



Amitraz induced cytotoxic effect on bovine cumulus cells and impaired oocyte maturation

Noelia Nikoloff¹ · Ana C. Carranza Martin¹ · Mariana C. Fabra¹ · Cecilia C. Furnus¹

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Abstract

The aim of this study was to evaluate the genotoxic and cytotoxic effects of amitraz (AMZ) on the primary culture of bovine cumulus cells (CC) and oocyte nuclear maturation. Cytotoxicity was evaluated by assessing mitochondrial activity with the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Genotoxicity was estimated using the alkaline single cell gel electrophoresis (SCGE) assay. Apoptosis was detected with the Annexin V-affinity assay. The in vitro maturation test was performed in bovine oocytes. To understand AMZ action, glutathione content, superoxide dismutase enzyme activity, and lipid peroxidation were evaluated in CC. Results showed that AMZ lethal concentration (LC 50_{24h}) for bovine CC was 32.55 µg/mL (MTT assay). A 25 µg/mL induced late apoptosis and necrotic cells ($p < 0.05$); however, DNA damage was decreased at the same concentration (SCGE assay; $p < 0.05$). A decrease in metaphase II was observed at 25 µg/mL, and degenerate oocytes were observed at 15 and 25 µg/mL ($p < 0.05$). None of the oxidative stress parameters evaluated showed significant differences. This study contributes to a better understanding of AMZ in this model, suggesting its potential cytotoxicity and impact on bovine reproduction.

Keywords Formamide pesticide · Cumulus-oocyte complex · Apoptosis · MTT · Single-cell gel electrophoresis · Oocyte nuclear maturation

Introduction

Amitraz {N, N-[(methylamino) dimethylidylidene] di-2, 4-xylidine} (AMZ) is a formamidine pesticide widely used as an insecticide and an acaricide. Pesticides are defined as substances that are used for preventing, destroying, repelling, or mitigating a pest with potential cell damage (Padula et al. 2012). Since 1974, this broad-spectrum antiparasitic agent has been used in veterinary medicine and agriculture all over the world. Veterinary medicine uses AMZ to control ectoparasites in pigs, cattle, sheep, goats, and dogs (Gupta and

Milatovic 2014). In bovine, farmers control ticks using AMZ in dipping baths (Moyo and Masika 2009). Amitraz is a liposoluble compound that is rapidly absorbed through the skin and mucous membranes, having rapid contact with all body tissues (Elinav et al. 2005; Marafon et al. 2010; Amizadeh et al. 2017). Its side and toxic effects have been extensively reported considering that AMZ exposure is potentially dangerous for human beings and animals (Grossman et al. 1993; Proudfoot 2003)

Additionally, human exposure to AMZ may occur through the consumption of food products containing residues and through contact with treated animals. The toxicity of AMZ has been studied by different agencies like The United States Environmental Protection Agency (US EPA 1996) and the Joint Meeting on Pesticide Residues in Food, World Health Organization (WHO 1998). The US EPA classifies AMZ as slightly toxic by the oral and inhalation routes (category III), moderately toxic by the dermal route (category II), and not a dermal irritant, only slightly irritant to the eyes and not a dermal sensitizer (category IV).

The potentially negative effects of AMZ to induce cell damage and death have been studied before. Human

Noelia Nikoloff and Ana C. Carranza Martin contributed equally to this work.

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✉ Cecilia C. Furnus
cfurnus@fcv.unlp.edu.ar; cfurnus@gmail.com

¹ IGEVET – Instituto de Genética Veterinaria “Ing. Fernando N Dulout” (UNLP-CONICET LA PLATA), Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, Calle 60 y 118, B1904AMA, La Plata, Buenos Aires, Argentina

luteinized cells treated with 34.1 $\mu\text{g}/\text{mL}$ AMZ for 24 h presented cytotoxic damage, resulting in cell death after 72 h (Young et al. 2005). Culture of Chinese hamster ovary (CHO)-K1 cells with 3.75 $\mu\text{g}/\text{mL}$ AMZ for 16 h showed increased apoptosis compared with the control group (Padula et al. 2012). Kim et al. (2007) reported that 30 mg AMZ/kg/day administered to rats for 19 days produced embryotoxicity and teratogenicity. Also, AMZ exposure was associated with prolonged estrus in rats and prolongation of proestrus, and shortening of diestrus in mice (Del Pino et al. 2017).

Mammalian cell culture is the biological model used in toxicology studies because it represents a pivotal *in vitro* system to define xenobiotic toxicity. The advantage of primary cell cultures is their species and organ specificity. Primary cells express the same proteins and genes as their cell types *in vivo* (Carney and Settivari 2013). As regards reproductive toxicology, several primary methods of the oviduct, cumulus, and luteal cell culture have been developed in recent years to reduce the use of animals in toxicological tests. This is important progress in the area of toxicology since these systems have the potential to become widely used compared with the commonly used *in vivo* tests (Guerreiro et al. 2019).

The main points of human and bovine reproductive processes are extremely close, making the bovine model interesting to be studied (Campbell et al. 2003). Bovine cumulus cells (CC) play a fundamental role in reproductive processes such as oocyte maturation and fertilization (Tanghe et al. 2002; Krisher 2004; Yuan et al. 2005). Numerous studies have recognized the predictive potential of bovine oocytes on the adverse effects that may occur during the maturation and fertilization processes and have validated these tests scientifically for use in reproductive toxicology. It has been shown that several chemical or pharmacological products act directly on the oocytes during the maturation process, causing oocyte cell cycle disruption and inducing DNA and oxidative damage (Lazzari et al. 2008; Beker van Woudenberg et al. 2012; Piersma et al. 2013).

From the above, AMZ appears as an interesting pesticide to study not only because of its possible effect on bovine reproduction—one of the main aspects of meat production—but also because the results of this study could be used in other exposed organisms, including human beings. The aim of this study was to evaluate the genotoxic and cytotoxic effect of AMZ on the primary culture of CC and the reproductive process of bovine oocyte nuclear maturation. Also, AMZ potential to induce oxidative stress was studied.

Materials and methods

Chemicals

Pure AMZ (N-methyl bis (2,4-xylyminomethylamine) (#45323; CAS 33089-61-1), cycloheximide (#C1988),

dimethyl sulfoxide (DMSO; #4540; CAS 67-68-5), methanol (#34860; CAS 67-56-1), acetic acid (#A6283; CAS 64-19-7), Ethylenediaminetetraacetic sodium salt (EDTANa2; #E5134), Tris (#T1503), Tris HCL (#T5941), Triton X-100 (#T9284), NaOH (#S8045) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; #M5655; CAS 57360-69-7) and all reagents for media preparation, were purchased from Sigma Chemical Co. (St. Louis). Follicle-stimulating hormone (FSH) was purchased from Bioniche (Belleville, Ontario, Canada). Annexin V-fluorescein isothiocyanate (FITC) was purchased from Biosource International, Inc. (USA), ethanol (ETOH; #1.00983.1000_T; CAS 64-17-5) was purchased from Merck KGaA (Darmstadt, Germany) and bleomycin (BLM) was kindly provided by Gador S.A. (Nippon Kayaku Co., Japan). Normal (#50000) and low (#S32830) melting agarose were obtained from Carlsbad (CA, USA), and SYBR Green I (#4402953) was obtained from Molecular Probes (Eugene, OR, USA).

Experimental models

Bovine ovaries were obtained from an abattoir and transported to the laboratory within 3 h of slaughter in sterile NaCl solution (9 g/mL) supplemented with antibiotics at 37 °C. The ovaries were pooled regardless of the estrous cycle stage of animals. Cumulus oocyte complexes (COCs) were aspirated from follicles (3–8 mm) using an 18-G needle. We only selected cumulus-intact oocytes with an evenly granulated cytoplasm using a low-power (20–30x) stereomicroscope (Nikon, Tokyo, Japan).

Primary culture of CC

Each primary CC culture was obtained isolating CC from oocytes after maturation of 20 COC by repeated pipetting with a narrow-bore glass pipette. The CC were cultured at a starting density of 5×10^4 cells/mL per well in 12-well plates (NuncTM #150628) in TCM 199 medium with 10% fetal bovine serum (FBS) at 39 °C in 5% CO₂ in the air with saturated humidity for 4 days until the cells were nearly confluent.

In vitro maturation

The COCs were washed twice in TCM 199 buffered with 15 mM HEPES containing 10% FBS and twice in *in vitro* maturation (IVM) medium (TCM 199, 10% FBS, 0.2 mM sodium pyruvate, 1 mM glutamine, 1 $\mu\text{g}/\text{mL}$ FSH, 1 $\mu\text{g}/\text{mL}$ 17 β -estradiol, antibiotics). Groups of 10 COCs were transferred to 50- μL IVM medium under mineral oil (Squibb, USA) preequilibrated in a CO₂ incubator. The COCs were cultured in IVM medium at 39 °C, 5% CO₂ in the air, and saturated humidity for 24 h.

AMZ preparation

The pure drug AMZ first was diluted in DMSO to obtain the stock solutions. For the MTT assays the concentrations of the stock solutions were 1 mg/mL, 5 mg/mL, 20 mg/mL, and 60 mg/mL. TCM 199 medium with 10% FBS was used to obtain final working concentrations of 1, 2.5, 5, 10, 15, 25, 50, 100, 250, 500, and 750 $\mu\text{gAMZ/mL}$. For the cyto and genotoxic assays, oocyte nuclear maturation, and oxidative stress the stock solution concentration was 5 mg/mL. TCM 199 medium with 10% FBS was used to obtain the final working concentration of 10; 15 and 25 $\mu\text{gAMZ/mL}$. Those stocks were stored at $-20\text{ }^{\circ}\text{C}$ until use. The final DMSO concentration in all treatments was 0.5%, it was included as solvent control.

Preliminary study: determination of AMZ lethal concentration 50 (LC50)

The $\text{LC}_{50_{24\text{h}}}$ of AMZ was evaluated using the MTT assay following the protocol of Wu et al. (2013). Briefly, 1×10^4 CC/well was cultured in TCM 199 on 96-well microplates (Corning #3599) for 4 days until the cells were nearly confluent. Afterward, the culture medium was removed and cells were treated with 1, 2.5, 5, 10, 15, 25, 50, 100, 250, 500, and 750 $\mu\text{g/mL}$ AMZ (3.40 to 2556.15 μM). The MTT stock solution was added at a final concentration of 0.25 mg/mL and incubated at $37\text{ }^{\circ}\text{C}$ for 3 h on 96-well microplates. Later, 100- μL DMSO was added to dissolve formazan blue crystals. Absorbance was measured at 550 nm with a microplate spectrophotometer (Multiskan™ GO (Thermo Fisher Scientific)). Negative (NC) (TCM 199 medium), solvent (DMSO 0.5%), and positive (PC) (ETOH 10%) controls were conducted and run simultaneously with AMZ treatments. Data were expressed as the mean of absorbance (550 nm) of three independent experiments.

The LC50 value was determined from the dose-response curve, where the 50% cytotoxicity intercepts with the AMZ concentration on the x-axis. Experiments were performed in triplicate.

Genotoxicity and cytotoxicity assays

Experiments were performed using primary cultures of bovine CC. Cultured cells were exposed to three different concentrations of the tested compound for 24 h (10, 15, and 25 $\mu\text{g/mL}$ AMZ), equivalent to 25, 50, and 75% of the LC50 value, respectively, and corresponding to 34.08, 51.12, and 85.20 μM AMZ. Genotoxicity and cytotoxicity assays were performed at the end of the treatments. Bleomycin (1 $\mu\text{g/mL}$) was used as a PC for single-cell gel electrophoresis (SCGE) and 10% ETOH was the PC for apoptosis. Cells grown in medium without the addition of any other compound were used as the negative control, and 0.5% DMSO was used as

solvent control. All the experiments were performed in triplicate to allow an accurate estimate of the interexperimental variation.

SCGE assay

Genotoxicity was evaluated with SCGE and performed using the alkaline version described by Singh et al. (1988) with some modifications. Briefly, slides were covered with a first layer of 180- μL 0.5% normal agarose. An amount of 75 μL 0.5% low melting point agarose was mixed with approximately 1.5×10^4 CC and then layered onto the slides, which were immediately covered with coverslips. After agarose solidification at $4\text{ }^{\circ}\text{C}$ for 10 min, coverslips were removed and slides were immersed overnight at $4\text{ }^{\circ}\text{C}$ in fresh lysis solution (1% sodium sarcosinate, 2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, pH 10.0, 1% Triton X-100, and 10% DMSO). The slides were equilibrated in alkaline solution (1 mM EDTA Na₂, 300 mM NaOH) (pH 13) for 20 min. Electrophoresis was carried out for 30 min at 25 V and 300 mA (1.25 V cm^{-1}). Afterward, slides were neutralized by washing three times with 0.4 M Tris HCL buffer (in distillate water) (pH 7.5) every 5 min and subsequently washed in distilled water. Slides were stained with a 1/1,000 SYBR Green I solution. Two hundred randomly selected comet images were analyzed per treatment per replicate. Scoring was performed at $\times 40$ by one researcher using a fluorescent microscope (Olympus BX40 equipped with a 515–560-nm excitation filter). Nucleoids (cells without nuclear membrane) were classified based on the extent of strand breakage, according to their tail length, into five types: grade 0, no visible tail; grade I, comets with a tiny tail; grade II, comets with a dim tail; grade III, comets with a clear tail; and grade IV, comets with a clear decrease in the diameter of the head and a clear tail (Fig. 1a) (Cavaş and Könen 2007). Genetic damage index (GDI) was obtained according to Pitarque et al. (1999) using the formula $\text{GDI} = \{[1(\text{I}) + 2(\text{II}) + 3(\text{III}) + 4(\text{IV})]/\text{N}(\text{I-IV})\} \times 100$, where I–IV represent the nucleoid type and NI–NIV represent the total number of nucleoids scored. Visual scoring (arbitrary units) is rapid as well as simple, and there is very close agreement between this method and computer image analysis (percentage of DNA in the tail) (Heaton et al. 2002). Three replicates were done, and samples were performed in duplicate for each experimental point.

Annexin V affinity assay

The Annexin V assay was performed to evaluate cytotoxicity. This assay is based on annexin V fluorescein isothiocyanate (FITC) binding ability to expose phosphatidylserine, an early indicator of apoptosis (van Engeland et al. 1998). At the end of treatments, 1×10^6 CC were washed in PBS, resuspended in 250 μL of $1 \times$ binding buffer (10 mM HEPES, 140 mM

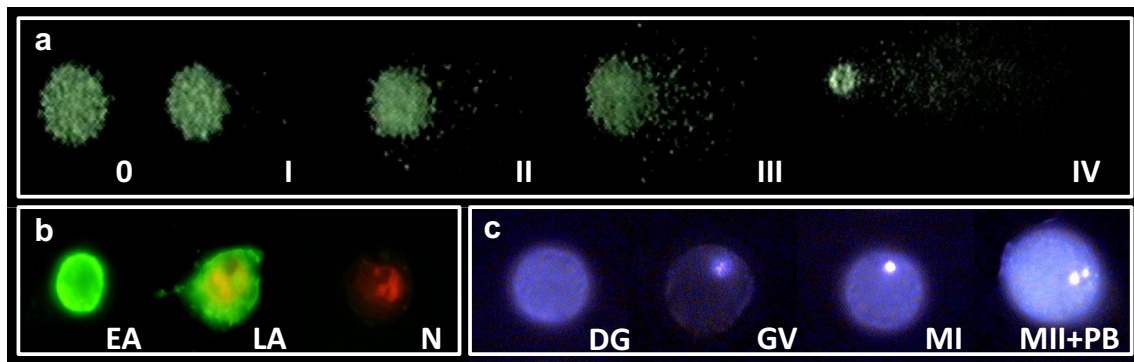


Fig. 1 Images that represent the classification used in each assay; **a** DNA damage at individual bovine cumulus cell (CC) level. Grade 0 (no visible tail), Grade I (comets with tiny tail), Grade II (comets with a dim tail), Grade III (comets with a clear tail), Grade IV (comets with a clear decrease in the diameter of the head and a clear tail) ($\times 40$); **b** Apoptosis evaluated by annexin V-FITC assay in CC. Early apoptotic

(EA) (annexin-V positive/PI negative), late apoptotic (LA) (annexin-V positive/PI positive), and necrotic (N) cells (annexin-V negative/PI positive) (100X); **c** Bovine oocyte nuclear maturation evaluated with Hoechst 33342. Oocytes were classified as degenerate (DG), germinal vesicle (GV), metaphase I (MI), metaphase II + polar body (MII + PB) stage of maturation process ($\times 20$)

NaCl, and 2.5 mM CaCl₂), and exposed to 2- μ L annexin V-FITC for 15 min at room temperature in the dark. Afterward, cells were washed with a binding buffer and 2- μ L propidium iodide (PI) (stock solution, 10 μ g/mL) was added to each sample. Samples were analyzed under an Olympus BX40 epifluorescent microscope equipped with an appropriate filter combination. The CCs were classified following the criteria reported by Pläsier et al. (1999) as alive (annexin V negative/PI negative), early-apoptotic (annexin V positive/PI negative), late apoptotic (annexin V positive/PI-positive), and necrotic cells (annexin V negative/PI-positive) (Fig. 1b). Each experiment was repeated three times, and samples were performed in duplicate for each experimental point. The percentage of stained CC was determined by counting 100 cells per coverslip in at least four different fields (Lourenço et al. 2014). Results were expressed as the mean percentage of alive, early-apoptotic, late-apoptotic, and necrotic cells.

Oocyte nuclear maturation

The COCs were treated with 10-, 15-, and 25- μ g/mL AMZ for 24 h. After IVM, oocytes were placed in TCM 199 medium + 0.2% hyaluronidase at room temperature and then pipetted to remove the CC. Solvent and positive controls were 0.5% DMSO and 0.39 μ M cycloheximide (Lazzari et al. 2008), respectively. Oocyte nuclear maturation was assessed by mounting and staining the denuded oocytes with the fluorescent DNA-specific dye Hoechst 33342 and then examined under an epifluorescence microscope Olympus BX40 equipped with an appropriate filter combination. Oocytes were classified as a germinal vesicle (GV), metaphase I (MI), or metaphase II + polar body (MII + PB) stage of the maturation process (Fig. 1c) (Izadyar et al. 1997; Süs et al. 1998). Oocytes with an abnormal configuration of chromatin or without chromatin were classified as degenerate (DG). Results were expressed as oocyte percentages with different

status of nuclear maturation. Different COCs from the primary culture of CC were used. A total of 120 oocytes were evaluated per treatment, and 40 oocytes were examined per experiment. Three independent experiments were performed.

Oxidative stress

Redox balance

Negative controls and cultures of treated CC were used to measure oxidative stress. At the end of the treatments, the culture medium was discarded, and cells were homogenized on ice with a Teflon-glass homogenizer in microtubes containing 100- μ L phosphate buffer saline (PBS). Then, the suspension was centrifuged at 4500 $\times g$ for 10 min at 4 $^{\circ}$ C, and the supernatant was stored at -80 $^{\circ}$ C until use. Protein concentration, superoxide dismutase (SOD) activity, total reduced glutathione (GSH) concentration, and lipid peroxidation were determined with the same pool of CC, as described by Luchetti et al. (2017) using a 96-well microplate reader (Biochrom EZ Read 400 Microplate Reader) and Galapagos for EZRead Software (Biochrom Ltd., Cambridge, UK). Each assay was performed taking into account the standard assay and calibration curve. Cultures were performed in at least three independent experiments.

Protein quantification

The Bradford method (Bradford 1976) was used to determine protein concentration in each sample. Samples with 10 μ L and 100 μ L Bradford's reagent were used. The standard Bradford assay consisted of 1-mg/mL albumin. The blank was buffer without protein. The absorbance at 595 nm was recorded, and the protein concentration was determined by comparison to a standard curve of known albumin concentrations. The result was expressed as μ g protein/mL.

Total reduced glutathione/oxidized glutathione assay

Total intracellular GSH content was evaluated as described by Takahashi et al. (1993) with slight modifications. For this purpose, 10 μL of each sample was incubated in a phosphate buffer (pH 7) containing NADPH and GSH reductase. The enzymatic reduction of GSSG to GSH was measured with 5, 5-dithiobis 2-nitrobenzoic acid in a kinetic measurement for 5 min at 405-nm absorbance. Blanks consisted of PBS wash medium. Results were expressed as pmol GSH/ μg protein.

SOD activity

The RX MONZA RANSOD SD 125 kit was used to determine SOD activity. Xanthine and xanthine oxidase (XOD) generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) forming red formazan dye. Then, SOD activity is measured by the inhibition degree of this reaction. One unit of SOD is that which causes 50% inhibition of the INT reduction rate under the assay conditions. Sample and reagent volumes were adapted to the microplate, using one-tenth of the volumes indicated in the kit (Luchetti et al. 2017). In the kinetics assay performed according to the manufacturer's instructions, initial absorbance was read at 492 nm after the 30 s of mixing the reagents with the samples, and final absorbance was determined after 3 min. Results were expressed as units SOD/ μg protein.

Lipid peroxidation

Lipid peroxidation levels were measured using the thiobarbituric acid reactive substances (TBARS) method adapted to the microplate (Luchetti et al. 2017). The 30- μL homogenate was treated with 15% trichloroacetic acid (TCA) (w/v)–0.375% thiobarbituric acid (TBA) (w/v)–0.25 M HCl in microtubes and heated for 15 min in water at 100 °C. After cooling, the absorbance was determined at 492 nm. Results were expressed as nmol MDA/ μg protein.

Statistical analyses

The LC50 value calculated from the concentration-response curve was fitted using the Graph Pad Prism version 5.01 (GraphPad Software Inc., San Diego, CA, USA). For the rest of the experiments, a completely randomized block design was used. The statistical model included the random effects of blocks (replicate; $n = 3$) and the fixed effect of treatments: NC vs. DMSO vs. 10 $\mu\text{g}/\text{mL}$ vs. 15 $\mu\text{g}/\text{mL}$ vs. 25 $\mu\text{g}/\text{mL}$ vs. PC. Oocyte nuclear maturation, CC apoptosis, and comet assay rates were analyzed by logistic regression using the GENMOD procedure (SAS 9.4 Institute Cary, NC, USA). Results were expressed as percentage \pm standard error

(SEM). Results of the MTT assay, GDI, protein concentration, and oxidative status were analyzed using the GLMIX procedure (SAS 9.4 Institute, Cary, NC, USA) with gamma distribution when it was not normal. Results were expressed as mean \pm SEM. To evaluate the concentration-dependent response to treatments, simple linear regression, and correlation analysis were performed in MTT and apoptosis assays using the CORR procedure (SAS 9.4 Institute, Cary, NC, USA). Also, an orthogonal polynomial contrast was done using the GLM procedure (SAS 9.4 Institute, Cary, NC, USA) to evaluate the response of the concentration curve in the MTT assay. Statistical significance was set at $p < 0.05$.

Results

The toxicity of AMZ was examined in bovine cultured CC. First, the metabolic activity of the cultured cells after exposure to AMZ was examined using the MTT assay. This assay determines the mitochondrial respiratory activity by the succinate–tetrazolium reductase system, which converts the yellow tetrazolium salt into a blue formazan dye (Robb et al. 1990). Cumulus cells treated with ETOH showed a significant decrease in energetic cell metabolism compared with NC conditions ($p < 0.01$). The CC treated with AMZ showed inhibition of mitochondrial activity in the 10 to 750 $\mu\text{g}/\text{mL}$ exposure range ($p < 0.01$). Regression analysis revealed that energetic cell metabolism diminished as a negative function of the AMZ concentration ($r = -0.64$, $p = 0.05$; Fig. 2). GraphPad Prism analysis of CC mortality demonstrated that LC50_{24h} was 32.55 $\mu\text{g}/\text{mL}$ with 95% confidence intervals. We observed that mitochondrial activity decreased abruptly after treatment with 50 $\mu\text{g}/\text{mL}$ AMZ, with more than 70% CC mortality. Thereafter, differences in the 100–750- $\mu\text{g}/\text{mL}$ AMZ concentration range were not significant ($p \geq 0.08$). The statistical analysis demonstrated that the response of the curve was cubic ($p = 0.04$).

The alkaline version of SCGE was performed in bovine CC exposed for 24 h to different concentrations of AMZ. This version detects DNA migration caused by strand breaks, alkaline labile sites, and transient repair sites. Data showed no differences in DNA damage and GDI between negative controls (NC and DMSO, $p = 0.3$; Table 1). Treatment with 1 $\mu\text{g}/\text{mL}$ BLM (PC) significantly increased the proportion of damaged cells and incremented GDI values compared with NC values ($p < 0.01$; Table 1). The DNA damage decreased with 15 and 25 $\mu\text{g}/\text{mL}$ AMZ ($p < 0.01$; Table 1). The percentage of DNA damage decreased with 15 and 25 $\mu\text{g}/\text{mL}$ AMZ ($p < 0.01$; Table 1) due to a decrease of grade II, III and IV in 15 $\mu\text{g}/\text{mL}$ ($p < 0.05$; Table 1), and types I, II, III, and IV in 25 $\mu\text{g}/\text{mL}$ ($p < 0.05$; Table 1). The GDI values demonstrated that 15 and 25 $\mu\text{g}/\text{mL}$ had lesser values than NCs ($p < 0.01$; Table 1)

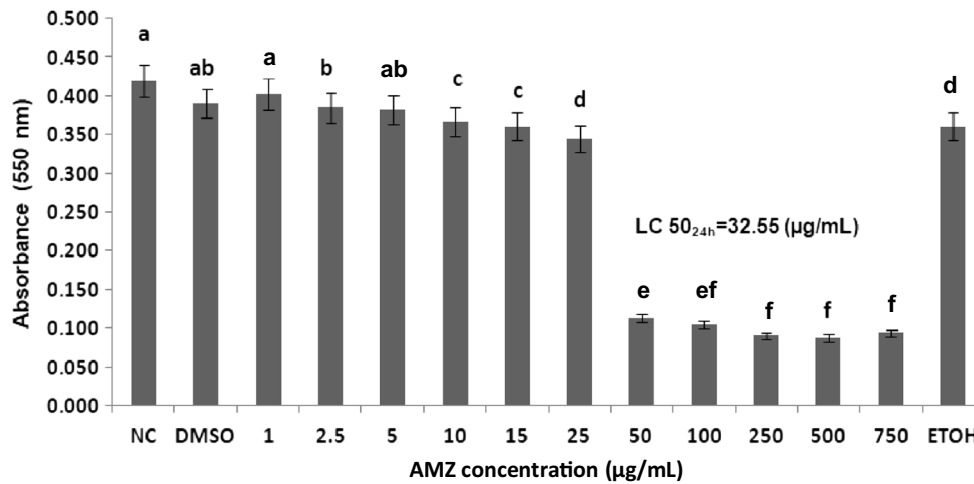


Fig. 2 Mitochondrial activity evaluated by the MTT assay in bovine cumulus cells treated with Amitraz (AMZ). Cultures were incubated for 3 h with MTT after 24 h of antiparasitic AMZ. Results are expressed as the mean absorbance (550 nm) of mitochondrial activity from three independent experiments (y-axis) and plotted against the AMZ

concentration (0–750 µg/mL concentration range; x-axis). The negative control (NC) was without solvent, the positive control was 10% ethanol (ETOH) and 0.5% dimethyl sulfoxide (DMSO) was used as solvent controls. **a–f** Bars with different letters differ ($P < 0.05$)

In the present study, apoptosis was detected by the Annexin V-FITC binding method. The proportions of apoptotic and necrotic cells were consistent with the observed frequency for NC cultures. Treatment with ETOH (PC) significantly increased the apoptosis rate, decreased the alive cellular proportion, and increased necrotic cells compared with DMSO ($p < 0.01$; Table 2). Comparison of the AMZ treatments with NC results showed that the total apoptosis rate was similar between NC, 10, and 15 µg/mL AMZ ($p \geq 0.2$; Table 2). On the other hand, a higher apoptosis rate was observed when CCs were treated with 25 µg/mL AMZ due to an increase in late apoptotic cells ($p < 0.01$; Table 2). This concentration also showed that the alive cell proportion was lower compared with NC, 10, and 15 µg/mL AMZ ($p < 0.01$; Table 2). We observed that when CCs were treated with 15 µg/mL AMZ, only late apoptosis was significantly higher

than with NC ($p < 0.01$; Table 2). The rate of necrosis was higher with 25 µg/mL AMZ as compared with NC ($p < 0.01$; Table 2). The regression analysis demonstrated that total apoptosis increased as a function of the AMZ concentration ($r = 0.62$; $p < 0.01$).

Achievement of the maturation stage (completion of meiosis up to the metaphase II) was selected as the toxicological endpoint of the IVM test on bovine oocytes. The NC results did not differ from the solvent control ($p \geq 0.7$; Table 3). The maturation rate significantly decreased when the PC was assayed ($p < 0.01$; Table 3). The PC increased the frequencies of GV, MI, and DG, while reducing the oocyte rate in MII + PB compared with the control ($p < 0.01$; Table 3). When COCs were matured in the presence of 25 µg/mL AMZ, we observed an impaired oocyte nuclear maturation, which was evidenced by a decrease in the percentage of MII + PB

Table 1 Analysis of DNA damage in cumulus cell (CC) exposed to amitraz (AMZ) determined by alkaline single cell gel electrophoresis

AMZ (µg/mL)	Damage proportion (%)					Damage (%) (II+III+IV)	GDI
	0	I	II	III	IV		
NC	57.0±1.6 ^a	21.5±1.7 ^a	13.2±1.2 ^a	5.0±1.1 ^a	3.3±0.3 ^{ab}	21.7±0.4 ^a	76.6±0.8 ^a
DMSO	51.1±4.5 ^a	25.1±2.8 ^a	14.8±1.7 ^a	6.3±0.9 ^a	2.8±0.9 ^a	24.1±2.4 ^a	85.0±0.9 ^a
10	54.5±4.6 ^c	27.08±3.7 ^a	11.8±2.8 ^b	5.2±0.9 ^a	1.5±0.8 ^b	18.5±3.9 ^a	72.3±10.4 ^a
15	61.5±0.76 ^a	27.5±1.8 ^a	7.2±0.9 ^c	2.8±0.6 ^b	1.0±0.3 ^c	11.0±1.5 ^b	54.7±2.4 ^b
25	79.0±4.0 ^a	14.5±2.3 ^b	5.8±2.2 ^c	0.5±0.3 ^c	0.2±0.2 ^c	6.5±2.3 ^c	28.7±5.8 ^c
BLM	8.0±1.0 ^b	22.7±1.2 ^b	17.5±2.0 ^d	27.2±0.7 ^d	24.7±0.3 ^d	69.4±1.9 ^d	238.0±3.5 ^d

Types I–IV indicate grades of DNA damage as a percentage of pooled data from three independent experiments. Genetic damage index (GDI) = $(1 \times I + 2 \times II + 3 \times III + 4 \times IV) / (I + II + III + IV) \times 100$. Bovine CC were incubated in TCM 199 medium and treated with AMZ for 24 h. NC Negative control; DMSO dimethyl sulfoxide; solvent control; bleomycin (BLM, 1 µg/mL) was used as positive control. A total of 600 cells were analyzed per treatment from three replicates performed on different days. Data are expressed in percentage ± SEM. ^{a–d} Columns without a common superscript differed ($p < 0.05$)

oocytes (NC: 71.7 ± 2.6 vs $25 \mu\text{g AMZ/mL}$: 32.5 ± 6.3) and an increase of GV rate (NC: 2.5 ± 0.0 vs $25 \mu\text{g AMZ/mL}$: 15.0 ± 5.8) with respect to the NC values ($p < 0.01$; Table 3). The results also demonstrated an increased rate of DG oocytes with 15 and $25 \mu\text{g/mL AMZ}$ compared with the NC ($p < 0.01$; Table 3).

To understand the AMZ mechanism of damage, the oxidative stress status of CC was evaluated. Data showed no differences in protein concentration ($\mu\text{g}/\mu\text{l}$), T-BARS (MDA/ μg protein), GSH-GSSH (nmol/ μg protein), and SOD (SOD/ μg protein) when AMZ treatments were compared with the solvent values ($p \geq 0.2$; Table 4).

Discussion

In the present study, we investigated the genotoxic and cytotoxic effect of AMZ on the primary culture of ovarian somatic cells and its impact on bovine oocyte maturation. As was mentioned before, AMZ is a widely used pesticide in bovines, dogs, pigs, bees, as well as in poultry. However, information about the levels of AMZ and its metabolites that reach different tissues from different species and genders in the range of toxic doses is scarce. In mammals, AMZ administered orally is rapidly absorbed, distributed, and metabolized, and it is eliminated primarily by urine (US EPA 1996; WHO 1998). Despite 55–74% of the dose is excreted in the urine within the first 24 h after administration (US EPA 1996; WHO 1998) in all species studied, the available data on AMZ toxicity in reproductive cells is also scarce.

A commonly used parameter to describe acute toxicity is LC50. Determination of LC50 allows finding the concentration that correlates with the extreme response since the lowest LC50 value is more toxic than the substance itself (Raj et al. 2013). In this study, AMZ LC50_{24h} was $32.55 \mu\text{g/mL}$. It is important to highlight that the range of concentrations used in this investigation would be expected to be found in the environment since the dosage used for the bovine bath was 250

$\mu\text{g/mL}$. According to previous studies, AMZ induces cytotoxicity. Young et al. (2005) assessed human luteinized granulosa cells with the MTT assay and found that viability decreased by 20% from $50 \mu\text{g/mL AMZ}$ at 24 h. We found the same cytotoxic effect in bovine CC from $10 \mu\text{g/mL}$, suggesting that the bovine CC model was more sensitive than human luteinized granulosa cells for detecting AMZ cytotoxicity. In this regard, the effectiveness of the use of bovine CC for detecting genetic damage and cytotoxicity induced by different types of xenobiotics is well known (Anchordoquy et al. 2019a; Anchordoquy et al. 2019b; Carney and Settivari 2013).

The most frequently applied methodology for determining induction of DNA damage and repair processes in isolated cells is the alkaline SCGE, which can be used in a multiplicity of experimental models (Nikoloff et al. 2014; Pereira et al. 2017; Laborde et al. 2020). For instance, Padula et al. (2012) found increased DNA damage in CHO-K1 cells treated with $3.75 \mu\text{g/mL AMZ}$ for 16 h; Radakovic et al. (2013) demonstrated that various AMZ concentrations (0.035, 0.35, 3.5, 35, and $350 \mu\text{g/mL}$) induced DNA damage in human lymphocytes, finding the highest DNA damage with $3.5 \mu\text{g/mL}$ while the highest concentrations of AMZ (35 and $350 \mu\text{g/mL}$) showed lower levels of DNA damage, they suggest that this is possibly due to formation of crosslinks. The induction of either DNA-DNA or DNA-protein cross-links reduces the ability of the DNA to migrate in the agarose gel (Tice et al. 2000). Our results are in agreement with Radakovic et al. (2013), we demonstrated that CC treated with 15 and $25 \mu\text{g/mL AMZ}$ elicited a lower DNA damage than the lowest concentration tested ($10 \mu\text{g/mL}$). A single bioassay is not enough to characterize the toxicity of a pesticide under study (Larramendy et al. 2015), for this reason, we performed a battery of genotoxic and cytotoxic assays. Thus, taking into account our results, we hypothesized that DNA damage results would be related to several aspects of cell cytotoxicity. Our results of the MTT assay revealed an alteration in the frequency of alive/dead cells among AMZ-treated cell cultures in the 10 to $25 \mu\text{g/mL}$ concentration range. Moreover,

Table 2 Analysis of apoptosis in bovine cumulus cells (CC) exposed to amitraz (AMZ) measured by annexin V-FITC

AMZ ($\mu\text{g/mL}$)	Live %	Early apoptosis %	Late apoptosis %	Necrotic %	Total apoptosis %
NC	81.5 ± 4.8^a	13.2 ± 4.7^a	2.7 ± 0.9^a	2.7 ± 1.2^a	15.8 ± 4.2^a
DMSO	78.7 ± 5.8^a	18.2 ± 6.6^a	2.7 ± 0.7^a	0.5 ± 0.5^b	20.8 ± 5.9^{ab}
10	67.33 ± 3.8^a	19.83 ± 1.8^a	6.7 ± 5.2^{ab}	6.17 ± 3.8^{ac}	26.5 ± 4.7^{bc}
15	62.7 ± 4.2^a	20.0 ± 6.3^a	8.83 ± 4.5^b	8.5 ± 2.0^{ac}	28.8 ± 6.1^{bc}
25	43.5 ± 1.3^b	26.2 ± 6.4^a	17.5 ± 4.7^b	12.8 ± 2.8^c	43.7 ± 1.6^c
ETOH	30.2 ± 13.3^b	16.8 ± 1.0^a	49.2 ± 13.6^c	3.8 ± 0.7^a	66.0 ± 12.9^d

Annexin V and propidium iodide were used to evaluate apoptosis and necrosis in CC. Bovine CC were incubated in TCM 199 medium and treated for 24 h with AMZ. Negative control (NC); dimethyl sulfoxide (DMSO): solvent control; ethanol (ETOH) (10%) was used as positive control. A total of 600 cells in three independent replicates from different days were analyzed per treatment. Data are expressed in percentage \pm SEM. ^{a-d} Columns without a common superscript differed ($p < 0.05$)

Table 3 Bovine oocyte nuclear maturation after 24 h exposure to amitraz (AMZ)

AMZ (µg/mL)	COCs (n)	GV %	MI %	MII+PB %	DG %
NC	120	2.5±1.4 ^a	23.3±5.1 ^a	71.7±2.6 ^a	2.5±0.0 ^a
DMSO	120	1.7±0.8 ^a	21.7±2.2 ^a	73.3±0.8 ^a	3.3±2.2 ^a
10	120	6.7±1.7 ^a	25.8±3.6 ^a	60.8±7.3 ^a	6.7±5.5 ^{ab}
15	120	6.7±1.7 ^a	21.6±2.2 ^a	56.7±0.8 ^a	15.0±2.5 ^b
25	120	18.3±3.0 ^b	34.2±8.8 ^a	32.5±6.3 ^b	15.0±5.8 ^b
PC	120	14.2±3.0 ^b	40.8±0.8 ^b	18.3±3.3 ^c	26.7±3.0 ^c

Bovine cumulus-oocyte complexes (COCs) were treated with AMZ in the IVM medium for 24 h. *NC* Negative control; *DMSO* dimethyl sulfoxide: solvent control; *PC* positive control: cycloheximide 0.39 µg/mL. *MI* metaphase I; *MII* metaphase II; *PB* polar body; *GV* germinal vesicle; *DG* degenerate. Data were expressed as percentage ± SEM of three independent experiments. ^{a-c} Columns without a common superscript differed (*p* < 0.05).

CC treated with 25 µg/mL AMZ showed higher rates of apoptosis. The induction of cytotoxic processes could also lead to a reduction of the frequency of damaged cells included in the final count. Due to the very low molecular weight of the DNA in terminal apoptotic and necrotic cells, the DNA of many of these cells could be lost from the gels under the typical electrophoretic conditions used (Vasquez and Tice 1997). Even though the alkaline comet assay (pH > 13) used in this experiment detects single-strand breaks, double-strand breaks, DNA-DNA/DNA- protein cross-linking, excision repair sites, and alkaline-labile sites, it is not as suitable for apoptosis detection as the comet assay under neutral conditions (Archana et al. 2013). However, further experiments are needed to confirm our hypothesis.

The detection of apoptotic cells can be done using different assays for apoptotic process evaluation. Annexin V is an early assay for apoptosis detection because it reveals the membrane phospholipid phosphatidylserine translocation from the inner to the outer leaflet of the plasma membrane. It is frequently used in conjunction with PI, which penetrates the plasma membrane when the lipid bilayers are altered, becoming more permeable, and binds to nucleic acids. Thus, the progression of apoptosis could be studied from cell viability to early-stage apoptosis and finally to late-stage apoptosis and cell death

(Archana et al. 2013). Our results showed that AMZ induced only late apoptosis from 15 µg/mL and necrosis from 25 µg/mL. Other studies describe that AMZ induces apoptosis in other biological models as well (Padula et al. 2012; Radakovic et al. 2013).

It is important to note that AMZ is applied in the same period as bovine reproduction management is done. Moreover, it is well documented that pesticides for veterinary use can affect gametes and reproductive processes and induce genotoxic and cytotoxic damage in different cell types and organisms (Soloneski et al. 2008; Carlsson et al. 2013; Anchordoquy et al. 2019a). Studies in vitro and in vivo have demonstrated that chemicals or pharmacological compounds can block or damage many processes that occur during maturation and subsequent fertilization. These alterations result in an infertile cycle or abnormal pregnancy outcome (Alm et al. 1998; Miller et al. 2004; Bremer et al. 2005). In vitro oocyte maturation is the first step in vitro embryo production. This technique is routinely applied in animal breeding with high success in the rate of viable embryos and offspring (Galli and Lazzari 2008). Lazzari et al. (2008) reported the successful development and optimization of bovine oocyte IVM for toxicity testing purposes. The IVM test analyzes the nuclear configuration changes after exposure of COC to testing

Table 4 Protein concentration and oxidative status of bovine cumulus cells (CC) treated with amitraz (AMZ) for 24 h

AMZ (µg/mL)	Protein (µg/µL)	TBARS (MDA/µg protein)	GSH/GSSG (nmol/µg protein)	SOD (USOD/ µg protein)
NC	1.2±0.1 ^a	433.1±176.1 ^a	0.4±0.0 ^a	1.2±0.1 ^a
DMSO	1.1±0.0 ^a	755.0±170.3 ^a	0.1±0.0 ^b	1.5±0.2 ^b
10	1.3±0.1 ^a	494.3±134.6 ^a	0.1±0.0 ^b	1.3±0.1 ^b
15	1.2±0.1 ^a	697.6±200.1 ^a	0.2±0.0 ^b	1.5±0.2 ^b
25	1.2±0.1 ^a	585.0±186.2 ^a	0.2±0.0 ^b	1.6±0.2 ^b

Bovine CC were incubated in TCM 199 medium and exposed during 24 h to AMZ. *NC* Negative control; *DMSO* dimethyl sulfoxide: solvent control. Protein, GSH, TBARS, and SOD results are expressed as mean ± SEM of SOD (USOD/µg protein), MDA (nmol MDA/µg protein) and GSH (pmol/µg protein) with respect to Bradford values from at least three independent replicates. ^(a-b) Values with different superscripts within each column differed (*p* < 0.05)

substances compared to nonexposed oocytes. Achievement of the maturation stage (completion of meiosis up to metaphase II) was selected as the toxicological endpoint of the IVM test, which is proposed as an alternative system to monitor the potential adverse effects caused by xenobiotics on the maturation process. Our findings indicate that the process of maturation was affected by AMZ (25 $\mu\text{g}/\text{mL}$), which increased degenerate and decreased MII+PB oocytes, suggesting that the oocyte represents a relevant model for reproductive toxicity detection. Thus, the meiotic process can reveal the toxic effects induced by AMZ. This is the first time that AMZ is evaluated in this model. We consider that when AMZ is used directly, it can affect bovine reproduction. Since the bovine is a model, this reproductive alteration could happen in other species.

Oxidative stress can be the result of either an increase of ROS production and/or a reduction of its elimination. Excessive intracellular ROS causes oxidative damage due to a modification of macromolecules producing lipid peroxidation, protein dysfunction, DNA breaks, and, finally, cell death (Cui et al. 2012). We did not find differences between treatments in any of the oxidative status assays performed. Our results are in contrast with Radakovic et al. (2013) findings, who showed that 3.5- $\mu\text{g}/\text{mL}$ AMZ significantly increased catalase reaction with DNA damage and the percentage of apoptotic and necrotic cells in comparison with antioxidant catalase (100 IU/mL or 500 IU/mL). A recent study on mouse hippocampal cells culture found that AMZ induces oxidative stress from 80 to 100 μM , measured with different endpoints (cytochrome P450 isoenzymes, KEAP1/NRF2 pathway, cotreatment with the antioxidant N-acetylcysteine) (Moyano et al. 2019). Many studies demonstrated that the concentration at which AMZ decreases cell viability depends on the model used (Ueng et al. 2004; Young et al. 2005; Padula et al. 2012; Radakovic et al. 2013). According to all exposed above, there is a discrepancy in the AMZ induce oxidative stress, which could be due to the use of different cell lines and oxidative stress endpoints used. Radakovic et al. (2013) used lymphocytes that could react differently than CC concerning the sensitivity to AMZ and its metabolites. Moreover, Anchordoquy et al. (2019b) observed that lymphocytes were more sensitive than CC to the antiparasitic doramectin. Since it is known that different mechanisms contribute to AMZ induce oxidative stress (Costa et al. 1991; Moyano et al. 2019), other mechanisms not investigated in this work could contribute to the generation of ROS and induce cell death, more studies are required to determine the cause of cell death in AMZ-treated CC. On the other hand, we cannot rule out that AMZ acts through another mechanism of action independently from the induction of ROS on bovine CC. One of them is the inhibition of histamine receptor 1 by AMZ that induces DNA damage and caspase-2 activation, inhibiting the mitochondrial pathway that ends in apoptotic cell death (Jangi et al. 2006).

Another mechanism that AMZ reduces cell viability is through α 2-adrenergic receptor agonist (Del Pino et al. 2015). The possible effects of these α 2-adrenergic receptors that could cause cell death are a decrease of cyclic AMP by inhibiting adenylyl cyclase (Chen and Hsu 1994), an increase of extracellular Ca^{2+} by activation of the Ca^{2+} channel (Shin and Hsu 1994). All these alterations lead to pathological effects such as cell death (Cui et al. 2012; Kloster et al. 2013; Jiang et al. 2011).

Antiparasitics and antimicrobials are two important groups of veterinary drugs used worldwide. However, the residues that they leave in the environment, and the toxicological effects that could be induced in exposed organisms is worrying. Normally, the parent compounds and/or metabolites are excreted through the urine and feces of the animals and released directly into the environment. These compounds can pollute the environment during manufacturing processes and packaging disposal in inappropriate places. Furthermore, animal feces are frequently used as a fertilizer due to their nutrient and organic matter content, but they also contain significant amounts of veterinary drugs that can affect other organisms, including human beings (Halling-Sørensen et al. 1998; Hamscher et al. 2002; Boxall et al. 2018). The concern about antiparasitic drugs is due to their high toxicity in soil, in terrestrial invertebrates such as insects and mites, in aquatic invertebrates, and bacteria (Wang et al. 2008; Boxall et al. 2018; Pathak and Navnet 2018). The degradation of AMZ to stable aniline products may be responsible for the environmental and health risks caused by its application and use (Brimecombe and Limson 2007). Residues of AMZ are found in tissues (Yu et al. 2010), milk (Li et al. 2018), and even in honey (Lozano et al. 2019). The Codex Alimentarius does not contain maximum residue limit values of AMZ in foods (WHO-FAO 2018). On the other hand, the European Food Safety Authority set maximum residue limits of AMZ in bovine products (0.2 mg/Kg in the fat tissue, liver, and kidney and 0.01 mg/kg in milk) (EFSA 2016). Although the concentration of AMZ residues in food would be controlled in some parts of the world, accidental exposure is still a matter of concern (Yilmaz and Yildizdas 2003). Currently, the use of treatments to avoid losses due to subclinical parasitosis is very frequent in livestock farming. Besides, in many countries, AMZ is not generally subject to professional control due to the easy availability of the product without prescription. No diagnosis is made before treatment, and the choice of antiparasitic agents is for commercial reasons. We, therefore, suggest that antiparasitic drugs require predictive techniques for evaluating their toxicity, and this work contributes with important information about AMZ reproductive, genotoxicity, and cytotoxicity.

Finally, the present study has shown for the first time that AMZ induces apoptosis and decreases viability in primary cultures of bovine CC. Also, AMZ impairs the oocyte

maturation process. Overall, the cytotoxic damage would not be due to oxidative stress, at least in CC. However, the underlying mechanism of action of AMZ in the oocyte maturation process is still beyond our knowledge, showing that the deleterious effects induced by AMZ need further studies. Unfortunately, there is no data on the levels of AMZ in blood, follicular liquor, or urine after animal application. Without this information, we have no idea if the hazard we have identified *in vitro* is anything to be concerned about (Jennings 2015). Our working group will carry out new studies taking into account these measurements.

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Authors' contributions NN created the methodology, conducted the investigation, made the formal analysis, and wrote the original draft. ACCM created the methodology, conducted the investigation, made the formal analysis, and wrote the original draft. MF participated in the investigation and made the data curation. CCF provided the research resources, reviewed, and edited the manuscript.

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Data availability The datasets used during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate Not applicable

Consent for publication Not applicable

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References

- Alm H, Torner H, Tiemann U, Kanitz W (1998) Influence of organochlorine pesticides on maturation and post-fertilization development of bovine oocytes *in vitro*. *Reprod Toxicol* 12:559–563. [https://doi.org/10.1016/s0890-6238\(98\)00033-1](https://doi.org/10.1016/s0890-6238(98)00033-1)
- Amizadeh M, Safari-Kamalabadi M, Askari-Saryazdi G, Amizadeh M, Reihani-Kermani H (2017) Pesticide exposure and head and neck cancers: a case-control study in an agricultural region. *Iran J Otorhinolaryngol* 29(94):275–285
- Anchordoquy JM, Anchordoquy JP, Nikoloff N, Gambaro R, Padula G, Seoane A, Furnus C (2019a) Doramectin induced cytotoxic and genotoxic effects on bovine peripheral lymphocytes and cumulus cells *in vitro*. *J Environ Sci Health Part B* 54(2):147–154. <https://doi.org/10.1080/03601234.2018.1559569>
- Anchordoquy JP, Anchordoquy JM, Nikoloff N, Gambaro R, Padula G, Furnus C, Seoane A (2019b) Cytotoxic and genotoxic effects induced by enrofloxacin-based antibiotic formulation Floxagen® in two experimental models of bovine cells *in vitro*: peripheral lymphocytes and cumulus cells. *Environ Sci Pollut Res* 26(3):2998–3005. <https://doi.org/10.1007/s11356-018-3776-2>
- Archana M, Yogesh TL, Kumaraswamy KL (2013) Various methods are available for the detection of apoptotic cells—a review. *Indian J Cancer* 50(3):274–283. <https://doi.org/10.4103/0019-509X.118720>
- Beker van Woudenberg A, Grollers-Mulderij M, Snel C, Jeurissen N, Stierum R, Wolterbeek A (2012) The bovine oocyte *in vitro* maturation model: a potential tool for reproductive toxicology screening. *Reprod Toxicol* 34:251–260. <https://doi.org/10.1016/j.reprotox.2012.05.098>
- Boxall ABA, Fogg LA, Blackwell PA, Kay P, Pemberton EJ (2018) Review of veterinary medicines in the environment. R&D Technical Report P6-012/8TR. Environment Agency, Bristol
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254. <https://doi.org/10.1006/abio.1976.9999>
- Bremer S, Balduzzi D, Cortvriendt R, Daston G, Eletti B, Galli A (2005) The effects of chemicals on mammalian fertility. The report and recommendations of ECVAM Workshop 53—the first strategic workshop of the EU ReProTect Project. *Altern Lab Anim* 33:391–416
- Brimecombe R, Limson J (2007) Voltammetric analysis of the acaricide amitraz and its degradant, 2,4-dimethylaniline. *Talanta* 71:1298–1303. <https://doi.org/10.1016/j.talanta.2006.06.037>
- Campbell BK, Souza C, Gong J, Webb R, Kendall N, Marsters P (2003) Domestic ruminants as models for the elucidation of the mechanisms controlling ovarian follicle development in humans. *Reprod Suppl* 61:429–443
- Carlsson G, Patring J, Kreuger J, Norrgren L, Oskarsson A (2013) Toxicity of 15 veterinary pharmaceuticals in zebrafish (*Danio rerio*) embryos. *Aquat Toxicol* 126:30–41. <https://doi.org/10.1016/j.aquatox.2012.10.008>
- Carney EW, Settivari R (2013) Chapter 33 - Predictive toxicology: biological assay platforms. *A Comprehensive Guide to Toxicology in Preclinical Drug Development* 777–806
- Cavaş T, Könen S (2007) Detection of cytogenetic and DNA damage in peripheral erythrocytes of goldfish (*Carassius auratus*) exposed to a glyphosate formulation using the micronucleus test and the comet assay. *Mutagenesis* 22:263–268. <https://doi.org/10.1093/mutage/gem012>
- Chen TH, Hsu WH (1994) Inhibition of insulin release by a formamidine pesticide amitraz and its metabolites in a rat beta-cell line: an action mediated by alpha-2 adrenoceptors, a GTP-binding protein, and a decrease in cyclic AMP. *J Pharmacol Exp Ther* 271:1240–1245
- Costa LG, Gastel J, Murphy SD (1991) The formamidine pesticides chlordimeform and amitraz decrease hepatic glutathione in mice through interaction with alpha2-adrenoceptors. *J Toxicol Environ Health, Part A Curr Issues* 33(3):349–358. <https://doi.org/10.1080/15287399109531532>
- Cui H, Kong Y, Zhang H (2012) Oxidative stress, mitochondrial dysfunction, and aging. *J Signal Transduct* 2012:13–13. <https://doi.org/10.1155/2012/646354>
- Del Pino J, Moyano-Cires PV, Anadon MJ, Diaz MJ, Lobo M, Capo MA, Frejo MT (2015) Molecular mechanisms of amitraz mammalian toxicity: a comprehensive review of existing data. *Chem Res Toxicol* 28(6):1073–1094. <https://doi.org/10.1021/tx500534x>
- Del Pino J, Moyano P, Ruiz M, Anadon MJ, Diaz MJ, García JM, Labajo-Gonzalez E, Frejo MT (2017) Amitraz changes NE, DA and 5-HT biosynthesis and metabolism mediated by alterations in estradiol content in CNS of male rats. *Chemosphere* 181:518–529. <https://doi.org/10.1016/j.chemosphere.2017.04.113>
- EFSA (2016) Setting of maximum residue levels for amitraz, coumaphos, flumequine, oxytetracycline, permethrin, and streptomycin in

- certain products of animal origin. doi:<https://doi.org/10.2903/j.efsa.2016.4570>
- Elinav E, Shapira Y, Ofra Y, Hassin T, Ben-Dov IZ (2005) Near-fatal amitraz intoxication: the overlooked pesticide. *Basic Clin Pharmacol Toxicol* 97:185–187. https://doi.org/10.1111/j.1742-7843.2005.pto_97399.x
- Galli C, Lazzari G (2008) The manipulation of gametes and embryos in farm animals. *Reprod Domest Anim* 43(2):1–7. <https://doi.org/10.1111/j.1439-0531.2008.01136.x>
- Grossman MR, Garvey MS, Murphy MJ (1993) Amitraz toxicosis associated with the ingestion of an acaricide collar in a dog. *J Am Vet Med Assoc* 203:55–57
- Guerreiro DD, Mbemba GT, Bruno JB, Faustino LR, de Figueiredo JR, Rodrigues APR (2019) *In vitro* culture systems as an alternative for female reproductive toxicology studies. *Zygote* 27(2):55–63. <https://doi.org/10.1017/S0967199419000042>
- Gupta RC, Milatovic D (2014) Insecticides. *Biomark Toxicol*:389–407. <https://doi.org/10.1016/b978-0-12-404630-6.00023-3>
- Halling-Sørensen B, Nors Nielsen S, Lanzky PF, Ingerslev F, Holten Lützhøft HC, Jørgensen SE (1998) Occurrence, fate, and effects of pharmaceutical substances in the environment a review. *Chemosphere* 36:357–393. [https://doi.org/10.1016/s0045-6535\(97\)00354-8](https://doi.org/10.1016/s0045-6535(97)00354-8)
- Hamscher G, Sczesny S, Höper H, Nau H (2002) Determination of persistent tetracycline residues in soil fertilized with liquid manure by high-performance liquid chromatography with electrospray ionization tandem mass spectrometry. *Anal Chem* 74:1509–1518. <https://doi.org/10.1021/ac015588m>
- Heaton PR, Ransley R, Charlton CJ, Mann SJ, Stevenson J, Smith BH, Rawlings JM, Harper EJ (2002) Application of single-cell gel electrophoresis (comet) assay for assessing levels of DNA damage in canine and feline leukocytes. *J Nutr* 132(6):1598–1603. <https://doi.org/10.1093/jn/132.6.1598>
- Izadyar F, Colenbrander B, Bevers MM (1997) Stimulatory effect of growth hormone on *in vitro* maturation of bovine oocytes is exerted through the cyclic adenosine 30,50-monophosphate signaling pathway. *Biol Reprod* 57:1484–1489. <https://doi.org/10.1095/biolreprod57.6.1484>
- Jangi SM, Diaz-Perez JL, Ochoa-Lizarralde B, Martin-Ruiz I, Asumendi A, Perez-Yarza G, Gardeazabal J, Diaz-Ramon JL, Boyano MD (2006) H1 histamine receptor antagonists induce genotoxic and caspase-2-dependent apoptosis in human melanoma cells. *Carcinogenesis* 27:1787–1796. <https://doi.org/10.1093/carcin/bgl021>
- Jennings P (2015) The future of *in vitro* toxicology. *Toxicol in Vitro* 29(6):1217–1221. <https://doi.org/10.1016/j.tiv.2014.08.011>
- Jiang Q, Bai T, Shen S, Li L, Ding H, Wang P (2011) Increase of cytosolic calcium induced by trichosanthin suppresses cAMP/PKC levels through the inhibition of adenyl cyclase activity in HeLa cells. *Mol Biol Rep* 38:2863–2868. <https://doi.org/10.1007/s11033-010-0432-4>
- Kim JC, Shin JY, Yang YS, Shin DH, Moon CJ, Kim SH, Park SC, Kim YB, Kim HC, Chung MK (2007) Evaluation of the developmental toxicity of amitraz in Sprague-Dawley rats. *Arch Environ Contam Toxicol* 52:137–144. <https://doi.org/10.1007/s00244-006-0021-7>
- Kloster MM, Naderi EH, Haaland I, Gjertsen BT, Blomhoff HK, Naderi S (2013) cAMP signaling inhibits p53 acetylation and apoptosis via HDAC and SIRT deacetylases. *Int J Oncol* 42:1815–1821. <https://doi.org/10.3892/ijo.2013.1853>
- Krisner RL (2004) The effect of oocyte quality on development. *J Anim Sci* 82:14–23. https://doi.org/10.2527/2004.8213_supplE14x
- Laborde MR, Larramendy ML, Soloneski S (2020) Cytotoxic and genotoxic assessments of 2, 4-dichlorophenoxyacetic acid (2, 4-D) in *in vitro* mammalian cells. *Toxicol in Vitro* 65:104783. <https://doi.org/10.1016/j.tiv.2020.104783>
- Larramendy ML, Nikoloff N, Ruiz de Arcaute C, Soloneski S (2015) Genotoxicity and cytotoxicity exerted by pesticides in different biotic matrices: an overview of more than a decade of experimental evaluation. *J Environ Anal Toxicol* 4:4 <https://doi.org/10.4172/2161-0525.1000225>
- Lazzari G, Tessaro I, Crotti G, Galli C, Hoffmann S, Bremer S, Pellizzer C (2008) Development of an *in vitro* test battery for assessing chemical effects on bovine germ cells under the ReProTect umbrella. *Toxicol Appl Pharmacol* 233:360–370. <https://doi.org/10.1016/j.taap.2008.08.019>
- Li P, Teng Y, Nie Y, Liu W (2018) SERS detection of insecticide amitraz residue in milk based on Au@ Ag core-shell nanoparticles. *Food Anal Methods* 11(1):69–76
- Lourenço B, Sousa AP, Almeida-Santos T, Ramalho-Santos J (2014) Relation of cumulus cell status with single oocyte maturity, fertilization capability, and patient age. *J Reprod Infertil* 15:15–21
- Lozano A, Hernando MD, Uclés S, Hakme E, Fernández-Alba AR (2019) Identification and measurement of veterinary drug residues in beehive products. *Food Chem* 274:61–70. <https://doi.org/10.1016/j.foodchem.2018.08.055>
- Luchetti CG, Cruzans PG, Lombardo DM (2017) Antioxidant effects of metformin plus ITS during *in vitro* maturation of porcine COC. *Medicina* 77(1):543
- Marafon CM, Delfim CI, Valadao CA, Menotti R, Andrade SF (2010) Analysis of amitraz in cats by gas chromatography. *J Vet Pharmacol Ther* 33:411–414. <https://doi.org/10.1111/j.1365-2885.2009.01144.x>
- Miller KP, Borgeest C, Greenfeld C, Tomic D, Flaws JA (2004) In utero effects of chemicals on reproductive tissues in females. *Toxicol Appl Pharmacol* 198:111–131. <https://doi.org/10.1016/j.taap.2003.07.016>
- Moyano P, Ruiz M, García JM, Frejo MT, Baselga MJ, Lobo M, García J, Del Pino J (2019) Oxidative stress and cell death induction by amitraz and its metabolite BTS-27271 mediated through cytochrome P450 and NRF2 pathway alteration in a primary hippocampal cell. *Food Chem Toxicol* 129:87–96. <https://doi.org/10.1016/j.fct.2019.04.042>
- Moyo B, Masika PJ (2009) Tick control methods used by resource-limited farmers and the effect of ticks on cattle in rural areas of the Eastern Cape Province, South Africa. *Trop Anim Health Prod* 1(4): 517–523. <https://doi.org/10.1007/s11250-008-9216-4>
- Nikoloff N, Natale GS, Marino D, Soloneski S, Larramendy ML (2014) Flurochloridone-based herbicides induced genotoxicity effects on *Rhinella arenarum* tadpoles (Anura: *Bufo*idae). *Ecotoxicol Environ Saf* 100:275–281. <https://doi.org/10.1016/j.ecoenv.2013.10.021>
- Padula G, Ponzinibbio MV, Picco S, Seoane A (2012) Assessment of the adverse effects of the acaricide amitraz: *in vitro* evaluation of genotoxicity. *Toxicol Mech Methods* 22(9):657–661. <https://doi.org/10.3109/15376516.2012.666683>
- Pathak VM, Navnet (2018) Handbook of research of microbial tools for environmental waste management; ed. IGI Global, India
- Pereira AF, Borges P, Fontbonne A, Cardoso L, Gaivão I, Martins-Bessa A (2017) The Comet assay for detection of DNA damage in canine sperm. *Reprod Domest Anim* 52(6):1149–1152. <https://doi.org/10.1111/rda.13042>
- Piersma AH, Bosgraf B, van Duursen MBM, Hermsen SAB, Jonkere LRA, Kroesef ED, van der Lindene SC, Mane H, Roelofs MJE, Schulpna SHW, Schwarzd M, Uibeld F, van Vugt-Lussenburge BMA, Westerhout J, Wolterbeek APM, van der Burge B (2013) Evaluation of an alternative *in vitro* test battery for detecting reproductive. *Toxicants Reprod Toxicol* 38:53–64. <https://doi.org/10.1016/j.reprotox.2013.03.002>
- Pitarque M, Vaglenov A, Nosko M, Hirvonen A, Norppa H, Creus A, Marcos R (1999) Evaluation of DNA damage by the comet assay in

- shoe workers exposed to toluene and other organic solvents. *Mutat Res* 441:115–127. [https://doi.org/10.1016/s1383-5718\(99\)00042-x](https://doi.org/10.1016/s1383-5718(99)00042-x)
- Pläsier B, Lloyd DR, Paul GC, Thomas CR, Al-Rubeai M (1999) Automatic image analysis for quantification of apoptosis in animal cell culture by annexin-V affinity assay. *J Immunol Methods* 229:81–95. [https://doi.org/10.1016/s0022-1759\(99\)00107-6](https://doi.org/10.1016/s0022-1759(99)00107-6)
- Proudfoot AT (2003) Poisoning with amitraz. *Toxicol Rev* 22(2):71–74. <https://doi.org/10.2165/00139709-200322020-00001>
- Radakovic M, Stevanovic J, Djelic N, Lakic N, Knezevic-Vukcevic J, Vukovic-Gacic B, Stanimirovic Z (2013) Evaluation of the DNA damaging effects of amitraz on human lymphocytes in the comet assay. *J Biosci (New Delhi, India)* 38:53–62. <https://doi.org/10.1007/s12038-012-9287-2>
- Raj J, Chandra M, Dogra TD, Pahuja M, Raina A (2013) Determination of the median lethal dose of the combination of endosulfan and cypermethrin in Wistar rat. *Toxicol Int* 20(1):1–5. <https://doi.org/10.4103/0971-6580.111531>
- Robb J, Norval M, Neill WA (1990) The use of tissue culture for the detection of mycotoxins. *Lett Appl Microbiol* 10:161–165. <https://doi.org/10.1111/j.1472-765x.1990.tb00105.x>
- Shin DH, Hsu WH (1994) Influence of the formamide pesticide amitraz and its metabolites on porcine myometrial contractility: involvement of alpha 2-adrenoceptors and Ca²⁺ channels. *Toxicol Appl Pharmacol* 128:45–49. <https://doi.org/10.1006/taap.1994.1178>
- Singh NP, McCoy MT, Tice RR, Schneider EL (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* 175:184–191. [https://doi.org/10.1016/0014-4827\(88\)90265-0](https://doi.org/10.1016/0014-4827(88)90265-0)
- Soloneski S, Reigosa MA, Molinari G, González NV, Larramendy ML (2008) Genotoxic and cytotoxic effects of carbofuran and furadan® on Chinese hamster ovary (CHOK1) cells. *Mutat Res Genet Toxicol Environ Mutagen* 656(1–2):68–73. <https://doi.org/10.1016/j.mrgentox.2008.07.007>
- Süss U, Stranzinger G, Wuthrich K (1998) Chromosome configurations and time sequence of the first meiotic division in bovine oocyte matured *in vitro*. *Biol Reprod* 38:871–880. <https://doi.org/10.1095/biolreprod38.4.871>
- Takahashi M, Nagai T, Hamano S, Kuwayama M, Okamura N, Okano A (1993) Effect of thiol compounds on *in vitro* development and intracellular glutathione content of bovine embryos. *Biol Reprod* 49(2):228–232. <https://doi.org/10.1095/biolreprod49.2.228>
- Tanghe S, Van Soom A, Nauwynck H, Coryn M, de Kruif A (2002) Minireview: Functions of the cumulus *oophorus* during oocyte maturation, ovulation, and fertilization. *Mol Reprod Dev* 61:414–424. <https://doi.org/10.1002/mrd.10102>
- Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF (2000) The single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. *Environ Mol Mutagen* 35:206–221. [https://doi.org/10.1016/0014-4827\(88\)90265-0](https://doi.org/10.1016/0014-4827(88)90265-0)
- Ueng TH, Hung CC, Wang HW, Chan PK (2004) Effects of amitraz on cytochrome P450-dependent monooxygenases and estrogenic activity in MCF-7 human breast cancer cells and immature female rats. *Food Chem Toxicol* 42(11):1785–1794. <https://doi.org/10.1016/j.fct.2004.06.010>
- US EPA (1996) Amitraz Reregistration Eligibility Decision (RED), Case 0234 (EPA-738-F-96-031), US Environmental Protection Agency, Office of Prevention, Pesticide and Toxic Substances, Washington
- van Engeland M, Nieland LJ, Ramaekers FC, Schutte B, Reutelingsperger CP (1998) Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry* 31:1–9. [https://doi.org/10.1002/\(sici\)1097-0320\(19980101\)31:1<1::aid-cyto1>3.0.co;2-r](https://doi.org/10.1002/(sici)1097-0320(19980101)31:1<1::aid-cyto1>3.0.co;2-r)
- Vasquez M, Tice RR (1997) Comparative analysis of apoptosis versus necrosis using the single-cell gel (SCG) assay. *Environ Mol Mutagen* 29(S28):53
- Wang HY, Yang Y, Su JY, Shen JL, Gao CF, Zhu YC (2008) Assessment of the impact of insecticides on *Anagrus nilaparvatae* (Pang & Wang) (Hymenoptera: Mymaridae): an egg parasitoid of the rice planthopper *Nilaparvata lugens* (Hemiptera: Delphacidae). *Crop Prot* 27(3–5):514–522. <https://doi.org/10.1016/j.cropro.2007.08.004>
- WHO (1998) Pesticide residues in food: 1998: toxicological evaluations (No. WHO/PCS/99.18). World Health Organization, Geneva
- WHO-FAO (2018) Codex Alimentarius. http://www.fao.org/fao-who-codexalimentarius/codex-texts/DBS/vet_drugs/veterinary-drugs/en/. Accessed July 2018
- Wu J, Tu D, Yuan LY, Yuan H, Wen LX (2013) T-2 toxin exposure induces apoptosis in rat ovarian granulosa cells through oxidative stress. *Environ Toxicol Pharmacol* 36:493–500. <https://doi.org/10.1016/j.etap.2013.03.017>
- Yilmaz HL, Yildizdas DR (2003) Amitraz poisoning, an emerging problem: epidemiology, clinical features, management, and preventive strategies. *Arch Dis Child* 88(2):130–134. <https://doi.org/10.1136/adc.88.2.130>
- Young FM, Menadue MF, Lavranos TC (2005) Effects of the insecticide amitraz, an α 2-adrenergic receptor agonist, on human luteinized granulosa cells. *Hum Reprod* 20(11):3018–3025. <https://doi.org/10.1093/humrep/dei194>
- Yuan YQ, Van Soom A, Leroy JL, Dewulf J, Van Zeveren A, de Kruif A, Peelman LJ (2005) Apoptosis in cumulus cells but not in oocytes may influence bovine embryonic developmental competence. *Theriogenology* 63:2147–2163. <https://doi.org/10.1016/j.theriogenology.2004.09.054>
- Yu H, Tao Y, Le T, Chen D, Ishsan A, Liu Y, Yuan Z (2010) Simultaneous determination of amitraz and its metabolite residue in food animal tissues by gas chromatography-electron capture detector and gas chromatography-mass spectrometry with accelerated solvent extraction. *J Chromatogr B* 878(21):1746–1752. <https://doi.org/10.1016/j.jchromb.2010.04.034>

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