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Genotoxicity of pesticides: the role of non-active ingredients

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Abstract
Amitraz is a formamidine-based insecticide and acaricide used in veterinary applications. Despite the use of various genetic assessment criteria and testing systems to investigate amitraz poisoning, studies have yielded diverse and inconclusive results. This study aimed to analyze the genotoxic potential of the insecticide amitraz and compare the effects of the active constituent and a commercial product containing amitraz. Chinese hamster ovary cells were cultured during one cellular cycle in Ham F12 medium containing 1.25, 2.5 and 3.75 µg mL^{-1} of amitraz. Quantitative comet and cytokinesis-block micronucleus assay were employed to evaluate the potential genotoxic effect. All genotoxicity parameters evaluated clearly demonstrated the capability of the commercial amitraz formulation to negatively affect DNA, inducing both cytogenetic and cytomolecular damage. After culturing the cells with the active component of the formula, only a slight, non-significant increase in damage was found. Since our findings showed that the active component of the formula is not the sole responsible for the genotoxic effect of the commercial product, we emphasize the importance of considering the adverse effects of the solvents used in commercial pesticide formulations.

Key words: Amitraz, cell viability test, genotoxicity, quantitative comet assay, micronucleus test, cell cultures.

Genotoxicidad de pesticidas: el papel de los ingredientes no activos

Resumen. Amitraz es un insecticida y acaricida a base de formamidina de uso veterinario. A pesar de que se han utilizado diversos criterios de valoración genéticos y sistemas de prueba para la investigación sobre el envenenamiento por amitraz, los resultados que surgieron de estas investigaciones fueron diversos y no concluyentes. El objetivo de este estudio fue analizar el potencial genotóxico del insecticida amitraz y comparar el efecto del componente activo y un producto comercial que contiene amitraz. Se cultivaron células de ovario de hámster chino durante un ciclo celular en medio Ham F12 con concentraciones de 1,25; 2,5 y 3,75 µg ml^{-1} de amitraz. Se emplearon los ensayos de micronúcleos con bloqueo de la citocinesis y cometa cuantitativo para evaluar el posible efecto genotóxico. Todos los parámetros de genotoxicidad evaluados mostraron claramente la capacidad de la formulación comercial de amitraz para afectar negativamente al ADN, induciendo daño tanto citogenético como citomolecular. Después de cultivar las células con el componente activo de la fórmula, solo se encontró un ligero aumento no significativo del daño. Como nuestros hallazgos demostraron que el componente activo de la fórmula no es el único responsable del efecto genotóxico del producto comercial, enfatizamos la importancia de tener en cuenta el efecto adverso de los solventes utilizados en las formulaciones comerciales de pesticidas.

Palabras clave: Amitraz, viabilidad celular, genotoxicidad, ensayo cometa cuantitativo, ensayo de micronúcleos, cultivo celular.

INTRODUCTION

Pesticide toxic exposure occurs when chemicals intended to control a pest affect non-target organisms; virtually all people are inevitably exposed to pesticides through environmental contamination or occupational use. Amitraz {N,N_-[(methylimino)dimethylidyne]di-2,4-xylidine}, is a formamidine insecticide and acaricide commonly used to prevent tick and mite infestation in fruit, cotton, and hops, and as a veterinary medicine for the

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treatment of ectoparasites in pigs, cattle, sheep, goats, and dogs (Khan et al. 2008, Nanjundappa et al. 2021, Fereydooni et al. 2023). It is a highly liposoluble compound quickly absorbed through the skin and mucous membranes making this exposure potentially dangerous for humans and animals (Marafon et al. 2009, Dhooria and Agarwal 2016). Some human populations are exposed because of their activities associated with the agricultural use of amitraz, while others are exposed through the diet (Bolognesi 2003). The EPA (Environmental Protection Agency), according to acute toxicity studies, classifies amitraz as Class III-slightly toxic by the oral and inhalation routes and as Class II-moderately toxic by the dermal route (US EPA 1996).

The α_2-adrenoreceptor agonist potency of amitraz may be the major cause of central nervous system toxicity of this drug in animals and humans (Hu et al. 2019). Incidences of amitraz poisoning have been enlarged due to its increased production and use (Ulukaya et al. 2001, Proudfoot 2003). There are several cases of poisoning by amitraz reported in the literature in dogs, cats, horses, and humans (Westermann et al. 2004, Avsarogullari et al. 2006, Caprotta et al. 2009, Dhooria and Agarwal 2016).

Diverse genetic end-points and test-systems have been utilized for research on amitraz poisoning and results emerging from these investigations were diverse and inconclusive (del Pino et al. 2015). Osano et al. (2002) claimed that it should be considered a potential genotoxicant capable of altering gene functions in *Xenopus laevis*; and Young et al. (2005) reported that exposing WIL2NS cells to 0.035% of amitraz resulted in a significant reduction of cell numbers after human lymphocytes exposure. Results obtained in our laboratory by means of the qualitative alkaline comet assay in *in vitro* cultured Hamster ovary cells showed that a commercial formulation of amitraz significantly increased DNA damage at concentrations ranging from 2.50 to 3.75 µg mL^{-1} (Padula et al. 2012). Similar findings were reported by Radakovic et al. (2013) in human lymphocytes incubated with varying concentrations of amitraz (0.035 - 350 µg mL^{-1}), and by Giorgini et al. (2023), who founded significantly reduced cell viability in HepG2 cells exposed to 156.25 µM of amitraz. Furthermore, Nikoloff et al. (2021) documented late apoptosis, necrosis induction and degenerate oocytes, but no DNA damage in bovine cells treated with 25 µg mL^{-1} of amitraz; and Carranza-Martin et al. (2024) found decreased viability, reduced mitochondrial activity and alterations in membrane integrity in bovine sperm treated with the same doses.

In addition, the data available, both human and animal, do not allow clear separation of the features of toxicity of amitraz from those of the hydrocarbon solvents in which it is commonly dissolved (Proudfoot 2003).

The aim of this study was to analyze the genotoxic potential of the insecticide amitraz *in vitro* in Hamster ovary cells by using quantitative alkaline single-cell gel electrophoresis (comet assay) and cytokinesis-block micronucleus assay as cytomolecular and cytogenetic damage biomarkers respectively. Comparison was made between the effect of the active constituent and a commercial product containing amitraz and between the employed techniques as tools for the analysis of pesticide-induced genotoxicity.

MATERIALS AND METHODS

Cells. CHO cells obtained from the American Type Culture Collection (Manassas, VA, USA) were cultured in Ham's F12 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Notocor Laboratories, Province of Cordoba, Argentina) and antibiotics (50 IU penicillin and 50 µg mL^{-1} streptomycin) (Bagó Laboratories, Buenos Aires, Argentina) in a humidified atmosphere with 5% CO_2. Cells were cultured in Falcon T-25 (Nunc, Denmark).

Chemical reagents. Comercial product containing amitraz was Azadieno® (Merial, Argentina). The concentration of this product was 12%, it was dissolved in bidistilled water in order to obtain final concentrations of 1.25; 2.5 and 3.75 µg mL^{-1}.

A stock solution of amitraz (N-methylbis (2,4-xylyminomethyl) amine, Chem Service, USA) in DMSO was stored at room temperature in the dark. This solution was diluted in bidistilled water to obtain final working concentrations of 1.25; 2.5 and 3.75 µg mL^{-1}. The final solvent concentration was <1% for all treatments.

The highest concentration that allows survival of a sufficient number of cells to perform the analysis was selected. The concentrations of amitraz to which the cells were exposed were the same for the commercial formulation and the active constituent.

Experimental design. Commercial formulation and active constituent were tested. Treatments were performed during one cellular cycle when the cells were in the exponential growth phase. After treatment cells were trypsinized, resuspended and aliquots were obtained for cell viability test and comet assay. Another set of flasks added with B-cytochalasin at the end of the first cycle was used for micronucleus assay. Negative controls including untreated cells and solvent vehicle-treated cells were run simultaneously with pesticide-treated cultures. Each experiment was repeated three times and average values are shown in the tables. Analysis was carried out blinded by one investigator.

Cell Viability Test. Cell viability was estimated using the trypan blue exclusion method (Strober 2015). Briefly, cell suspensions were prepared by trypsinization, centrifugation, and resuspension. A mix of 100 µL of cell pellets and 100 µL of Trypan blue solution (4% Gibco BRL, Grand Island, NY, USA) was incubated for 3 min. Trypan blue penetrates the damaged membrane of dead cells and stains the nucleus. Then both the number of stained cells and the total number of cells were analyzed in a Neubauer chamber. The percentage of viable cells was determined by calculating the percentage of unstained cells.

Comet assay. Single cell gel electrophoresis was performed using the alkaline version described by Singh et al. (1988) with some modifications (Tice and Strauss 1995). Briefly, slides were covered with a first layer of 180 µl of 0.5% normal agarose (Carlsbad, Ca, USA). An amount of 75 µl of 0.5% low melting point agarose (Carlsbad, Ca, USA) was mixed with approximately

15,000 cells suspended in 25 µl of culture media and layered onto the slides, which were then immediately covered with coverslips. After agarose solidification at 4 °C for 10 min, coverslips were removed and slides were immersed overnight at 4 °C in fresh lysis solution. The slides were equilibrated in alkaline solution for 20 min. Electrophoresis was carried out for 30 min at 25 V and 250 mA (1.25 V/cm). Afterwards, slides were neutralized by washing them three times with Tris buffer (pH 7.5) every 5 min and subsequently washed in distilled water. Slides were stained with 1/1000 SYBR Green I (Molecular Probes, Eugene, Oregon, USA) solution (Olive 1999).

Scoring was made at 400x magnification using a fluorescent microscope (Olympus BX40 equipped with a 515-560 nm excitation filter) connected through a Sony 3 CCD-IRIS Color Video Camera. The DNA migration was determined with the CASP software: Comet Assay Software Project (Końca et al. 2003). DNA-damage was expressed as Olive Tail Moment (OTM arbitrary units, Tice et al. 2000) and as Tail DNA (percentage of DNA in the tail of the comet). From each of the two slides made for one dose, 50 randomly selected cells were measured, thus giving 100 cells per sample and 300 cells per dose in all the experiments (three determinations).

Micronucleus test. The cytokinesis-blocked micronucleus assay was modified from Fenech et al. (2003). Cells were cultured as monolayers during two cell cycles, 30 h. At the end of the first cycle, B-cytochalasin (3 µg mL^{-1} final concentration) (Sigma, St. Louis, MO, USA) was added to the cultures. Cells were then removed by trypsinization and agitation. The cell suspension was centrifuged and the pellet was resuspended in 5 mL of fixative (methanol:acetic acid 3:1), the cells were washed with fresh fixative three times, resuspended, dropped onto clean slides and stained with 5% Giemsa for 10 min. One-thousand binucleated cells were analysed per experimental point. Fenech (2007) scoring criteria for micronuclei (MNi) determinations were used.

Statistical Analysis. Data were presented as means ± SD and p values less than 0.05 were considered statistically significant. Results from micronuclei analysis were statistically analyzed using the X^2-test with the Statgraphics® 5.1 software. The statistical evaluation of the quantitative comet assay was made by using the non-parametric Kruskal-Wallis Contrast which tests the null hypothesis of equal medians of comet parameters within each of the four treatments. Linear regression analysis was made to compare the results obtained with comet assay and micronuclei analysis.

RESULTS

Table 1, Figures 1 and 2 show the results obtained when cells were treated with the amitraz-containing commercial formulation. All the tested genotoxicity parameters showed the ability of this chemical product to negatively affect DNA. The frequency of micronuclei in binucleated cells significantly increased in all amitraz containing treatments (X^2=12.94, p<0.001; X^2= 19.19, p<0.001 and X^2= 26.82, p<0.001 respectively). The same has been observed with Comet assay quantitative analysis, both Tail DNA (K= 36.40, p<0.001) and Olive tail moment (K= 35.08, p<0.001) increase statistically significantly in cells treated with the product. Viability decreased significantly with doses 2 and 3 (X^2= 13.77, p<0.001 and X^2= 26.73, p<0.001).

Table 1. Micronuclei frequency, Comet assay parameters and Viability in CHO cells treated with a commercial formulation with amitraz as active constituent at different final concentrations.

Treatment	Micronuclei (‰)	Tail DNA	Olive Tail Moment	Viable cells (%)
Neg. Control	15 (0.12)	1.48 (0.55)	0.37 (0.15)	96.4 (0.19)
1.25 µg mL^{-1}	43 (0.20)	6.93 (2.06)	3.09 (0.97)	96.3 (0.19)
2.50 µg mL^{-1}	51 (0.22)	8.70 (1.89)	3.69 (0.88)	92.5 (0.26)
3.75 µg mL^{-1}	60 (0.24)	11.43 (2.60)	4.63 (1.17)	90.6 (0.29)

Standard error of the mean is indicated between parentheses.

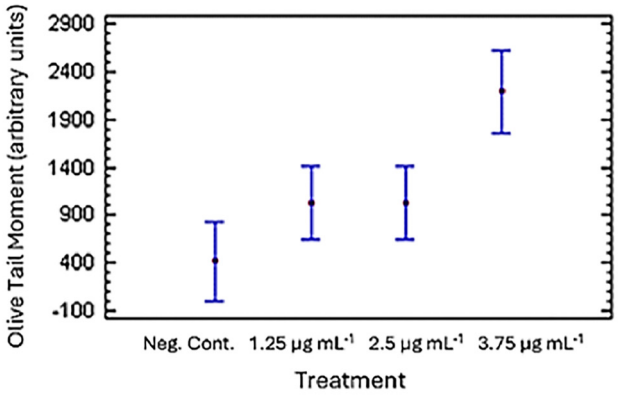

Figure 1. Average graph for Olive Tail Moment in CHO cells treated with a commercial formulation with amitraz as active constituent.

Table 2, Figures 3 and 4 abstract the results observed after culture the cells with the active constituent of the formulation. Both comet assay biomarkers: Tail DNA (K=9.11, p>0.05) and OTM (K=6.58, p>0.05) showed a non-significant slight increase. Micronuclei frequency showed similar behavior than comet parameters and non-significant differences between control and treated cells were observed (X^2=0.97, p>0.05). A low significant decrease in viability was detected only for cells treated with the dose 2 (X^2=10.86, p<0.01).

Despite the decrease in viability after treatment, a sufficient number of cells were obtained to develop the techniques, both in cells treated with the active component and in those cultured with the commercial product. The viability of cells treated with amitraz or DMSO in trypan blue exclusion assay was at least 90%.

Table 2. Micronuclei frequency, Comet assay parameters and Viability in CHO cells treated with amitraz at different final concentrations.

Treatment	Micronuclei (‰)	Tail DNA	Olive Tail Moment	Viable cells (%)
Neg. Control	18 (0.13)	1.24 (0.20)	0.52 (0.08)	97.7 (0.15)
DMSO	20 (0.13)	1.55 (0.45)	1.01 (0.25)	96.9 (0.18)
1.25 µg mL^{-1}	17 (0.13)	2.69 (0.72)	1.36 (0.41)	98.3 (0.13)
2.50 µg mL^{-1}	24 (0.15)	3.38 (0.92)	1.45 (0.41)	94.7 (0.22)
3.75 µg mL^{-1}	21 (0.14)	8.38 (1.32)	2.87 (0.83)	93.9 (0.24)

Standard error of the mean is indicated between parentheses.

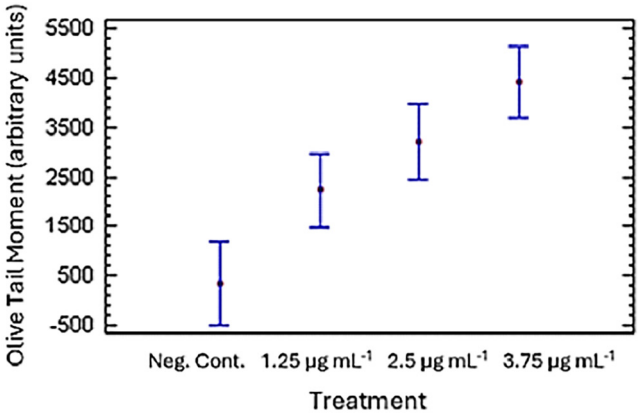

Figure 2. Average graph for Tail DNA in CHO cells treated with a commercial formulation with amitraz as active constituent.

Linear regression analysis was made to compare the results obtained with comet assay and micronucleus analysis. In cells treated with commercial product comparison for OTM and Tail DNA showed a significant relationship between both parameters (R^2=98.91, $p<0.001$), and the same occurs between OTM and MNi (R^2=99.77, $p<0.001$) and Tail DNA and MNi frequency (R^2= 98.93, $p<0.001$). Results for active constituent treated cells were statistically significant for the correlation between OTM and Tail DNA (R^2=98.95, $p<0.001$) but not for the others (R^2=19.13, $p>0.05$ for MNi vs. Tail DNA; and R^2=13.95, $p>0.05$ for MNi vs. OTM).

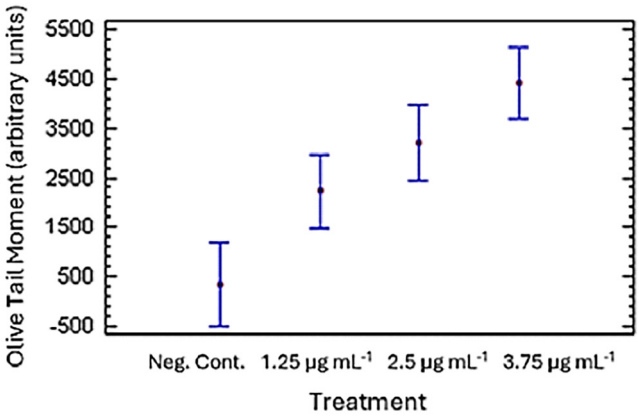

Figure 3. Average graph for Olive Tail Moment in CHO cells treated with amitraz.

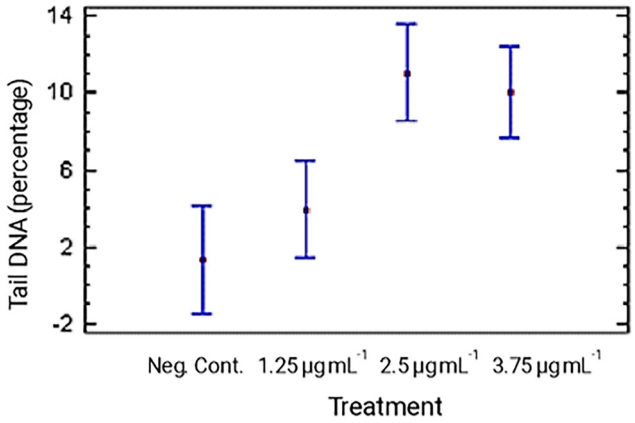

Figure 4. Average graph for Tail DNA in CHO cells treated with amitraz.

DISCUSSION

Long-term low-level exposure occurs when individuals are exposed to pesticide residues in the air, water, soil, sediment, or food; this kind of exposure has been the subject of great concern due to its possible role in the induction of congenital malformations and carcinogenesis (Mnif et al. 2011, Kim et al. 2017). The elucidation of the genotoxic potential associated with pesticides in human and animal populations plays a key role in cancer risk characterization because of the implications of mutagenesis processes in the early steps of carcinogenesis and reproductive toxicology.

Amitraz, a commonly used insecticide, is an effective tool for the prevention of animal pests. However, results obtained in this study highlight its potential adverse effects. Our findings clearly demonstrate the capability of the analyzed compounds to induce both cytogenetic and cytomolecular damage under experimental conditions. Both comet and micronucleus assays exhibited similar sensitivities, as evidenced by the statistical analysis of regression.

It is important to emphasize that the commercial formulation exhibited a significant genotoxic effect, whereas the active constituent did not cause statistically significant DNA damage. These findings align with previous studies, including those by Rojas-García et al. (2018), who reported that amitraz alters gene expression in vertebrates, and Nikoloff et al. (2021), Giorgini et al. (2023) and Carranza-Martin et al. (2024), who observed a reduction in cell viability in mammalian cells exposed to amitraz.

Amitraz's mechanism of action involves the activation of octopamine receptors in invertebrates, leading to neurological disruption. In mammals, amitraz functions as an agonist of alpha-2 adrenergic receptors and an inhibitor of monoamine oxidase (MAO), leading to neurotoxic and metabolic effects (del Pino et al. 2015, Giorgini et al. 2023). Amitraz has a short half-life in mammals, approximately 24 hours, as it is metabolized primarily in the liver into active metabolites such as N'-(2,4-dimethylphenyl)-N-methylformamidine (DMPF). These metabolites, including DMPF, are known to contribute to its biological activity and toxicity (Roberts and Reigart 2013). When amitraz interacts with the environment, it degrades into compounds such as 2,4-dimethylformanilide (DMF) and other by-products, which may persist and contribute to environmental toxicity (Ghosh et al. 2020).

Results from our laboratory, using the qualitative alkaline comet assay, showed that the commercial formulation of amitraz significantly increased DNA damage at concentrations ranging from 2.50 to 3.75 µg mL^{-1} (Padula et al. 2012). These findings are consistent with those reported by Radakovic et al. (2013), who observed similar effects in human lymphocytes exposed to concentrations between 0.035 and 350 µg mL^{-1}. Furthermore, the doses used in our study (1.25-3.75 µg mL^{-1}) are comparable to those reported by earlier studies, albeit much lower than those recommended by veterinary laboratories in Argentina (0.0125-0.025%).

Interestingly, the commercial product containing amitraz induced greater genotoxicity than the active constituent alone. This effect is likely attributable to other components of the formulation, such as impurities or solvents. Previous research has demonstrated the genotoxicity of common organic solvents, including toluene and xylene, which are often used in pesticide formulations (Moro et al. 2012, Prueitt et al. 2013, Wang et al. 2013). Although these inert ingredients lack pesticidal activity, they can be biologically active and, in some cases, more toxic than the active ingredient itself.

In Argentina, manufacturers of veterinary products do not disclose the complete list of components in commercial formulations, indicating only the proportion of the active ingredient. However, studies have shown that amitraz is frequently dissolved in hydrocarbon solvents, such as xylene and toluene (Caprotta et al. 2009, Moro et al. 2012, Prueitt et al. 2013, Wang et al. 2013). These solvents are known to enhance the absorption and bioavailability of amitraz but may also contribute significantly to its genotoxic effects.

In conclusion, our findings demonstrate that amitraz exhibits genotoxicity at doses ranging from 2.50 to 3.75 µg mL^{-1} and that this effect is significantly influenced by other components of the commercial formulation. Given the widespread exposure to pesticides through diet, environmental pollution, or occupational use, it is crucial to establish safe exposure limits. The presence of complex chemical mixtures, including impurities and solvents, in commercial pesticide formulations highlights the need for comprehensive toxicological evaluations. This underscores the importance of monitoring not only the active ingredients but also the so-called inert components, which may pose significant risks to human and environmental health.

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REFERENCES

1. Avsarogullari L, Ikizceli I, Sungur M, Sozuer E, Akdur O, Yucei M. Acute amitraz poisoning in adults: clinical features, laboratory findings, and management. *Clin. Toxicol.* 2006; 44: 19-24.
2. Bolognesi C. Genotoxicity of pesticides: a review of human biomonitoring studies. *Mutat. Res.* 2003; 543: 251-272.
3. Caprotta CG, Martinez M, Tiszler M, Guerra V. Amitraz poisoning. *Arch. argent. Pediatr.* 2009; 107: 449-458.
4. Carranza-Martin AC, Fabra MC, Urrutia Luna N, Farnetano N, Anchordoquy JP, Anchordoquy JM, Picco SJ, Furnus CC, Nikoloff N. *In vitro* adverse effects of amitraz on semen quality: Consequences in bovine embryo development. *Theriogenology*. 2023; 199:106-113.
5. del Pino J, Moyano-Cires PV, Anadon MJ, Díaz MJ, Lobo M, Capo MA, Frejo MT. Molecular mechanisms of amitraz mammalian toxicity: a comprehensive review of existing data. *Chem Res Toxicol*. 2015; 28(6):1073-1094.
6. Dhooria S, Agarwal R. Amitraz, an underrecognized poison: A systematic review. *Indian J Med Res.* 2016; 144: 348-358.
7. Fenech M, Chang WP, Kirsch-Volders M, Holland N, Bonassi S, Zeiger E. HUuman MicronNucleus project. HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. *Mutat. Res.* 2003; 534: 65-75.
8. Fenech M. Cytokinesis-block micronucleus cytome assay. *Nat. Protoc.* 2007; 2: 1084-1104.
9. Fereydooni S, Arfaee F, Youssefi M, Zahra F, Gharib F, Tabari M. *In vitro* toxicity of combination of amitraz and carvacrol on *Demodex canis*. *Open Vet. J.* 2023; 13: 894-902.
10. Giorgini M, Taroncher M, Tolosa J, Ruiz MJ, Rodríguez-Carrasco Y. Amitraz and its metabolites: oxidative stress-mediated cytotoxicity in hepG2 cells and study of their stability and characterization in honey. *Antioxidants*. 2023; 12(4): 885.
11. Ghosh S, Ojha PK, Carnesecchi E, Lombardo A, Roy K, Benfenati E. Exploring QSAR modeling of toxicity of chemicals on earthworm. *Ecotoxicol Environ Saf.* 2020; 190: 110067.

12. Hu S, Benner C, White J, Martin R, Feenstra K. Pharmacokinetics and brain distribution of amitraz and its metabolites in rats. *Environ Toxicol Pharmacol.* 2019; 65: 40-45.
13. Khan SJ, Roser DJ, Davies CM, Peters GM, Stuetz RM, Tucker RNJ. Ashbolt chemical contaminants in feedlot wastes: concentrations, effects and attenuation. *Environ. Int.* 2008; 34: 839-859.
14. Kim K, Kabir E, Jahan S. Exposure to pesticides and the associated human health effects. *Sci Total Environ.* 2017; 575: 525-535.
15. Końca K, Lankoff A, Banasik A, Lisowska H, Kuszewski T, Góźdź S, Koza Z, Wojcik A. A cross-platform public domain PC image-analysis program for the comet assay. *Mut. Res.* 2003; 534: 15-20.
16. Marafon CM, Delfim CI, Valada CA, Menotti R, Andrade SF. Analysis of amitraz in cats by gas chromatography. *J. Vet. Pharmacol. Ther.* 2009; 33: 411-414.
17. Mnif W, Hassine A, Bouaziz A, Bartegi A, Thomas O, Roig B. Effect of endocrine disruptor pesticides: A review. *Int J Environ Res Public Health.* 2011; 8(6): 2265-2303.
18. Moro AM, Brucker N, Charão M, Bulcão R, Freitas F, Baierle M, Nascimento S, Valentini J, Cassini C, Salvador M, Linden R, Thiesen F, Buffon A, Moresco R, Garcia SC. Evaluation of genotoxicity and oxidative damage in painters exposed to low levels of toluene. *Mut. Res.* 2012; 746: 42-48.
19. Nanjundappa S, Narayanan S, Darsana Udayan N, Kanapadinchareveetil S, Jacob M, Ravindran R, Juliet S. Disposition Kinetics of Amitraz in Lactating Does. *Molecules.* 2021; 26: 4769-4772.
20. Nikoloff N, Carranza Martin A, Fabra C, Furnus C. Amitraz induced cytotoxic effect on bovine cumulus cells and impaired oocyte maturation. *Environ Sci Pollut Res.* 2021; 28: 29188-29199.
21. Olive P. DNA damage and repair in individual cells: applications of the comet assay in radiobiology. *Int J Radiat Biol.* 1999; 75: 395-405.
22. Osano O, Oladimeji AA, Kraak MH, Admiraal W. Teratogenic effects of amitraz, 2,4-dimethylaniline, and paraquat on developing frog (*Xenopus*) embryos. *Arch Environ Contam Toxicol*. 2002; 43: 42-49.
23. Padula G, Ponzinibbio MV, Picco S, Seoane A. Assessment of the adverse effects of the acaricide amitraz: in vitro evaluation of genotoxicity. *Toxicol. Mech. Methods.* 2012; 22: 657-666.
24. Proudfoot AT. Poisoning with amitraz. *Toxicol. Rev.* 2003; 22: 71-74.
25. Prueitt RL, Rhomberg LR, Goodman JE. Hypothesis-based weight-of-evidence evaluation of the human carcinogenicity of toluene diisocyanate. *Crit. Rev. Toxicol.* 2013; 43: 391-435.
26. Radakovic M, Stevanovic J, Djelic N, Lakic N, Knezevic-Vukcevic J, Vukovic-Gacic B, Stanimirovic Z. Evaluation of the DNA damaging effects of amitraz on human lymphocytes in the Comet assay. *J. Biosci.* 2013; 38: 53-62.
27. Roberts J, Reigart R. Recognition and Management of Pesticide Poisoning, US EPA Office of Pesticide Programs, 2013; Sixth Edition.
28. Rojas-García A, Solís-Heredia M, Paredes S, Pérez-Maldonado I. Occupational exposure to pesticides and DNA damage in workers from the state of Nayarit, Mexico. *Environ Sci Pollut Res*. 2011; 18: 987-996.
29. Singh NP, Mc Coy M, Tice R, Schneider E. A simple technique for quantization of low levels of DNA damage in individual cells. *Exp. Cell Res.* 1988; 175: 184-191.
30. Strober W. Trypan blue exclusion test of cell viability. *Curr Protoc Immunol.* 2015; 111: A3.B.1-A3.B.3.
31. Tice R, Strauss G. The single cell gel electrophoresis/comet assay: a potential tool for detecting radiation-induced DNA damage in humans. *Stem Cells*. 1995; 13: 207-214.
32. Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu J, Sasaki Y. Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. *Environ. Mol. Mutagen.* 2000; 35: 206-221.
33. USEPA (United States Environmental Protection Agency). Amitraz Reregistration Eligibility Decision (RED), Case 0234. (EPA-738-F-96-031), US Environmental Protection Agency, Office of Prevention, Pesticide and Toxic Substances, Washington, DC. 1996.
34. Ulukaya S, Demirag K, Moral AR. Acute amitraz intoxication in human. *Intensive Care Med.* 2001; 27: 930-933.
35. Wang F, Li C, Liu W, Jin Y. Oxidative damage and genotoxic effect in mice caused by sub-chronic exposure to low-dose volatile organic compounds. *Inhal. Toxicol.* 2013; 25: 235-242.
36. Westermann CM, Boerma S, van Nieuwstadt RA. Amitraz intoxications in the horse: cases and backgrounds. *Tijdschr Diergeneeskd.* 2004; 129: 438-441.
37. Young FM, Phungtamdet W, Sanderson BJS. Modification of MTT assay conditions to examine the cytotoxic effects of amitraz on the human lymphoblastoid cell line, WIL2NS. *Toxicol. in Vitro.* 2005; 9: 1051-1059.