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EXAMINATION OF GENOTOXIC EFFECTS OF FUMAGILLIN *IN VIVO*

Milan KULIĆ[1], Nevenka ALEKSIĆ[2], Zoran STANIMIROVIĆ[2], Siniša RISTIĆ[1], and Snežana MEDENICA[1]

[1] Faculty of Medicine, University of Sarajevo, East Sarajevo, Bosnia and Herzegovina
[2] Faculty of Veterinary Medicine, University of Belgrade, Belgrade, Serbia

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Fumagillin is an antibiotic derived from the fungus *Aspergillus fumigatus*. It has been used successfully for the treatment of intestinal microsporidiosis in HIV-positive humans, as well as in those suffering from intestinal amebiasis and microsporidial keratoconjunctivitis. In veterinary medicine it is approved for the treatment of microsporidiosis in bees and fish. In this research fumagillin was tested for the ability to provoke

Corresponding author: Dr Zoran Stanimirović, Department of Biology,Faculty of Veterinary Medicine, University of Belgrade,11000 Belgrade, Serbia,Blvd. oslobodjenja 18,e-mail: zoran@vet.bg.ac.rs

chromosomal aberrations in mouse bone marrow cells. BALB/c mice were administered fumagillin by gastric probe in doses of 5, 10 and 20 mg/kg b.w. Water-sugary syrup was the negative and cyclophosphamide (15 mg/kg b.w.) the positive control. Significantly increased frequencies ($p<0.01$ or $p<0.001$) of numerical chromosomal aberrations (aneuploidies and poliploidies) was observed both in the medium (10 mg/kg b.w.) and the highest (20 mg/kg b.w.) dose of fumagillin. Structural chromosomal aberrations (gaps, breaks and insertions) were noticeably more frequent in comparison to negative control only in the highest experimental dose of dyciklohеksilamine. These results clearly showed that fumagillin in concetrations 10 and 20 mg/kg b.w. had a genotoxic potential *in vivo*.

Key words: chromosomal aberrations, genotoxicity, fumagillin (dicyclohexylamine)**.**

INTRODUCTION

Fumagillin is an antibiotic derived from the fungus *Aspergillus fumigatus*. It has widely been used both in human and veterinary medicine. It has appeared to be the most effective medicine in supressing cryptosporidiosis and microsporidiosis caused by *Enterocytozoon bieneusi* which can be fatal in HIV-infected persons (MOLINA *et al*., 2000; CONTEAS *et al*., 2000). Due to its antiparasitic efficacy fumagillin has also been widely applied in veterinary medicine against microsporidiosis of bees and fish (KATZNELSON and JAMIESON, 1952; BAILEY, 1953; EL-MATBOULI and HOFFMAN, 1991; MORRIS *et al*., 2003).

Fumagillin is very stable in honey (FURGALA, 1962) even at higher temperatures; for example it was detectable after having been kept at 80^{o}C for 35 days (ASSIL and SPORNS, 1991). Mixed with syrup it is very effective in supressing *Nosema* in hibernating honeybee colonies. However, it has not been effective against dormant spores of *Nosema apis*, thus has never eliminated the disease in the bee colony entirely.

Till now some adverse effects of fumagillin were discovered. After the treatment of *Nosema apis*-infected bees with fumagillin the electron-density of the mitochondrial matrix in corpora allata increased and the dimensions of the mitochondria diminished in comparison with untreated infected bees. (LIU, 1990a). Fumagillin produced certain effects on secretion granules of hypopharyngeal glands of bees which increased and had homogeneous structure, which is explained by the changes in secretory activities of glands (LIU, 1990b). It was confirmed that fumagillin largely increased mortality in bees as well as the number of fungi (RADA *et al*., 1997).

Fumagillin has also been used in the treatment of microsporidiosis in fish including the ones caused by *Myxobolus cerebralis* (EL-MATBOULI and HOFFMAN, 1991; KARAGOUNI *et al*., 2005) and *Tetracapsuloides bryosalmona* (HEDRICK *et al*., 1988; KENT and DAWE, 1994; LE GOUVELO *et al*., 1999; MORRIS *et al*., 2003).

Fumagillin is effective in curing microsporidial keratoconjunctivitis (ROSEGER *et al*., 1993, WILKINS *et al*., 1994). Taken in oraly it has appeared highly effective against chronic *Enterocytozoon bieneusi* infections in patients with AIDS and other types of immunodeficiencies (MOLINA *et al.,* 2002). For further administration, however, appropriate therapeutical models should be completely improved and side effects of the treatment should be eliminated (CONTEAS *et al*., 2000).

Data on genotoxic effects of fumagillin obtained from *in vitro* studies showed discrepancy, being either positive (STOLTZ *et al*., 1970; STANIMIROVIĆ *et al*., 1999; STEVANOVIĆ *et al*., 2000 and 2008; KULIĆ 2006) or negative (PURCHASE *et al*., 1978; MORTELMANS *et al*., 1986; HEIL, 1996). On the other hand, there are insufficient data on genotoxic effects of fumagillin *in vivo*.

Having considered the aforementioned, testing genotoxic effects of fumagillin *in vivo* and *in vitro* should be continued, especially because of the fact that these effects varied among various tests and depended largely on the doses of fumagillin and because of its interaction with endogenous and exogenous factors (AMES, 1989; ALBERTINI *et al*., 2000; NORPPA, 2003, STANIMIROVIĆ *et al*., 2005).

The aim of this research was to evaluate the possible *in vivo* cytogenetic effects of fumagillin in various doses used habitually in bee-keeping.

MATERIALS AND METHODS

The genotoxic effects of dicyclohexylamine (CAS No. 101-83-7, Fumagillin–ET®, Evrotom, Ruma, Serbia) were observed in the following three doses: 5, 10 and 20 mg/kg b.w. The medium experimental dose (10 mg/kg) corresponded to the therapeutical dose of fumagillin in bee-keeping.

Fumagillin does not dissolve readily in water. Therefore, it was stirred in a small quantity of warm water (≥32-35°C) until turned into paste and prior to gradual adding of water-sugar syrup and mixing together. The solution was administered to the mice in experimental groups seven consecutive days.

Five groups of animalswere tested: three groups treated with fumagillin, the positive and negative control. Each group comprised six six-month-old BALB/c male mice weighing approximately 20 g. The animals were kept in unchanged conditions under 12/12-h light-dark periods at constant temperature (21°C) with free access to food and water.

The animals in the negative control group were treated with water-sugar syrup (1:1). Cyclophosphamide, a well-known clastogene and mutagene (ANDERSON *et al*., 1995), was used as the positive control and administered i.p. in the dose of 15 mg/kg b.w. for seven consecutive days.

Cytogenetic analyses were carried out on the bone marrow cells obtained from the long bones (femur and tibia) according to HSU and PATTON (1969), modified by ZIMONJIĆ *et al*., (1990). Preparations were flame-dried and stained with Giemsa solution (Sigma Chemical Co., St. Louis, MO). G-banding was carried out by the tripsin method of SEABRIGHT (1971) and RONNE (1991). Chromosomes and sets of chromosomes were identified on the basis of criteria established by the COMMITTEE

ON STANDARDIZED GENETIC NOMENCLATURE FOR MICE (1979) and Cowell's photoatlas of mice chromosomes (COWELL, 1984).

Six-hundred well spread metaphases per treatment were analysed for the presence of chromosomal aberrations. Statistical analyses was performed by Statistica 6.0 software programme, ANOVA, Student's t-test and LSD-test.

RESULTS

Genotoxic effects of fumagillin were analysed assessing chromosomal aberrations in the mouse bone marrow cells. The doses tested were 5.0, 10.0 and 20.0 mg/kg b.w.The obtained results showed that in certain doses fumagillin is capable of provoking chromosomal abberations.In the lowest dose, 5 mg/kg b.w., fumagillin did not seem to influence neither structural nor numeric chromosomal abberrations in mouse bone marrow cells, since the mean number of gaps, breaks and insertions remained similar to the numbers in the negatove control; the same was true for aneuploidy and poliploidy (Table 1).Twice the minimum dose of fumagillin (10 mg/kg b.w.) succeded in inducing numerical abberrations only. The increase in the average number of aneuploidies rose to 6.37±0.92, which was significant ($p<0.01$) in comparison to the negative control (5.00±0.76). Poliploidy was not more frequent than expected. Fumagillin administered to BALBc mice in the dose of 20 mg/kg for seven consecutive days had major consequences for the frequency of both numerical and structural chromosomal aberrations in the bone marrow cells (Figure 1 and Figure 2). The average number of gaps more than doubled increasing from baseline 2.00±0.74 to 5.75±0.89 ($p<0.001$). The frequency of acentric chromosomes plummeted from negligable (0.75±0.71) to 4.37±0.74 ($p<0.001$) and the increase in insertions was dramatic ($p<0.001$) having reached 3.25±0.71 on average. The mean number of numerical chromosomal abberrations, both aneuploidies and poliploidies rose considerably ($p<0.001$) to 31.75±1.28 and 5.37±0.7440, respectively.

Table 1. Cytogenetic parametres in the cells of bone marrow in BALB/c mice in control and experimental groups of animals treated by fumagillin

Chromosal aberrations	No. of analyzed metaphases	Negative control		Fumagillin 5 mg/kg b.w.		Fumagillin 10 mg/kg b.w.		Fumagillin 20 mg/kg b.w.		Positive control	
		Mean±SE	(%)	Mean±SE	(%)	Mean±SE	(%)	Mean ± SE	(%)	Mean ± SE	(%)
Aneuploidies	600	5.00±0.76	0.83	5.25±0.71	0.87	6.37±0.92	1.06**	31.75±1.28	5.29***	105.87±2.95	17.65***
Poliploidies	600	0.25±0.46	0.04	0.50±0.53	0.08	0.75±0.89	0.12	5.37±0.74	0.89***	16.25±0.89	2.71***
Gaps	600	2.00±0.93	0.33	2.37±0.52	0.39	2.62±0.74	0.44	5.75±0.89	0.96***	17.62±1.06	2.94***
Acentrics	600	0.75±0.71	0.13	0.87±0.83	0.16	1.25±0.71	0.21	4.37±0.74	0.73***	6.62±0.52	1.10***
Insertions	600	0.00±0.00	0.00	0.00±0.00	0.00	0.25±0.46	0.04	3.25±0.71	0.54***	6.37±1.19	1.06***
Total cytogenetic changes	600	8.00±1.20	1.33	9.00±1.07	1.50	11.25±1.83	1.87**	50.5±1.93	8.41***	152.75±2.76	25.46***

*** Statistically significant difference in comparison to negative control $p<0.001$

** Statistically significant difference in comparison to negative control $p<0.01$

Significantly increased frequencies ($p<0.01$ or $p<0.001$) of numerical chromosomal aberrations (aneuploidies and poliploidies) was observed both in the medium (10 mg/kg b.w.) and the highest (20 mg/kg b.w.) dose of fumagillin.

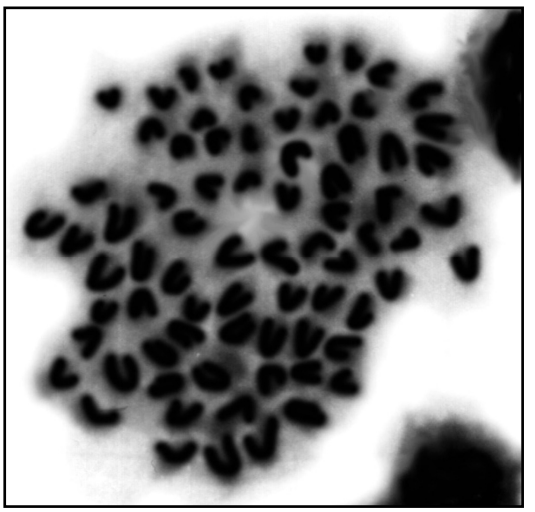

Figure 1. Poliploidy in a mouse bone marrow cell (BALB/c mice treated with fumagillin, 20 mg/kg bw)

Structural chromosomal aberrations (gaps, breaks and insertions) were noticeably more frequent in comparison to negative control only in the highest experimental dose of dycikloheksilamine.

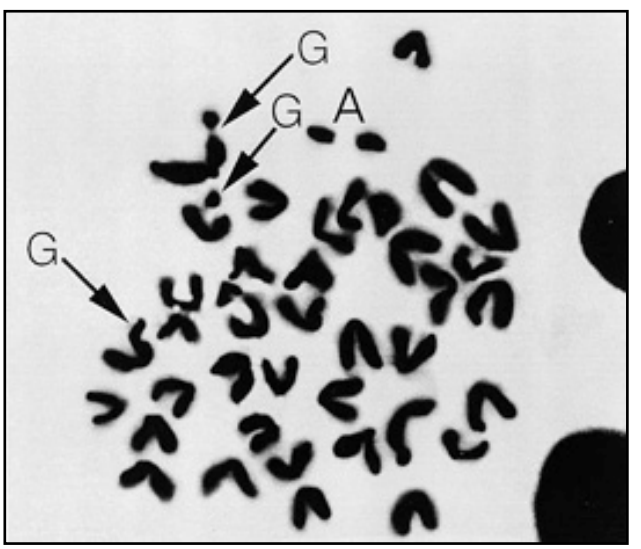

Figure 2. Chromosome gaps (G) and an acentric (A) in a bone marrow cell of a BALB/c mouse treated with the 20-mg/kg-dose of fumagillin

When taking into consideration total cytogenetic changes, a significant increase ($p<0.01$) in comparison to negative control (8.00 ± 1.19) was noticed in the medium (11.25±1.83) and in the highest (50.50±1.93) dose group ($p<0.001$).

DISCUSSION

Fumagillin (dicyclohexylamine) has the widespread use in suppression of fungus *Nosema apis* infections in honey bees (KATZNELSON and JAMIESON, 1952; BAILEY, 1953). Due to its high stability in bees'food (ASSIL and SPORNS, 1991) residua of this antibiotic in honey or other products of bees which are used as food can easily reach final users including children, adolescents, convalescents, chronic patients and the elderly (STANIMIROVIĆ *et al*., 1999, 2005, 2006, 2007; STEVANOVIĆ *et al*., 2000, 2006, 2008; KULIĆ 2006). Fumagillin has been recommended for curing microsporidial eye infections (ROSEGER *et al*., 1993, WILLKINS *et al*., 1994). In addition, it is effective in the treatment of chronic *Enterocytozoon bieneusi* infections common in HIV patients (MOLINA *et al*., 2000, 2002; CONTEAS *et al*., 2000). Having analysed the aforementioned it is evident that the evaluation of genotoxic effects of fumagillin (dicyclohexylamine) is necessary. In addition, the data on its genotoxic effects *in vitro* are ambiguous. The results of a group of authors are positive (STOLTZ *et al*., 1970; STANIMIROVIĆ *et al*., 1999; STEVANOVIĆ *et al*., 2000; KULIĆ 2006; STEVANOVIĆ *et al*., 2008), which is contradictory to the findings of some others (PURCHASE *et al*., 1978; MORTELMANS *et al*., 1986; HEIL, 1996). Furthermore, there is very little information on the genotoxic effects of fumagillin *in vivo* which were obtained with much higher doses (STEVANOVIĆ *et al*., 2006; STANIMIROVIĆ *et al*., 2007).

The results of the present study point to the capability of fumagillin in certain doses of causing numerical and structural chromosomal aberrations *in vivo*. The highest tested dose (20 mg/kg b.w.) resulted in the increase in the frequency of aneuploidies, poliploidies, gaps, breaks and insertions with the $p<0.001$ level of significance. These results are in accordance with the previous *in vivo* findings of STANIMIROVIĆ *et al*. (2007). However, the fumagillin concentrations investigated by STANIMIROVIĆ *et al*. (2007) (25 mg/kg b.w., 50 mg/kg b.w., and 75 mg/kg b.w.) were much higher than those used in the current work (5 mg/kg b.w., 10 mg/kg b.w. and 20 mg/kg b.w.). Our *in vivo* findings of chromosome aberrations agree with the previous *in vitro* results of STANIMIROVIĆ *et al*., (1999), STEVANOVIĆ *et al*. (2000 and 2008) and KULIĆ (2006), which claimed that fumagillin significantly increased the frequencies of structural chromosomal aberrations.

Indeed, *in vitro* research on dicyclohexylamine has not shown indicators of its genotoxic potential in Salmonella test using *Salmonella typhimurium* types TA 98, TA 100, TA 1535 and TA 1538 (PURCHASE *et al*., 1978; MORTELMANS *et al*., 1986). However, in the research carried out by STOLTZ *et al*., (1970) samples of human lymphocytes incubated in the presence of dicyclohexylamine sulphate for 5 and 24 hours showed a marked dose-dependent increase in aberrations.

National Toxicology Program (2006a,b) reports on the positive genotoxic effects of dicyclohexylamine nitrate in Salmonella test (National Toxicology

Program 2006a) while dicyclohexylamine seemed not to induce genotoxicity (NATIONAL TOXICOLOGY PROGRAM 2006b). Besides, there are certain data on genotoxic effects of secondary metabolites (gliotoxin and verruculogen) of *Aspergillus fumigatus* which fumagillin is derived from. Gliotoxin causes changes in the DNA (GOLDEN *et al*., 1998) and it appeared to be genotoxic in *in vitro* test systems (NIEMIEN *et al*., 2002); meanwhile, verruculogen produced effects in Salmonella/microsomal mutagenicity assays (SABATER-VILAR *et al*., 2003).

The results of our research clearly showed an increase in the frequency of chromosomal aberrations provoked by fumagillin.

Finally, although there is no reliable information regarding fumagillin residue levels in food except those of MLADJAN and JOVIĆ (2000) and KULIĆ (2006), our results concerning the increased frequencies of chromosomal abberrations (poliploidy, aneuploidy, gaps, breaks and insertions) induced by fumagillin lead to the conclusion that fumagillin residues in food may have genotoxic effects that could increase risk for cancer and chromosomal aberrations (STANIMIROVIĆ *et al*., 2006, 2007; STEVANOVIĆ *et al*., 2006, 2008). Moreover, beekeepers who are occupationally exposed to fumagillin may also be at genotoxic risk. Besides, there is a sheer necessity for compulsory education of beekeepers concerning consumers' safety. 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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ISPITIVANJE GENOTOKSIČNIH EFEKATA FUMAGILLINA *IN VIVO*

Milan KULIĆ[1], Nevenka ALEKSIĆ[2], Zoran STANIMIROVIĆ[2], Siniša RISTIĆ[1] i Snežana MEDENICA[1]

[1]Medicinski fakultet, Univerzitet u Sarajevu, Istočno Sarajevo, Republika Srpska
[2]Fakultet veterinarske medicine, Univerzitet u Beogradu, Beograd, Srbija

I z v o d

Fumagillin (diciklo-heksil-amin) je antibiotik izolovan iz gljive *Aspergillus fumigatus*. Koristi se u humanoj medicini u lečenju intestinalnih mikrosporidioza mahom kod obolelih od AIDS-a, kao i kod intestinalne amebioze i keratokonjunktivitisa prouzrokovanog mikrosporidijama. U veterinarskoj medicini se primenjuje u lečenju mikrosporidioza pčela i riba. U radu je ispitivana genotoksičnost fumagilina praćenjem hromozomskih aberacija u ćelijama kostne srži miša. Miševi BALB/c soja tretirani su fumagilinom gastričnom sondom u dozi od 5, 10 i 20 mg/kg t.m. Vodeno-šećerni sirup je predstavljao negativnu, a ciklofosfamid (15 mg/kg tm) pozitivnu kontrolu. Utvrđeno je da srednja (10 mg/kg tm) i najveća doza fumagilina (20 mg/kg tm) signifikantno ($p<0,01$, odnosno $p<0,001$) povećavaju učestalost numeričkih aberacija. Najviša testirana doza fumagilina, u poređenju sa negativnom kontrolom, indukovala je i strukturne i numeričke aberacije vieoko signifikantno ($p<0,001$). Ovi rezultati nedvosmisleno govore da fumagilin (dicikloheksilamin) u opsegu srednje i najveće doze ispoljava genotoksični potencijal.

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