

FREQUENCY OF CHROMOSOMAL ABERRATIONS IN THE EVALUATION OF GENOTOXIC POTENTIAL OF DICYCLOHEXYLAMINE (FUMAGILLIN) IN VIVO

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*Dicyclohexylamine (fumagillin), as an antibiotic produced by fermentation of *Aspergillus fumigatus*, is used in human medicine for the treatment of intestinal microsporidiosis in patients with HIV infection, intestinal amebiasis and microsporidial keratoconjunctivitis. In veterinary medicine, fumagillin is effective in suppressing microsporidiosis of bees and fish. In this study, the genotoxicity of fumagillin was evaluated in mouse bone marrow cells using chromosome aberrations (CA) assay. Dicyclohexylamine was administered to mice by gavage in a dose of 25, 50, 75 mg/kg b.w., with water-sugar syrup as the negative control and cyclophosphamide as the positive control (40 mg/kg b.w.) Significantly increased frequency ($p < 0.01$ or $p < 0.001$) of numerical chromosomal aberrations (aneuploidies and polyploidies) and structural chromosomal aberrations of gaps, breaks and centric rings were observed only at the highest experimental dose of dicyclohexylamine, compared with the negative control. However, in point of induction of Rb translocations, both the median (50 mg/kg b.w.) and highest (75 mg/kg b.w.) experimental dose showed a significant ($p < 0.001$) increase (7.12 ± 0.26 and 9.00 ± 0.10 , respectively) in comparison with the negative control (0.00 ± 0.00). Chromosomes 4 and 19 participated in these Rb translocations. These results suggest that dicyclohexylamine (fumagillin) has genotoxic potential in mammal in vivo chromosomal aberration (CA) test system.*

Key words: dicyclohexylamine (fumagillin), honey bee, nosema-disease, genotoxicity, chromosome aberrations (CA), Robertsonian chromosome Rb (4.19).

INTRODUCTION

Dicyclohexylamine (fumagillin) is an antibiotic isolated from *Aspergillus fumigatus*. Currently, it is the only drug effective in suppressing cryptosporidiosis and microsporidiosis caused by *Enterocytozoon bieneusi*. These are common and frequently fatal infections in AIDS sufferers (Molina *et al.*, 2000; Conteas *et al.*,

2000). Fumagillin is also a strong antiparasitic and amoebicidal compound (McCowen *et al.*, 1951; Killough *et al.*, 1952) and has acquired importance in veterinary medicine against microsporidiosis of bees and fish (Katznelson and Jamieson, 1952; Bailey, 1953; El-Matbouli and Hoffmann, 1991; Morris *et al.*, 2003).

It is known that dicyclohexylamine 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 (EMA, 2000; FDA, 2005), but the maximum residue level (MRL) is not affirmed neither in the EU, nor in the USA.

Fumagillin, fed in syrup, has proven to be especially effective in suppressing nosema in overwintered honey bee colonies and newly established packages, 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,b). In addition, adverse effects on bees after treatment with fumagillin were described. Mitochondria from the corpora allata of *Nosema*-infected honeybees, after treatment with fumagillin, had a higher electron density in the mitochondrial matrix and smaller diameter than those from untreated honey bees (Liu, 1990a). After treatment with fumagillin, secretion granules in the hypopharyngeal glands of *Nosema*-infected honey bees were increased in size, slightly electron dense, and the granular content was homogenous. Some of these granules were also partially crystallized. These ultrastructural changes in the secretion granules of fumagillin treated bees are probably associated with a change in secretory activity of the glands (Liu, 1990b). Moreover, fumagillin significantly increases the mortality of bees and significantly increases the counts of yeasts in comparison with the control (Rada *et al.*, 1997).

Dicyclohexylamine has also been proposed for suppression of other microsporidian parasites in the invertebrates and vertebrates. Thus, Zbinden *et al.* (2005) presented a protocol for inhibiting vertical transmission of the microsporidian parasite *Octosporea bayeri* in the freshwater crustacean *Daphnia magna*. Fumagillin, administered in the diet, has been used to treat microsporidiosis in fish, because studies found it to be effective against some myxozoan parasites, including *Myxobolus cerebralis* (Myxosporea), the causative agent of whirling disease in many fish species (El-Matbouli and Hoffmann, 1991; Karagouni *et al.*, 2005) and *Tetracapsuloides bryosalmonae* (Malacosporea), the causative agent of proliferative kidney disease of chinook salmon *Oncorhynchus tshawytscha* (Hedrick *et al.*, 1988; Kent and Dawe, 1994) and rainbow trout *Oncorhynchus mykiss* (Le Gouvello *et al.*, 1999; Morris *et al.*, 2003). However, in the 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 was used more than 40 years ago for the treatment of intestinal amebiasis (McCowen *et al.*, 1951; Killough *et al.*, 1952). Fumagillin is effective when used topically in the treatment of microsporidial

keratoconjunctivitis, as well (Roseger *et al.*, 1993; Wilkins *et al.*, 1994). The results of Molina *et al.* (2000, 2002) supported the use of oral fumagillin as an effective treatment for chronic *Enterocytozoon bieneusi* infection in patients with AIDS and other types of immunodeficiency, but further work is necessary to fully establish proper therapeutic protocols and manage side effects of the treatments (Conteas *et al.*, 2000).

However, according to EMEA (2000), indications for use of fumagillin are only nosemosis of honey bees and proliferative kidney disease of trouts. For treatment of *Nosema* infections in honey bees fumagillin is the only chemical registered.

Data referring to genotoxic effects of fumagillin were obtained by *in vitro* investigations and the results were 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).

Bearing in mind all the aforementioned, investigations of the genotoxic effects of fumagillin *in vivo* are necessary, even more as these effects significantly depend on test models, on chosen doses of the tested substance, and its interaction with other endogenous and exogenous factors (Ames, 1989; Albertini *et al.*, 2000; Norppa, 2003; Stanimirovic *et al.*, 2005).

The objective of this study was to evaluate and characterize possible genotoxic effects of dicyclohexylamine (fumagillin) *in vivo* using different doses which are in the therapeutic range used in beekeeping. In the undertaken cytogenetic assay the frequency of chromosome aberrations (CA) was monitored. In addition, we identified chromosomes which take part in the formation of chromosomal rearrangements which occurred as a consequence of dicyclohexylamine activity.

MATERIAL AND METHODS

The genotoxic effects of dicyclohexylamine (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 chromosomal aberration (CA) assay.

Three experimental doses of dicyclohexylamine (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 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 container occasionally. The antibiotic mixture should be admixed with water-sugar syrup shortly before use.

The experimental design for chromosomal aberration (CA) test included three groups: positive control, negative control, and experimental group. The experimental group was divided into three subgroups based on selected doses of

fumagillin. All groups had six animals per dose/group (male sex). Our experimental design was conducted in two cycles. We used BALB/c mice 6 weeks of age with 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 dicyclohexylamine (fumagillin) in 1:1 water-sugar syrup, as in the formulation usually used in beekeeping, and orally administered in mice. Since tested doses of fumagillin were 25, 50 and 75 mg/kg b.w., each mice received 0.55, 1.1 and 1.65 mg/kg b.w. respectively in the 7-day treatment, that is 0.08, 0.157 and 0.236 mg/kg b.w. respectively per mice per day.

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 volume injected was 0.01 mL/g b.w. All animals received daily i.p. treatments for a 7-day period.

Cytogenetic analysis was performed by direct method of rinsing long bone marrow (femur and tibia) according to Hsu and Patton (1969) as modified by Zimonjic *et al.* (1990). Slides were made by the flame-dried technique and later stained with Giemsa (Sigma Chemical Co., St. Louis, MO). G-banding of chromosomes was done by the trypsin method of Seabright (1971) and Ronne (1991). Chromosomes and chromosomal bands were identified on the basis of criteria established by the Committee on Standardized Genetic Nomenclature for Mice (1979) and Cowell's photoatlas of mouse chromosomes (Cowell, 1984), in order to identify chromosomes which take part in the formation of Robertsonian (Rb) metacentric chromosomes.

We analyzed 600 well-spread metaphases for each treatment to detect the presence of chromosomal aberrations (CA). Statistical analyses was 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 dicyclohexylamine (fumagillin) were evaluated in bone marrow cells of BALB/c strain mice by monitoring the chromosome aberrations (CA). It was established by cytogenetic analysis that there was an increase in the frequency of numerical and structural chromosome aberrations in the mouse bone marrow cells after 7-day per os treatment with increasing doses of fumagillin (25, 50 and 75 mg/kg b.w.) in comparison with the negative control. Fumagillin showed the ability to induce numerical aberrations of aneuploidy and poliploidy type and the following types of structural chromosome aberrations: gaps, breaks, centric rings and Robertsonian (Rb) translocations (Table 1) (Figures 1, 2 and 3).

Significantly increased frequency ($p < 0.01$ or $p < 0.001$) of numerical chromosomal aberrations (aneuploidies and polyploidies) and structural chromosomal aberrations of gaps, breaks and centric rings were observed only at

Table 1. Types of different chromosomal aberrations in mouse marrow cells of control groups and cells treated with increasing doses of fumagillin

CA	No. of MA	Negative control		Fumagillin 25 mg/kg b.w.		fumagillin 50 mg/kg b.w.		fumagillin 75 mg/kg b.w.		Positive control	
		Mean±SD	%	Mean±SD	%	Mean±SD	%	Mean±SD	%	Mean±SD	%
A	600	5.00±0.75	0.83	5.25±0.70	0.87	6.37±0.91	1.06	31.75±1.28***	5.29	105.87±2.94***	17.64
P	600	0.00±0.00	0.00	0.00±0.00	0.00	0.00±0.00	0.00	5.37±0.74***	0.89	16.25±0.88***	2.70
G	600	2.00±0.92	0.33	2.37±0.51	0.39	2.62±0.74	0.44	5.75±0.88***	0.96	17.62±1.06***	2.94
B	600	0.00±0.00	0.00	0.00±0.00	0.00	1.25±0.71	0.21	4.37±0.74***	0.73	6.62±0.51***	1.10
CR	600	0.00±0.00	0.00	0.00±0.00	0.00	0.00±0.00	0.00	3.25±0.71**	0.54	6.37±1.19***	1.06
Rb	600	0.00±0.00	0.00	0.00±0.00	0.00	7.12±0.26***	1.19	9.00±0.10***	1.50	14.17±0.41***	2.36
Tcc	600	7.00±1.35	1.17	7.62±1.06	1.27	17.36±1.83***	2.89	59.49±1.92***	9.91	166.90±2.76***	27.82

CA, Chromosomal aberrations; A, aneuploidy; P, polyploidy; G, gaps; B, breaks; CR, Centric rings; Rb, Robertsonian translocations; Tcc, total cytogenetic changes

***Significantly different from negative control at $p < 0.001$

**Significantly different from negative control at $p < 0.01$



Figure 1. G-band of chromosomes of the mouse treated with the highest dose of fumagillin (75 mg/kg b.w.) – arrow indicates the Robertsonian chromosome Rb (4.19)

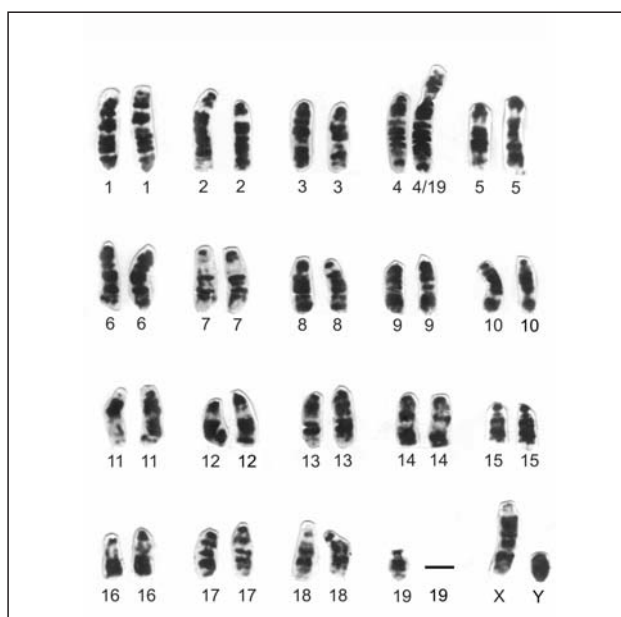


Figure 2. G-banded karyogram of the mouse heterozygous for the Robertsonian translocation Rb (4.19) (animal treated with the highest dose of fumagillin – 75 mg/kg b.w.)

the highest experimental dose of dicyclohexylamine (75 mg/kg b.w.) compared with the negative control (Table 1). However, in point of induction of Rb translocations, both the median (50 mg/kg b.w.) and the highest (75 mg/kg b.w.) experimental dose showed a significant ($p < 0.001$) increase (7.12 ± 0.26 and 9.00 ± 0.10 , respectively) in comparison with the negative control (Table 1). After the completion of G-band analyses and chromosome identification it was observed that chromosomes 4 and 19 had participated in the formation of Rb translocations (Figures 1, 2 and 3). The noticed structural Rb (4.19) aberration was in a heterozygous state and identified in 5 animals: two of 12 animals treated with the median dose (50 mg/kg b.w.) of fumagillin and 3 of 12 animals treated with the highest dose of fumagillin (75 mg/kg b.w.).

Regarding total cytogenetic changes (Table 1), a significant increase ($p < 0.001$) was observed in both, the median dose group (17.36 ± 1.83) and the highest dose group (59.49 ± 1.92) compared to the negative control group (7.00 ± 1.35).

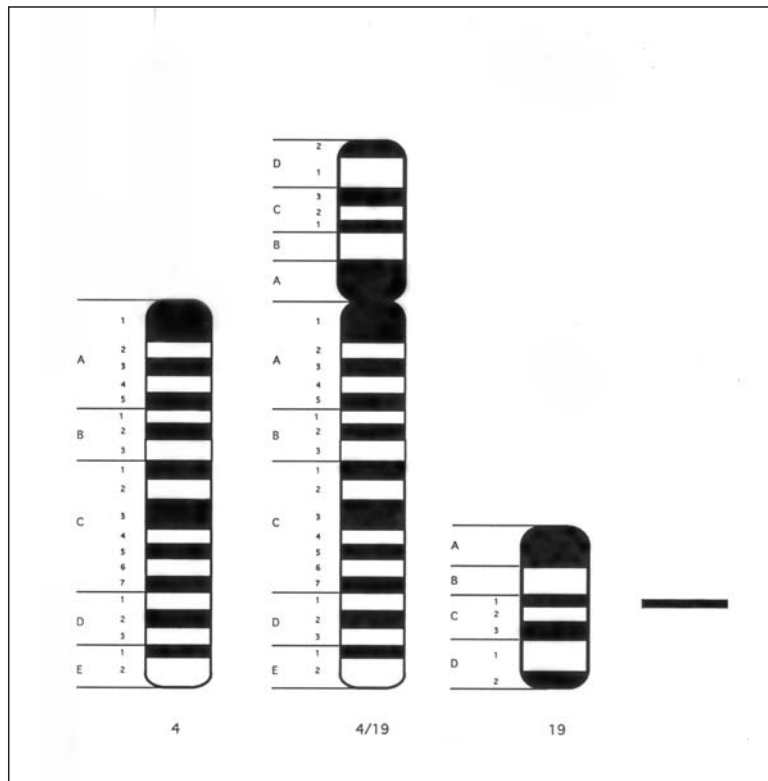


Figure 3. Distribution of G-bands in the idiogram of the Robertsonian chromosome Rb (4.19) of the BALB/c mouse

DISCUSSION

Dicyclohexylamine (fumagillin) is an antibiotic originally used against fungal *Nosema apis* infections in honey bees (Katznelson and Jamieson, 1952; Bailey, 1953) and its residues from honey 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) considering its high stability in such diets (Assil and Sporns, 1991) Furthermore, topical fumagillin is suggested for treatment of ocular infection caused by microsporidia (Roseger *et al.*, 1993; Wilkins *et al.*, 1994); whilst oral fumagillin is an effective treatment for chronic *Enterocytozoon bieneusi* infection in patients with AIDS (Molina *et al.*, 2000, 2002; Conteas *et al.*, 2000). Having in mind all specified informations, it is obvious that evaluation of genotoxic effects of fumagillin is needful, even more as the available data referring to genotoxic effects of fumagillin (dicyclohexylamine) obtained by *in vitro* investigations are equivocal, i.e. 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).

The results of this study points to an ability of tested doses of dicyclohexylamine (fumagillin) to induce numerical and structural chromosome aberrations *in vivo*, particularly the median and the highest doses (Table 1). Thus, the median dose of fumagillin (50 mg/kg b.w.) significantly ($p < 0.001$) increased the frequency of Rb translocations; whilst the highest dose of fumagillin (75 mg/kg b.w.) increased the frequency of aneuploidy, polyploidy, gaps, breaks and Rb translocations with a signification level of $p < 0.001$, and centric rings with a signification level of $p < 0.01$.

Since data regarding genotoxic effects of dicyclohexylamine (fumagillin) *in vivo* are lacking (Toxicological Evaluation, 2000) there is no possibility for direct comparasion of our results. However, if we compare them to the results of Van Went-de Vries (1975) who investigated cyclohexilamin, we can notice certain agreement, but some disagreement as well. Van Went-de Vries *et al.* (1975) observed a significant increase in structural chromosome aberrations (ring chromosome, exchange figures, fragments and breaks) in Chinese hamsters treated with cyclohexilamine (CHA) at a dose of 200mg/kg/day. However, there was no significant increase in aneuploid or polyploid cells in the same study (Van Went-de Vries *et al.*, 1975); whilst fumagillin (dicyclohexylamine) in our study showed the ability to induce aneuploidies and polyploidies. Our results showed a significant increase ($p < 0.001$) in frequency of these aberrations only in the highest dose group (75 mg/kg b.w.). These findings are in accordance with the previous *in vitro* findings of Stanimirovic *et al.* (1999) and Stevanovic *et al.* (2000).

Furthermore, *in vitro* investigations of dicyclohexylamine gave no indication of its genotoxic potential in the Salmonella/microsome assay using *Salmonella typhimurium* strains TA 98, TA 100, TA 1535 and TA 1538 (Purchase *et al.*, 1978; Mortelmans *et al.*, 1986). Moreover, investigations of the DNA-damaging effect of dicyclohexylamine in the UMU test, using *Salmonella typhimurium* strain TA

1535/pSK1002 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 Hussein, 1969) thereby establishing conditions for damaging DNA. However, in a cytogenetic study obtained by Stoltz *et al.* (1970) lymphocytes from human blood samples incubated with dicyclohexylamine sulfate for 5- and 24-hours showed a concentration-dependent increase in aberration rate from approx. 6% in the controls to approx. 16% in the experimental groups.

In addition, the National Toxicology Program (National Toxicology Program 2006a,b) reports a positive genotoxic effect in the *Salmonella* test for dicyclohexylamine nitrite (National Toxicology Program 2006a) and negative for dicyclohexylamine (National Toxicology Program 2006b). Besides, there are references about genotoxic effects of secondary metabolites (gliotoxin and verruculogen) of *Aspergillus fumigatus*, the fungus that produces fumagillin, too. Namely, gliotoxin induced DNA adduct formation (Golden *et al.*, 1998) and has been proven 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).

In our study, in a certain number of animals treated with the median and the highest doses of fumagillin (50 and 75 mg/kg b.w), after G-band analyses, we observed that acrocentric chromosomes 4 and 19 participated in the formation of unusual Rb metacentrics. However, even though such a type of chromosome aberration was not found in the negative control group, we cannot claim for certain that the observed Rb (4.19) chromosome is a consequence of genotoxic activity of fumagillin, considering the fact that some Rb chromosomes can be found in the genome of laboratory mice, and are probably derived from a feral mouse (Gropp and Winking, 1981). This is supported by the statement that nucleolus organizer regions (NORs) do have some influence upon the frequency of Rb translocations, since in mouse cell lines chromosomes with active NORs are significantly represented in Rb chromosomes (Miller *et al.*, 1978). From laboratory mice it is known that chromosomes 12, 15, 16, 18 and 19 may bear NORs proximal to the centromeric heterochromatin (Winking *et al.*, 1988).

In addition, according to Gropp and Winking (1981), there are breakpoints prior to Rb translocation located within the paracentromeric heterochromatin or C-band positive material. This fact seems encouraging with regard to our finding of Rb chromosome 4.19, since that in our study a significant increase ($p.001$) in the frequency of gaps and breaks has been observed in animals treated with the highest dose of fumagillin (75 mg/kg b.w.). These structural changes may have occurred in paracentromeric heterochromatin or C-band positive material of chromosome 4 and 19 which could consequently result in subsequent occurrence of Rb(4.19).

However, our attempts to identify chromosomes which took part in the formation of centric rings failed.

Finally, our results considering the increases 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 drugs already used because ageing and various xenobiotics reduce the capacity for drug-metabolising enzymes (Ingelman-Sundberg, 2001; Bajic *et al.*, 2004). It should be also emphasized that the possible ability of dicyclohexylamine (fumagillin) to induce Rb translocations should not be neglected. The induction of these aberrations in germ cells of sexually matured individuals - consumers of honey with residues - could bring about reproductive problems as chromosome segregations in trivalent mitotic figures, which enclose heterozygous Rb chromosome, could arise 6 theoretically possible combinations of gametes (1/6 normal, 1/6 balanced and 4/6 unbalanced gametes with asomy or disomy), all this in view of known effects of Rb translocations on mammal fertility (Gustavson, 1977; Soldatovic *et al.*, 1994; Stanimirovic, 1995; Vucinic *et al.*, 1996; Eaker *et al.*, 2001).

In a conclusion, it is necessary to educate beekeepers with regard to the use of fumagillin for the purpose of consumer safety, even more that fumagillin has the ability to induce Rb translocations and other types of structural chromosomal aberrations which consequently could result in reproductive problems in honey consumers. A similar caution should be taken with patients treated with fumagillin against microsporidia. Additionally, it is necessary to perform further investigations of dicyclohexylamine in order to reveal other potential adverse effects of the substance, even more as MRL for dicyclohexylamine is not affirmed neither in the EU, nor in the USA.

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UČESTALOST HROMOZOMSKIH ABERACIJA U FUNKCIJI EVALUACIJE GENOTOKSIČNOG POTENCIJALA FUMAGILINA *IN VIVO*

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SADRŽAJ

Dicyclohexylamin (fumagilin), kao antibiotik koga proizvodi gljivica *Aspergillus fumigatus*, se koristi u humanoj medicini u borbi protiv intestinalnih mikrosporidioza kod pacijenata sa HIV infekcijom, ali i za tretman obolelih od intestinalnih amebijaza i mikrosporidijanog keratokonjunktivitisa. U veterinarskoj medicini efikasan je u lečenju mikrosporidioza pčela i drugih insekata uzrokovanih sa *Nosema apis*, *N. cerani*, *N. bombici*, ali i mikrosporidioza riba uzrokovanih sa *Myxobolus cerebralis* i *Tetracapsuloides bryosalmonae*. U ovom radu ispitivana je genotoksičnost dicitloheksilamina kvantifikacijom i kvalifikacijom hromozomskih

abracija u ćelijama kostne srži miševa soja BALB/c. Dicikloheksilamin je aplikovan miševima intragastrično u dozama od 25, 50, 75 mg/kg b.w., pri čemu je vodeno-šećerni sirup predstavljao negativnu, a cyclophosphamid (40 mg/kg b.w.) pozitivnu kontrolu. Utvrđeno je da samo najveća doza dicikloheksilamina signifikantno povećava učestalost ($p < 0.01$ ili $p < 0.001$) numeričkih hromozomskih abracija (aneuploidija i poliploidija) i strukturnih hromozomskih abracija tipa gapova, prekida i centričnih ringova, u poređenju sa negativnom kontrolom. Međutim, srednja (50 mg/kg b.w.) i najviša (75 mg/kg b.w.) eksperimentalna doza izazvale su signifikantno ($p < 0.001$) povećanje (7.12 ± 0.26 i 9.00 ± 0.10 , respektivno) Robertsonovih (Rb) translokacija u poređenju sa negativnom kontrolom (0.00 ± 0.00). U formiranju ovih Rb translokacija utvrđeno je da učestvuju hromozomi 4 i 19. Ovi rezultati sugerišu da je dicikloheksilamin (fumagilin) ispoljio visok genotoksični potencijal sa sposobnošću indukcije i Rb. translokacija.