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Effects of Pyriproxyfen on Viability and Increase of Intracellular Lipids in HepG2 Cell Line

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Abstract

Introduction: Pyriproxyfen, (2-[1-methyl-2-(4-phenoxyphenoxy) ethoxy] pyridine) (PPF) is an insecticidal used in household, agricultural, and horticultural applications to control many insect species. We tested its hepatic toxicity in hepatoma HepG2 cell line, we also evaluate if PPF could induce nonalcoholic fatty liver disease.

Materials and methods: The hepatoma HepG2 cell line was exposed for 24-48 hrs with serum-free DMEM to the active principles at different concentrations. The cell viability was assessed by measuring reduction of the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). For the evaluation of in vitro steatosis, the cells were rinsed with cold phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde. Images of cell were captured using an optic microscope and stained lipid droplets were then extracted with isopropanol (60%) for quantification by measuring its absorbance at 510 nm.

Results: The MTT-test showed that PPF is cytotoxic at all concentrations tested both at 24 h and 48 h. Cell viability is below 50% for concentrations 1-10 ppm while the viability is less than 10% for the concentration 100 ppm. PPF induces the increasing intracellular lipids from 1 ppm concentration. The maximum effect is observed at 100 ppm.

Discussion: In our in vitro study we found a loss of cell viability of about 50% for concentrations from 1-10 ppm by the MTT-Test that measures mitochondrial enzyme activity. Because the mitochondrial enzyme activity affected major changes at the starting/beginning of the apoptotic this condition suggested that PPF is strongly cytotoxic to human hepatocytes in the presented assays. Already at 1 ppm concentration PPF induces the increasing intracellular lipids, in HepG2 *in vitro* culture.

Keywords: Pyriproxyfen; Agricultural pesticides; HepG2 cell line

Introduction

Agricultural pesticides include those chemicals intended to kill insects, plants, fungi, rodents, and other organisms that interfere with the production, storage, and distribution of agricultural products. Most agricultural pesticides now being used can have detrimental effects on human health. Despite their popularity and extensive use, pesticides are now raising serious concerns about health risks arising from the exposure to farmers when mixing and applying pesticides or working in treated fields and from residues on food and in drinking water for the general population [1].

Several researchers have found associations between pesticide exposure and chronic health effects that include lung cancer, prostate cancer, lymphohemopoietic cancers, pancreatic cancer, and colorectal cancer [2-5]. Other scholars reported a significant association between neurological symptoms and pesticide exposure [6]. Bronchitis, asthma, wheezing, farmer's lung, and rhinitis have also been reported to be associated with respiratory morbidity especially among farmworkers [7]. In fact, farmworker, pesticide exposure must be considered separately compared to the exposure of the general population because of the extensive exposure which farmers must endure [8,9].

Pyriproxyfen, (2-[1-methyl-2-(4-phenoxyphenoxy) ethoxy] pyridine,) (PPF) is an insecticidal used in household, agricultural, and horticultural applications to control many insect species, including the common housefly (Musca domestica), mosquitos, imported red fire ants (Solenopsis invicta), and silver leaf whitefly (Bemisia argentifolii) [10].

It is used on citrus fruit in Israel, South Africa, Spain and Italy and it has also been considered by WHO for vector control under its Pesticides Evaluation Scheme. PPF is a juvenile hormone analogue (JHA) that interrupts normal development and metamorphosis of targeted mosquitoes [11]. It also alters parthenogenic reproduction in non-target cladoceran species as it induces male production that can lead to a decrease in fecundity, a reduction in population density, and subsequent ecological effects [12]. Highly potent in terms of activity and specificity, PPF degrades rapidly in soil under aerobic conditions, with a half-life of 6.4-36 days and it disappeared from aerobic lake water–sediment systems with half-lives ranging from 16 to 21 days. Pyriproxyfen is also relatively lipophilic as it has an octanol/water partitioning coefficient of Kow 5.6. In short- and long-term studies of the effects of pyriproxyfen in mice, rats and dogs, the liver was assessed as the main toxicological target (increases in liver weight and changes in plasma lipid concentrations, particularly cholesterol) [13].

In this paper, also in consideration of the chemical and physical

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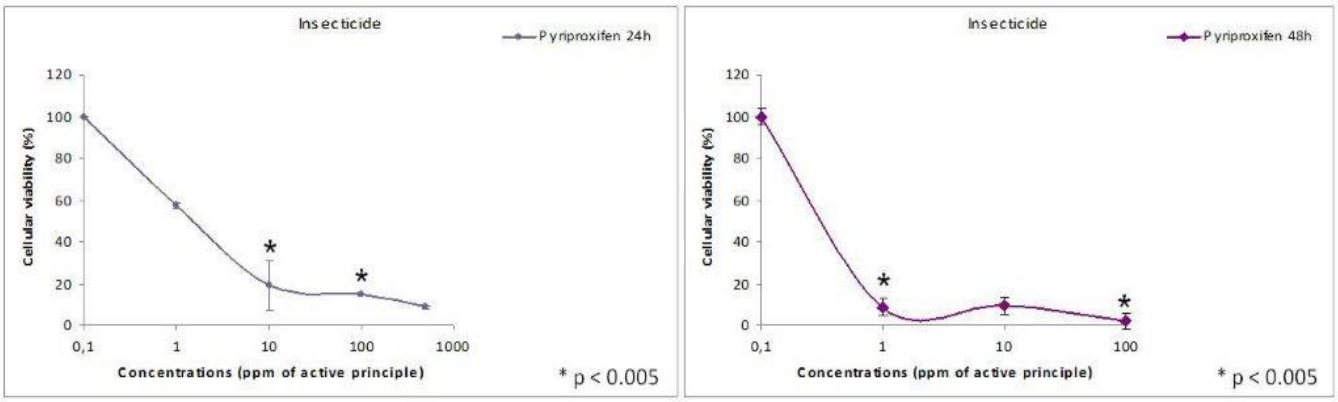

Figure 1: Dose-dependent effect of Pyriproxifen on HepG2 cell viability at different concentrations (0.1-500 ppm) for 24 hours (A) and (0.1-100 ppm) for 48 hours (B).

characteristics of the substance, we tested its hepatic toxicity in hepatoma HepG2 cell line, we also evaluate if PPF can induce nonalcoholic fatty liver disease (NAFLD).

Materials and Methods

Materials

The hepatoma HepG2 cell line was provided by American Type Culture Collection (ATCC HB 8065). Cell viability was performed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTT-test) (Sigma Aldrich, Milan, Italy). In vitro steatosis was induced by incubating hepatocytes with 6mM of a mixture of oleic (18:1) and linoleic acid (18:2) ratio 1:1. Fat accumulation was determined using Oil red O staining (Sigma Aldrich, Milan Italy).

Methods

Cell culture: The hepatoma HepG2 cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/ml streptomycin and 100 µg/ml antimycotic. All cell culture materials were purchased from Gibco, Invitrogen, Milan Italy. The cells were grown on tissue culture plates (BD Bioscience-Falcon, San Jose, USA), using an incubator with a humidified atmosphere (95% air/5% CO_2 v/v) at 37°C during 48 h to 80% confluence, than washed, and exposed for 24-48 hrs with serum-free DMEM to the active principles at different concentrations (1-10-100-500 ppm). Before treatment, all the pesticides were solubilized in a 100% DMSO solution, then diluted in serum-free medium to reach 0.5% DMSO.

MTT assay: HepG2 (1.0x105) cultured in a 24-well plate in DMEM medium were treated with AP (active principle) for 24-48 hrs at different concentrations. In particular the cells were treated with Pyriproxifen 0.1-1-10-100-500 ppm for 24 hrs and with Pyriproxifen 1-10-100 ppm for 48hrs. The cell viability was assessed by measuring reduction of the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Mitochondrial dehydrogenates of living cells reduce the tetrazolium ring, yielding a blue formazan product which can be measured spectrophotometrically. The optical densities obtained are directly proportional to the number of living cells. The cytotoxic effect of a sample is evaluated by the percentage of living cells present in the sample, in relation to the cells treated only with the solutions [14]. After treatment, the medium was replaced by a solution of MTT 1 mg/ml in DMEM medium without phenol-red. After 3 h of incubation, the liquid was aspirated and the insoluble formazan produced was dissolved in HCl 0.1 M in isopropanol. The optical densities of the obtained solutions were measured at 570 nm using a Beckman DU 640 spectrometer (Beckman, Milan, Italy).

Oil red O staining: For the evaluation of in vitro steatosis, 1.0x105 HepG2 cells were seeded in a 24-well plate and treated for 24 hrs with fat acid. After treatment, the cells were exposed for 24hrs to the pesticides. Than the cells were rinsed with cold phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde (Sigma Aldrich, Milan Italy) for 30 min and stained with Oil Red O solution (0.5%) (Sigma Aldrich, Milan Italy). Images of cells were captured using an optic microscope and stained lipid droplets were then extracted with isopropanol (60%) for quantification by measuring its absorbance at 510 nm.

Results

The MTT-test showed that PPF is cytotoxic at all concentrations tested both at 24 hrs and 48 hrs (Figure 1). Cell viability is below 50% for concentrations 1-10 ppm while the viability is less than 10% for the concentration 100ppm and over.

In order to evaluate the levels of in vitro steatosis, Oil red staining was performed. Pyriproxifen induces the increasing intracellular lipids from 1 ppm concentration. The maximum effect is observed at 100 ppm as reported in Figure 2.

Discussion

According to the US Environmental Protection Agency (EPA), approximately five billion pounds of pesticides are used annually worldwide [15] A cross-sectional study of pesticide handling practices among Cambodian farmers suggested that most of the pesticides used in agriculture belonged to the World Health Organization (WHO) class I (extremely and highly dangerous) and class II (moderately dangerous) categories [16]. Therefore, one immediate aim might be to reduce the use of WHO Classes I and II pesticides, and replace these pesticides with less-toxic alternatives, especially in developing countries [17].

Pyriproxyfen is a juvenile hormone mimicking insecticide, used for the control of flies, beetles, midges and mosquitoes that inhibit larval development and maturation as a juvenile hormone III mimic.

The World Health Organization has classified pyriproxyfen as "product that improbably can cause acute risk in normal use" [18]. It is a solid (melting range 48-50°C) of low volatility and only slightly soluble

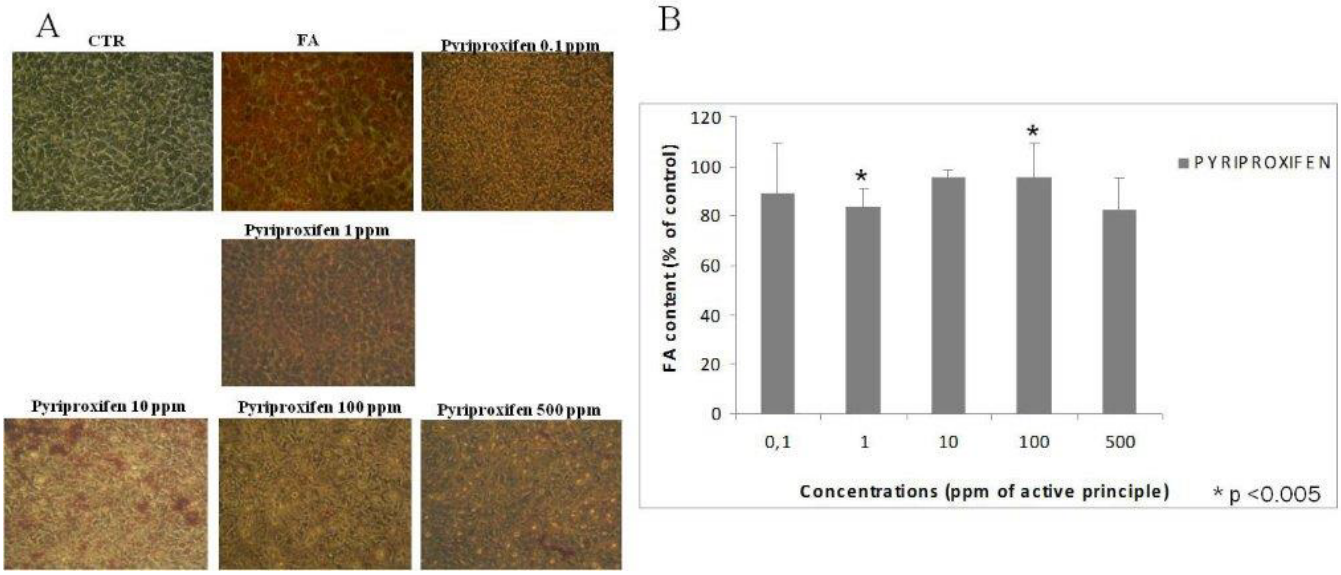

Figure 2: Panel A showed *in vitro* HepG2 steatosis images. The droplets stained positive for lipids by the Oil-Red-O staining. Panel B showed fatty acid content of HepG2 treated with different concentrations (0.1-500 ppm) of Pyriproxifen and evaluated by the Oil-Red-O staining.

in water. It has no discernible acidic or basic characteristics and is stable to hydrolysis at pH 4-9 at 25°C, but it is prone to slow photolysis.

The toxicity of pyriproxyfen was evaluated, for the first time, in the study WHO/FAO Joint Meeting on Pesticdes Residues [18] that showed low acute oral toxicity of pyriproxyfen, with LD50 values >5000 mg/kg body weight of mice, rats and dogs. The daily dose absorbed (ADI) by man was evaluated between 0 and 0.1 mg of active substance per kg of body weight. This assessment is derived from the determination of a value of NOAEL of 10 mg/kg of body weight per day, in one study with the duration of a year, performed on dogs, this value has been applied a safety factor of 100. The pyriproxyfen in these concentrations has been shown to be neither genotoxic nor carcinogenic.

Although Chang et al. [19] showed that the population of microbial community decreased rapidly after incubation with 10 mg Kg (-1) of pyriproxyfen for 91 days, indicating the toxicity of pyriproxyfen toward bacterial communities in a closed soil ecosystem [19].

Koyama et al. showed a decrease in body rats weight exposed at 10,000 ppm of PPF [20]. In blood tests they found an increase in bilirubin, GOT, triglyceride and total cholesterol. In particular, they showed that the weight of rat liver increased with an exposition of only 2,000 ppm with a higher incidence of blackish brown coloration of the liver [20]. This study aimed to the evaluation of the hepatotoxic effect of PPF on HepG2 cell line.

In our in vitro study we found a loss of cell viability of about 50% for concentrations 1-10 ppm by the MTT-Test that measures mitochondrial enzyme activity. Because the mitochondrial enzyme activity affected major changes at the starting/beginning of the apoptotic process [21], this condition suggested that pyriproxifen is strongly cytotoxic to human hepatocytes in the presented assays.

The outcome of this experimental research showed that PPF induces the increasing intracellular lipids, in HepG2 in vitro culture, already at 1 ppm concentration. Pesticides cause the impairment or inhibition of enzyme responsible for oxidation and synthesis of fatty acids or the receptor molecules controlling these enzymes. The result is the lipid accumulation in the cells [22]. This finding may correlate in vivo with increased liver damage toward the possibility to induce nonalcoholic fatty liver disease (NAFLD). The latter, is characterized by fat accumulation in the liver that start with simple hepatic steatosis and may progress toward inflammation (nonalcoholic steatohepatitis [NASH]) with progressive fibrosis and liver damage and is the most common reason for abnormal level (expression) of hepatic enzymes worldwide. Pyriproxifen may alter the hepatic function and thus can influence a xenobiotic response in the cells. We observed moderate steatosis in the hepatocytes when the cells are treated with 10 and 100 ppm of Pyriproxifen. Microscopic image analyses relative to HepG2 treated at different concentrations of pyriproxifen revealed accumulation of small lipid droplets after 24 hrs of treatment indicating that the doses (1 and 10 ppm) of active principle caused steatosis or abnormal accumulation of lipids.

The morphologic analysis obtained by inverted microscope images capture, confirmed by quantification spectrophotometrically at 510 nm of the vesicles stained positive for lipids (Oil Red O) shows a significant increase in triacylglyceride in the 100 ppm exposed hepatocytes compared to the control.

Conclusions

The current in vitro study indicates that pyriproxifen causes cytotoxicity and steatosis at the concentrations evaluated. Workers in the agricultural sector, or people involved in gardening or disinfestations may inhalate this active principle and even at low doses this may increase the severity of liver disese or even induce the liver cell damage and steatosis in healthy people.

For these reasons pesticide management and regulations, educational programs on safety precautions, reinforcement of safety behaviors, especially the proper use of personal protection equipment (PPE) in the workplace, are effective approaches for preventing diseases related to occupational pesticide exposures regardless of pesticides risk class of which we do not know fully the possible toxicity.

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