Pakistan J. Zool., vol. 45(4), pp. 1103-1112, 2013

Evaluation of Genotoxic and Cytotoxic Effects of Natamycin in Mice Bone Marrow Cells

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Abstract - Natamycin, a food preservative, is widely used in food industry against yeast and fungi. The potential genotoxicity of a commercial formulation of natamycin (Delvocid, containing 50% natamycin as the active ingredient) in mice bone marrow cells was investigated *in vivo* by chromosome aberrations (CA) and micronucleus assays (MA). Animals were intraperitoneally treated with 200, 400 and 800 mg/kg of natamycin for 6, 12 and 24 h in CA assay, for 24, 48 and 72 hours in MN assay. In the present study, natamycin did not increase chromosome aberrations. It significantly induced frequency of micronucleus at all concentrations for both 24 and 48 h in female mice, at the two highest (400 and 800 mg/kg) concentrations for both 24 and 48 h in male mice when compared with negative control. Mice bone marrow erythrocytes exposed to all concentrations of natamycin showed significant decreases in mitotic index for all treatment periods. In addition, natamycin reduced polychromatic erytrocyte/normochromatic erytrocyte ratio at all concentrations for 48h and at the highest (800 mg/kg) concentration for 24 and 72h in female mice; at all concentrations for 24 and 48h in male mice. The results of the present study show that a commercial formulation of natamycin was aneugenic and cytotoxic to mice bone marrow *in vivo*. For this reason it is necessary to be careful when using these chemicals in food as preservatives.

Key words: Natamycin, food additive, chromosome aberration, micronucleus, mice bone marrow

INTRODUCTION

Nowadays, use of food additives has become widespread because of developing industry, increasing population and food consumption. Therefore, it is crucial to find new food sources and preserve them for a long time without molding. Becuse of this reason, various methods were developed to preserve, flavour and improve taste and appearance of foods. And, food additives were classified as antioxidants, preservatives, food colors, food flavours, emulsifiers-stabilizers and sweeteners based on their functions in foods. It was noticed that food additives, especially food preservatives, are genotoxic in different test systems (Njagi and Gopalan, 1982; Luca *et al.*, 1987; Pagano and Zeiger, 1987; Munzer *et al.*, 1990; Akin and Sumer, 1991; Kayraldiz and Topaktas, 2007; Turkoglu, 2007; Rencuzogullari *et al.*, 2001; Onyemaobi *et al.*, 2012). However, there are many food preservatives whose genotoxic effects are still unknown (Rencuzogullari *et al.*, 2001; Turkoglu, 2007; Arslan *et al.*, 2008).

Natamycin has been used as a food preservative worldwide for over 40 years (WHO TRS 430) and is approved as a food additive/ preservative by the European Union, the World Health Organization and individual countries use as a fungistat to suppress mold on cheese, meats and sausage (EPA, 2012).

There are many negative results on the effects of natamycin, in that some of these reports suggest that natamycin do not induce mutation and it is not clastogenic and mutagenic in various test systems such as *Salmonella*/mammalian microsome mutation assay in *Salmonella typhimurium*, *Escherichia coli* strains, in a mouse lymphoma mutation assay, in chromosome aberration assay with CHO cells (Cox *et al.*, 1973; EMEA, 1998; WHO, 2006; EPA, 2012; PRD, 2012). Rencuzogullari *et al.* (2009) indicated that natamycin has genotoxic effect in human lymphocytes.

No report is found on the genotoxicity of natamycin in *in vivo* mice bone marrow in the literature. Sarikaya and Solak (2003) reported that the widespread use of the food preservatives cause serious problems of health. Therefore, it is very important to assess genotoxicity and cytotoxicity of these chemicals. According to OECD (2005)

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0030-9923/2013/0004-1103 $ 8.00/0

chromosome aberations (CA) and micronuclears (MN) assays in bone marrow could provide to assess the potential of a test chemical to cause DNA damage. Many researchers reported that CA (Carrano and Natarajan, 1988; Hagmar *et al.,* 2001; Ieradi *et al.,* 2003) and MN (Wakata *et al.,* 1989; Vanparys *et al.,* 1992; Hayashi *et al.,* 1994; EPA, 1996) assays are the most commnly used short-term *in vivo* assays for detecting of chemical toxicity. MN can be formed from acentric chromosomal fragments which arise as a result of chromosome breaks after clastogenic effect or whole chromosomes that do not migrate during anaphase as a result of aneugenic affects (Heddle *et al.,* 1991). Hence, both clastogenic and aneugenic effects can be detected with MN assay (Kirsch-Volders *et al*., 1997; Norppa and Falck, 2003).

The aim of the present study was to investigate the genotoxic effect of natamycin in mice bone marrow cells using CA and MN assays. The mitotic index (MI) and polychromatic erytrocyte/Normochromatic erytrocyte (PCE/NCE) ratio were also calculated to evaluate cytotoxic effect of natamycin in mice bone marrow cells.

MATERIALS AND METHODS

Test chemicals

In the present study, the commercial formaulation of natamycin (Delvocid which has 50% natamycin as active ingredient and % 50 lactose) (CAS No 7681-93-8) was used as the test material. It was purchased from Maysa Food Industry in Turkey. The chemical structure of natamycin is shown in Figure 1. Giemsa (Cat. No. 109204, CAS No 51811-82-6) and May Grunwald (Cat. No. 101424) were obtained from Merck. Fetal calf serum (Cat. No. N4762,) and Mitomycin C (Cat. No. M0503, CAS No 50-07-7) were purchased from Sigma Aldrich. MMC was used as positive control, distilled water was used as negative control.

The animals and concentrations

In this study, male and female mice (*Mus musculus*), (8-12 weeks of age, with average body weight of 20-25g), were used and purchased from Trakya University Scientific Research Center. The animals were maintained in closely inbred colony under conventional laboratory conditions at a room temperature of 25±5°C and in 12h dark and 12h light cycles. Food pellets and water were provided ad libitum. Five groups were prepared for the chromosome aberration assay (5 animals each) and micronucleus assay (5 animals each). Three of these were experiment groups. One of these was the positive, and the other one was the negative control group.

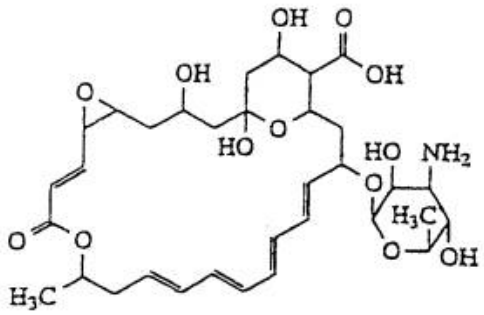


Fig. 1. Chemical structure of natamycin

According to van Eeken and Wubs (1976), the LD_{50} of natamycin (intraperitoneal) was found to be 1600 mg/kg bw (WHO, 2006). In the study, mice were injected with 200, 400 and 800 mg/kg bw (1/8, 1/4, ½ LD_{50}, respectively) concentrations of natamycin intraperitoneally (ip).

Chromosomal aberrations (CAs) assay

The method of Preston *et al*. (1987) was followed in the preparation of chromosome aberration (CA) assay. Natamycin was dissolved in distilled water and was injected intraperitoneally to female and male mice (10-12 weeks) in 6, 12 and 24h periods. In order to arrest cells at metaphase, colchicine (0.01%) was injected intraperitoneally 3h before servical dislocation. Then, the bone marrow from a femur was flushed out in 1% sodium citrate, the suspension was centrifuged for 5 min at 1000 rpm. The cells were incubated at 37°C for 25 min with hypotonic solution (1% sodium citrate) and fixed with fixative (1:3 acetic acid:methanol) three times. The cells were spread on glass slides and left to dry. The slides were stained with 10% Giemsa in Sörensen buffer for 10 min.

One hundred well-spread metaphase were examined for each concentration and treatment

period. Chromosomal aberrations were investigated at x1000 magnification. Gaps were not evaluated as CA, according to Mace *et al.* (1978). Mitotic index (MI) was determined by scoring 3000 cells from each animal. The MI explained the effects of the chemicals on the G_2 stage of the cell cycle (Rencuzogullari *et al.*, 2008).

Micronucleus (MN) assay

For the analysis of micronucleus assay in mice bone marrow cells, female and male mice (8-10 weeks) were treated with the same concentrations intraperitoneally for 24, 48 and 72h. Bone marrow smears were done according to the methods of Schmid (1975) and Aaron *et al.* (1989) with minor modifications. The bone marrow cells were flushed out with fetal calf serum, and the suspension was centrifuged for 10 min at 2000 rpm. The pellets were spread on a slide glass and fixed with methanol. The slides were stained with May Grunwald for 3 min, May Grunwald:distilled water (1:1) for 2 min, 10 % Giemsa in Sörensen buffer for 10 min. A total of 1000 erythrocytes were scored for each animal at a magnification of x1000. The numbers of micronucleated PCE and micronucleated NCE were counted. PCE/NCE ratio was calculated.

Statistical analysis

Variance analysis of data was done using STATISTICA AXA 7.1 computer program. Fisher's exact test and X^2 test were used for CA and MI, respectively. Kalmogorov Smirnov test was used, and the significance between groups was determined using the one-way analysis of the variance (ANOVA), followed by a post hoc test. If ANOVA was significant, Dunnett's test was performed (Zar, 1999). For non parametric data, the Kruskal-Wallis test was carried out followed by the Mann-Whitney U test. Dose-response relationship was determined using Pearson correlation analysis. $P \leq 0,05$ was considered as the level of significance.

RESULTS

In order to determine the genotoxic and cytotoxic effects of natamycin in mice bone marrow cells was used three different concentrations (200, 400 and 800 mg/kg) and four different parameters (CA, MN, MI and PCE/NCE ratio) for different periods (6, 12, 24, 48 and 72 h).

The results of CA assay are given Table I. Natamycin increased the frequency of CA for all concentration ranges (200-800 mg/kg) and exposure times (6, 12 and 24 hours) both in male and female mice bone marrow cells when compared with the negative control. But this increase was not significant. In addition, natamycin significantly reduced MI at all concentrations both in male and female mice for 6, 12 and 24 h treatment periods (Fig. 2). These decreases were concentration-dependent (In female mice, for 24h: $r^2= 1$, $P \leq 0.05$; in male mice, for 12h: $r^2= 0.997$, $P \leq 0.05$, for 24h: $r^2= 0.9345$, $P \leq 0.05$) (Fig. 3). The correlation between CAs and MI values of female and male mice was shown in Figures 4 and 5. This correlation was significant in male mice for 6 and 12h treatment periods. But in female mice, it was not significant.

The results of MN assay are given in Table II. Natamycin was found to significantly induce MN formation at all concentrations in female mice, at the highest two concentrations (400 and 800 mg/kg) of this compound in male mice for 24 and 48 hours (for 48h: $r^2=0.9922$, $P \leq 0.01$; for 72h: $r^2=0.916$, $P \leq 0.05$) (Fig. 6). Natamycin also reduced the PCE/NCE ratio at all concentrations for 48 h in female mice, for 24 and 48 h in male mice. At the highest concentration (800 mg/kg), natamycin reduced PCE/NCE ratio for 24 and 72 h in female mice. In female mice, these decreases were concentration-dependent.

DISCUSSION

The results of the present study revealed that natamycin increased the frequency of CA for all concentration ranges (200-800 mg/kg) and exposure times (6, 12 and 24 hours) in mice bone marrow cells. But this increase was not significant. This finding suggets that natamycin was not clastogenic. These results are in agreement with previous reports about natamycin. PRD (2012) reported that natamycin was considered to be non-mutagenic in bacterial reverse mutation assay and in on in vitro mammalian CA assay. In the report of WHO (2006), it was stated that Delvocid which is a commerical

Table I. Frequency of chromosome aberrations and mitotic index in bone marrow cells of female and male mice exposed to natamycin.

Test substance	Periods (h)	Concentrations (mg/kg)	Female mice		Male mice	
			Total abnormality (-gap) (%)	MI (%)	Total abnormality (-gap) (%)	MI (%)
Distilled water	6	-	0	3.17	0	3.97
MMC	6	2	12***	2.0**	13***	2.40***
Natamycin	6	200	2	2.26*	1	2.73**
	6	400	2	2.07**	1	2.50**
	6	800	4	1.96**	2	2.26***
Distilled water	12	-	1	3.63	0	4.53
MMC	12	2	12*	2.30**	19***	2.73***
Natamycin	12	200	1	2.66*	2	3.20**
"	12	400	2	2.37**	3	2.53***
"	12	800	3	2.17***	5	2.13***
Distilled water	24	-	1	3.93	2	4.37
MMC	24	2	9*	2.67**	21***	2.53***
Natamycin	24	200	1	3.00*	0	3.03**
	24	400	1	2.37***	3	2.96**
	24	800	2	1.93***	7	1.87***

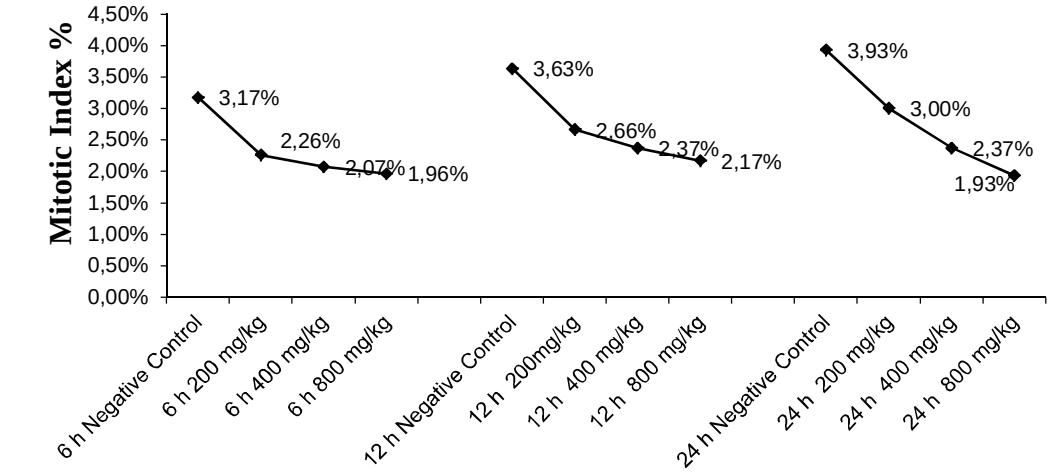
*P≤0,05; **P≤0,01; ***P≤0,001

Table II.- Frequency of micronucleus and PCE/NCE in bone marrow cells of female and male mice exposed to natamycin.

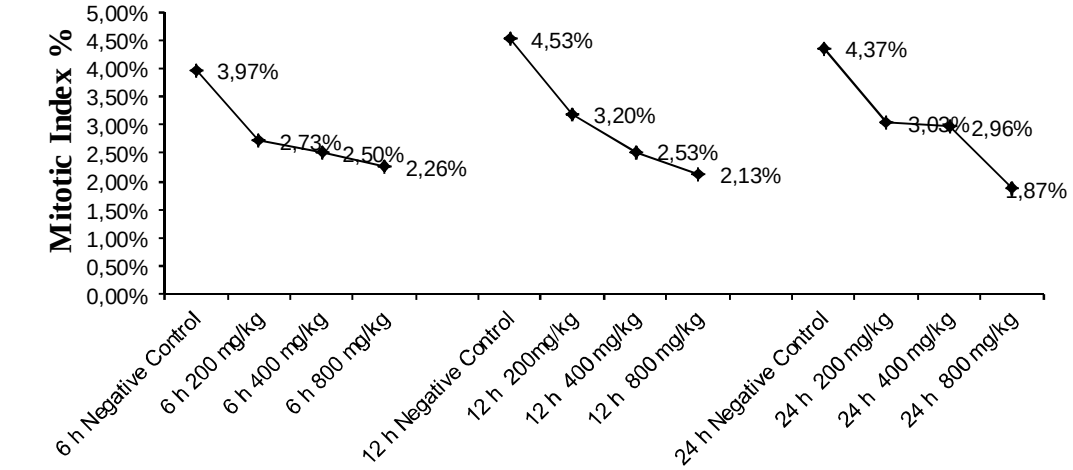
Test substance	Total cell No./mice number	Periods (h)	Concen. (mg/kg)	Female mice		Male mice	
				Total MNPCE % ±S.E.	PCE/NCE ±S.E.	Total MNPCE % ±S.E.	PCE/NCE ±S.E.
Distilled water	5000/5	24	-	6.20±2.20	1.67±0.20	19.60±0.75	1.48±0.03
MMC	5000/5	24	2	56.40±6.65***	0.98±0.12,	37.20±3.83***	0.91±0.02***
Natamycin	5000/5	24	200	21.20±3.72*	1.10±0.14	23.60±3.54	1.26±0.09*
	5000/5	24	400	28.80±2.06***	1.05±0.28	34.00±2.28*	0.96±0.05***
	5000/5	24	800	30.40±0.98***	0,88±0,14*	30.00±1.79*	0.86±0.05***
Distilled water	5000/5	48	-	5.20±1.02	1.90±0.19	16.00±2.28	1.70±0.11
MMC	5000/5	48	2	51.20±2.58***	0.90±0.08**	37.60±2.71***	0.81±0.03***
Natamycin	5000/5	48	200	12.40±1.72*	1.01±0.29**	19.20±2.58	1.00±0.05***
	5000/5	48	400	16.00±1.79**	0.84±0.09***	35.20±4.32***	0.97±0.03***
	5000/5	48	800	26.80±3.32***	0.99±0.10**	34.40±1.94***	0.81±0.03***
Distilled water	5000/5	72	-	9.20±0.80	1.51±0.18	15.20±1.36	1.52±0.05
MMC	5000/5	72	2	33.60±7.55***	1.06±0.20	36.80±2.87***	0.82±0.08***
Natamycin	5000/5	72	200	12.80±2.15	1.39±0.13	19.20±1.96	1.35±0.10
	5000/5	72	400	10.80±2.42	1.31±0.09	16.80±0.80	1.23±0.02
	5000/5	72	800	23.20±3.32*	1.01±0.10*	19.20±1.96	1.26±0.11

MNPCE: Micronucleated polychromatic erythrocyte, MNNCE: Micronucleated normochromatic erythrocyte, SE: Standart error, *P≤0,05; **P≤0,01; ***P≤0,001

form of natamycin was not mutagenic in *S. typhimurium, E. coli* and *B. subtilis* and natamycin was not clastogenic both in male and female mice (Cox *et al.*, 1973). Contrary to these, Rencuzogullari *et al.* (2009) indicated that natamycin increased frequency of SCE and CA in human lymphocytes.



A



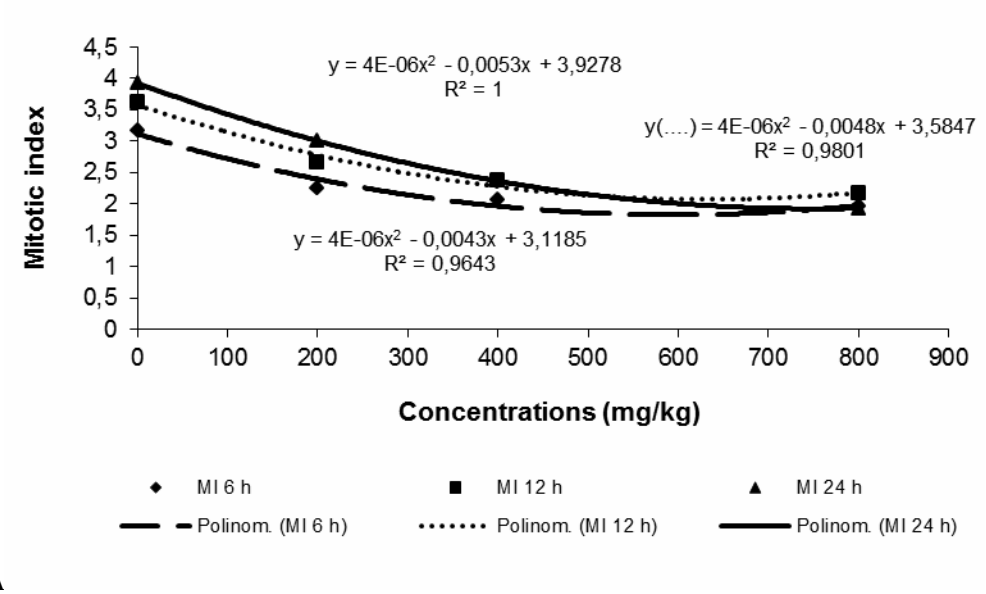
B

Fig. 2. Frequency of mitotic index observed in bone marrow cells of female (A) and male (B) mice exposed to natamycin.

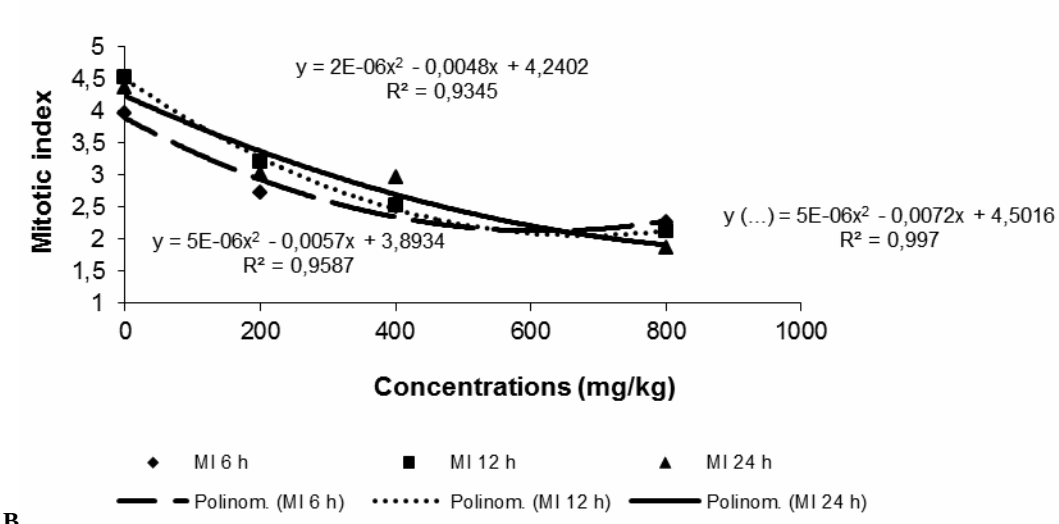
This statement is inconsistent with the results of the present study.

In the present study, natamycin was found to significantly induce MN formation at all concentrations in female mice, at the highest two concentrations (400 and 800 mg/kg) of this compound in male mice for 24 and 48 hours. These results support previous findings obtained by Rencuzogullari *et a.l* (2009) for natamycin, which significantly induced MN frequency in human lymphocytes.

The induction of CAs and micronucleated erythrocytes by chemical compound suggest the clastogenic potential of this chemical. MN may be formed from clastogenic and aneugenic effects. But, in this study, a clastogenic effect was not observed in CA assay. So, formation of MN suggests that natamycin might be aneugenic.



A



B

Fig. 3. Regression lines and correlation coefficient (r) of mitotic index in bone marrow cells of female (A) and male (B) mice at 6 h, 12 h and 24 h following treatment with natamycin.

MI is used to indicate cytotoxicity of chemicals. A decreased MI reflects the inhibition of cell cycle and effects the cell division negatively (Amorim *et al.,* 2000). The results of this study revealed that natamycin significantly decreased MI at all concentrations both in male and female mice for 6, 12 and 24 h treatment periods. Negative correlation was found between MI and concentrations and between MI and CA frequency. But the increase in the frequency of CAs was not significant. This finding suggests that toxicity increased with the increasing of concentrations. Rencuzogullari *et al.* (2009) reported that natamycin showed cytotoxic effect by decreasing the RI, MI and NDI in human lymphocytes. This statement is in agreement with results of the present study.

The PCE/NCE ratio is used to obtain information about the cell cycle specific action of a positive chemical. A decrease in PCE/NCE ratio reflects a cytotoxic effect or alterations in

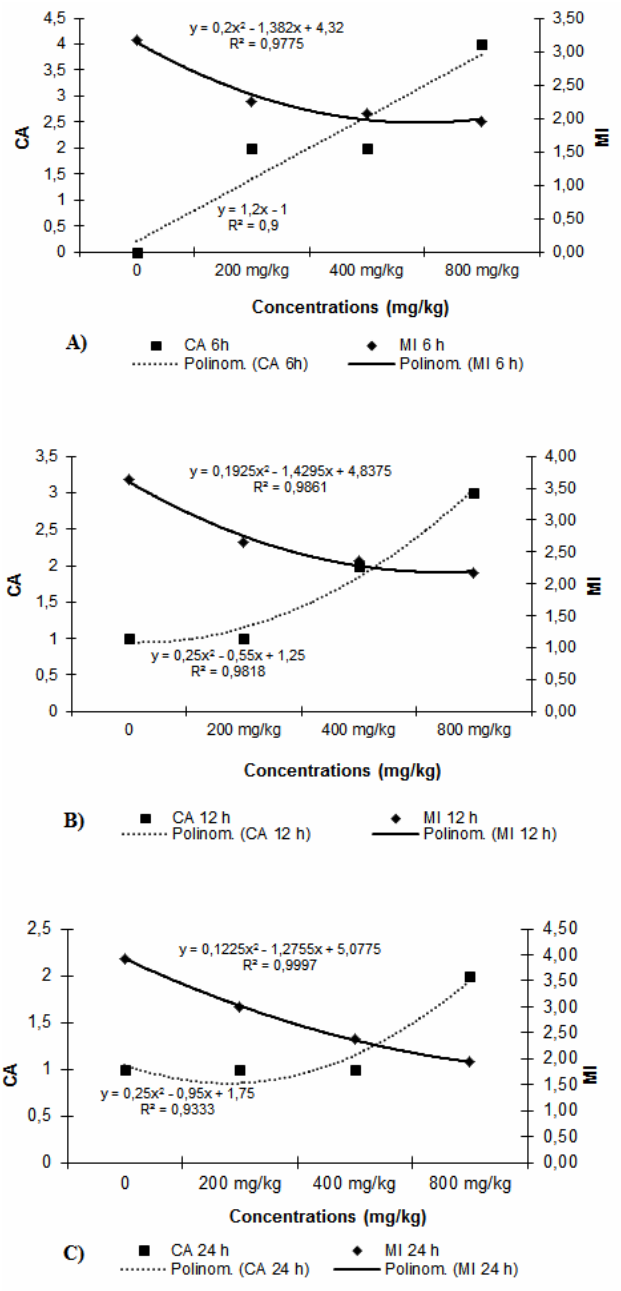


Fig. 4. Regression lines and correlation coefficient (r) of CAs and mitotic index in bone marrow cells of female mice at 6 h (A), 12 h (B) and 24 h (C) following treatment with natamycin.

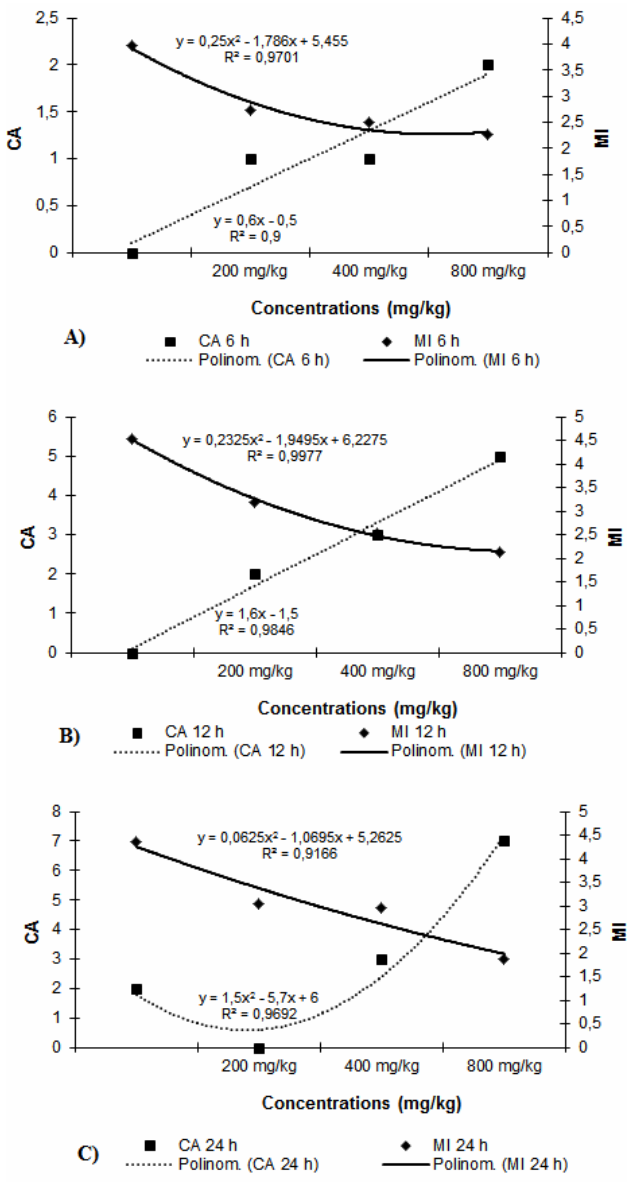


Fig. 5. Regression lines and correlation coefficient (r) of CAs and mitotic index in bone marrow cells of male mice at 6 h (A), 12 h (B) and 24 h (C) following treatment with natamycin.

erythropoiesis. The PCE/NCE ratio is decreased because of the cavity formation in bone marrow when there are cytotoxic effects on the cell division and/or maturation of the nucleated cells (Gollapudi *et al.*, 1984). Natamycin also decreased the PCE/NCE ratio at all concentrations for 48 h in female mice, for 24 and 48 h in male mice. At the highest concentration (800 mg/kg), natamycin decreased PCE/NCE ratio for 24 and 72 h in female mice. Significant decreases in the MI and PCE/NCE ratio, as observed in the present study, reflect the

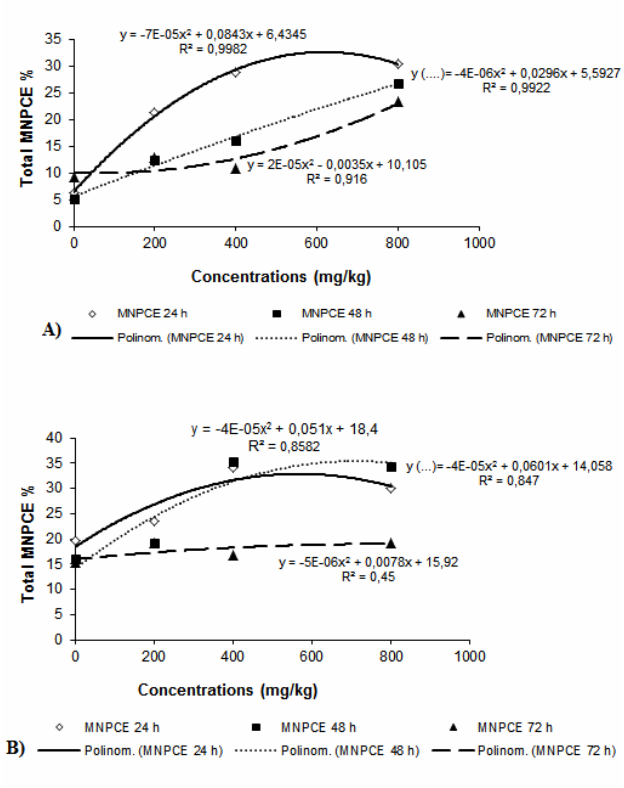


Fig. 6. Regression lines and correlation coefficient (r) of total MNPCE (%) in bone marrow cells of female (A) and male (B) mice at 6 h, 12 h and 24 h following treatment with natamycin.

cytotoxic potential of natamycin. Decreases in the MI (Meng and Zhang, 1994, 2002; Rencuzogullari *et al.*, 2001; Kumar and Panneerselvam, 2007; Rencuzogullari *et al.*, 2009) and PCE/NCE ratio (NTP, 1993; Shelby *et al.*, 1993; Isbrucker *et al.*, 2006) were also reported in previous researches on the cytotoxic effects of food preservatives.

In conclusion, the results of the present study show that a commercial formulation of natamycin was aneugenic and cytotoxic to mice bone marrow *in vivo*. For this reason it is necessary to be careful when using these chemicals in food as preservatives. However, additional *in vivo* and *in vitro* mutagenicity studies measuring different levels of DNA damage are still necessary.

ACKNOWLEDGEMENT

This study was supported by Trakya University Scientific Research Fund with the project number TUBAP-751 and it is part of doctoral thesis titled “Toxic effects of Delvocid on *Mus musculus*”.

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(Received 4 April 2013, revised 3 August 2013)