

Original article

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Abnormal sperm morphology in mouse germ cells after short-term exposures to acetamiprid, propineb, and their mixture

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Pesticides are one of the most potent environmental contaminants, which accumulate in biotic and abiotic components of ecosystems. Acetamiprid (Acm), a neonicotinoid insecticide, and Propineb (Pro), a dithiocarbamate fungicide, are widely used to control sucking insects and fungal infections on crops, respectively. The present study was undertaken to investigate the genotoxic effects of these compounds, individually and in mixtures, in mouse germ cells by using the sperm morphology assay. Mice were injected intraperitoneally with 0.625, 1.25, and 2.50 $\mu\text{g mL}^{-1}$ of Acm, 12.5, 25, and 50 $\mu\text{g mL}^{-1}$ of Pro, and their mixture at the same concentrations over 24 and 48 h. Acm did not significantly increase the percentage of abnormal sperm at any concentration. The frequency of abnormal sperm significantly increased after 24 and 48 h of exposure to 50 $\mu\text{g mL}^{-1}$ of Pro. The mixtures of 2.50 $\mu\text{g mL}^{-1}$ of Acm and 50 $\mu\text{g mL}^{-1}$ of Pro induced sperm abnormalities antagonistically both after 24 and 48 h of exposure. Results suggest that Acm was non-genotoxic for mouse germ cells, while Pro may have been a germ cell mutagen due to the observed increase in the frequency of sperm abnormalities. However, to gain better insight into the mutagenicity and DNA damaging potential of both of these pesticides, further studies at molecular level should be done.

KEY WORDS: *combined effects; pesticide mixture; sperm morphology assay*

At present times, the consumption and types of pesticides used in agricultural production is increasing considerably throughout the world due to the rise in population and crop production. As much as 4.6 million tons of pesticides are released annually into the environment and approx. 500 types of these pesticides are considered a threat to the environment and human beings (1).

Pesticides are commonly used in agricultural areas as single compounds or in mixtures. However, many are non-biodegradable and therefore inevitably expose people occupationally or environmentally to pesticide residues, through air, water, and food. Therefore, studies on the mutagenic activity of pesticide mixtures

using different assays on the genetic system of a living organism are vital.

Many investigations have focused on the evaluation of genotoxicity of pesticides in occupational or environmental settings using different methods (2-12). It has been reported that exposure to environmental contaminants including pesticides causes major pathological effects in the human male reproductive system as well as that of experimental animals (13, 14). The sperm morphology assay is one of the most widely used genetic toxicology assays. Furthermore, the development of sperm head abnormality has been used as a reliable short-term biological indicator in the evaluation of chemical genotoxicity (15-17). The

mouse sperm morphology test also has potential in identifying chemicals that induce spermatogenic dysfunction and perhaps heritable mutations (18).

Acetamiprid (Acm), a neonicotinoid insecticide, is widely used to control sucking insects on crops (19). Neonicotinoids are crucially potent neurotoxic insecticides that act as agonists of nicotinic acetylcholine receptors (nAChR) (20). Propineb (Pro), a dithiocarbamate fungicide, is commonly used for disease control in a wide range of crops (21). Dithiocarbamate fungicides are mainly used for the eradication of fungal infections on plants, fruit, and vegetables (22).

Commercial formulations of Acm and Pro are commonly used on agricultural crops such as tomato, potato, melon, apple, and tobacco, either separately or in mixture (23). Mixtures of chemicals are often well-known to be considerably more toxic than its individual components and their combined effect can be additive, synergistic, or antagonistic (9).

Many researchers have reported that pesticides caused genotoxic effects when used individually (8, 10, 11, 15). However, in reality all living organisms are exposed to not only to individual chemicals, but also to their mixtures. Additive, synergistic or antagonistic effects of pesticide mixtures are due to the interactions between the chemicals that comprise them. Thus, in mutagenicity and carcinogenicity studies, the combined use of substances investigated provides results that are much more significant for establishing genotoxic effects. Although there are a few studies focused on the genotoxicity of Acm and Pro (9, 21, 24, 25), the mutagenicity of their mixtures *in vivo* in mouse germ cells of mice has not been investigated, which motivated us to use the sperm morphology assay to perform precisely this type of study.

MATERIALS AND METHODS

Chemicals

In the present study, commercial formulations of Acm (Mosetam 20 SP[®], containing 20 % acetamiprid as active ingredient; CAS No. 135410-20-7) and Pro (Antracol 70 WP[®], containing 70 % propineb as active ingredient; CAS No. 12071-83-9) were used as the test materials. Mosetam 20 SP[®] was obtained from Safa Agriculture (Turkey) and Antracol 70 WP[®] from

Bayer (Turkey). The chemical structures of Acm and Pro are shown in Figures 1 and 2. Giemsa dye (CAS No. 51811-82-6) was obtained from Merck[®] (Darmstadt, Germany). Mitomycin C (MMC; CAS No. 50-07-7) was obtained from Sigma[®] (Taufkirchen, Germany). All test solutions were freshly prepared prior to each experiment.

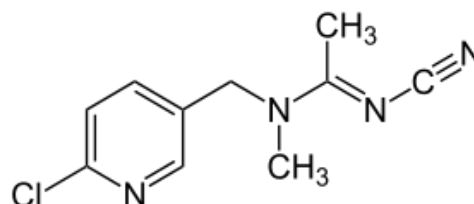


Figure 1 Chemical structure of acetamiprid

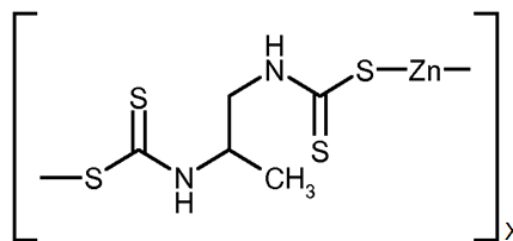


Figure 2 Chemical structure of propineb

Experimental animals

In this study, 90 male mice (*Mus musculus*, 8-10 weeks of age, with average body weight of 20-25 g), were used. They were purchased from Abant Izzet Baysal University Experimental Animals Applications and Research Center, Turkey. The animals were maintained in a closely inbred colony under conventional laboratory conditions at a room temperature of 25 ± 5 °C and in 12 h dark and 12 h light cycles. Food pellets and water were provided *ad libitum*. The experiment was approved by the Ethics Committee of Abant Izzet Baysal University in Turkey.

Study design

The used concentrations of pesticides were selected according to the results of a preliminary study. Based on the pesticide concentrations used to treat plant disease (23). Acm and Pro were dissolved in water in order to obtain the following mixtures: $0.625 \mu\text{g mL}^{-1}$ of Acm + $12.5 \mu\text{g mL}^{-1}$ of Pro; $1.25 \mu\text{g mL}^{-1}$ of Acm + $25 \mu\text{g mL}^{-1}$ of Pro; $2.5 \mu\text{g mL}^{-1}$ of Acm + $50 \mu\text{g mL}^{-1}$ of Pro; $5 \mu\text{g mL}^{-1}$ of Acm + $100 \mu\text{g mL}^{-1}$ of Pro; $10 \mu\text{g mL}^{-1}$ of Acm + $200 \mu\text{g mL}^{-1}$ of Pro. It was observed that the mixtures of Acm and

Pro exhibited high cytotoxic effects at two of the highest concentrations (5 µg mL⁻¹ of Acm + 100 µg mL⁻¹ of Pro; 10 µg mL⁻¹ of Acm + 200 µg mL⁻¹ of Pro) and also decreased the ratio of dividing cells at these same concentrations over a 48 h treatment period. Therefore, these two mixtures were discarded and the remaining three were selected to be tested in this study. In addition, Acm and Pro were also tested separately in order to determine whether these pesticides are spermatotoxic individually.

In the present study, mice were intraperitoneally (*i.p.*) injected with Acm and Pro at concentrations of (0.625, 1.25, and 2.50) µg mL⁻¹ and (12.5, 25, and 50) µg mL⁻¹, respectively, over 24 and 48 h. In addition, their mixture was administered at the same test concentrations over the same period.

MMC was used as a positive control, as it was previously confirmed as a mutagenic agent (15). The significant increase in the number of sperm abnormalities in this group showed that the method was correctly applied. Distilled water was used as a negative control. The *i.p.* route was favoured since it is one of the fastest and most efficient means of delivering test chemicals in a short-term assay (26). Ninety male mice were randomly allocated into fifteen groups (n=3 per group) and treated over 24 and 48 h. These groups are given in Table 1.

Sperm morphology assay

After the treatment, the animals were sacrificed by cervical dislocation. Both of the cauda epididymises were dissected out, cut into pieces in 5 mL of saline, filtered, and smears were made according to the standard protocol for sperm morphology assay, as proposed by Wyrobek (18, 27). The smears were fixed in methanol and stained with 10 % Giemsa in Sörensen buffer for 10 min. A total of 1000 sperms per animal were scored under a microscope (Olympus CX21®, Japan) with 100x10 magnification. Sperm head abnormalities were determined as having either normal or abnormal morphology according to Wyrobek and Bruce (27). According to these criteria, a “hookless head” does not have a spherical spot at the tip of the sperm head; a “banana head” has a banana-like form; an “amorphous head” lacks the usual hook and is deformed; and a “folded sperm” is folded on itself.

Statistical analysis

The data were analysed by using SPSS 20 for Windows (SPSS Inc., Chicago, IL, USA) and results

Table 1 Study design

Test group	Chemical	Concentration (µg mL ⁻¹)
I	Distilled water	-
II	MMC	0.200
III	Acm	0.625
IV	Acm	1.250
V	Acm	2.500
VI	Distilled water	-
VII	MMC	0.200
VIII	Pro	12.50
IX	Pro	25.00
X	Pro	50.00
XI	Distilled water	-
XII	MMC	0.200
XIII	Acm + Pro	0.625 + 12.50
XIV	Acm + Pro	1.250 + 25.00
XV	Acm + Pro	2.500 + 50.00

All of the treatments were performed over 24 and 48 h

obtained were expressed as mean±standard error (SE) of three individual analyses. The Kruskal-Wallis test was carried out followed by the Mann-Whitney U test to compare the statistical significance of the differences between the treated and control groups. The dose-response relationship was determined using Pearson correlation analysis. $P < 0.05$ was considered significant. The measured values were also compared with the expected values. The expected mean value and SE were calculated as follows (28): mean % (expected for Acm + Pro) = mean % (Acm) + mean % (Pro) = 100 % (control); SE (expected for Acm + Pro) = [(SE for Acm)² + (SE for Pro)²]^{1/2}.

Non-parametric Mann-Whitney U test was used to detect the significance of difference between the expected and measured values. Additive, synergistic and antagonistic effects were evaluated to interpret effects of mixtures. When the measured values were insignificantly higher than the expected values, there was an additive effect. When the measured values were significantly higher than the expected values, there was a synergistic effect, whereas when the measured values were significantly lower than the expected ones, there was an antagonistic effect (28).

RESULTS

The results obtained by the sperm morphology assay are presented in Table 2. Acm induced different types of sperm abnormalities such as “hookless”, “banana”, “amorphous”, and “folded” sperms at all concentrations for 24 and 48 h when compared with the negative control (Figure 3), but these increases were not statistically significant. Pro also increased the frequency of abnormal sperm during the same treatment periods. The frequency of abnormal sperm induced by Pro was significant at the highest concentration ($50 \mu\text{g mL}^{-1}$) for 24 and 48 h when compared with the negative controls. As shown in Figure 4, these increases were concentration-dependent (for 24 h: $R^2=0.8033$, $p<0.01$ and for 48 h: $R^2=0.9488$, $p<0.05$). “Hookless head” was the most common abnormality observed at the highest concentration for 24 and 48 h.

It was also observed that the mixture of Acm and Pro did not induce sperm head abnormalities at any of the concentrations over 24 and 48 h when compared with the negative controls. In addition, there was no significant difference between the two time points

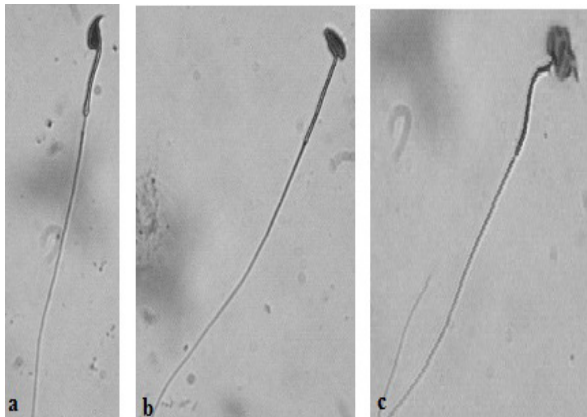


Figure 3 a) Normal sperm; b) Without hook (at $50 \mu\text{g mL}^{-1}$ of Pro for 24 h); c) Amorphous head sperm (at the highest concentration of mixture for 48 h) (x1000)

(Acm 24 h vs. Acm 48 h, Pro 24 h vs. Pro 48 h, etc.) regarding abnormal sperm frequency.

The results of individually applied pesticides were also compared to results for their mixture at the same concentrations. Interestingly, it was found that the highest concentration ($2.5 \mu\text{g mL}^{-1}$ of Acm + $50 \mu\text{g mL}^{-1}$ of Pro) significantly decreased the percentage of abnormal sperm for 24 and 48 h when compared with the individual concentrations of Pro ($50 \mu\text{g mL}^{-1}$) in both treatment periods.

In order to detect the possible combined actions of the pesticides, the expected mean value and SE were calculated and measured values were compared to the expected values. According to the results, the mixture of Acm and Pro at treatment durations

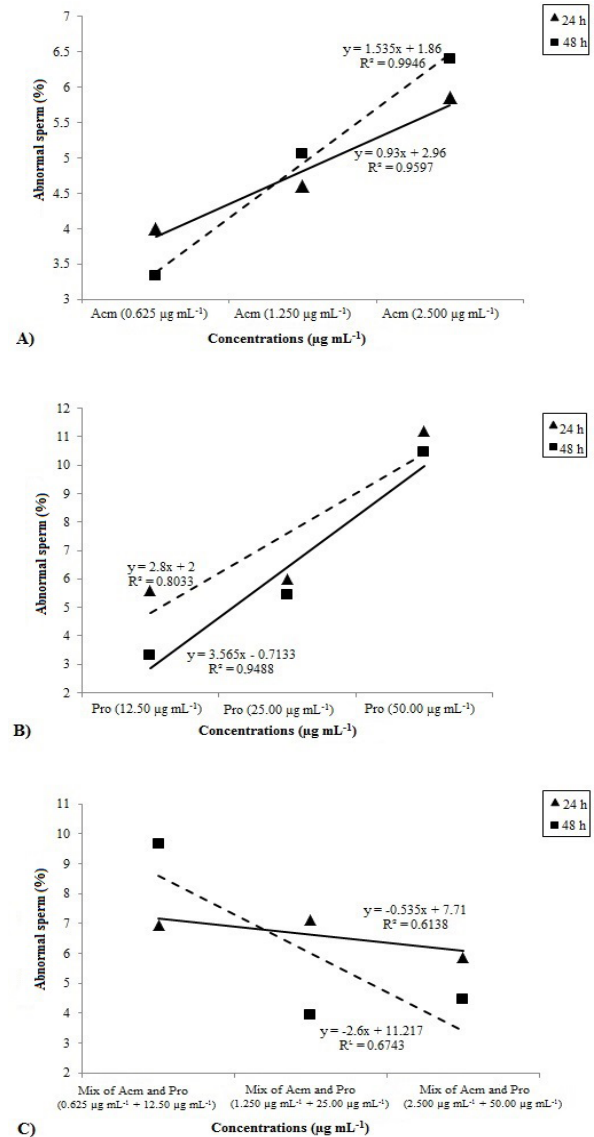


Figure 4 Graphs showing concentration-response curve slopes and correlation coefficients (R^2) of % abnormal sperm in mice germ cells treated with Acm, Pro, and their mixture for 24 and 48 h

produced mostly additive effects. However, it was found that the measured abnormal sperm (%) was significantly above the expected values, that is, a synergistic effect was observed at the lowest concentration of the mixture ($0.625 \mu\text{g mL}^{-1}$ of Acm + $12.5 \mu\text{g mL}^{-1}$ of Pro). Also, an antagonistic effect was observed at the highest mixture concentration (Figure 5).

Table 2 Effects of acetamiprid, propineb, and their mixture on sperm morphology in *Mus musculus*

Test group	Abnormal sperm (%)	
	24 h	48 h
I	5.10±0.30	5.13±0.37
II	26.63±0.63*	28.60±0.66*
III	4.00±0.80	3.33±0.69
IV	4.60±0.30	5.06±0.40
V	5.86±0.40	6.40±1.30
VI	4.86±0.35	5.20±0.30
VII	26.70±0.60*	28.60±0.69*
VIII	5.60±0.50	3.33±0.48
IX	6.00±0.50	5.46±0.29
X	11.20±0.91*	10.46±1.23*
XI	4.76±0.39	5.33±0.37
XII	26.40±0.50*	28.80±1.15*
XIII	6.93±2.68	9.66±2.36
XIV	7.13±1.27	3.93±0.69
XV	5.86±1.00 ^a	4.46±0.33 ^a

Data are reported as mean value ± SE

The treatments are explained in detail in Table 1

* p<0.001, when compared with negative controls

^a (p<0.01) each pesticide alone as compared to the mixture of Acm and Pro

DISCUSSION

The present study reports for the first time *in vivo* genotoxicity of combinations of Acm and Pro on sperm in germ cells of mice. The results of the present study revealed that Acm slightly, but not significantly, increased the percentage of abnormal sperm in mouse germ cells at all concentrations (0.625, 1.25 and 2.50) µg mL⁻¹ and exposure times (24 and 48 h). These findings suggest that Acm might not be a germ cell mutagen. According to the United States Environmental Protection Agency (US EPA) (29), Acm is classified as an “unlikely” human carcinogen. It has relatively low acute and chronic toxicity in mammals and there is no evidence of carcinogenicity, neurotoxicity, mutagenicity, and/or endocrine disruption. However, studies (8, 9) have reported genotoxic effects for Acm and other neonicotinoid insecticides (30-33). For instance, Bal et al. (34) reported that the neonicotinoid clothianidin induced impairment of the male mouse reproductive system. Such claims are inconsistent with the results of this study.

In the present study, Pro increased the percentage of abnormal sperm in mouse germ cells at the highest concentration (50 µg mL⁻¹) for 24 and 48 h. Quinto and De Marinis (35, 36) reported that Pro did not increase the percentage of abnormal sperm in mice. These reports are also in disagreement with our results.

However, the results of the present study support previous findings obtained by many other researchers. Prasad et al. (37) have reported that thiram increased the frequency of abnormal sperm in germ cells of mice. Hemavathi and Rahiman (38) stated that the commonly used dithiocarbamate fungicides ziram, thiram, and dithane M-45 induced a significant increase in the frequency of abnormal sperm at all tested doses. Giri et al. (39) have observed that the highest concentration of carbosulfan induced a >7-fold increase in the frequency of abnormal sperm. Ma et al. (40) found that the total sperm morphological abnormality was significantly higher in carbon disulphide-exposed workers.

There are no available investigations about the mutagenicity of Acm and Pro mixtures in germ cells of mice. Kocaman and Topaktas (9) have reported that the mixture of Acm and α-cypermethrin synergistically induced genotoxicity/cytotoxicity in human peripheral blood lymphocytes. Many researchers have found various pesticides, including Pro, to cause genotoxicity in peripheral blood lymphocytes of people applying pesticides in agriculture (2, 41-44). Furthermore, there are many investigations about the genotoxic effects of combinations of different pesticides (45-52).

For the sperm morphology assay, the treatment period had a wide time range due to the process of spermatogenesis. An understanding of the short-term, as well as the long-term, effects is essential if the causes of infertility and testicular atrophy are to be revealed cytogenetically. Many investigations evaluated effects of different chemicals using short-term exposure (14, 53-56). This was why, in the present study, 24 and 48 h applications were chosen for the short-term assay.

The exact reason of the increase in the frequency of abnormal sperm was not clear and opinions on this subject differ. The induction of abnormal sperms was assumed to be a result of an abnormal chromosome (57), minor alteration in testicular DNA (15), and point mutation (58). According to several studies (59-62), small deletions, point mutations, and abnormal chromosomes were proposed as possible genetic causes of such alterations. Bruce and Heddle (63) attributed the occurrence of sperm head abnormalities

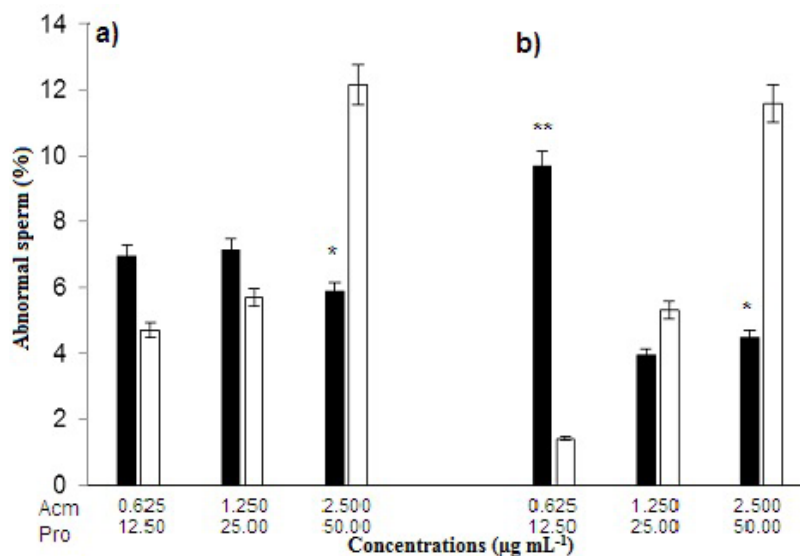


Figure 5 Percentage of abnormal sperm (Mean±SE) in germ cells of mice exposed to mixtures of acetamiprid (*Acm*) and propineb (*Pro*) for 24 (a) and 48 h (b). Dark and white bars represent the measured values and the expected values, respectively. * and ** represent significant antagonistic and synergistic effects, respectively ($p < 0.05$)

to the chromosomal aberrations that occur during the packaging of genetic material in the sperm head or occurrence of point mutation in testicular DNA. Several studies (64-67) reported that abnormalities may also arise as a consequence of mistakes in the spermatozoa-differentiating process during spermatogenesis. Another two studies (66, 67) reported that abnormalities in sperm heads may occur by physiological, cytotoxic or genetic mechanisms or alterations in testicular DNA which in turn disrupts the process of differentiation of spermatozoa.

Conclusion and limitations

The results of the present study suggest that under the experimental conditions studied, *Acm* was non-genotoxic for mouse germ cells. *Pro*, however, may be a germ cell mutagen due to the observed increase in frequency of sperm abnormalities. Furthermore, higher concentrations of the *Acm* and *Pro* mixture antagonistically induced genotoxic effects of them on germ cells of mice. However, it also has to be mentioned that this study was limited by its use of commercially available pesticide formulations. Some of the effects observed in the present study might have been mediated by the other constituents of the pesticide formulations used. Although the biological activity of a pesticide is mostly determined by its active substance, the pesticide formulations available

on the market often consist of a pure active ingredient and different technical materials, which are usually used to improve the properties of a chemical for handling, storage, and application (68). In many cases, such materials contain various ingredients, some of which are protected by patents, which make it difficult to fully understand the results obtained with commercially available pesticides, as was the case in the present study.

Nevertheless, the findings of present study suggest that the sperm head abnormality assay may be used as a short-term and reliable biological indicator in the genotoxic bioassay of pesticides. However, to obtain better insight into the mutagenicity and DNA damaging potential of *Acm* and *Pro*, further studies at molecular level should be done.

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Sažetak

Abnormalna morfologija mišjih spermija nakon kratkotrajne izloženosti acetamipridu, propinebu i njihovoj mješavini

Pesticidi su snažni zagađivači okoliša, a akumuliraju se i u biotičkim i abiotičkim sastavnicama ekosustava. Acetamiprid (Acm), insekticid iz skupine neonikotinoidea, i propineb (Pro), fungicid iz skupine ditiokarbamata, imaju široku primjenu u kontroli sisajućih insekata, odnosno gljivičnih infekcija, na usjevima. Ovim se radom istražuju genotoksični učinci spomenutih spojeva, pojedinačno i u mješavini, na mišje spolne stanice testom morfološke analize spermija. Miševima je intraperitonealno ubrizgano 0,625, 1,25 i 2,50 $\mu\text{g mL}^{-1}$ Acm-a, 12,5, 25,00 i 50,00 $\mu\text{g mL}^{-1}$ Pro-a, te njihova mješavina pri istim koncentracijama tijekom 24 i 48 sati. Acm nije povećao postotak abnormalnih spermija ni pri jednoj koncentraciji. Učestalost abnormalnih spermija značajno je porasla nakon 24 i 48 sati izloženosti 50 $\mu\text{g mL}^{-1}$ Pro-a. Mješavina od 2,50 $\mu\text{g mL}^{-1}$ Acm-a i 50,00 $\mu\text{g mL}^{-1}$ Pro-a uzrokovala je abnormalnosti spermija antagonistički i nakon 24 i nakon 48 sati izloženosti. Naši rezultati pokazuju kako Acm nije bio genotoksičan za mišje spolne stanice, a primijećeni porast učestalosti abnormalnosti spermija nakon izlaganja Pro upućuje na to da bi on mogao biti mutagen. Međutim, kako bi se stekao bolji uvid u mutagenost i potencijal obaju pesticida za oštećenje DNA, nužna su daljnja istraživanja na molekularnoj razini.

KLJUČNE RIJEČI: kombinirani učinci; mješavina pesticida; test morfološke analize spermija

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