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In vitro acute cytotoxicity of neonicotinoid insecticide imidacloprid to gill cell line of flounder *Paralichthy olivaceus**

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Abstract *In vitro* acute cytotoxicity of neonicotinoid insecticide imidacloprid (IMI) to the gill cell line of flounder (FG) that collected in the gill of *Paralichthys olivaceus*, was examined by 3 widely used endpoint bioassays: NR (neutral red), MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) and TCP (total cell protein). The result shows that the IMI increased at concentrations ≥0.5 μg/ml. The IC_{50} value of NR, MTT, and TCP was 41.86, 38.46, and 39.08 μg/ml, respectively. The ultrastructural observation revealed that the mitochondria of the cells exposed to 60 μg/ml IMI for 48 h were severely damaged, swollen or disrupted, while their nuclei and rough endoplasmic reticulum (RER) remained normal. This would suggest that the mitochondria are probably the primary target of IMI.

Key word: imidacloprid, cell line, cytotoxicity, flounder; *Paralichthy olivaceus*

1 INTRODUCTION

Imidacloprid 1-(6-chloro-3-pyridylmethyl)-*N*-nitroimidazolidin-2-ylideneamine (IMI), is a quite new class of neonicotinoid insectide, which acts by binding to pharmacologically diverse nicotinic acetylcholine receptor (nAChR) subtypes (Buckingham et al., 1997). IMI is currently used in agriculture to control insects including rice hoppers, aphids, thrips and white flies (Elbert et al., 1990; 1998; Worthing, 1994). It has also been used against soil insects, termites and some species of biting insects such as rice water weevil and Colorado beetle (Tomlin, 1994). The increasing use of IMI demands detailed studies to evaluate its potential toxic risks to non-target organisms. Recently, the toxicity of IMI to earthworms and amphibians was investigated (Luo et al., 1999; Zang et al., 2000; Feng et al., 2004). However, the effects of IMI on aquatic vertebrates such as fish have not been fully studied.

IMI is persistent, and would enter aquatic environments causing pollution (UNEP, 1991; Flores-Cespedes et al., 2001). Previous eco-toxicity data of aquatic pollutants are usually derived from LC_{50} bioassays at adult fish by determining the concentration of the pollutant that is lethal to 50% of the animal being tested against certain pollutant usually 96 h of exposure. Such *in vivo* bioassays are, however, very costly and time consuming, requiring the model fish and specially designed aquatic laboratory facilities. There is also a need for rapid and short-term *in vitro* bioassays to screen the potential eco-toxicity of the pollutants. Rachlin and Perlmutter (1968) first suggested the application of cultured fish cells to *in vitro* acute cytotoxicity assay of aquatic pollutants, which was then widely employed by many other authors (Kocan et al., 1979; Bols et al., 1985; Huuskonen et al., 1998; Li and Zhang, 2001; 2002). This study is to test the feasibility of utilizing FG (gill cell line of flounder) cell line to evaluate *in vitro* acute toxicities of the neonicotinoid insecticide IMI.

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2 MATERIALS AND METHODS

2.1 Cells and chemicals

A continuous cell line FG established from gills of flounder *Paralichthys olivaceus* (Tong et al., 1997) was used in the experiment. The cells were cultured at 20ºC in minimal essential medium (MEM; HyClone), supplemented with 10% bovine calf serum (BCS; HyClone), 100 U/ml penicillin and 100 μg/ml streptomycin. Three duplicated cultures were tested for each concentration of IMI.

IMI with 96% of its active ingredient was purchased from Hailir Pesticides and Chemicals Company, Qingdao of China, and bovine serum albumin (BSA), neutral red (NR), and 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were from Sigma, St. Louis, MO, USA.

2.2 Cytotoxicity assays

The cells were harvested then diluted to 1×10^6 cells/ml in MEM with 10% BCS. The cell suspension was agitated evenly and 200 μl aliquot was added to each well of 96 well tissue culture plates (Costar, Cambridge, MA, USA) for overnight incubation at 20ºC. The medium was removed next day and the cells were re-fed with unmodified medium (the control) or with the medium containing IMI in various doses from 0.5 to 80 μg/ml. The toxic endpoints were determined 48 h after exposure.

The NR uptake assay was done according to the method of Borenfreund and Puerner (1985). After 48 h exposure, the test medium was replaced with 200 μl of 50 μg/ml NR solution, which had been pre-incubated overnight at 20ºC and filtered beforehand to remove fine precipitates of dye crystals. After *in situ* incubation for 3 h at 20ºC, the plates were rinsed with warm phosphate-buffered saline (PBS), and the cells were de-stained with 200 μl de-staining solution (glacial acetic acid: 96%-ethanol:water=1:50:49). After 10 min of agitation at room temperature, the absorbance of the solution in each well was measured at 540 nm with a microplate reader (Unico, CA, USA) and the IC_{50} value, the concentration of test agent causing a 50% inhibition in NR uptake, was determined.

The MTT assay was done in the procedure described by Borenfreund et al. (1988). After 48 h of exposure, the test medium was replaced with 20 μl of 5 mg/ml MTT in PBS. After staining for 4 h at 20ºC, the staining solution was carefully removed and the cells were rapidly rinsed twice with PBS. Then, 150 μl/well DMSO was added to solubilize the purple formazan crystals produced. The absorbance of each well was measured at 490 nm with a microplate reader and the IC_{50} value was calculated.

The cell protein assay was performed according to the method adopted from Shopsis and Eng (1985). After 48 h incubation, the medium was removed, and the cells were washed with PBS. Then the medium was lysed in 50 μl of 0.1 mmol/L NaOH. The plate was incubated for an hour at 20ºC, and then 200 μl Coomassie blue staining solution was added to each well. After an initial 20 min incubation at room temperature, the absorbance of each well was measured at 590 nm using a microplate reader. A series of dilution from 1 to 100 μg/ml BSA dissolved in 0.1 mol/L NaOH were used for the protein standard.

All the experiments were performed at least 3 times, and the average absorbance at each dose was calculated in percentage of absorbance of cells incubated at different doses of IMI against the control.

2.3 Transmission electron microscopy

Ten 25 cm^2 cell culture flasks were each seeded with 1.25×10^6 cells and incubated in MEM with 10% BCS in the presence of 0 and 60 μg/ml of IMI. After being cultured at 20ºC for 2 days, the medium was discarded, and the cell sheets were flushed twice with PBS and trypsinized with 0.3 ml 0.25% trypsin solution. The detached cells were suspended in PBS, collected by centrifugation at 300 g for 10 min and fixed in 2% glutaraldehyde in PBS (pH 7.3) for one hour at room temperature. The glutaraldehyde-fixed cells were then post-fixed in 1% osium tetroxide. After dehydration, the cells were embedded in Epon 812, sectioned, and stained with uranyl acetate and lead citrate. The stained sections were observed and photographed under an H-7000 electron microscope.

3 RESULTS

3.1 Cytotoxic activity

A total of 8 doses of IMI ranging from 0.5 to 80

μg/ml were tested using 3 basal cytotoxicity endpoints (NR, MTT and cell protein assays). The cytotoxic activity of IMI to flounder cells is shown in Fig.1. Cytotoxicity was similar in all systems, regardless of the toxic endpoints employed. The IMI was already toxic to FG cells at 0.5 μg/ml, the lowest concentration tested, and the toxicity to the cells increased with the concentration. However, no obvious gross change in the external morphology and in the attachment of the cells were observed under a microscope in the conditions tested, although their growth was markedly inhibited (Fig.2).

IC_{50} values, the concentrations of IMI resulting in 50%-inhibition of cytotoxicity parameters after 48 h exposure to IMI, were calculated (Fig.1) being 41.86, 38.46 and 39.08 μg/ml for NR, MTT, and cell protein assays, respectively.

3.2 Ultrastructural alterations

The control cell had a conspicuous nucleus with a single electron-dense nucleolus. A large number of mitochondria and numerous cisternae of rough endoplasmic reticulum (RER), and they are usually located at one side of the periphery of the nucleus (Fig.3A, B). The cell contained also some granules and vacuoles scattered in the cytoplasm. In contrast, the mitochondria of the cells exposed to 60 μg/ml IMI for 48 h were severely damaged as the cristae swelled or disrupted, while the nuclei and

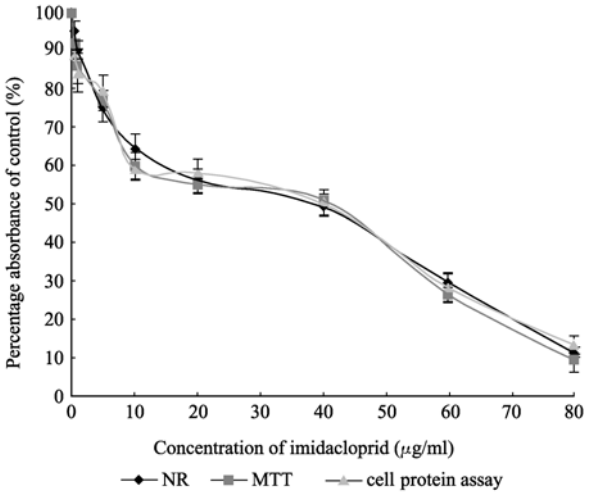

Fig.1 *In vitro* cytotoxicity of IMI to FG cells as determined by NR assay, MTT assay, and cell protein assay

The individual data points are expressed in arithmetic mean percentage of control ± SE

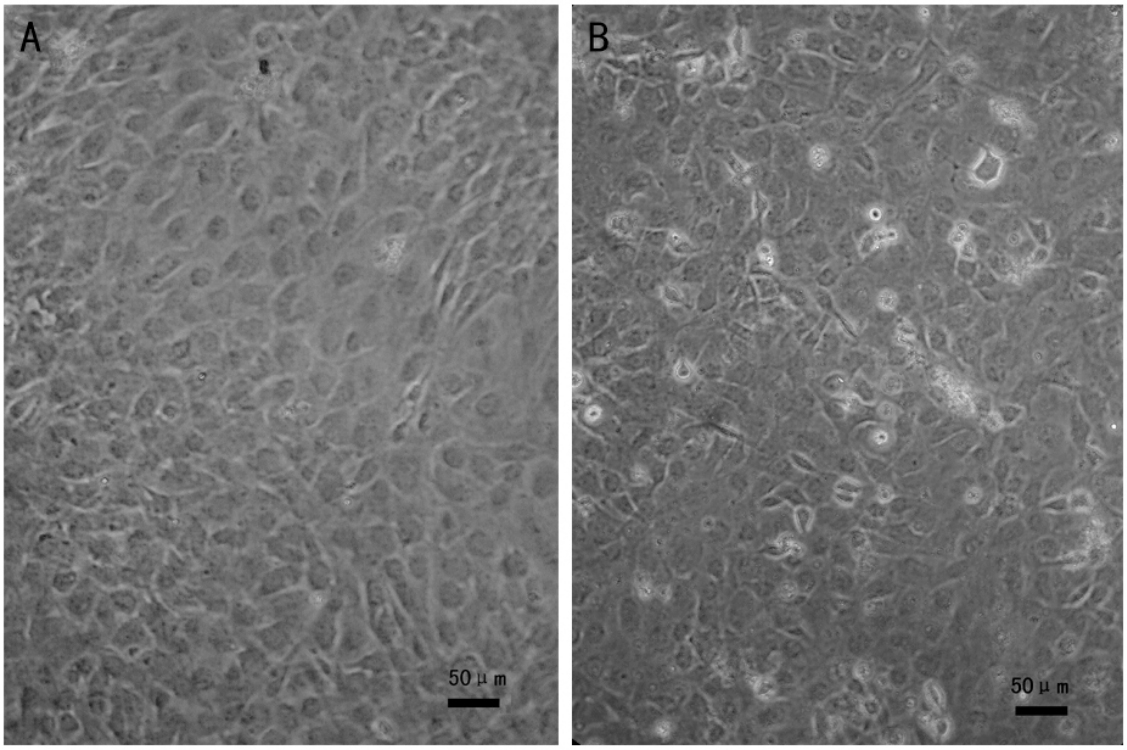

Fig.2 Micrographs of FG cell morphology

A: Cells grown in the absence of IMI for 48 h; B: Cells grown in the presence of 60 μg/ml IMI for 48 h

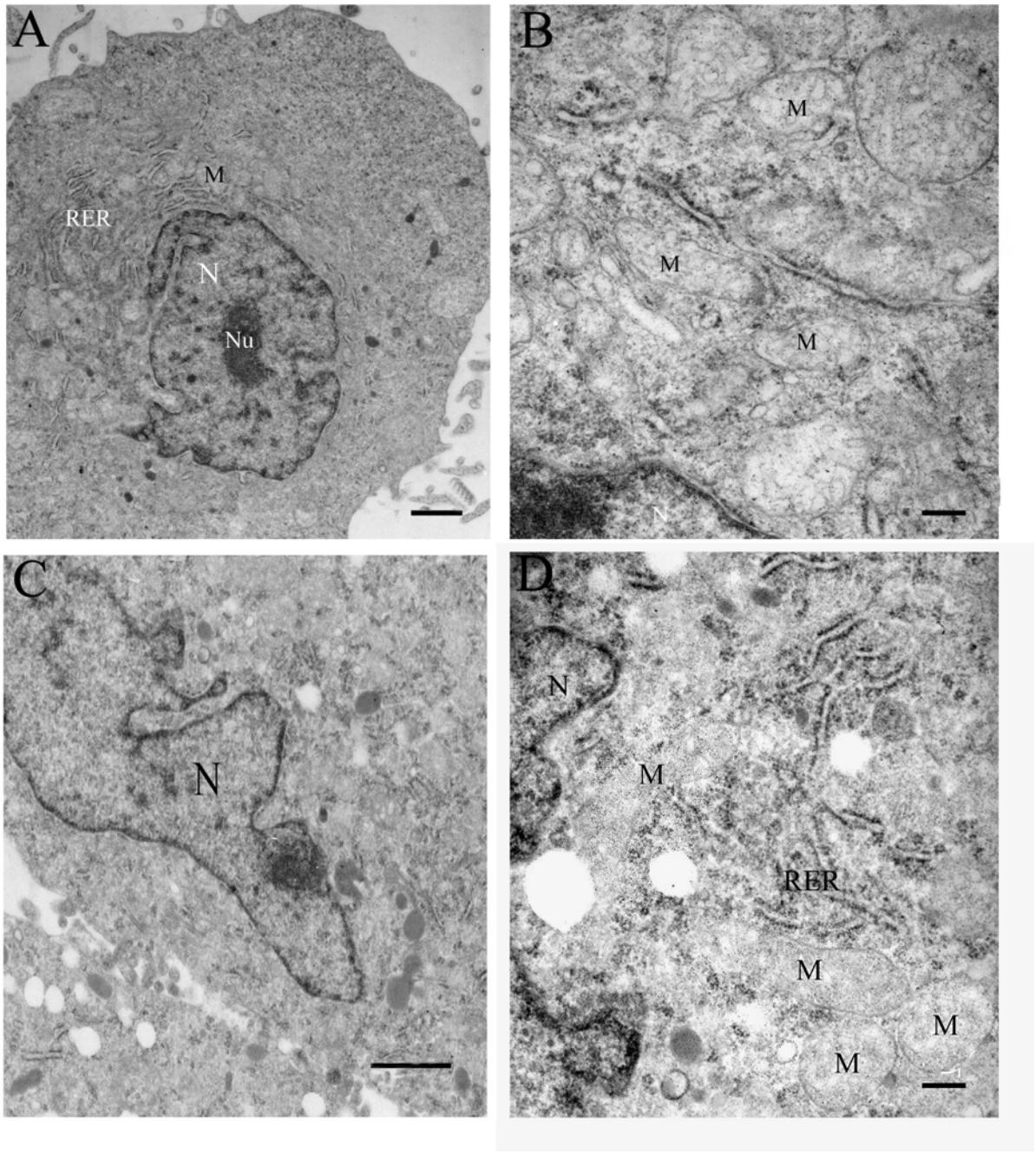

Fig.3 The ultrastructures of acetamiprid-exposed and control FG cells

A and B: Micrographs showing nucleus with a single nucleolus, and mitochondria in the control cell. C and D: Micrographs showing nucleus with a single nucleolus, and swelled mitochondria in the cell exposed to 60 μg/ml IMI for 48 h. N: nucleus; Nu: nucleous; RER; rough endoplasmic reticulum; M: mitochondrion; Bars represent 1 μm in A and C; 200 nm in B and D

rough endoplasmic reticulum appeared fairly normal (Fig.3C, D). Morphological alterations of apoptosis, such as membrane blebbing and nuclear fragmentation, were not observed.

4 DISCUSSION

Three widely used endpoint bioassays-NR, MTT, and cell protein assays were employed in this study to measure the *in vitro* acute cytotoxicity of IMI. The main finding was that the *in vitro* cytotoxicity of IMI to FG cells was closely correlated in all 3 systems of the toxic endpoints employed independently. This not only supports the idea of Ekwall (1995) that most cell lines have a similar response to toxicants when toxicity is

measured by various endpoints, corresponding to the inhibition or destruction of basal functions and structures, but also suggests that both NR and MTT as well as cell protein assays can be equally used to predict acute cytotoxicity.

It has been shown that FG cells are suitable candidates for evaluating *in vitro* acute cytotoxicity of organophosphorus pestictides such as parathion and methylparathion (Li and Zhang, 2001; 2002) and the organotin compound like bis-(tri-*n*-butyltin)oxide (Su et al., 2005). Here we extend the use of FG cells to evaluate *in vitro* acute cytotoxicity of neonicotinoid insecticide IMI. The FG cells are very susceptible to IMI because it apparently inhibits the cell growth at doses ranging from 0.5 to 80 μg/ml. This makes FG cells an excellent candidate for quickly evaluating the *in vitro* acute cytotoxicity of neonicotinoid insecticides like IMI.

The primary mode of IMI action is via blocking diverse nicotinic acetylcholine receptors in the insect's central nervous system (Buckingham et al., 1997). Here we show that IMI is capable of inhibiting the FG cell growth and causing severe damage to the mitochondria, and to RER to a lesser extent, hinting that the toxic effect of IMI is neither restricted to nAChR blocking nor to insects. The mitochondria are organelles that produce energy with which the cell can use for its various functions. The ultrastructural alteration in the mitochondria may affect their energy production, which then results in the slow-down of cell growth as observed in IMI-exposed FG cells. It needs further study to clarify if mitochondria are the primary target of IMI.

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References

Bols, N. C., S. A. Boliska and D. G. Dixon, 1985. The use of fish cell cultures as an indication of contaminant toxocity to fish. *Aquat Toxicol* **6**: 147-155.

Bornfreund, E. and J. A. Puerner, 1985. Toxicity Determined In Vitro by Morphological Alterations and Neutral Red Absorption. *Toxicol. Lett.* **24**: 119-124.

Bornfreund, E., H. Babich and N. Martin-Alguacil, 1988. Comparison of two in vitro cytotoxicity assays: the neutral red (NR) and tetrazolium (MTT) Tests. *Toxicol. in Vitro* **2**: 1-6.

Buckingham, S. D., B. Lapied, H. L. E. Corronc et al., 1997. Imidacloprid actions on insect neuronal acetylcholine receptors. *The Journal of Experiment Biology* **200**: 2 685-2 692.

Ekwall, B., 1995. The Basal Cytotoxicity Concept. In Goldberg A, and L Zutphen eds, Alternative Methods in Toxicology, New York, Mary Ann Liebert, USA. P. 721-725.

Elbert, A., R. Nauen, M. Cahill et al., 1996. Resistance management with chloronicotinyl insecticides using IMI as example. *Pflanzenschutz-Nachrichten Bayer* **49**: 5.

Feng, S. L., Z. M. Kong, X. M. Wang et al., 2004. Acute toxicity and genotoxicity of two novel pesticides on amphibian, Rana N. Hallowell. *Chemosphere* **56**: 457-463.

Flores-Céspedes, F., M. González-Pradas, M. Fernández-Pérez et al., 2002. Effects of dissolved organic carbon on sorption and mobility of imidacloprid in soil. *J. Environ. Qual.* **31**: 880-888.

Guez, D., S. Suchail, M. Gauthier et al., 2001. Contrasting Effects of Imidacloprid on Habituation in 7-and 8-Day-Old Honeybees (Apis mellifera). N*eurobiology of Learning and Memory***76**: 183-191.

Huuskonen, S. E., M. E. Hahn and P. Lindström-Seppä, 1998. A fish hepatoma cell line (PLHC-1) as a tool to study cytotoxicity and CYP1A induction properties of cellulose and wood chip extracts. *Chemospher* **36**: 2 921-2 932.

Kocan, R. M., M. L. Landolt and K. M. Sabo, 1979. *In vitro* toxicity of eight mutagens/carcinogens for three fish cell lines. *Bull Environ. Contam. Toxicol.* **23**: 269-274.

Li, H. Y. and S. C. Zhang, 2001. *In vitro* cytotoxicity of the organophosphorus pesticide parathion to FG-9307 cells. *Toxicol in Vitro* **15**: 643-647.

Li, H. Y. and S. C. Zhang, 2002. *In vitro* cytotoxicity of the organophorus insecticide methylparathion to FG-9307, the gill cell line of flounder (*Paralichthys olivaceus)*. *Cell Boil. Toxicol.* **18**: 235-241.

Luo, Y. Z., Y. Zhong et al., 1999. Toxicological Study of Two Novel Pesticides on Earthworm *Eisenia Foetida. Chemosphere* 39(13): 2 347-2 356.

Rachlin, J. W. and A. Perlmutter, 1968. Fish cells in culture for study of aquatic toxicants. *Water Res.* **2**: 409-414.

Shopsis, C. and B. Eng, 1985. Rapid Cytotoxicity Testing Using a Semi-automated Protein Denaturation on Cultured Cells. *Toxicol. Lett.* **26**: 1-8.

Su, F., S. C. Zhang, H. Y. Li and L. Y. Zhu, 2005. *In vitro* cytotoxicity of the organotin compound bis-(tri-n-butyltin) oxide to FG cells. *Toxicology Mechanisms and*

Methods **15**(3): 205-209

Tomlin, C., 1994. The Pesticide Manual: A World Compendium, 10th ed. British Crop Protection Council, Croydon.

Tong, S. L., H. Li and H. Z. Miao, 1997. The establishment and partial characterization of a continuous fish cell line FG-9307 from the gill of flounder Paralichihys olivaceus. *Aquaculture* **156**: 327-333.

UNEP (United Nations Environment Programme), 1991. Assessment of the state of pollution of the Mediterranean Sea by organophosphorous compounds. *MAP Technical Reports Series* No.58.

Worthin, K. D., 1994. The Pesticide Manual: A World Compendium, 10th ed. British Crop Protection Council, Croydon.

Zang, Y., Y. Zhong, Y. Luo et al., 2000. Genotoxicity of two novel pesticides for the earthworm *Eisenia fetida*. *Environment Pollution* **108**: 271-278.