>12.02***</td>
<td>0.37</td>
<td>54.99</td>
</tr>
</tbody>
</table>

MN, micronuclei; ***\(p<0.001\) (ANOVA, Student’s \(t\) test); SD, standard deviation; Xk, percentage of mean value of MN of positive control group

DISCUSSION

Fumagillin is the only chemical registered for the treatment of Nosema infections in honey bees, whilst residues of fumagillin from honey, due to its high stability in honey (Assil and Sporns, 1991) 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 treatment of ocular infections caused by microsporidia (Roseger et al., 1993; Wilkins et al., 1994); whilst recent investigations support the use of oral fumagillin as an effective treatment for chronic Enterocytozoon bieneusi infection in immunocompromised patients (Molina et al., 2000, 2002; Conteas et al., 2000). All the above mentioned indicates the need to evaluate genotoxic properties of fumagillin. The available data referring to genotoxic effects of fumagillin (dicyclohexylamine) obtained by in vitro investigations are equivocal, i.e. either positive (Stoltz et al., 1970; Stanimirovic et al., 1999; Stevanovic et al., 2000) or negative (Purchase et al., 1978; Mortelmans et al., 1986; Heil et al., 1996) and there are no references regarding genotoxic effects of fumagillin in vivo (Toxicological Evaluation, 2000).

In our in vivo study the mitotic index (MI) and micronuclei (MN) were monitored in bone marrow cells of BALB/c strain mice treated with selected doses of fumagillin \((25, 50 \text{ and } 75 \text{ mg/kg b.w.})\). The results show that all experimental
doses of fumagillin induced a significant decrease ($p<0.001$) in MI (Table 1) in comparison with the negative control. Moreover, a significant decrease ($p<0.001$) in MI was also observed in all experimental doses in comparison with the positive control (Table 1). These results are in accordance with the findings of other authors considering the antiproliferative effects (antiangiogenic effects) of fumagillin. Ingber et al. (1990) were the first to report that the fungal metabolite fumagillin potently suppresses angiogenesis due to its strong inhibition of endothelial cell proliferation in vitro and tumor-induced neovascularization in vivo. Wang et al. (2000) revealed that fumagillin inhibits human umbilical vein endothelial cells (HUVEC) proliferation at low nM concentration, and has partial or no effect on non-endothelial cells at concentrations up to 1 µM. The molecular target of fumagillin and its analogue TNP-470 is methionine aminopeptidase-2 (MetAP-2) (Sin et al., 1997; Griffith et al., 1998; Liu et al., 1998). Fumagillin binds MetAP-2 on His-231, thus 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, hence its inhibition results in the cell cycle arrest and apoptosis (Fardis et al., 2003). These findings provided a starting point for the rational design of novel fumagillin analogues with fewer side-effects than fumagillin (Fardis et al., 2003).

Keezer et al. (2003) reported that fumagillin and its synthetic derivative, TNP-470, target the endothelial cell cytoskeleton through modification of the phosphorylation state and subcellular localization of coflin and hsp27, two proteins involved in actin cytoskeleton and focal adhesion of endothelial cells. Mazzanti et al. (2004) confirmed that the antiangiogenic agent fumagillin is specific for the inhibition of endothelial cell proliferation. Moreover, these authors investigated the early effects of the antiangiogenic agents, endostatin and fumagillin, on the gene expression profiles in HUVEC by microarray analysis in order to elucidate pathways common to the effects of these agents. Their results supported the notion that genes DOC1, KLF4, and TC1 are specific for the endothelial cells response to endostatin and fumagillin, therefore they suggested that these genes might be involved in the initial pathways of angiogenesis inhibition. Nevertheless, Mazzanti et al. (2004) mentioned that further studies should be necessary to clarify these early mechanisms and present a better understanding of the functions of these genes.

Some reports indicate that the synthetic analogue of fumagillin TNP-470 inhibits tumor growth in vivo, but without an unambiguous corresponding decrease in tumor vasculature (Katzenstein et al., 1999; Shusterman et al., 2001; Morales et al., 2002). In addition, TNP-470 initial clinical trials produced inconclusive results, without a clear evidence of either reduced tumor growth or vascularity (Logothetis et al., 2001). In particular, alterations in serum levels of surrogate markers for antiendothelial activity were not detected (Logothetis et al., 2001). Moreover, Huang et al. (2004) detected a unique pattern of remodelling of tumor vasculature after TNP-470 administration, characterized by rapid and striking proliferation of microvessels. In addition, TNP-470 rapidly induced tumor cell apoptosis. These findings suggest that TNP-470 does not have antitumor
effects due to antiangiogenic activity, but rather due to a direct action on tumor cells (Huang et al., 2004).

Recently, after observations of short-term toxicity of TNP-470 in the B16F10 murine melanoma cell line in vitro and investigations of the mechanism of action, Okroj et al. (2005) concluded that TNP-470 could induce intracellular generation of reactive oxygen species (ROS), which act toxically inside B16F10 cells. One may suggest that this novel activity of TNP-470 might be beneficial in some cases, but it could also be responsible for some undesirable side-effects.

Stimulation of angiogenesis may have a place in the treatment of cardiovascular disease. Reestablishing blood flow to ischemic tissues through angiogenesis may provide a biologic "bypass" for patients with ischemic heart disease or peripheral vascular disease (Gibaldi, 1998). Thus, the possibility of fumagillin and its residues to reach consumers and patients through honey (Stanimirovic et al., 1999; Stevanovic et al., 2000) would be a disturbing factor of the specified beneficial effects of angiogenesis due to antiangiogenic activities of fumagillin and its analogues. Taking this into account, as well as the fact that fumagillin is suggested for topical treatment of ocular infection caused by microsporidia (Roseger et al., 1993; Wilkins et al., 1994) and oral treatment of chronic Enteroctozoon bieneusi infection in patients with AIDS and other types of immunodeficiency (Molina et al., 2000, 2002; Conteas et al., 2000) and our results which show a decrease in the MI in the bone marrow cells of BALB/c mice raise a question: what would happen if the named consumers had ischemic heart disease or peripheral vascular disease?

Furthermore, in vitro investigations of dicyclohexylamine gave no indication of its genotoxic potential in the Salmonella/microsome assay (Purchase et al., 1978; Mortelmans et al., 1986). Moreover, investigations of 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), which is rather surprising considering that fumagillin has primarily two epoxide structures capable of alkylating proteins involved in the packaging of DNA (Birch and Hussain, 1969) thereby establishing conditions for DNA damage. However, in cytogenetic studies carried out by Stoltz et al. (1970) dicyclohexylamine sulfate showed a concentration-depended increase in the aberration rate from approx. 6% in the controls to approx. 16% in the experimental groups. Besides, there are no references regarding the ability of dicyclohexylamine to induce micronuclei.

In addition, the National Toxicology Program (National Toxicology Program 2006a,b) reports a positive genotoxic effect in Salmonella test for dicyclohexylamine nitrite (National Toxicology Program 2006a) and negative for dicyclohexylamine (National Toxicology Program 2006b). Besides, there are references about the exerted genotoxic effects of secondary metabolites (gliotoxin and verruculogen) of Aspergillus fumigatus, the fungus that produces fumagillin, as well (Golden et al., 1998; Nieminen et al., 2002; Sabater-Vilar et al., 2003).

Our in vivo investigations of genotoxic effects of fumagillin, using the micronucleus (MN) assay, showed that all tested doses significantly \( p<0.001 \)
increased the frequency of MN in PCE. The results clearly show a correlation between dose and effect, i.e. increased doses of fumagillin induced an increase in the frequency of MN (Table 2; Figure 1). This dose effect relationship points to the genotoxic properties of fumagillin. There is no possibility for direct comparison of these results since data regarding the ability of fumagillin to induce MN are lacking. Nevertheless, our results are in accordance with the published findings, considering the ability of other drugs and pesticides to increase the frequency of MN in PCE (Çelik et al. 2003; Bajić et al., 2004; Andrighetti-Fröhner et al., 2006; Itoh et al., 2006).

Finally, our results related to the decrease in MI, and the increases in MN frequency 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 drugs already used as ageing and various xenobiotics reduce the capacity for drug-metabolising enzymes (Ingelman-Sundberg, 2001; Stanimirovic et al., 2005).

In order to insure the safety of consumers, it is necessary to educate beekeepers regard the use of fumagillin, even more as fumagillin has antiproliferative properties as well as the ability to increase the frequency of MN in PCE. A similar caution should be taken with patients treated with fumagillin against microsporidia.

Moreover, additional studies of the adverse effects of fumagillin should be undertaken in order to provide all the necessary data to define an MRL for this substance; our results should not be disregarded in any case.

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Fumagilin (dicyclohexylamine) je prirodni antibiotik koga produkuje Aspergillus fumigatus. Upotrebljava se u veterinarskoj medicini protiv mikrosporidioza pčela i riba, ali i u humanoj medicini za tretman intestinalnih amebijaza, microsporidijalnih keratokonjunktivitisa i intestinalnih mikrosporidioza uzrokovanih sa Enteroxotozoon bieneusi kod pacijenata obolelih od AIDS-a i drugih tipova imunodeficiencije. U ovom radu procenjivan je genotoksični potencijal fumagilina u celijama kostne srži miševa praćenjem mitotskog indeksa i učestalosti pojave mikronukleusa u polihromatofilnim eritrocitima. Fumagilinom su u 7-dnevnom tretmanu tretirani BALB/c miševi intragastrično u dozama 25, 50, 75 mg/kg TM, u intervalima od 24h. Kao negativna kontrola korišćen je vodeno–šećerni sirup, dok je ciklofosfamid (40 mg/kg TM) korišćen kao pozitivna kontrola. Sve eksperimentalne doze fumagilina su izазвale signifikantno smanjenje ($p<0.001$) mitotskog indeksa ($3.47 \pm 0.04\%$, $3.17 \pm 0.01\%$ i $2.27 \pm 0.02\%$, respektivno) u poređenju sa negativnom kontrolom ($6.00 \pm 0.01\%$), kao i u odnosu na pozitivnu kontrolu ($14.78 \pm 0.09$). Fumagilin je signifikantno ($p<0.001$) povećao frekvenciju pojave mikronukleusa ($4.98 \pm 0.35$, $8.45 \pm 0.57$ i $12.02 \pm 0.37$), respektivno, u odnosu na negativnu kontrolu ($1.04 \pm 0.28$). Ovi rezultati ukazuju da fumagilin (dicyclohexylamine) ispoljava antiproliferativni (citotoksični) i genotoksični potencijal u sisarskom in vivo testu.