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Evaluation of cytotoxicity, genotoxicity and embryotoxicity of insecticide propoxur using flounder gill (FG) cells and zebrafish embryos

Manish Raj Pandey [a], Huarong Guo [b,*]

[a] Laboratory of Evolution & Development, Institute of Evolution & Marine Biodiversity, Ocean University of China, Qingdao 266003, China
[b] Department of Marine Biology, Ocean University of China, Qingdao 266003, China

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ABSTRACT

Cytotoxicity, genotoxicity and embryotoxicity of carbamate insecticide propoxur were evaluated using flounder gill (FG) cells and zebrafish embryos. The cytotoxicity of propoxur in FG cells was analyzed by MTT, neutral red uptake (NRU), lactate dehydrogenase (LDH) release and Hoechst 33342 and propidium iodide double staining, and acute cytotoxic effects were observed in a concentration-dependent manner. The 24 h-IC_{50} values of 89.96 ± 1.04, 103.4 ± 1.14 and 86.59 ± 1.13 μg/ml propoxur were obtained by MTT, NRU and LDH assays, respectively. The lethal effects were induced in FG cells mainly through necrosis but not apoptosis as evidenced by double fluorescence staining. Comet assay showed weak genotoxic effects and statistically significant DNA damages were recorded in the cells exposed to highest tested concentration of 75 μg/ml propoxur ($p < 0.05$). Propoxur exerted obvious acute toxic effects on the survival, spontaneous movement, hatching and heart rate, and development (yolk and pericardial sac edema) of zebrafish embryos in both time- and concentration-dependent manner only at ⩾100 μg/ml. The corresponding 24 h-, 48 h- and 96 h-LC_{50} values of propoxur in zebrafish embryos were 166.4 ± 1.06, 146.3 ± 1.07 and 134.8 ± 1.06 μg/ml, respectively. The above data obtained suggest a low acute toxicity of propoxur to the *in vitro* cultured FG cells and zebrafish embryos.

1. Introduction

Propoxur (2-isopropoxyphenyl methylcarbamate) is a *N*-methylcarbamate insecticide and acaricide. It was introduced to the market in 1959 and has been widely used against turf, forestry, and household pests and fleas. It is also used in pest control for other domestic animals, mosquitoes, bugs, ants, gypsy moths, and other agricultural pests (Tomlin, 1994). The main toxic mechanism of action for propoxur in target species is to inhibit the activity of acetylcholinesterase (AChE), resulting in the disruption of normal nervous system function causing a rapid "knockdown" effect and possible death (Baron, 1991; WHO, 2005). In addition, propoxur has also been shown to be toxic to non-target species like honeybees, birds and even mammals including humans, though its toxicity varies according to the species (CDPR, 1997; FAO, 2006; Lakota et al., 1981). Propoxur is classified to be moderately toxic (Toxicity Category II) for oral exposure and slightly toxic (Toxicity Category III) via the dermal and inhalation routes of exposure by the U. S. Environmental Protection Agency (EPA). It is also classified as a Group B_2 probable human carcinogen (USEPA, 1997). Propoxur may remain in the environment for weeks to several months, longer than most carbamates. It is also likely to be moderately persistent and mobile in soils, having characteristics which could produce leaching to groundwater (USEPA, 1997) and may impose adverse effects on human health.

Despite the obsolete and restrictive use of propoxur in some regions, the widespread application of propoxur have been found increasing particularly in the developing countries, propoxur has attracted increasing concerns on the safety of aquatic organisms as this pesticide eventually ends up into the aquatic environment. Fish are often used for monitoring toxicity in the aquatic environments. Accumulating evidences showed that propoxur is moderately to slightly toxic to freshwater fish (FAO, 2006). Lakota et al. (1981) determined the behavioral changes and median lethal concentration (LC_{50}, 7.34 μg/ml propoxur) of five month-old common carp (*Cyprinus carpio*) fries after exposure to propoxur. Srivastava and Singh (1982) reported the 96 h-LC_{50} value of 6.50 μg/ml propoxur for adult female Indian catfish (*Heteropneustes fossilis*) and the acute toxicity of propoxur on carbohydrate metabolism. The reported 96 h-LC_{50} values of propoxur in rainbow trout (*Salmo gairdneri*), fathead minnow (*Pimephales promelas*), bluegill (*Lepomis macrochirus*) and goldfish (*Carassius auratus*) were 8.13, 25.12, 4.78 and 36.2 μg/ml, respectively (Vittozzi and De Angelis, 1991; Wang

* Corresponding author. Tel.: +86 532 82031932.
E-mail address: huarongguo@ouc.edu.cn (H. Guo).

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et al., 2009). Hanson et al. (2007) examined the toxic effects of propoxur on the growth and reproduction, as shown by gonadosomatic indices, of three freshwater fish species of Nile tilapia (*Oreochromis niloticus*), Bagrid catfish (*Chrysicthys nigrodigitatus*) and African catfish (*Clarias gariepinus*). Recently, sublethal toxicity and *in vivo* genotoxicity of propoxur in the common carp (*C. carpio*) were evaluated by hematological/biochemical biomarkers and histopathological examination and indicated the genotoxic potential of this insecticide (Gul et al., 2012). However, all the above-mentioned studies have focused on the toxicity of propoxur on juvenile and adult fish species, and the embryotoxicity of propoxur on fish is lacking.

Fish embryos and larvae are generally the most sensitive stages in the life cycle of teleosts (Laale and Lerner, 1981; Lele and Krone, 1996) and they are ideal for determining the toxic responses to environmental pollutants. Thus fish embryo toxicity (FET) tests have been developed to evaluate the developmental toxicity of toxicants in fish, and most of these tests were carried out in zebrafish embryo because of its transparency and easy maintenance. The transparent nature of eggs and embryos allows the visualization of morphological and structural abnormalities in the whole body following exposure to chemicals (Brannen et al., 2010; Fraysse et al., 2006; OECD, 2006). Up to date, studies on the developmental toxicity of carbamate pesticides in zebrafish embryos are scarce. Lin et al. (2007) reported the toxic effects of carbaryl, a kind of carbamate pesticide, on zebrafish embryos including the viability, malformations in the tail region, pericardial edema, red blood cell accumulation and bradycardia. Schock et al. (2012) also observed the defects in heart formation, decreased heart rate and developmental delay/defect in cardiac looping in zebrafish embryos after exposure to carbaryl. The toxic effects of another carbamate insecticide aldicarb and its metabolite aldicarb-sulfoxide to zebrafish embryos demonstrated sublethal effect, with the significant increase of heart rate at lower concentrations up to 1 μM and decrease at test concentrations above 30 μM, only for aldicarb-sulfoxide on the organismic level (Kuster and Altenburger, 2007). Thus investigating the embryotoxicity of propoxur in zebrafish will markedly contribute to the safety assessment of propoxur for aquatic organisms.

In vitro cell cultures, as an alternative of whole animals, provide us a useful tool to examine the cytotoxic and genotoxic effects of chemicals and environmental pollutants in a rapid and cost-effective way (Bols et al., 2005). Although negative carcinogenic and teratogenic toxic effects were observed in live rats and rabbits (FAO, 2006), cytotoxic and inconsistent genotoxic effects of propoxur have been reported in cultured mammalian cells. Wang et al. (1998) found that the propoxur has less cytotoxic effect than its N-nitroso derivative in the hamster lung fibroblast (V79) cells and primary rat tracheal epithelial (RTE) cells. However, no mutagenic effects of propoxur were recorded on either type of the above-mentioned cells. Maran et al. (2010) also reported the growth inhibitory effect of propoxur in Chinese Hamster Ovary cell line (CHO-K1). But Ündeğer and Başaran (2005) reported that the propoxur significantly induced DNA damage in human peripheral lymphocytes at all concentrations tested. However, information regarding the cytotoxicity and genotoxicity of propoxur to fish cell line is unknown.

Up to now, various *in vitro* cultured fish cells have been used for the safety assessment of chemicals and environmental pollutants in fish (Babich and Borenfreund, 1991; Castaño et al., 2003; Segner, 1998). The continuous marine flatfish cell line of flounder gill (FG), maintained in our laboratory since its establishment by Tong et al. (1997), has already been successfully used to evaluate the cytotoxicity and genotoxicity as well as the corresponding toxic mechanism of action of an array of environmental pollutants (Guo and Zhang, 2002; Li and Zhang, 2001; Na et al., 2009; Xiao et al., 2011, 2007; Yang et al., 2010; Yin et al., 2007). However, the toxic effects of propoxur have not yet been evaluated in FG cells.

The main purpose of the present study was therefore to examine the toxic effects of propoxur in the *in vitro* cultured FG cells and zebrafish embryos with a view to record its cytotoxicity, genotoxicity and embryotoxicity. In specific, *in vitro* studies were performed using FG cell line to determine the cytotoxic effects of propoxur by MTT reduction, neutral red (NR) uptake, lactate dehydrogenase (LDH) release, and Hoechst 33342 and propidium iodide (PI) double staining assays; genotoxic effect was evaluated by comet assay. The embryotoxicity test dealt with the assessment of propoxur in developing zebrafish embryos by observing diverse general morphological endpoints.

2. Material and methods

2.1. Chemicals

Propoxur (2-isopropoxyphenyl methylcarbamate, 99.8% pure), MTT (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide), neutral red (NR), dimethyl sulphoxide (DMSO), low melting point agarose (LMPA), 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), ethylene diamine tetraacetic acid (EDTA), trypsin and tris base were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals used were of analytical grade. Plastic cell culture flasks (25 cm^2) and 24- and 96-well culture plates were from Corning Incorporation (NY, USA).

A 100 μg/μl stock solution of propoxur was prepared in DMSO and stored at 4 °C before being used. Working solutions were prepared by dilution of the stock solution into culture media immediately before use. The final concentration of DMSO in the working solutions was always $\leqslant$0.5% (v/v).

2.2. Cell line

The continuous flounder gill (FG) cell line, derived from the gill tissues of flounder (*Paralichthys olivaceus*) and maintained in this laboratory since 1993 (Tong et al., 1997), was used for cytotoxicity and genotoxicity assays. Briefly, the cells were cultured at 20 °C in minimal essential medium (MEM; Gibco BRL, New York), supplemented with 10% bovine calf serum (BCS; Hyclone, USA), 100 IU/ml penicillin, and 100 μg/ml streptomycin, buffered to pH 7.4 in plastic cell culture flasks at 20 °C.

2.3. Zebrafish and eggs

Adult zebrafish (*Danio rerio*) were purchased locally from a fish dealer and acclimatized and kept in five glass aquaria of 10 l each filled with matured water. They were maintained at 26 ± 1 °C under a 14/10 h (light/dark) photoperiod cycle (Westerfield, 2000). Fish were fed twice daily with live nematodes and a part of the water was exchanged every day. In the evening, male and female fish (2:1) were placed in a spawning box. Spawning was triggered once the light was turned on the next morning and the fertilized eggs were collected and examined under a stereomicroscope.

2.4. MTT assay

MTT assay, described by Borenfreund et al. (1988), is based on the reduction of soluble yellow MTT tetrazolium salt to a blue insoluble MTT formazan product by mitochondrial succinic dehydrogenase in cells. MTT assay was carried out according to the method described previously (Yang et al., 2010). Briefly, FG cells were exposed to fresh MEM medium containing 0 (control), 0.5, 1, 10, 25, 50, 75, 100, 150, and 200 μg/ml propoxur, respectively