# Evaluation of the DNA damaging effects of amitraz on human lymphocytes in the Comet assay

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Amitraz is formamidine pesticide widely used as insecticide and acaricide. In veterinary medicine, amitraz has important uses against ticks, mites and lice on animals. Also, amitraz is used in apiculture to control *Varroa destructor*. It this study, the alkaline Comet assay was used to evaluate DNA damaging effects of amitraz in human lymphocytes. Isolated human lymphocytes were incubated with varying concentrations of amitraz (0.035, 0.35, 3.5, 35 and 350 μg/mL). The Comet assay demonstrated that all concentrations of amitraz caused statistically significant increase in the level of DNA damage, thus indicating that amitraz possesses genotoxic potential. The concentration of amitraz that produced the highest DNA damage (3.5 μg/mL) was chosen for further analysis with the antioxidant catalase. The obtained results showed that co-treatment with antioxidant catalase (100 IU/mL or 500 IU/mL) significantly reduced the level of DNA damage, indicating the possible involvement of reactive oxygen species in DNA damaging effects of amitraz. Flow cytometric analysis revealed increase of the apoptotic index following treatment with amitraz. However, co-treatment with catalase reduced the apoptotic index, while treatment with catalase alone reduced the percentage of apoptotoc cells even in comparison with the negative control. Therefore, catalase had protective effects against ROS-mediated DNA damage and apoptosis.

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## 1. Introduction

Amitraz [*N*-methylbis (2,4-xylyliminomethyl) amine] is formamidine pesticide marketed worldwide as an acaricide and insecticide since 1974 (Crofton *et al.* 1989). It is non-systemic insecticide with contact and respiratory action. In veterinary medicine, amitraz has important uses for demodectic manage (*Demodex canis*) in dogs (Farmer and Seawright 1980) and it is also used against the ticks, mites and lice on cattle, sheep, pigs and goats (Tomlin 1994). In some countries, diluent form of amitraz is applied in humans to treat pediculosis or scabies (Kalyoncu *et al.* 2002). Also, amitraz is used by beekeepers to control *Varroa destructor*.

Amitraz exerts toxic effects on ectoparasites by interaction with the octopamine receptors of arthropods (Evans and Gee 1980; Dudai *et al.* 1987). Although mammals do not have octopamine receptors, amitraz exerts side effects in mammals through activation of $\alpha_2$-adrenoceptors (Hsu 1996). Amitraz has been shown to induce cytochrome P450-dependent monooxygenases in the liver of treated rats (Ueng *et al.* 2004) and decrease hepatic glutathione activity in mouse (Costa *et al.* 1991). In bovine seminal vesicle, amitraz inhibited prostaglandin $E_2$ synthesis (Vim *et al.* 1978). At high dose levels, amitraz can cause tumours in female mice and it is classified as a Group C possible human carcinogen (US EPA 1996). In dogs, following a topical application, amitraz increased plasma glucose and inhibited insulin secretion (Hsu and Schaffer 1988). Evidence from animal studies also suggests that amitraz is potential reproductive toxicant (Hayes and Laws 1991; Cooper *et al.* 1999). Moreover, amitraz exhibits toxic effects in the human reproduction cells *in vitro* and inhibits the production of the

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steroid hormone progesterone (Young *et al.* 2005). In humans, amitraz intoxication has been reported and exposure effects include CNS depression, hypothermia, bradycardia, hypotension, hyperglycemia, glycosuria, vomiting and respiratory failure (Kennel *et al.* 1996; Kalyoncu *et al.* 2002; Yilmaz and Yildizdas 2003).

Due to its excellent miticidal activity, amitraz is widely used in apiculture for the obligatory annual control of *Varroa destructor*. In beehives, amitraz is used in the form of smoke or vapour (Marchetti and Barbattini 1984). The lack of residues in honey is related to the instability of amitraz in an acidic medium like honey (Berzas Nevado *et al.* 1990). According to EMEA (European Agency for the Evaluation of Medicinal Products) residues of amitraz in honey were stable for up to 4 months when samples were stored at –20°C. Amitraz also does not remain stable in beeswax (Wallner 1999; Korta *et al.* 2001). It degrades over a period of 2–4 weeks in honey, but in beeswax it completely degrades within 1 day (Korta *et al.* 2001). On the other hand, this acaricide is easily hydrolysed to toxic 2,4-dimethylaniline (2,4-DMA) and various products containing the 2,4-DMA moiety (Jiménez *et al.* 2002). Unlike amitraz, its degradation products are stable in honey and can be found as residues in food and in surface water samples (Corta *et al.* 1999). Maximum Residue Limit (MRL) in honey is 0.2 mg/kg as defined in No. 2377/90/EC regulation for amitraz. On the other hand, no MRL is fixed for beeswax even when it is used for pharmaceutical purposes, food packaging or cosmetics. In honey collected from colonies treated with amitraz, varroacide residues can be found below the MRL value (Floris *et al.* 2001; Martel *et al.* 2007; Lodesani *et al.* 2008).

Exposure to amitraz would be expected to pose a greater hazard to pet owners, agricultural workers and beekeepers because of the continual exposure to this acaricide. Therefore, it is very important to evaluate possible genotoxic effects of amitraz in view of health protection. Genotoxicity of the amitraz was explored using different test systems from bacteria to mammals. Osano *et al.* (2002) reported genotoxic effects of amitraz and its metabolite at very low concentrations (<0.005 mM) in the Vibrio test. Likewise, amitraz caused genotoxic effect by induction of chromosomal aberrations in bone marrow cells of mice (Pejin *et al.* 2006). In contrast, amitraz and its metabolites were found to be negative for mutation in the Ames test (Tudek *et al.* 1988) and did not induce DNA strand breaks on rat hepatocytes (Grilli *et al.* 1991).

On the basis of available data we cannot obtain a clear idea of the possible genotoxic effects of amitraz. Besides, it is worthwhile to investigate the mechanisms of amitraz action on DNA. It has been shown that some pesticides may induce oxidative stress through the generation of reactive oxygen species, leading to lipid peroxidation and DNA damage (Abdollahi *et al.* 2004; Vidyasagar *et al.* 2004; Shadnia *et al.* 2005). There is evidence that free oxygen radicals are produced during AMZ oxidation (Kruk and Bounias 1992). This data suggest that amitraz may be able to induce oxidative DNA damage via reactive oxygen species (ROS).

In this study, the Comet assay was used to assess the DNA damaging effect in human lymphocytes exposed to amitraz in order to provide additional genotoxicological data for this compound. In addition, we used catalase to determine whether the mechanism underlying DNA damage of amitraz is mediated by ROS. In order to determine an apoptotic index we performed the annexin V–propidium iodide (AnnV–PI) staining apoptosis test.

## 2. Materials and methods

### 2.1 *Amitraz and controls*

Varamit® (0.2 g/mL of Amitraz dissolved in xylol) was purchased from Evrotom (Ruma, Serbia; CAS No 33089–61–1). The chemical structure of amitraz is shown in figure 1. Amitraz stock standard solution was prepared in propylen glycol to obtain a concentration of 100 mg/mL. We used propylen glycol as a solvent instead of xylol, because pure xylol and amitraz in xylol produce unacceptable levels of cytotoxicity (data not shown). The stock standard solution of amitraz was appropriately diluted with propylen glycol to prepare working solutions to obtain 0.035, 0.35, 3.5, 35 and 350 μg/mL doses of amitraz. Hydrogen peroxide (100 μM) was used as a positive control to verify the sensitivity of the test system. Negative control was composed of all ingredients except an active one (amitraz). Therefore, in a final volume of 1000 μL, the negative control contained 940 μL of PBS, 50 μL of RPMI 1640 with isolated lymphocytes, 7 μL of propylenglycol and 3 μL of xylol.

### 2.2 *Isolation of lymphocytes*

Heparinised blood samples (4 mL) were obtained by venepuncture from two healthy male donors under 25 years of age. The study was approved by the local Medical Ethics Committee, performed in accordance with Declaration of Helsinki, and informed donor consent was also obtained. Lymphocytes were isolated from whole blood with Ficoll-Paque medium and centrifuged at 1900*g* 15 min. The lymphocytes forming a layer were directly above Ficoll-Paque. The isolated lymphocytes were washed twice in RPMI 1640 medium, each wash was followed by a centrifugation 10 min at 1800*g*. Finally, the supernatant was removed as carefully as possible without

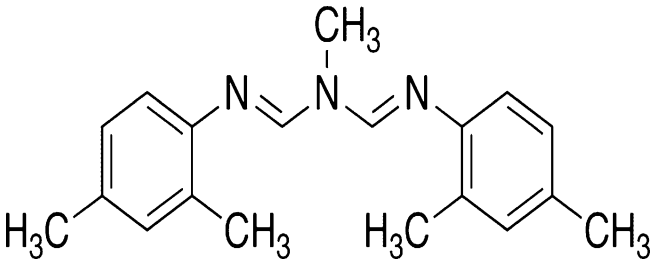

**Figure 1.** The chemical structure of amitraz.

disturbing the pellet. An aliquot of 1 mL of RPMI 1640 was added and the pellet was re-suspended. A manual cell count and an estimate of cell viability were performed using Trypan blue exclusion test.

### 2.3 *The Comet assay*

Alkaline Comet assay was performed according to the Singh *et al.* (1988) and Tice *et al.* (1991) technique with slight modifications. Microscope slides were precoated with 1% normal melting point agarose and allowed to air-dry at room temperature for at least 48 h. After incubation with the tested compound for 1 h, the cell viability was evaluated using Trypan blue exclusion test. After centrifugation (5 min at 2000 rpm), 100 µL of cell suspension was mixed with 100 µL of 1% low melting point agarose (LMPA). The suspension was rapidly pippeted onto the first agarose layer and spread using a coverslip, and placed in the fridge to solidify. After removal of the coverslip, the 90 µL of 0.5% LMPA was added as the third layer, spread using a coverslip and allowed to solidify at 4°C for 5 min. Afterwards, the slides were immersed in cold lysis solution at pH 10 (2.5 M NaCl, 100 mM EDTA, 10 mM Tris pH 10, 1% Triton X–100, 10% DMSO) overnight at 4°C. After lysis, the slides were placed in a horizontal gel electrophoresis tank to allow DNA unwinding in cold alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH>13) for 30 min. Electrophoresis was done at 4°C with electric current of 25 V and 300 mA for 30 min. All these steps were performed under dimmed light (tank was covered with a black cloth) to prevent additional DNA damage. The slides were then neutralized with 400 mM Tris-HCl (pH 7.5) for 5 min. The neutralization was repeated three times. Then, the slides were fixed with cold methanol, dried and stored. Before analysis, the slides were rehydrated with ice cold distilled water and stained with 50 µL of 20 µg/mL ethidium bromide. Various levels of DNA damage on human lymphocytes are presented in figure 2. In the co-treatment we used as an antioxidant catalase from bovine liver (CAS No 9001–05–2), Sigma Chemical Co., St. Louis, USA.

### 2.4 *Scoring of Comets*

Slides were examined at 400× magnifcation on a fluorescent microscope (Leica, UK) and image analysis software (Comet Assay IV Image Analysis system, PI, UK). From each replicate slide, 50 nuclei were scored (a total of 100 nuclei per donor) and the percentage of tail DNA was used to evaluate the extent of DNA migration.

### 2.5 *Annexin V–propidium iodide (AnnV–PI) staining apoptosis test*

This assay is based on the ability of the protein anexxin V to bind to phosphatidylserine (PS) exposed on the outer membrane leaflet in apoptotic cells (PS also appears on the necrotic cell surface). In viable cells, PS is located in the inner membrane leaflet, but upon induction of apoptosis it is translocated to the outer membrane leaflet and becomes available for annexin V binding. The addition of propidium iodide (PI) enabled viable ($AnnV^{neg}/PI^{neg}$), early apoptotic ($AnnV^{poz}/PI^{neg}$) and necrotic ($AnnV^{neg}/PI^{poz}$) cells to be distinguished. The annexin V assay was performed following the instructions provided by the manufacturer of the ANNEXIN V FITC kit (Beckman Coulter, CA, USA). Briefly, after the treatment of isolated lymphocytes for 1 h, cells ($1\times10^6$ cells/mL) were washed in cold PBS, suspended in binding buffer and then added with 1 µL annexin-V FITC and 5 µL propidium iodide (100 µg/mL) for 15 min. Finally, 400 µL 1× binding buffer was added to each tube and cells were read by flow cytometry (Partec Cyflow SL) by differentiation of at least 20,000 cells. Flow cytometric analysis was performed using Partec FloMax software. The apoptotic index (AI) was calculated as the percentage of Annexin V positive and PI-negative cells divided by the total number of cells in the gated region.

### 2.6 *Statistical analysis*

Statistical analysis of the results obtained in the experiment was carried out using software STATISTICA v. 6. In the Comet assay, Levene's test for homogeneity of variance and Kolmogorov-Smirnov test for normality of distribution were used prior to statistical analysis. Considering that the data were not in line with the requirements for the application of parametric tests, differences between treatments were tested using Kruskal-Wallis test and Mann-Whitney U test. As for the flow cytometric analysis of apoptosis we used the *z*-test for proportions.

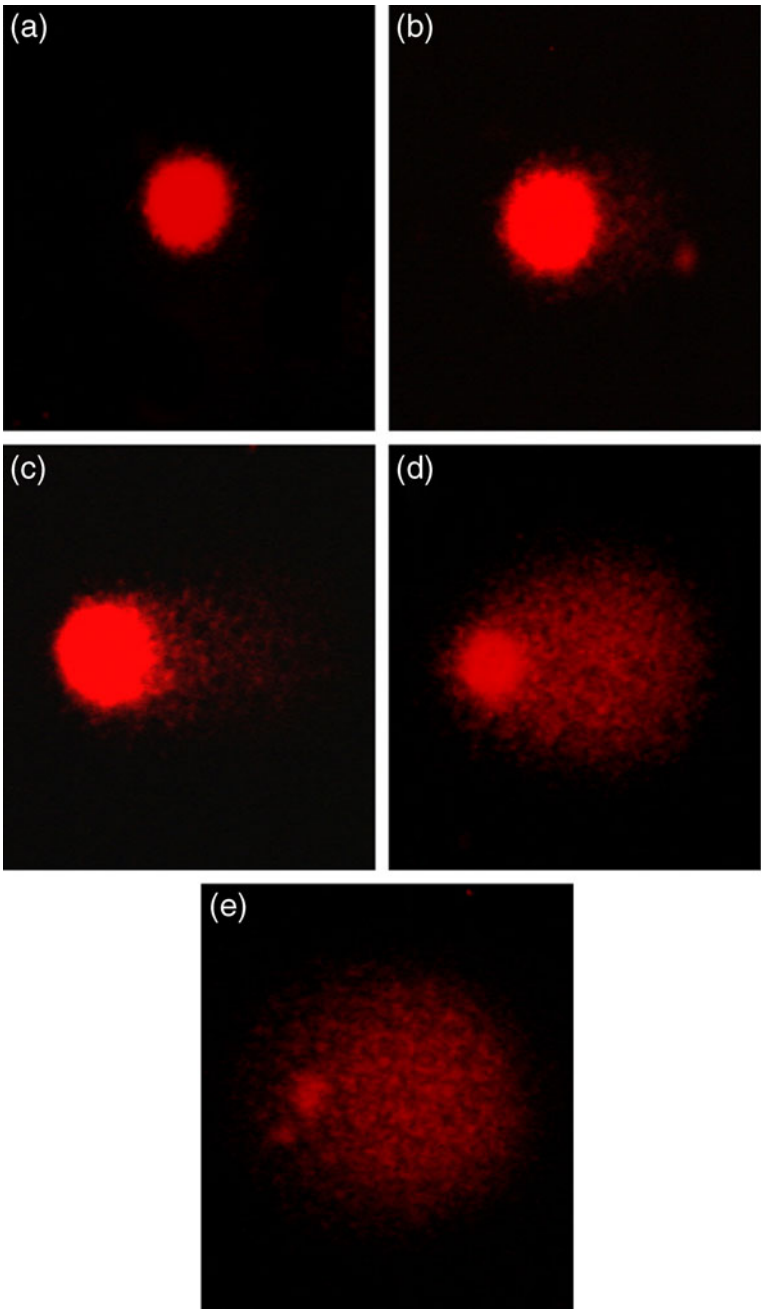


**Figure 2.** Various level of DNA damage on human lymphocytes in the Comet assay: (**a**) none, (**b**) low, (**c**) medium, (**d**) high and (**e**) total.

## 3. Results

### 3.1 *Trypan blue exclusion assay*

The viability of lymphocytes treated with amitraz or xylol for 1 h in Trypan blue exclusion assay was at least 90%. In experiments with catalase, viability of lymphocytes was also over 90%. In all experimental groups, the viability of lymphocytes treated with the positive control (100 μM $H_2O_2$) was at least 82%. Therefore, in all our experiments level of cytotoxicity was acceptable.

### 3.2 *Treatment with amitraz*

The minimal percentage of tail DNA at all treatments was 0%, while maximal DNA damage ranged from 28.09% (at

**Table 1.** Statistical parameters in the experiment with various concentrations of amitraz

| Treatments | | Valid n | Median | Minimum | Maximum | Lower quartil | Upper quartil |
|---|---|---|---|---|---|---|---|
| Amitraz (μg/mL) | 0 | 200 | 1.304 | 0.000 | 28.344 | 0.166 | 3.503 |
| | 0.035 | 200 | 2.947 | 0.000 | 39.288 | 0.886 | 8.251 |
| | 0.35 | 200 | 2.287 | 0.000 | 28.091 | 0.311 | 5.914 |
| | 3.5 | 200 | 4.553 | 0.000 | 89.110 | 0.508 | 11.334 |
| | 35 | 200 | 2.620 | 0.000 | 47.904 | 0.356 | 8.968 |
| | 350 | 200 | 2.822 | 0.000 | 100.000 | 0.401 | 8.768 |
| $H_2O_2$ (100 μM) | | 104 | 3.525 | 0.000 | 93.622 | 0.204 | 14.605 |

Negative control: 0 μL/mL of amitraz; positive control: 100 μM $H_2O_2$.

0.35 μg/mL of amitraz) to 100.0% (at 350 μg/mL) (table 1). DNA damage in all treatments was heterogenous (coefficients of variations ranged from 118.84% at 0.35 μg/mL of amitraz to 158.31% at 350 μg/mL of amitraz). Maximal interquartile range of 14.40% was achieved after the treatment with the positive control ($H_2O_2$), while a minimum of 3.34% was obtained for the negative control (table 1; figure 3). Bearing in mind the character of experimental data, we used medians to quantify average values. The average percentage of tail DNA ranged from 1.3% in the negative control group to 4.55 in cells treated with 3.5 μg/mL of amitraz. The results of Kruskal-Wallis test ($H_{6,1304}$=43.769; $p$<0.001, figure 3) points to very high statistical significance of percentage of tail DNA between various concentrations of amitraz and controls (0 μg/mL of amitraz and 100 μM $H_2O_2$). The results

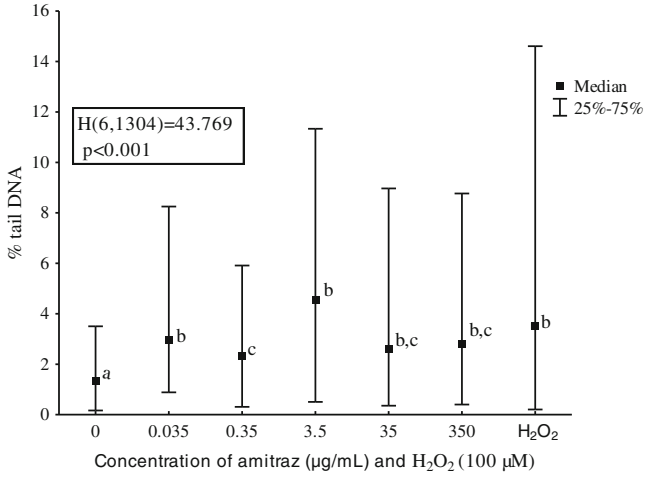


**Figure 3.** Effects of various amitraz concentrations on purified human lymphocytes. Negative control: 0 μg/mL of amitraz; positive control: 100 μM $H_2O_2$. For each treatment the average % of tail DNA is shown with lower and upper quartiles, as well as the results of Kruskal-Wallis analysis of variance for all treatments. The same letters denote medians which do not differ significantly ($p$>0.05).

of the Mann-Whitney U test (table 2) clearly showed that this difference is due to statistically highly significant difference between the negative control and all other treatments with amitraz, as well as the diffference between treatment with 0.35 μg/mL of amitraz and treatments with 0.035 μg/mL and 3.5 μg/mL of amitraz and the positive control (figure 3). In experiment with amitraz, 100 μM $H_2O_2$ used as the positive control gave rise to a significant DNA damage ($p$<0.001).

### 3.3 *Co-treatment with catalase*

In order to understand the mechanism of genotoxic effect of amitraz, we used the antioxidant catalase. The concentration of amitraz (3.5 μg/mL) that produced the highest DNA damage was chosen for co-treatment with this antioxidant. In all treatments, the minimal percentage of tail DNA was 0% (table 3). The maximum percentage of tail DNA was in the range from 6.82% in the negative control to 96.79% in the positive control (100 μM $H_2O_2$). The data obtained in this experiment was non-homogenous – the coefficients of variations were in the range 90.46% (positive control) to 143.07% (500 IU/mL catalase). Minimal interquartile range was observed in the negative control (1.55%) and the maximal in the positive control (22.83%) (table 3; figure 4). The results of Kruskal-Wallis test showed very highly statistical significance ($H_{5,1200}$=427.352; $p$<0.001) (figure 4) between medians which were in the range from 0.47% for the treatment with 500 IU/mL of catalase to 12.30% for cells treated with the positive control. On the other hand, the results of Mann-Whitney U test (table 4) showed that percentage of tail DNA in the negative control was significantly lower compared to all other treatments, except for the 500 IU/mL of catalase alone. In the co-treatment with 3.5 μg/mL of amitraz and catalase, the average precentage of tail DNA was lower in comparison to cultures treated with 3.5 μg/mL alone (table 4; figures 4 and 5), at both concentrations of catalase used in this experiment.

**Table 2.** Levels of statistical significance in Mann-Whitney U test in experiment with various concentrations of amitraz

| Treatments | | Amitraz (μg/mL) | | | | | $H_2O_2$ (100 μM) |
|---|---|---|---|---|---|---|---|
| | | 0.035 | 0.35 | 3.5 | 35 | 350 | |
| Amitraz (μg/mL) | 0 | <0.001*** | 0.008** | <0.001*** | <0.001*** | 0.001** | <0.001*** |
| | 0.035 | | 0.015* | 0.257 | 0.385 | 0.637 | 0.560 |
| | 0.35 | | | 0.001** | 0.122 | 0.055 | 0.039* |
| | 3.5 | | | | 0.065 | 0.175 | 0.893 |
| | 35 | | | | | 0.682 | 0.249 |
| | 350 | | | | | | 0.409 |

*$p \leq 0.05$; **$p \leq 0.01$; ***$p < 0.001$.

Negative control: 0 μL/mL of amitraz; positive control:100 μM $H_2O_2$.

### 3.4 *Annexin V–propidium iodide (AnnV–PI) staining apoptosis test*

The results of evaluation of apoptosis by flow cytometry are presented in figure 6. In the negative control, we observed 89.87% of viable cells, 5.42% of cells in early apoptosis and 4.72% of necrotic cells. After the tretment with amitraz, viable cells dropped to 70.86% ($p<0.001$), while there was a significant ($p<0.001$) increase of early apoptotic (14.72%) and necrotic cells (14.41%). However, concomitant treatment with 3.5 μg/mL of amitraz and catalase resulted in reduction of the apoptotic index (percentage early apoptotic cells) in comparison with cells treated only with amitraz, and this effect was more profound with higher concentration of catalase applied (500 IU/mL). Thus, we observed 11.34% of early apoptotic cells co-treated with 100 IU/mL of catalase ($p<0.001$) and 8.72% of necrotic cells. The co-treatment with 500 IU/mL of catalase caused further decrease ($p<0.001$) of the percentage of early apoptotic cells (7.25%) and necrotic cells (6.18%) in comparison with the treatment with amitraz alone (3.5 μg/mL). Interestingly, lymphocytes treated only with 500 IU/mL of catalase had significantly ($p<0.05$) lower percentage of early apoptotic cells (5.13%) in comparison with all other treatments including the negative control. This finding indicates that catalase had protective effects against apoptosis. Finally, the positive control (100 μM $H_2O_2$) produced the most profound effect ($p<0.001$) in comparison with all other treatments, with only 67.62% of viable cells and highest percentages of early apoptotic (15.74%) and necrotic cells (16.65%).

## 4. Discussion

Amitraz is broad spectrum formamidine pesticide used against ticks and mites. It is widely used in veterinary medicine and agriculture, and therefore, there is an increased risk of contact with this acaricide. In mammals, toxic effects of amitraz are well understood, but little is known about possible genotoxic effects, because available data are insufficient and equivocal. Therefore, it is important to examine the effects of amitraz on the integrity of DNA.

In our *in vitro* study the responses of human lymphocytes after treatment with varying concentrations of amitraz were evaluated by the Comet assay. The results show that all selected concentrations of amitraz (0.035, 0.35, 3.5, 35 and 350 μg/mL) induced significant increase in the level of DNA damage ($p<0.05$) in comparison with the negative control. The highest concentrations of amitraz (35 and 350 μg/mL)

**Table 3.** Statistical parameters in co-treatment of amitraz with catalase

| Treatments | Valid n | Median | Minimum | Maximum | Lower quartil | Upper quartil |
|---|---|---|---|---|---|---|
| Amitraz 0 μg/mL | 200 | 0.487 | 0.000 | 10.376 | 0.099 | 1.650 |
| Catalase 500 IU/mL | 200 | 0.466 | 0.000 | 10.603 | 0.108 | 2.207 |
| Amitraz 3.5 μg/ml | 200 | 5.142 | 0.000 | 39.648 | 1.327 | 11.334 |
| Amitraz 3.5 μg/ml and catalase 100 IU/mL | 200 | 0.962 | 0.000 | 6.820 | 0.128 | 3.040 |
| Amitraz 3.5 μg/ml and catalase 500 IU/mL | 200 | 0.851 | 0.000 | 8.222 | 0.128 | 3.089 |
| $H_2O_2$ (100 μM) | 200 | 12.297 | 0.000 | 96.792 | 7.282 | 30.110 |

Negative control: 0 μL/mL of amitraz; positive control:100 μM $H_2O_2$.

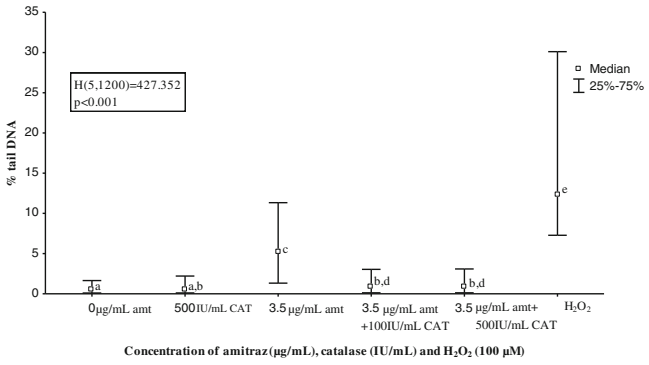


**Figure 4.** The effects of catalase on reduction of DNA damage caused by amitraz. Negative control (0 µg/mL) was the solvent and the positive control was 100 µM $H_2O_2$. For each treatment average % of tail DNA is shown with lower and upper quartiles, as well as the results of Kruskal-Wallis analysis of variance. The same letters denote medians which do not differ significantly ($p>0.05$).

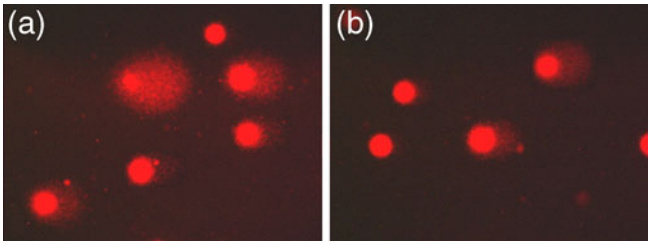


**Figure 5.** (**a**) Comets after the treatment with 3.5 µg/mL of amitraz. (**b**) Reduced DNA damage in cells treated with 3.5 µg/mL of amitraz and 500 IU/mL catalase.

showed lower levels of DNA damage, in comparison with the intermediate concentration of 3.5 mg/mL, possibly due to formation of cross-links (Tice *et al.* 2000). These results are in agreement with the *in vivo* findings of Pejin *et al.* 2006. In that study, all tested doses of amitraz (3.6 mg/kg b. wt., 1.8 mg/kg b. wt., 0.9 mg/kg b. wt.) induced numerical and structural chromosomal changes in bone marrow cells of mice. It is considered that degradation products of amitraz are responsible for manifested genotoxic effects. Similarly, Osano *et al.* (2002), reported positive genetic response of amitraz and its degradation products in the Vibrio test at very low concentrations (<0.005 mM).

As expected, the positive control (100 µM of $H_2O_2$) caused increased level of DNA damage compared with the negative control ($p<0.05$). Therefore, the experimental conditions were appropriate for detection of DNA damage.

Also, we evaluated the influence of catalase on DNA damage to establish whether the induction of DNA damage is caused by oxidative stress. Numerous data in the literature suggest that some pesticides produce oxidative stress through the generation of reactive oxygen and nitrogen species, leading to lipid peroxidation and DNA damage (Stanimirovic *et al.* 2003, 2005, 2006, 2007; Abdollahi *et al.* 2004; Vidyasagar *et al.* 2004; Shadnia *et al.* 2005). Catalase is one of the enzymes that protect the cell from free-radical-induced damage by degradation of hydrogen peroxide. Our study showed that antioxidant catalase significantly reduced ($p<0.05$) DNA damage caused by amitraz. These result indicated the involvement of free radicals in DNA damaging effect of tested pesticides. It is in good agreement with findings in which oxidation of amitraz leads to the generation of $OH^{\bullet}$ and $O_2$ (Kruk and Bounias 1992). The highly reactive $OH^{\bullet}$ has potential to react with DNA and cause the formation of strand breaks that can be detected by the Comet test. On the other hand, there is evidence that degradation products of amitraz exert genotoxic effect (Zimmer *et al.* 1980; Przybojewska 1997; Osano *et al.* 2002). We assume that aromatic amines of degradation products can be activated through obligatory N-hydroxylation, as well as subsequent conjugation by sulfation and/or acetilation. During these transformations reactive nitrenium ions and quinine imines can arise and cause DNA damage (Skipper *et al.* 2010). The mechanism of DNA damage responsible for genotoxicity of aniline remains to be identified.

**Table 4.** Levels of statistical significance in Mann-Whitney U test in co-treatment of amitraz with catalase (CAT)

| Treatments | Catalase 500 IU/mL | Amitraz 3.5 µg/mL | Amitraz 3.5 µg/mL and catalase 100 IU/mL | Amitraz 3.5 µg/mL and catalase 500 IU/mL | $H_2O_2$ (100 µM) |
|---|---|---|---|---|---|
| Amitraz 0 µg/mL | 0.383 | <0.001*** | 0.006** | 0.007** | <0.001*** |
| Catalase 500 IU/mL | | <0.001*** | 0.096 | 0.113 | <0.001*** |
| Amitraz 3.5 µg/mL | | | <0.001*** | <0.001*** | 0.001*** |
| Amitraz 3.5 µg/mL and catalase 100 IU/mL | | | | 0.979 | <0.001*** |
| Amitraz 3.5 µg/mL and catalase 500 IU/mL | | | | | <0.001*** |

*$p\leq0.05$; **$p\leq0.01$; ***$p<0.001$.

Negative control: 0 µL/mL of amitraz; positive control: 100 µM $H_2O_2$.

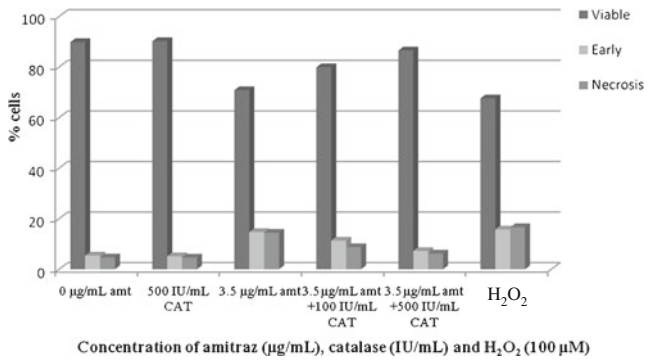


**Figure 6.** Annexin V–PI flow cytometry analysis of isolated human lymphocytes in the co-treatment with catalase. The cells are divided into three groups: viable, early apoptosis and necrosis.

In mammals, metabolism of amitraz occurs via hydrolisis to *N*-(2,4-dimethylphenylf)-*N*-methyl formamidine and 2,4 dimethylphenyl formanilide product containing the 2,4 DMA moiety (Knowles and Benezet 1981). We assume that amitraz exerts genotoxic effect probably due to its degradation products, since there are findings that confirmed genotoxic potential of 2,4 DMA (Zimmer *et al*. 1980; Przybojewska 1997; Osano *et al.* 2002). The mechanism of DNA damage responsible for genotoxicity of aniline remains to be identified. The degradation products of amitraz include aromatic amines which can be activated through obligatory N-hydroxylation, as well as subsequent conjugation by sulfation and/or acetilation. During these transformations reactive nitrenium ions and quinine imines can arise and cause DNA damage (Skipper *et al.* 2010).

Taken together, out results suggests that amitraz may induce oxidative stress by production of ROS. It is well known that oxidative stress plays a very important role in pathogenesis of several diseases (Banerjee *et al.* 2001; Abou-Donia 2003; Djelic and Anderson 2003). For this reason it should be noted that the highest tested concentration of amitraz is only 1.75 times higher than MRL for amitraz (200 ppm). while the lowest tested dose of amitraz (0.035 µM/L) is 5.75 times lower than that allowed. All tested concentrations of amitraz are lower than the recommended doses in beekeeping. The risk of direct exposure is related to the amount of the active ingredients used by beekeepers, and current findings indicate the importance of education of beekeepers on the use of amitraz and to implement protection measures, especially because beekeepers are at increased risk of amitraz exposure due to application of amitaz by fumigation. Besides the results presented here, additional experimental data are needed in order to elucidate all possible mechanisms underlying genotoxic effects of amitraz.

Finally, the results of Annexin V–propidium iodide (AnnV–PI) staining apoptosis test clearly showed that co-treatment with catalase caused significant decrease in percentage of apoptotic and necrotic cells in comparison with treatment with 3.5 µg/mL of amitraz alone. Furthermore, even the cells treated with 500 IU/mL of catalase alone exhibited lower percentage of apoptotic cells in comparison with all other treatments including the negative control. This result is indicative of the protective effects of catalase not only against genotoxic effects, as discussed above, but also against an apoptosis. This result is consistent with the previous report showing role of catalase in protection from oxidative stress and apoptosis in a human lens epithelial cell line HLEB-3 (Zheng *et al.* 2010). Namely, the ROS induced in oxidative stress are recognized as an important mediator of apoptosis in lens epithelial cells (Truscott 2005). Furthermore, numerous experimental data have shown that the process of apoptosis depends on increased ROS and intracellular catalase activity (Oral *et al.* 2000; Wang *et al.* 2006). Therefore, on the basis of all our experimental results it can be concluded that amitraz-induced oxidative stress opens a very intriguing field of study which could be complemented by further genetic, cytobiochemical and genomic analysis.

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