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Differential effects of herbicides atrazine and fenoxaprop-ethyl, and insecticides diazinon and malathion, on viability and maturation of porcine oocytes in vitro

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ABSTRACT

Exposure to pesticides may be a major cause of reproductive dysfunction in humans and animals. Atrazine and fenoxaprop-ethyl, widely used herbicides, and malathion and diazinon, organophosphate insecticides, are considered only slightly toxic to vertebrates; however, there is evidence of greater effects on reproductive function. The aim of this study was to evaluate the effect of these pesticides on oocyte viability and in vitro maturation. Gametes were matured in increasing concentrations of the pesticides and then stained with MTT to evaluate viability and bisbenzimide to assess the maturation stage, in the same oocyte. Atrazine had no effect on viability but maturation was significantly reduced, while fenoxaprop-ethyl affected both parameters. The insecticides affected viability and maturation but to a different degree. The four pesticides showed a more pronounced effect on maturation than on viability, due to a blockage at germinal vesicle stage.

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1. Introduction

To enhance food production, pesticides have been intensively used to control plagues and diseases in livestock and crops. Furthermore, they have contributed to improved human health by diminishing the number of disease-carrying insects (Saunders and Harper, 1994).

Unfortunately, their indiscriminate use produces damage to the environment (plants, animals, water, ground or soil), and creates resistance to the pesticides themselves. These chemicals can be dangerous for humans, causing acute and chronic intoxications, carcinogenesis, mutagenesis, teratogenesis, sterility, etc. (Saunders and Harper, 1994).

Although several pesticides have been banned or restricted because of their high toxicity, they persist in the environment and constitute potential hazards for wildlife and human health, particularly for populations that depend on sea products for subsistence (Campagna et al., 2001, 2007). Exposure to these chemicals may cause alterations in reproductive behavior and contribute to subfecundity, infertility, pregnancy loss (Auger et al., 1995; Zinaman et al., 1996), growth retardation, intrauterine fetal death and birth defects in humans (Shepard, 1986) or ovarian failure in cattle (Pocar et al., 2003).

Even in Arctic region, where the use of pesticides is not common, their presence is detectable in the blood and breast milk in populations, such as the Inuit. Pollutants are dispersed in the environment and bioaccumulate in sea and coastal animals, and the staple for these people (Campagna et al., 2002). In the last few decades, human fertility has decreased; it is estimated that one in every five couples is involuntarily sterile and there is an increased incidence of gonadal tumors (Nunziata, 1998).

Atrazine, a triazine, is one of the most effective and inexpensive herbicides used to control weeds in crop fields, and it is widely used despite it has recently been banned in some countries (Sass and Colangelo, 2006). Atrazine is considered only slightly toxic, though there are several studies showing it has an effect on the reproductive system. It may potentially be a disruptor of normal sexual development in frogs, affecting sexual development by inducing aromatase, which results in the increased conversion of androgens to estrogens (Hayes et al., 2002; Freeman and Rayburn, 2004; Murphy et al., 2006).

Fenoxaprop-ethyl (FE) is a more recently formulated herbicide for weed control in wheat, rice, and broad-leafed crops. It is an aryloxyphenoxyalcanoic acid, it inhibits the biosynthesis of fatty acids in plant meristems, affecting acetyl-coenzyme A carboxylase (Labrada et al., 1996; Waller et al., 2003). FE is not considered

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carcinogenic or mutagenic and there are no reports indicating that it could be harmful to human fertility or reproduction (Peterson et al., 2001).

Diazinon is an organophosphate (OP) insecticide, formerly used in residential buildings. Though it has a low persistence in soil, its half-life being 2–6 weeks, the domestic use was banned in the USA in 2004. Currently, it is only approved for agricultural use in the USA (US EPA, 2006), but in Mexico and some other countries, it is still widely used. It has been reported to impact the reproductive organs in rats by decreasing genital weights, reducing sperm motility and viability, and increasing sperm morphological abnormalities (Abd el-Aziz et al., 1994). It has been reported that diazinon alters sperm chromatin structure in mice (Piña-Guzman et al., 2005).

Malathion is another of the most commonly used OP insecticides around the world (Pluth et al., 1998). It is used to control pests affecting agricultural crops, ornamental plants, greenhouses, livestock, stored grain, forests, buildings, and gardens.

The main toxic effect of OP on insects is the irreversible inhibition of acetylcholinesterase by phosphorylation of serine residues in its active center, which leads to the increase of acetylcholine (Blasiak et al., 1999). However, for mammals it is believed to be only slightly toxic because it is rapidly broken down into non-toxic α-monoacids by carboxylesterases, enzymes that hydrolyze malathion and its metabolites into non-toxic intermediates that can be easily eliminated (Jonakovic, 2001). There is concern about the effect on vertebrate reproductive functions caused by the widespread use of malathion, as it can elicit alterations in germ and somatic cells (Bustos-Obregón and González-Hormazabal, 2003).

Although in vivo studies provide information about the toxic effects on experimental animals, field workers and communities exposed to pesticides, in vitro models, under controlled conditions, can provide information to understand the basic mechanisms of toxicity that are difficult to identify in whole animals (Kimmel et al., 1995). The aim of the present study was to evaluate the effect of two insecticides and two herbicides on viability and in vitro maturation of porcine oocytes. Since oocyte maturation is a critical prerequisite for subsequent fertilization and early embryo development, this in vitro system was used to explore the possible mechanisms of damage produced by pesticides.

2. Materials and methods

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo).

2.1. Collection and maturation of oocytes

Ovaries were collected from slaughtered 6-month-old gilts and transported to the laboratory, within 1 h, in sterile saline solution (0.9% NaCl) with 75 μg/mL of penicillin and 50 μg/mL of streptomycin, at 25–28 °C. Cumulus-oocyte complexes (COCs) were aspirated from 3 to 5 mm follicles, using an 18-gauge needle and a 10 mL disposable syringe. Follicular fluid was collected in a centrifuge tube and allowed to settle. Supernatant was discarded and the cell pellet washed twice with TRIS buffered medium (TBM: 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2$–$2H_2O$, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate).

Oocytes surrounded by a compact cumulus mass and homogeneous granular cytoplasm, were selected and washed three times with TBM. Thirty oocytes were transferred into each well of a 4-well multidish (Nunc, Denmark), containing 500 μL of culture media (TCM-199; In vitro, Mexico) supplemented with 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 0.1% (w/v) polyvinyl alcohol, 0.57 mM L-cysteine, 10 ng/ml EGF, 75 μg/ml penicillin, 50 μg/ml streptomycin, 1 U/ml FSH and 1 U/ml of LH. Maturation was carried out at 38.5 °C in a humidified atmosphere with 5% CO_2 for 44 h (Abeydeera et al., 1998).

2.2. Pesticides treatment

In order to evaluate the effect of pesticides on viability and oocyte maturation, technical grade herbicides atrazine and FE, (Aventis Cropscience, México), and commercially-formulated insecticides malathion (Agricultura Nacional, México) and diazinon (Tridente, México) were used.

A 10 mM stock solution was prepared in absolute ethanol for herbicides, and in deionized water for insecticides. Preliminary assays demonstrated that stock solution solvents, added to the culture media at equivalent concentrations where the pesticides were dissolved, did not affect viability and oocyte maturation (data not shown). At the beginning of the 44 h maturation period, aliquots from the stock solution were added in a range from 0 to 500 μM final concentrations to each well containing oocytes, in order to test the effect on viability. According to these preliminary results on viability (data not shown), atrazine and FE were added in 0, 50, 100 and 500 μM concentrations, while for diazinon 0, 25, 50 and 100 μM, and for malathion 0, 0.5, 1, 10, 25, 50, and 100 μM concentrations were used.

2.3. Viability and maturation assessment

Viability and in vitro maturation were evaluated simultaneously in the same oocyte. After in vitro maturation, cumulus cells were removed with 0.1% hyaluronidase in phosphate buffered saline (PBS). The oocytes were washed in TBM, and incubated in 0.5 mg/ml methylthiazolyldiphenyl-tetrazolium bromide (MTT) for 2 h to evaluate the viability. To assess maturation, oocytes were treated with 10 μg/ml bisbenzimide (Hoechst 33342) for 40 min, then washed and incubated for 20 min in sodium citrate 0.075%. Finally, the oocytes were fixed overnight with 2% paraformaldehyde and mounted in PBS-glycerol (1:9).

Purple stained oocytes were considered alive when evaluated under a light microscope. Maturation was analyzed with an epifluorescence microscope (Zeiss Axiostar). Those oocytes having a germinal vesicle (GV) were considered immature, those on first metaphase (MI) were judged to be maturing, while only those showing a second metaphase (MII) and a polar body were classified as matured.

2.4. Statistical analysis

Since data are discrete variables, non-parametric statistical tests were performed. All assays were done at least four times. The correlation among pesticide concentration and its effect on oocyte viability and maturation was assessed using Spearman's rank correlation coefficient.

The concentration required to produce death in 50% of the oocytes (lethal concentration 50, LC_{50}) and the concentration needed to inhibit the maturation in 50% of the oocytes (inhibition of maturation 50, IM_{50}) were calculated by a non-linear regression. Normalized data were used, considering controls as 100% for both, Spearman correlation and regression.

Meiotic phases percentage in each concentration was compared to the control using the χ^2 test.

A probability of $p < 0.05$ was considered statistically significant.

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