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Assessment of the Combined Effects of Acetamiprid and Propineb in Vivo

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ABSTRACT: Pesticides are among commonly used chemicals in agriculture and are one of major environmental pollutants. Acetamiprid and Propineb are widely used to control sucking insects and fungal infections on crops, respectively. The study presented aimed to research genotoxic effects of mixture of Acetamiprid and Propineb, in vivo. It was observed that mixture of Acetamiprid+Propineb increases the frequency of micronucleated polychromatic erythrocytes (MNPCE) at all concentrations for 24 and 48 h depending on concentrations. But these increases were not significant. The combined effect of the Acetamiprid and Propineb on bone marrow cells of mice in vivo was found to be antagonistic in terms of percentage of MNPCE. In addition, mixture of Acetamiprid and Propineb significantly decreased polychromatic erythrocytes/normochromatic erythrocytes (PCE/NCE) ratio at all concentrations. The results of the present investigation revealed that Acetamiprid was non-genotoxic, while mixture of the Acetamiprid and Propineb may have cytotoxic effects for mice bone marrow cells. But, additional in vivo and in vitro mutagenicity studies measuring different levels of DNA damage are still necessary.

Keywords: Mice bone marrow, micronucleus assay, mutagenicity, pesticide mixture

Acetamiprid ve Propinebin Kombine Etkilerinin in Vivo Değerlendirilmesi

ÖZET: Pestisitler tarımda yaygın olarak kullanılan kimyasallardır ve önemli çevre kirleticilerindendirler. Acetamiprid ve Propineb ürünlerde görülen fungal hastalıkları ve entomolojik patojenleri kontrol etmek için yaygın olarak kullanılırlar. Bu çalışmada Acetamiprid ve Propineb pestisit karışımlarının genotoksik etkilerinin in vivo araştırılması amaçlanmıştır. Acetamiprid+Propineb karışımının tüm konsantrasyonlarının 24 ve 48 saatlik muamelelerde konsantrasyonlara bağlı olarak mikronukleuslu polikromatik eritrosit (MNPCE) frekansını arttırdığı gözlendi. Fakat bu artışlar anlamlı bulunmamışlardır. Acetamiprid ve Propineb'in fare kemik iliği hücreleri üzerine olan kombine etkisinin MNPCE yüzdesi bakımından antagonistik olduğu bulunmuştur. Ayrıca, Acetamiprid ve Propineb karışımı polikromatik eritrosit/normokromatik eritrosit (PCE/NCE) oranını tüm konsantrasyonlarda anlamlı olarak azaltmıştır. Çalışmanın sonuçları Acetamiprid'in non-genotoksik olduğunu, Acetamiprid ve Propineb karışımının fare kemik iliği hücreleri için sitotoksik etkilere sahip olabileceğini göstermiştir. Fakat DNA hasarının farklı seviyelerini ölçen ilave in vivo ve in vitro mutajenite çalışmaları yapılması gereklidir.

Anahtar Kelimeler: Fare kemik iliği, mikronukleus yöntemi, mutajenite, pestisit karışımı

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INTRODUCTION

Pesticides have remained to be threat to the human, environment and other organisms for many years due to their bioaccumulations and persistence in the ecosystems. As much as 4.6 million tons of pesticides are released annually into the environment and this situation becomes a serious health concern, which has resulted in the adverse effects for all living creatures and environment (Zhang et al., 2011). Occupationally or incidentally, all living organisms are exposed to most of chemicals such as pesticides, their mixtures, and harmful gases in the air. The undesired effects of pesticides such as genotoxic, cytotoxic and carcinogenic have been showed (Costa et al., 2006; Pandey, 2008; Blair and Freeman, 2009; Kaymak and Rasgele, 2009; Kumar, 2010; Muranli and Guner, 2011).

Many investigations have been carried out on the genotoxic effects of individual pesticides (Giri et al., 2002; Rasgele and Kaymak, 2006; Kocaman and Topaktas, 2007; Costa et al., 2009; Kocaman et al., 2012; Srivastava et al., 2012). In addition, it is well known that the different effects such as additive, synergistic or antagonistic ones can be observed in the pesticide mixtures (Amorim et al., 2012; Schnug et al., 2014; Shaik et al., 2016; Taillebois and Thany, 2016). Therefore, the determination of genotoxic effects of pesticides mixtures as well as individual pesticides by using different organisms is crucial in environmental studies and combined effects of them should be considered to evaluate the genetic risk.

Acetamiprid (N-[(6-chloro-3-pyridyl) methyl]-N'-cyano-N-methyl-acetamidine) is a neonicotinoid insecticide and is used to control sucking insects on crops. Neonicotinoid insecticides are crucially potent neurotoxic insecticides that act as agonists on the nicotinic acetylcholine receptors (Tomizawa and Yamamoto, 1993). Although classified as an "unlikely" carcinogen for human, it has been reported to be clastogen in Chinese hamster ovary (CHO) cells (EPA, 2002). Furthermore, Kocaman and Topaktas (2007) have reported that Acetamiprid induced chromosome aberration (CA), sister chromatid exchange (SCE) and micronucleus (MN) formation in human peripheral lymphocytes. Propineb (Polymeric zinc 1,2-propylenebis (dithiocarbamate), belongs to the dithiocarbamate group of fungicides, is used as an effective agent in the control of plant diseases in a wide range of crops in agriculture (Soloneski et al., 2003). There are many negative results on the effects of Propineb in various test systems such as in Ames test with *Salmonella typhimurium*, in unscheduled DNA synthesis (UDS) test with rat hepatocytes, in the Hypoxanthine-guanine phosphoribosyltransferase (HGPRT) test with CHO cells, and in dominant lethal mutation test with mice (Watson, 1993). Furthermore, Rolandi et al. (1984) reported that it was observed no statistically significant increase in the frequency of micronuclei at any of tested doses of Propineb. Although classified as an "unlikely" acute hazard in normal use by World Health Organization, Propineb has moderate to low acute toxicity in mice, rats, hamsters, cats and sheep (Watson, 1993). It was observed that many pesticides in which propineb is implicit cause a significant increase in CA and MN frequencies of many people who use pesticides in agricultural areas (Bolognesi et al., 1993; Pasquini et al., 1996; Falck et al., 1999; Pastor et al., 2001; Pazy-Mino et al., 2002). In our earlier publication (Rasgele et al., 2014), it was showed in mice that Propineb induced significantly formation of micronucleus at 25 and 50 µg mL^{-1} concentrations for 24 h and at the highest (50 µg mL^{-1}) concentration for 48 h. Moreover, significant decline for PCE/NCE ratio was obtained at the same concentrations for 24 and 48 h. Numerous genotoxicity markers such as gene mutation assay, chromosome aberration assay and DNA damage assay have been developed for the detection of early biological effects induced by pesticides (Sato and Tomita, 2001). Micronucleus (MN) assay is a tool of great interest in toxicity risk assessment due to its simplicity, accuracy, wide tissue applicability and has been recently used for identification of genotoxic effects (Heddle, 1973; Schmid, 1975; Decordier and Kirsch-Volders, 2006). An increase in the frequency of MNPCE and a decrease in PCE/NCE ratio in treated animals determine genotoxicity and cytotoxicity, respectively (Heddle, 1973).

Acetamiprid and Propineb are commonly used on agricultural crops such as tomato, potato, melon, apple, tobacco, either separately or in combination (Karaca et al., 2009). But, there are a few studies on the genotoxicity of Acetamiprid and Propineb (Rolandi et al. 1984; Barrera et al., 2008; Kocaman and Topaktas, 2010; Cavas et al., 2012), there is no available investigation about mutagenicity of mixtures of Acetamiprid and Propineb *in vivo* in bone marrow cells of *Mus musculus*. The aim of this study was to investigate the frequencies of micronucleated erythrocytes following exposure mixtures of Acetamiprid and Propineb in bone marrows cells of mice using micronucleus assay due to commonly use of these pesticides and lack of information about their genotoxicities *in vivo*. Although we have published our results related to effect of Propineb (Rasgele et al., 2014), the data from that publication will be used to be able to make clear explanations and discussions in this paper because pesticide mixture we used has Propineb.

MATERIAL AND METHODS

Chemicals: In this study, the trading formula of Acetamiprid (containing 20% as active agent; CAS No. 135410-20-7) and Propineb (containing 70% as

active agent; CAS No. 12071-83-9) were used as the test materials. The commercial formulations of Acetamiprid and Propineb were purchased from Safa Agriculture and Bayer from Turkey, respectively. The chemical structures of Acetamiprid and Propineb are shown in Figures 1 and 2. Giemsa (CAS No. 51811-82-6) and May Grunwald (CAS No.17372-87-1) was obtained from Merck®. Mitomycin C (MMC; CAS No. 50-07-7) was used as the positive control while distilled water was used as the negative control. All test solutions were prepared just before each experiment.

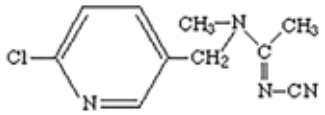

Figure 1. Chemical structure of acetamiprid

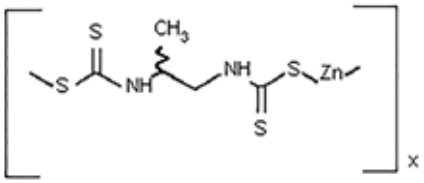

Figure 2. Chemical structure of propineb

Selection of Concentrations: The concentrations used were selected according to the results of a preliminary study. In the preliminary study, the concentrations were selected on the basis of doses used against diseases on crops such as tomato, potato, melon, apple, and tobacco (Karaca et al., 2009). The concentrations of mixtures of Acetamiprid and Propineb that were dissolved in water (0.625+12.5; 1.25+25; 2.5+50; 5+100; 10+200) μg mL^{-1} were used. In the preliminary study, it was observed that the mixtures of Acetamiprid and Propineb exhibited high cytotoxic effects in their two highest concentrations (5+100; 10+200) μg mL^{-1} and decreased the ratio of dividing cells at these concentrations in 48h treatment period. Based on the cytotoxicity of the test chemicals, the first three concentrations (0.625+12.5; 1.25+25; 2.5+50) μg mL^{-1} were determined as the concentrations to be tested in this study. In addition, pesticides were also tested separately in order to determine whether these pesticides would become effective or not when they are alone.

Experimental Animals: Experiments were performed on 8-10 week old male *Mus musculus* obtained from Abant Izzet Baysal University Experimental Animals Applications and Research Center, Turkey. Mice kept in polyethylene boxes, in controlled environment of temperature, humidity and light provided by the Abant Izeet Baysal Center where experiments were carried out. The experiment was approved by the Ethics Committee of Abant Izzet Baysal University in Turkey.

Fifteen groups were set with 180 mice were randomly allocated. Each group had 12 mice which half of them kept treated 24 hours the other half 48 hours. The groups were as follows:

* *Acetamiprid test*
- Group I (negative control) with distilled water
- Group II (positive control) with MMC (0.2 μg mL^{-1})
- Group III with Acetamiprid (0.625 μg mL^{-1})
- Group IV with Acetamiprid (1.25 μg mL^{-1})
- Group V with Acetamiprid (2.50 μg mL^{-1})

* *Propineb test*
- Group VI (negative control) with distilled water
- Group VII (positive control) with MMC (0.2 μg mL^{-1})
- Group VIII with Propineb (12.5 μg mL^{-1})
- Group IX with Propineb (25 μg mL^{-1})
- Group X with Propineb (50 μg mL^{-1})

* *The mixture of Acetamiprid and Propineb test*
- Group XI (negative control) with distilled water
- Group XII (positive control) with MMC (0.2 μg mL^{-1})
- Group XIII with mixture of Acetamiprid and Propineb (0.625+12.5 μg mL^{-1})
- Group XIV with mixture of Acetamiprid and Propineb (1.25+25 μg mL^{-1})
- Group XV with mixture of Acetamiprid and Propineb (2.50+50 μg mL^{-1})

Micronucleus (MN) Assay: Acetamiprid at concentrations of (0.625, 1.25 and 2.50) μg mL^{-1} and and Propineb at c oncentrations of (12.5, 25 and 50) μg mL^{-1} was given by i.p. route a single injection (0.01 mL per gram of animal) for 24 and 48h. In addition, their mixture was administrated at the same test concentrations over the

same period. Slides were prepared by the method described by Schmid (1975) and Aaron et al. (1989) with minor revisions as shown below. The cells were removed from bone marrow with fetal calf serum, and the homogenate was centrifuged for 10 min at 2000 rpm. The pellets were resuspended in a drop of serum, plastered it on a slide glass, fixed with methanol and stained with May Grunwald for 3 min, May Grunwald:distilled water (1:1) for 2 min, 10 % Giemsa in Sorensen buffer for 10 min (Rasgele et al., 2014).

Measurement of Micronucleus and PCE/NCE: A total of 2000 erythrocytes were scored for each animal at a magnification of x1000. The numbers of MNPCE and MNNCE were counted. PCE/NCE ratio was calculated.

Statistical Analysis: The data were analysed by using SPSS 20 for Windows and results obtained were expressed as mean ± SE. The Kruskal-Wallis test was performed followed by the Mann-Whitney U test to compare the statistical significance of the differences between treated and control groups.

Exposure-response relationship was identified using Pearson correlation analysis. $P<0.05$ was considered as the level of significance. Measured values were compared with expected values. The expected mean value and SE were calculated as following (Klaric et al., 2008). Mean % (expected for Acetamiprid+Propineb) = mean % (Acetamiprid) + mean % (Propineb) - 100% (control)

SE (expected for Acetamiprid+Propineb) = $[(SE \text{ for Acetamiprid})^2 + (SE \text{ for Propineb})^2]^{1/2}$

The non-parametric Mann Whitney U test was used to detect significance of difference between expected and measured values. Additive, synergistic and antagonistic effects were evaluated to interpret effects of mixtures.

Measured values	>	Expected values (insignificantly)	→ Additive effect
Measured values	>	Expected values (significantly)	→ Synergistic effect
Measured values	<	Expected values (significantly)	→ Antagonistic effect

RESULTS and DISCUSSION

Experiments were carried out to determine the effects of mixture of two pesticides on micronucleus formation on bone marrow cells as well as sole application of these pesticides.

An increase in the frequency of MNPCE in treated animals determines genotoxicity (Heddle, 1973). According to this, Acetamiprid did not significantly increase MNPCE frequency at any concentrations. In our earlier publication (Rasgele et al., 2014), Propineb induced significantly formation of micronucleus at 25 and 50 µg mL^{-1} concentrations for 24 h and at the highest (50 µg mL^{-1}) concentration for 48 h. In the present study, the mixture of Acetamiprid and Propineb showed an antagonistic effect at the all treatment times and concentrations except 0.625+12.5 µg mL^{-1} mixture treatment for 24h (Table 1). Increases in the frequency of MNPCE were in a dose-dependent.

A decrease in PCE/NCE ratio is indicative for bone marrow cytotoxicity (Heddle, 1973). 1.25 µg mL^{-1} concentration of Acetamiprid significantly decreased PCE/NCE ratio for 24h compared with negative control, but not in others. In our earlier publication (Rasgele et al., 2014), significant reduction for the PCE/NCE ratio was observed at 25 and 50 µg mL^{-1} concentrations of Propineb for 24 h and at the highest (50 µg mL^{-1}) concentration for 48 h. Moreover, the mixture of Acetamiprid and Propineb showed a significant synergistic effect at the all concentrations and treatment times due to reduction of PCE/NCE ratio compared to negative control (Table 1).

The data were underwent to linear regression analysis which fit well to define the exposure-response for 24 and 48 h. Acetamiprid, Propineb and mixture of them caused a significant dose-dependent decrease of the PCE/NCE ratio for 24 and 48h (Figure 3).

In order to detect the combined actions such as additive, and antagonistic of compounds, the expected mean value and SE were calculated and measured values were compared to expected values (Klaric et al., 2008). The measured % MNPCE was significantly below the expected values, that is, antagonistic effect was observed at all concentrations of mixture for 24 and 48 h treatments (Figure 4). The measured % PCE/NCE ratio was significantly below the measured values, that is, synergistic effect was observed at all concentrations of mixture for 24 and 48 h treatments (Table 1).

The study presents the first in vivo evidence for the genotoxicity of mixtures of Acetamiprid and Propineb in bone marrow cells of mice. The results of the present study revealed that mixture of Acetamiprid+Propineb increase the frequency of MNPCE at all concentrations for 24 and 48 h depending on concentrations. But these increases were not significant.

Table 1. A comparison of the between combined and individual effects of Acm and Pro in bone marrow cells of mice for 24 and 48 h treatment times

Test substance	Total cell number/ mice number	Concentrations (µg mL-1)	% MNPCE (mean±SE)		PCE/NCE (mean±SE)	
			24 h	48 h	24 h	48 h
Negative control	12000/6	-	24.00 ± 1.46	20.66 ± 0.84	1.78 ± 0.08	1.75 ± 0.07
Positive control	12000/6	0.2	49.66 ± 1.81**	51.66 ± 1.74**	0.66 ± 0.03**	0.64 ± 0.02***
Acm	12000/6	0.625	17.33 ± 2.23	20.00 ± 2.00	1.81 ± 0.10	1.60 ± 0.11
	12000/6	1.25	21.66 ± 1.58	22.66 ± 1.22	1.51 ± 0.06*	1.48 ± 0.13
	12000/6	2.5	29.66 ± 3.44	27.33 ± 2.71	1.87 ± 0.15	1.80 ± 0.16
Negative control	12000/6	-	17,66 ± 0,95	18,66 ± 0,84	1,66 ± 0,11	1,59 ± 0,06
Positive control	12000/6	0.2	51,66 ± 5,64**	45,00 ± 3,37***	0,71 ± 0,05***	0,67 ± 0,02***
Pro[a]	12000/6	12.5	17,00 ± 1,52	20,33 ± 1,89	1,39 ± 0,17	1,66 ± 0,06
	12000/6	25	21,33 ± 1,11*	21,33 ± 1,68	1,01 ± 0,10**	1,65 ± 0,03
	12000/6	50	50,66 ± 6,60**	33,00 ± 2,11***	0,94 ± 0,15**	1,19 ± 0,08**
Negative control	12000/6	-	16.00 ± 0.73	16.33 ± 1.40	1.68 ± 0.10	1.61 ± 0.06
Positive control	12000/6	0.2	53.33±4.72***	46.33 ± 3.59***	0.67 ± 0.03***	0.66 ± 0.01***
Mix of Acm and Pro	12000/6	0.625 + 12.5	9.33 ± 1.52	7.33 ± 1.90*[c]	1.29 ± 0.10**	1.22 ± 0.12*
	12000/6	1.25 + 25	8.33 ± 0.95[d]	10.66 ± 2.71[c]	0.92 ± 0.61***	0.67 ± 0.03***
	12000/6	2.5 + 50	12.00 ±1.03[d]	14.33 ± 1.81[d]	0.82 ±0.65***	0.65 ± 0.03***

[a]Rasgele et al. (2014). Acm: Acetamiprid; h: hour; MNPCE: Micronucleated polychromatic erythrocyte; NCE: Normochromatic erythrocyte; PCE: Polychromatic erythrocyte: Pro: Propineb; SE: Standard error.
*[b]p≤0.05; **[c]p≤0.01; ***[d]p≤0.001
* As compared to the negative control value,
b, c, d Each substance alone as compared to a combination of two pesticide.

There are many studies on pesticide mixtures' poisoning because of occupational and environmental reasons; but, no available investigation about genotoxicity of mixtures of Acetamiprid and Propineb in vivo in bone marrow cells of mice had not been found in the literature. The mixture of Acetamiprid and Propineb showed antagonistic action in bone marrow cells of mice. Our results were in parallel with the reports of Santamaria et al., (1997) and Piatti et al., (1994), which mixture of different insecticides and fungicides showed antagonistic effect. In the contrary many researches have been reported that the combinations of pesticide showed synergistic effect in bone marrow cells of mice (Meisner et al., 1992; Chauhan et al., 2005; Karabay and Oguz, 2005; Demsia et al., 2007; Sekeroglu et al., 2013) and human peripheral blood lymphocytes (Dolara et al., 1992; Roloff et al., 1992; Das et al., 2007; Demsia et al., 2007; Kocaman and Topaktas, 2010; Muranli et al., 2015). The differences of chemical structure of pesticides and different test systems used in these investigations may be responsible for the different genotoxic results of pesticide mixtures.

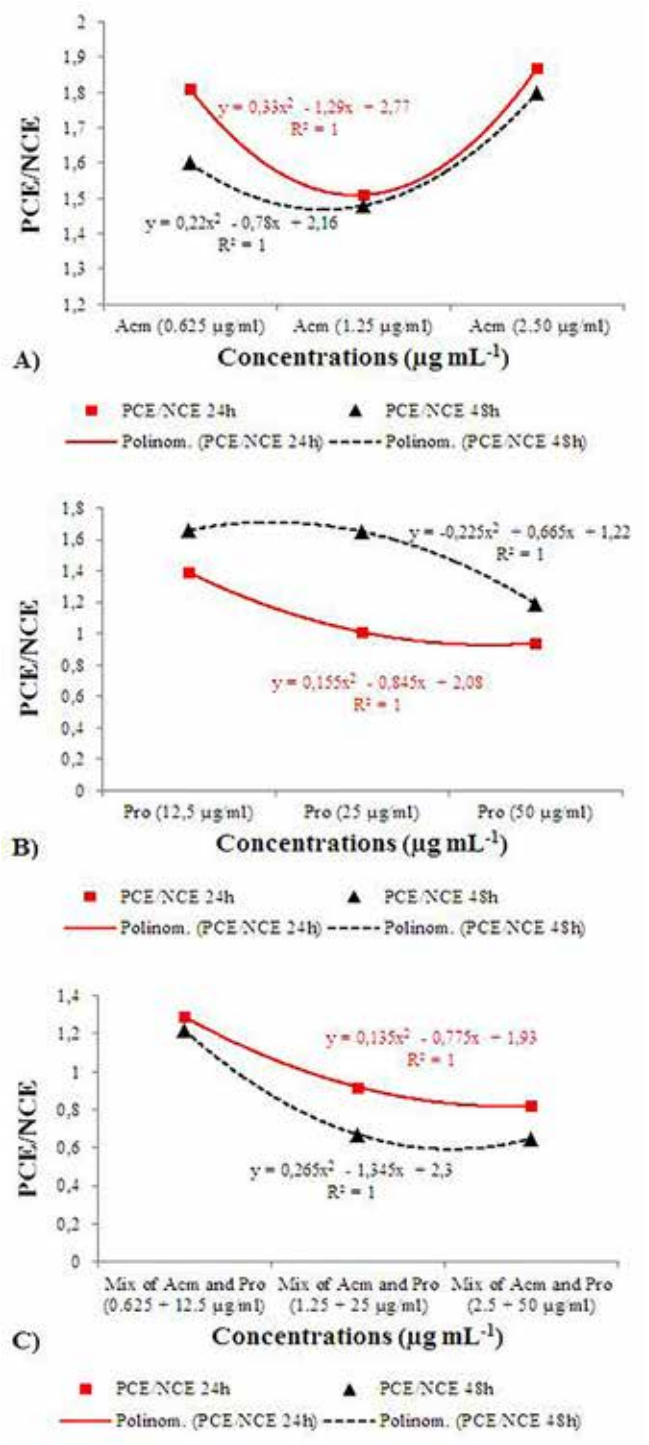

Figure 3. Dose related decrease of PCE/NCE ratio after exposure of Acetamiprid, Propineb and mixture of them for 24 and 48 h.

Propineb individually showed genotoxic effect in mice bone marrow cells (Rasgele 2014); but, Acetamiprid did not show any genotoxic affect. It can be found similar results in earlier literature (Rasgele et al., 2014). These findings could be attributed to different mechanisms involved pesticide action and chemistry.

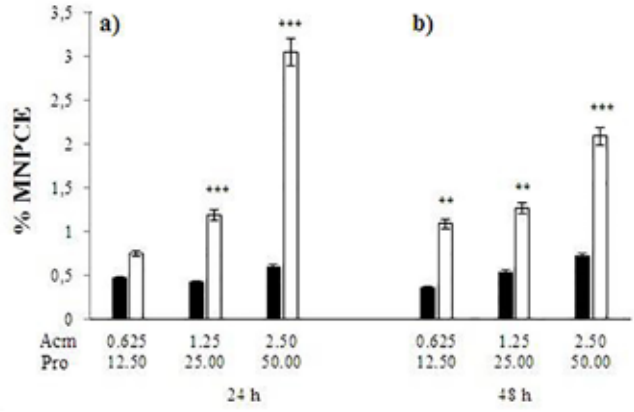

Figure 4. Percentage of micronucleated polychromatic erythrocytes (MNPCE) (Mean ± S.E.) in bone marrow cells of mice exposed to mixtures of Acetamiprid and propineb (Propineb) for 24 (a) and 48 h (b). Dark and white bars represent the measured values and the expected values, respectively. ** and *** represent significant antagonistic effects, respectively ($P < 0.05$)

The mechanisms of genotoxicity of Acetamiprid and Propineb are not yet known. It was reported that Acetamiprid induced reactive oxygen species (ROS) generation in three bacteria species (Yao et al., 2006) and plants (Ford et al., 2011). Moreover, Jie et al., (2003) have indicated that Acetamiprid might interact with DNA through a non-intercalative way. Guven et al. (1998) have reported that Propineb, as all dithiocarbamates, interferes with the synthesis and metabolism of proteins, due to its isocyanic metabolites and so these intermediates cause the activation/inactivation of sulphidril groups (-SH) present in aminoacids, proteins and enzymes (Lages et al., 2009). In addition, Rath et al., (2011) have noticed that the dithiocarbamate anions are highly reactive which can conjugate with other molecules containing SH groups and form metal chelates. The multisite interactions of dithiocarbamate give them advantage to influence the biological activities of different proteins, enzymes, and exert toxic effects. However, it is also known that the effects of mixtures of pesticide may differ from the individual effects of each pesticide (Marinovich et al., 1996).

CONCLUSIONS

The combined effect of the Acetamiprid and Propineb on bone marrow cells of mice in vivo was found non genotoxic in spite of genotoxic effect of Probineb alone. But, Acetamiprid and Propineb pesticides have cytotoxic effect when used in combination. Mixtures studies are very important to evaluate exposure to these compounds.

Occupational and environmental pesticide intoxication have threatened both public and environment health because of their excessive and unconscious uses. For this reason, it should be necessary to be careful when using these chemicals in agricultural areas and should take precautions.

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