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The effects of permethrin on rat ovarian tissue morphology

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ABSTRACT

All organisms are exposed to chemical agents during their lifetime. One of these agents is a pesticide that is used as fly killer. In this study we investigated the effects of permethrin on rat ovaries using light and electron microscopy. We used 24 Wistar albino female rats and divided them into 3 groups. Dosages 20 and 40 mg/kg/day permethrin were administered by gavage for 14 days. Normal saline was given to control rats. After treatment, ovarian tissues were collected and prepared for light and electron microscopy evaluation. Negative effects of permethrin were detected on follicular and corpus luteum cell morphology in a dose dependent manner when compared with the control group. Picnotic cellular appearance and condensed chromatin were detected as evidence of apoptotic cell death. Furthermore, degenerative changes were seen in the ultrastructure of mitochondria and endoplasmic reticulum. Thus, these findings suggested that permethrin caused degenerative effects on ovarian morphology in a dose dependent manner.

1. Introduction

Pesticides are economic agricultural poisons used to control or kill pests that act on the eggs or larvae of insects respectively in agriculture, medicine, industry and the household. These are believed to be the major factors behind the increase in agricultural productivity in the twentieth century. The abundant use of pesticides constitutes a danger not only for aquatic and terrestrial biodiversity but also for humans because of their presence in food chain. Depending on the target, pesticides are sub-classified into categories. Pyrethroids as one of these categories, is a group of synthetic chemical analogs of the naturally occurring toxin pyrethrin. It is derived from the dried flowers of *Chrysanthemum cinerariaefolium*. They are very stable in sunlight and generally effective against most agricultural insect pests when used at very low doses (Pine et al., 2008; Ngoula et al., 2012). Pyrethroids are considerably less toxic to mammals than other classes of insecticides like organochlorines, organophosphates, and carbamates. Because of their low human toxicity, pyrethroids are widely used to control insects in agriculture and in the home (Freeman et al., 2004). In this study, we used permethrin. Permethrin is an example of the pyrethroid insecticides, which are used to control a wide variety of insects in agricultural, veterinary and domestic uses. It is used in sprays, pet flea shampoos, lice shampoos, municipal mosquito abatement products, and aerosol bombs. It is also used on agricultural crops, particularly fruits and vegetables. Permethrin and other pyretroid insecticides have also been measured in high proportion of household dust samples (Meeker et al., 2009). The European Union classifies chemicals and labeled permethrin as hazardous to the environment (http://echa.europa.eu). Many studies have reported undeniable links between insecticides and serious health consequences including endocrine disruption, fertility problems (Colborn et al., 1993; Andersen et al., 2000), cancers (Ben Rhouma et al., 2001; Cabello et al., 2001; Clark et al., 2002; Roberts et al., 2003). There are some data, albeit limited, regarding altered reproduction or endocrine function due to pyrethroid insecticides in animal and in vitro studies that showed that some pyrethroid insecticides and their metabolites may cause endocrine disruption and have adverse effects on semen quality (Meeker et al., 2009). Also, some reports have shown destructive effects on hormones of the endocrine system. The aim of this study is to investigate the morphological effects of sub-lethal permethrin concentrations on rat ovaries at the histological and ultrastructural level.

2. Materials and methods

The experiments were carried out on 24 adult female Wistar Albino rats obtained from Istanbul University, Institute for Experimental Medical Research (DETAE). Animals were housed in

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metallic cages under regular light–dark conditions. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the Istanbul University, Institute for Experimental Medical Research. The rats were divided into 3 groups comprising 8 rats. We used permethrin as an insecticide with a 25/75 cis/trans isomer proportion (sc-201319A). We treated the 2 groups with 20 mg/kg/day, 40 mg/kg/day permethrin by gavage for 2 weeks. The remaining group was the control group and we treated them with normal saline.

At the end of the treatment the animals were sacrificed by overdose of intramuscular ketamine (90 mg/kg) (Sigma-K4138) injection. The ovaries were processed for light and electron microscopy by routine processes.

For light microscopic evaluation, tissue samples were fixed within 10% buffered formalin. The samples were then processed through graded ethanol for paraffin embedding. Serial sections (3–4 μm) were taken by microtome (Leica RM2255) and stained with hematoxylin and eosin, periodic acid-Schiff's reagent (PAS) and Masson trichrome. The sections were observed under light microscopy (Olympus BX40F4). Image samples were taken using objectives at 40× and 100× magnifications. Atretic follicles were counted from sections using a 40× magnification. The results were then analyzed statistically using Graph Pad software and graphs were plotted using Excel software.

For electron microscopic study, tissues were fixed using 2.5% glutaraldehyde (Fluka-49630) at +4 °C, and secondary fixation was done using 1% osmium tetroxide (EMS-19134) for 1 h at +4 °C. After washing with phosphate buffered saline, tissues were treated with 1% uranyl acetate at +4 °C for an hour. After an ascending acetone (Riedel-de Haen-24201) series (30, 50, 70, 90, 100, 100%) tissues were treated with 1:1 acetone:epon, 1:3 acetone:epon mixtures and pure epon at room temperature for 1 h then embedded in capsules filled with pure epon (Fluka 45359). The polymerization capsules were incubated at 60 °C for 18 h. Semi-thin (0.5 μm) and thin (0.06 μm) sections of tissue samples were cut by ultra microtome (Leica EM UC7). Semi-thin sections were taken and stained with toluidine blue and evaluated under light microscope. Thin sections were taken on nickel grids and stained on drops of uranyl acetate for 30 min and a drop of lead nitrate for 6 min. Sections were evaluated under a Jeol Jem 1011 transmission electron microscope. The images were transferred to Soft Imaging System Analysis programme by Megaview III digital camera and pictures were taken.

For evaluation of apoptosis, caspase 3 was detected, which is the last antigen to appear in the apoptosis process. The 10% formaldehyde fixed tissues were processed and embedded into paraffin blocks using a routine light microscopy processing schedule. Sections 2–3-μm thick were taken using a microtome (Leica RM2255) and put on charged slides. Sections were incubated overnight at 56 °C (Heraeus FB420). Rehydration was performed in decreasing concentration alcohol series. Sections were incubated in darkness for 15 min in 3% hydrogen peroxide (Merck-536092) in methanol (Merck-596470) to block endogenous peroxidase reaction. Sections were washed with PBS for 5 min. For antigen retrieval, sections were incubated in a 10% citrate buffer in a steamer until the buffer boiled. The sections were left to cool to room temperature for 20 min. After treatment with Ultra V block (Invitrogen Histostain Broad Spectrum HRP – 1029337A) for 20 min sections were incubated with 1/100 diluted rabbit monoclonal cleaved caspase 3 primary antibody (Cell Signaling (ASP175-5A1E)) overnight at 4 °C. After washing with PBS, sections were covered with biotin-labeled secondary antibody (Invitrogen Histostain Broad Spectrum HRP – 1029337A) for 30 min. Sections were washed with PBS, incubated with streptavidin-peroxidase (Invitrogen Histostain Broad Spectrum HRP – 1029337A) for 30 min. For visualization, the immunoreactivity sections were incubated in AEC chromogen solution (Zymed Laboratories AEC Substrate kit – 00-2007) for 20 min, washed with distilled water and counterstained with Mayer's hematoxylin (Bio-Optica – 3412). After washing with tap water sections were cover-slipped, examined and scored under light microscopy (Olympus BX40F4). Immunoreactive granulosa cells from antral follicles were counted in 20 random areas from each section using a 40× magnification objective. The results were analyzed statistically using Graph Pad software and graphs were plotted using Excel software.

Table 1
Compares the number of atretic follicles and caspase-3 reactivity in control and experimental groups. Significant changes 20 mg versus 40 mg-treated group and control versus 40 mg-treated group. (◊ $P < 0.001$, *** $P < 0.001$).

Groups	Atretic follicles; mean ± S.E.M. (n: 8)	Caspase-3; mean ± S.E.M. (n: 20)
Control	8.9 ± 0.35	6.85 ± 3.1
20 mg	9.2 ± 0.3	9.9 ± 2.8
40 mg	14.6 ± 0.4◊,***	16.1 ± 3.2◊,***

◊ $P < 0.001$: 20 mg versus 40 mg.
*** $p < 0.001$: control versus 40 mg.

3. Results

3.1. Light microscopic evaluation

Normal morphological features were detected in the primordial, primary, secondary and graaf follicles, corpus luteum, and cortex of the ovaries of the control group (Fig. 1a). The granulosa cell layer of all types of follicles showed intact appearance with clear cellular contacts (Fig. 1b). Luteal cells of corpus luteum showed normal morphological features with polygonal shape and intact cellular contacts (Fig. 1c). In the experimental groups although there was normal morphology in the follicles and corpus luteum, we detected atretic and degenerated follicles and degenerated oocytes (Fig. 1d and g). In the experiment groups, degenerative changes were dose dependent. In the granulosa cell layer, loss of cellular contacts was visible. Pyknotic cell morphology and condensed chromatin were seen (Fig. 1e and h). In contrast to the control group, vacuolization, dispersed cytoplasm and degenerated cellular contacts were detected in the luteal cell layer in a dose dependent manner (Fig. 1f and i). We detected weak caspase-3 reactivity in cells of large atretic follicles and no staining in growing follicles of the control group (Fig. 2a and b). In the 20 mg-treated group, moderate staining was seen in atretic follicles (Fig. 2c and d). Both the atretic follicles and growing follicles showed intense immunoreactivity of caspase 3 in the 40 mg-treated group, which was as a sign of apoptosis (Fig. 2e and f).

Statistical analysis showed significant quantitative changes in the number of atretic follicles and caspase-3 immunoreactivity between the control group and the 40 mg-treated group ($P < 0.001$) and also the 20 mg-treated group versus the 40 mg-treated group ($P < 0.001$). There is no significant difference between the control group and the 20 mg-treated group ($P > 0.05$) (Table 1 and Graph 1).

3.2. Electron microscopic evaluation

In the control group, oval or spherical shaped graaf follicle cells had oval or spherical nuclei that contained euchromatic genetic material (Fig. 3a). Cells had contacts with cytoplasmic extensions and cellular attachments were clear and intact (Fig. 3b). Cytoplasmic organelles like mitochondria with clear cristae, expansive rough endoplasmic reticulum (RER) cisternae and smooth endoplasmic reticulum (SER) tubules and Golgi apparatus were detected (Fig. 3c). Graaf follicle cells of the 20 mg-treated group showed disrupted cytoplasm with mitochondrial degeneration (Fig. 3d–f).