EFFECTS OF FUNGICIDE IMAZALIL ON THE EARLY DEVELOPMENT OF SEA URCHIN EGGS

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Abstract: The cytotoxic effects of the fungicide imazalil on the early development of fertilized eggs of sea urchins were investigated. Toxicity was estimated as the ratio of the inhibition of first cell division and gastrula formation. An addition of imazalil to two species of the sea urchin, Scaphechinus mirabilis (S. mirabilis) and Strongylocentrotus nudus (S. nudus), at a concentration greater than 7 ppm resulted in acute cell death. For these two species of sea urchins, the IC50 values for the first cell division with imazalil were 3.9 ppm and 1.8 ppm. The IC50 for the 24h-embryo (gastrula) for S. mirabilis and S. nudus was 0.8 ppm and 3 ppm, respectively. These values are comparable to the residue levels in fruits and vegetables, suggesting that the residual amount of imazalil is toxic for development of sea urchin embryos.

Key words: Imazalil, fungicide, sea urchin, egg, cell division

Introduction

Azole derivatives such as thiabendazole (TBZ) or imazalil, 1-[2-(2,4-dichloro-phenyl)-2-(2-propenyl-oxy)ethyl]-1H-imidazole are the examples of typical postharvest fungicides1,2). These fungicides are widely used in agriculture of foreign countries because they are very effective to controlling postharvest decay during storage and transport1,2). However, most fungicides being developed today are associated with lower potential for negative impact on the environment, including non-target organisms and specifically linked to a wide variety of human health hazards, ranging from headaches to endocrine disruption3–5). The maximum residue limits (MRLs), therefore, are set and registered by the government authorities for food safety6,7). However, it has not been fully elucidated what kind of chronic effects will occur in the presence of lower concentrations of pesticides or fungicide including imazalil. Although imazalil is widely used in citrus fruits imported from foreign countries to Japan, toxicities are partly described in vertebrates, but hardly in invertebrates.

The embryonic and larval stages of marine invertebrates are less tolerant to toxicants than adults and have been used for assessing the biological quality of marine water and sediments8–10). It is well known that sea urchin eggs and embryos are often used as bioindicators for potential water pollution, as they are very sensitive and convenient to evaluate the effect of biologically toxic substrates on the early development11). The environmental effects of some previously used biocides, such as tributyltin oxide and other antifouling compounds, on the marine environment are well studied11,12).

The imidazole fungicide imazalil, which was chosen as being representative of azole derivatives, is intensively used in the agronomic field and its teratogenic effect is well elucidated in vertebrates but not in invertebrates. In the present study, we used sea urchin eggs for assessing the impact of imazalil on the early development of eggs.
Materials and Methods

Materials

Imazalil and other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan). Imazalil was solubilized in DMSO and stored at −20°C until use.

Sea urchins (Scaphechinus mirabilis and Strongylocentrotus nudus) were collected at Mutsu Bay (Aomori, Japan) during the breeding seasons and cultured in aquaria at 16°C. Eggs and sperms of the sea urchins were obtained by conventional methods using artificial sea water (ASW: 460.3 mM NaCl, 10.1 mM KCl, 9.2 mM CaCl₂, 35.9 mM MgCl₂, 17.5 mM MgSO₄, 0.05% NaHCO₃, pH 8.2 by NaOH). Briefly, gamete shedding was induced by intracoelomic injection of either 1 mM acetylcholine dissolved in ASW or 0.6 M KCl. Eggs were collected and kept in ASW. Semen was pipetted dry sperm from the gonopores of males and stored undiluted at 4°C until use.

Cell Culture and Bioassay

The effects of imazalil were determined on the stage of first cleavage and the 24 h-embryo (gastrula). Fertilized eggs were transferred to 24-well microplates (Iwaki, Tokyo, Japan) at 5 min after fertilization and cultured at 23°C. Each well was filled with 1 ml of ASW containing imazalil in various concentrations (0–50 ppm). The cells from S. mirabilis were fixed with 10% formalin at 45 min and 24 hr after fertilization and observed under a microscope (Nikon Eclipse TS100). The eggs from S. nudus were fixed and observed after 60 min and 24 hr of incubation with imazalil.

For obtaining eggs without fertilization membranes (denuded eggs), fertilized eggs were treated with 1% (v/v) thioglycolic acid in ASW adjusted to pH 9.5 at 3 min after fertilization. Eggs were then washed and developed in Ca²⁺-free ASW (473 mM NaCl, 10.1 mM KCl, 35.9 mM MgCl₂, 17.5 mM MgSO₄, 0.05% NaHCO₃, pH 8.2 by NaOH) containing imazalil in various concentrations (0–50 ppm).

The IC₅₀ value was defined as the concentrations of imazalil required for a 50% inhibition of first cleavage or gastrula-formation and used as a parameter for the toxicity of imazalil to the development of sea urchin eggs.

Results

Fertilized eggs of sea urchins, S. mirabilis and S. nudus were placed in ASW containing imazalil in various concentrations (0–50 ppm) and the effects on the first cleavage were examined. Figure 1 shows the ratio (%) of cleaved eggs of S. mirabilis estimated at 45 min after fertilization. At 45 min, more than 90% eggs had finished the first cleavage in the absence of imazalil (0 ppm). In contrast, the acute toxicity was observed in the presence of more than 7 ppm of imazalil (Fig. 1). At the low imazalil concentrations (1–5 ppm), cleaved eggs (2 cells) were observed together with abnormal eggs. However, in the presence of imazalil more than 20 ppm, cleaved eggs were hardly observed (Fig. 2). The IC₅₀ value for the first cell division with imazalil was estimated to be 3.9 ppm (Fig. 1).

Since the eggs of S. nudus finish the first cleavage at about 60 min after fertilization, the effects of imazalil on the first cleavage of the eggs of S. nudus were examined at 60 min after fertilization (Fig. 3). The result showed that the effect of imazalil in S. nudus was slightly sensitive than that obtained in S. mirabilis. Namely, the acute
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Fig. 2. Effect of imazalil on the first cleavage of the eggs of sea urchin, *S. mirabilis*. The fertilized eggs were incubated in the absence (a) or presence of 1 (b), 2 (c), 5 (d), 7 (e), 10 (f), 20 (g), 50 (h) ppm of imazalil for 45 min and observed by a phase-contrast microscope. Scale bar: 100 µm.

Fig. 3. Effect of imazalil on the first cleavage of the eggs of sea urchin, *S. nudus*. The fertilized eggs were incubated with or without imazalil for 60 min. Percentage of 2 cell embryos were examined. Dotted lines show the IC50 values of imazalil. The results presented are mean values of 6 different batches. Error bars represent standard deviations.

Toxicity on the eggs of *S. nudus* was observed in the presence of more than 5 ppm of imazalil, and the IC50 value for the first cell division with imazalil was estimated to be 1.7 ppm.

To examine the effect of fertilization membrane, imazalil was also added to denuded eggs of *S. mirabilis* (Fig. 4). The result was coincidental with the data obtained in Fig. 1. The IC50 value for the first cell division of denuded eggs was estimated to be 3.3 ppm.

Next, the inhibitory effect of imazalil on gastrula formation was estimated to the two species of the sea urchin. Figure 5 shows the data of *S. nudus* at 24 h after fertilization. The gastrula formation was also inhibited in the presence of more than 5 ppm of imazalil, the same as the inhibition of first cleavage. The IC50 value for the gastrula formation of *S. nudus* and *S. mirabilis* was estimated to be 3.1 ppm (Fig. 5) and 0.8 ppm (data not shown), respectively. As shown in Figure 6, phase microscopic observation revealed that
Fig. 4. Effect of imazalil on first cleavage of the denuded eggs of sea urchin, *S. mirabilis*. The denuded eggs were incubated with or without imazalil for 45 min. Percentage of 2 cell embryos was examined. Dotted lines show the IC₅₀ values of imazalil. The results presented are mean values of 4 different batches. Error bars represent standard deviations.

Fig. 5. Percentage of sea urchin *S. nudus* normal gastrula obtained after 24 h exposure of fertilized eggs to different concentrations of imazalil. Dotted lines show the IC₅₀ values of imazalil. The results presented are mean values of 24 different batches. Error bars represent standard deviations.

Fig. 6. Effect of imazalil on *S. mirabilis* development. The eggs were incubated in the absence (a) or presence of 1 (b), 2 (c), 7 (d), 10 (e), 20 (f) ppm of imazalil for 24 h and observed by a phase-contrast microscope. Scale bar: 100 µm.
gastrulation in *S. mirabilis* was markedly retarded in the presence of 1 ppm imazalil and the embryos developed abnormally at higher concentration of imazalil (2 to 20 ppm).

**Discussion**

In the present study, we found that imazalil has the inhibitory effects on the early development of fertilized eggs of sea urchins. The IC$_{50}$ values for the first cell division and the 24 h-embryo (gastrula) of *S. mirabilis* with imazalil were 3.9 ppm and 0.8 ppm, respectively. The effects of imazalil were further examined using another kind of sea urchin, *S. nudus*. The IC$_{50}$ for the first cell division and the 24h-embryo (gastrula) for *S. nudus* was 1.7 ppm and 3.1 ppm, respectively.

Based on the lowest observed effect concentrations (LOEC) from the previous study, the organometallic antifoulant tributyltin (TBT) was very toxic to the sea urchin, *Paracentrotus lividus* (0.3 to 16.1 µg/l; 1 µg/l = 10$^{-3}$ ppm) and *Anthocidaris crassispina* (0.1 µg/l), followed in decreasing order of toxicity by the alternative antifouling compound Sea-Nine to *P. lividus* (28 to 36 µg/l)$^{13}$ and *A. crassispina* (10 fg/l; 10$^{-9}$-fold lower concentration than that to *P. lividus*)$^{12}$), the herbicide diuron to *A. crassispina* (1000 µg/l) and the surfactant sodium dodecyl sulfate (SDS) to *P. lividus*, *Lytechinus variegates*, and *Echinometra lucunter* (3000 to 4157 µg/l) and the insecticides chlorpyrifos to *Strongylocentrotus droebachiensis* and *S. purpuratus* (5400 µg/l) (reviewed by Bellas, J., et al.$^{14}$). Despite the methodological variability among experiments, this order of toxicity except for antifouling compounds is consistent with present toxicity data of imazalil.

The cytotoxic studies using mouse fibroblast cells$^{15}$ and marine chordates$^{16}$ revealed that imazalil was found to be toxic at the concentration of 14 ppm, and 7 ppm, respectively. In 2000, the Joint FAO/WHO (Food and Agriculture Organization / World Health Organization) Meeting on Pesticide Residues (JMPR) reported that, at a dose range of 5–20 ppm (mg/kg body weight), imazalil was fetotoxic in rats, mice, and rabbits$^{17,18}$. In our preliminary experiment, the gastrula formation of starfish, *Asterina pectinifera*, was also inhibited at 7 ppm of imazalil. These results indicate that sea urchin is more sensitive to imazalil than higher vertebrates, such as mammals, or starfish. In contrast, our previous study using green paramecia, *Paramecium bursaria*, revealed that the IC$_{50}$ values of proliferation for 1-day and 5-day are 0.3 ppm and 0.07 ppm, respectively$^{19}$, suggesting that the fresh water protist is highly sensitive to imazalil. Further information is required for the understanding of the accumulation effect of imazalil to higher vertebrates or marine invertebrates.

There have been many studies reporting pesticide or fungicide residue levels in fruits and vegetables. In oranges and tangerines from Valencia (Spain), imazalil was detected in the concentration range of 0.02–1.2 ppm (mg/kg)$^{20}$. In grapefruit imported from South Africa and the USA, lemon imported from Chile and the USA and orange imported from the USA, the imazalil residue was also reported as 0.1–2.2 ppm, 0.3–3.5 ppm, and 1.1–1.7 ppm, respectively$^{21}$. In these fruit samples, the concentration of imazalil residues did not exceed the maximum residue limit in FAO/WHO$^{7}$. However, our studies revealed that 0.8–3.9 ppm imazalil showed toxic effects on the early development of sea urchins. Further, Pennati et al. reported that imazalil, from 1 to 10 µM (1 µM = 0.297 ppm) doses, induced malformations in more than 70% of larvae of the ascidian *Phallusia mammilata*, when applied to two-cell stage embryos$^{16}$. These results suggest that the contamination levels of imazalil residues in agricultural products show serious problems for marine invertebrates including sea urchins, even if they are less than the maximum residue limit.

It has been elucidated that imazalil belongs to the demethylation inhibitor group of fungicides, which inhibit the cytochrome P-450 dependent enzyme$^{22}$. Earlier experiments dealing with nitrosamines, which are carcinogenic, seem to indicate that the sea urchin embryo does not possess active microsomal enzymes, or at least not until the gastrula stage$^{23}$. Results obtained in Fig. 1 to 4 revealed that, in the early developmental stages, while toxicant transformation by the cytochrome P450 has not been observed, imazalil can affect the first cell division of sea urchin embryo. Therefore, it would appear that a direct
effect of imazalil to sea urchin development should be taken into consideration.

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References


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