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Acute toxicity and genotoxicity of two novel pesticides on amphibian, *Rana N. Hallowell*

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Abstract

Imidacloprid [1-(6-chloro-3-pyridylmethyl)-*N*-nitro-imidazolidin-2-ylideneamine] and RH-5849 [2′-benzoyl-l′-tert-butylbenzoylhydrazinel] are two pesticides used in China since 1992. In the present study we conducted acute toxicity test, micronucleus (MN) test and comet assay of the two pesticides on amphibian, *Rana N. Hallowell*, a sensitive organism suitable for acting as the bio-indicator of aquatic and agricultural ecosystems. The values of LC_{50}-48 h of imidacloprid were found to be 165 mg l^{-1} for tadpoles of *Rana limnocharis* and 219 mg l^{-1} for tadpoles of *Rana N. Hallowell*. On the other hand, RH-5849 showed no acute toxicity to tadpoles during the 96 h exposure even it was saturated in the test solutions. There were significant differences in the MN frequencies between the negative controls and the treated groups at the dose of 8 mg l^{-1} for imidacloprid ($p < 0.05$) and 40 mg l^{-1} for RH-5849 ($p < 0.01$). Comet assay found significant differences ($p < 0.01$) in the distributions of DNA damage grades between the negative controls and groups treated in vitro with 0.05, 0.1, 0.2 and 0.5 mg l^{-1} of imidacloprid and 5, 25, 50 and 100 mg l^{-1} of RH-5849, respectively. DNA damage scores increased with the exposure levels of the two pesticides and dose–effect relationships were observed for both imidacloprid ($r^2 = 0.92$) and RH-5849 ($r^2 = 0.98$). The MN test and comet assay revealed potential adverse effects of the two pesticides on DNA in the erythrocytes of amphibians in aquatic and agricultural ecosystems.

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Keywords: Micronucleus; Comet assay; *Rana N. Hallowell*; Imidacloprid; RH-5849; Genotoxicity

1. Introduction

Toxicity, in particular genotoxicity, of pesticides on non-target organisms and their influences on ecosystems are of worldwide concern (Pimentel et al., 1998). Imidacloprid and RH-5849 (Fig. 1) are two pesticides used in China since 1992. Imidacloprid, as an agonist at the nicotinic acetylcholine receptor, is highly effective against many sucking insects including ricehoppers, 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). RH-5849, a nonsteroidal ecdysone agonist, which acts similar to 20-hydroxyecdysone by binding to the ecdysone receptor, has been found to be very effective against lepidopteran pests such as *Manduca sexta*, *Plodia interpunctella*, *Choritstoneura fumiferana*, *Carpocapsa pomonella*, *Spodoptera frugiperda*, *Heliothis zea*, *S. exigua*,

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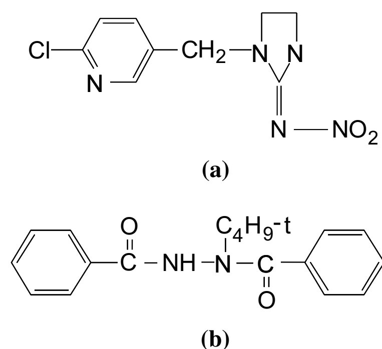

Fig. 1. The molecular structures of the two pesticides: (a) imidacloprid and (b) RH-5849.

S. littoralis and *S. exempta* in vegetables, cotton and cereals (Wing, 1988; Sundaram et al., 1998). As the use of the pesticides has become increasingly widespread in China, additional studies are needed to evaluate the potential toxic risks of the pesticides to non-target organisms. Their toxicity on earthworms, as well as their physical–chemical behavior in the soil environment, has been recently studied (Cox et al., 1996; Luo et al., 1999; Feng et al., 2000a; Zang et al., 2000). Their effects on non-target organisms in the aquatic and agricultural ecosystems, however, have not been fully understood.

Amphibians are important organisms in the aquatic and agricultural ecosystems; they are among the most important natural enemies of many agricultural pests. Because of their sensitivity to changes of their habitat and that their larvae live in the aquatic environment, amphibians have been regarded as bio-indicators of aquatic and agricultural ecosystems (Schuytema and Nebeker, 1999; Pollet and Bendell-Young, 2000; Marco et al., 2001), and were broadly used as typical test animals in evaluating the effects of chemicals on the aquatic and agricultural ecosystems (Cooke, 1973, 1977; Sundaram, 1995). Two kinds of amphibians, *Rana N. Hallowell* and *Rana limnocharis*, which are widely distributed in Southeast Asia, have been used as test organisms for the evaluation of eco-toxicity (Chen and Xia, 1993; Feng et al., 2000b; Sumida et al., 2002).

The micronucleus (MN) test and the single cell gel electrophoresis (or comet assay) are two most extensively used methods in the detection of genotoxicity of chemicals in the environment (Kassie et al., 2000). Compared to other assays, they are sensitive, rapid and easy to handle (Tucker and Preston, 1996; Kassie et al., 2000), and many comprehensive reviews are available concerning their methodologies and applications (Heddle et al., 1983; McKelvey-Martin et al., 1993; Fairbairn et al., 1995; Tice, 1995; Mitchelmore and Chipman, 1998; Rojas et al., 1999). A combination of MN test and comet assay enables comparison of the relative sensitivity of the two test systems, and may also give a clue about the fraction of DNA damage detected in the comet assay that will lead to fixed mutations (Goethem et al., 1997; Maluf and Erktmann, 2000).

The present study was initiated to combine acute toxicity test, MN test and comet assay to assess the acute toxicity and genotoxicity of imidacloprid and RH-5849 on the amphibians from different endpoints. The objective was to provide valuable information for a more comprehensive understanding of their effects on and potential risks to the non-target organisms in aquatic and agricultural ecosystems.

2. Materials and methods

2.1. Test organisms and chemicals

Tadpoles of two amphibians, *Rana N. Hallowell* and *Rana limnocharis*, and frogs of *Rana N. Hallowell* were selected as the test organisms. All of them were captured from one pool in the Zhijing Mountain area, about 10 km from Nanjing city in China. They were acclimated to the laboratory environment for seven days before the tests. The tadpoles of *Rana limnocharis* were about one month old, with body lengths of 33.2 ± 1.2 mm and body weights of 250 ± 20 mg; the tadpoles of *Rana N. Hallowell* were one and half months old, with body lengths of 37.5 ± 1.1 mm and body weights of 461 ± 60 mg. The frogs of *Rana N. Hallowell* with body weights of about 100 g were chosen for the tests.

Imidacloprid and RH-5849 were obtained from the Jiangsu Institute of Pesticides (Nanjing, China) and were over 95% pure.

2.2. Acute toxicity tests

Ten tadpoles were put in a 15 l glass tank containing 10 l of pesticide solution (20 ± 1 °C, dissolved oxygen (DO) > 8.5 mg l^{-1}). The pesticide solution in the tank was replaced every 24 h with freshly prepared solution of the same pesticide concentration. The tadpoles were visually examined once every 3 h, and the dead ones were removed in time. Tadpoles were considered dead when they did not respond to gentle touching. Dead tadpoles were counted after 24, 48, 72 and 96 h. The results were analyzed with the computer program "TSK" (Trimmed Spearman–Karber Program, Version 1.5), which is used by the Ecological Monitoring Research Division, Environmental Monitoring Systems Laboratory, United States Environmental Protection Agency (TSK, 1991) to calculate LC_{50} and its 95% confidence interval.

For acute toxicity test with *Rana limnocharis*, seven concentration levels (16.7, 30.0, 54.0, 97.2, 174.9, 314.9 and 566.8 mg l^{-1} in distilled water) of imidacloprid and six concentration levels (30, 57, 108.3, 205.8, 390.9 and

742.8 $mg\,l^{-1}$ in 0.5% Tween-80 solution) of RH-5849 were prepared. For acute toxicity test with *Rana N. Hallowell*, seven concentration levels (30, 45, 67.5, 101.2, 151.8, 227.8 and 341.7 $mg\,l^{-1}$ in distilled water) of imidacloprid and five concentration levels (30, 60, 120, 240 and 480 $mg\,l^{-1}$ in 0.5% Tween-80 solution) of RH-5849 were prepared. For the controls, 10 tadpoles were put in 10 l of 0.64% NaCl solution (for the osmotic equibalance) for imidacloprid and 0.5% Tween-80 solution for RH-5849, respectively.

2.3. Micronucleus test

The detailed MN test method was described by Feng et al. (2000b) and Chen and Xia (1993). Tadpoles of *Rana N. Hallowell* were exposed to different levels of the two pesticides in 15 l glass tanks containing 10 l solution (20 ± 1 °C; DO > 8.5 $mg\,l^{-1}$) for seven days. The solutions were replaced every 24 h with freshly prepared solutions at the same concentration levels. According to the results of the acute tests, three concentration levels of each pesticide were used (in $mg\,l^{-1}$): 2, 8, 32 for imidacloprid and 2.5, 10, 40 for RH-5849. Ten tadpoles were used for each group. 0.64% NaCl solution was used as the negative control and cyclophosphamide solution (30 $\mu g\,l^{-1}$) as the positive. Upon seven-day exposure, blood was taken from each tadpole by cardiac puncture and one smear was prepared for each animal. Fixed in methanol and stained with 5% of Giemsa in Sörensen buffer (pH 6.98), the smears were screened under a microscope (Model XSP-4C, Shanghai Shangguang Microscope Ltd., Shanghai, China; oil immersion lens, ×1000). The erythrocytes with one or more micronuclei were counted for a total of at least 2000 erythrocytes per tadpole and seven random animals were screened in each group. MN frequency (MN‰) was calculated as follows:

$$MN‰ = \frac{\text{Number of cells containing micronucleus}}{\text{Total number of cells counted}} \times 1000. \quad (1)$$

Statistical differences between control and exposed groups were determined with Dunnett's *t*-test.

2.4. Comet assay

The procedure used was basically the same as that described by Singh et al. (1988). Modifications due to the uniqueness of the biological material studied and due to the equipment available were relatively minor (Ralph et al., 1996; Zhong et al., 2001). The modified procedure was described in detail by Zhong et al. (2001).

Blood samples were collected from *Rana N. Hallowell* frogs, and erythrocytes were chosen because they are nucleated in amphibians. The survival rate of erythrocytes after the isolation was higher than 95% as examined by trypan blue exclusion test. The exposure concentrations (in $mg\,l^{-1}$) were 0.05, 0.1, 0.2 and 0.5 for imidacloprid and 5, 25, 50 and 100 for RH-5849. 0.5% Tween-80 solution was used as the negative control and Mitomycin C (100 $\mu g\,l^{-1}$) as the positive. Each level group was prepared in triplication. Upon 1-h exposure at 20 °C in a 5% CO_2 atmosphere, the cells were collected by centrifugation (10 min, 3000 rpm, at 4 °C) and washed twice with Hank's balanced salt solution at 4 °C to minimize possible damage repair. The survival rate of erythrocytes after the exposure was higher 90% as examined by trypan blue exclusion test. Samples were immediately placed on ice for comet assay. The essential steps of comet assay involve at least 1-h lysis of cells by detergent at high salt concentration and electrophoresis under alkaline conditions (300 mM NaOH and 1 mM Na_2EDTA, pH 13; 25 min unwinding; 50 min electrophoresis at 150 mA and 25 V). Nucleoids were stained with ethidium bromide and examined with a "TMD-EF" fluorescent microscope (Nikon, Japan). One hundred cells per slide were scored visually and given scores 0 (undamaged), 1, 2, 3 or 4 (maximally damaged) according to tail intensity (size and shape) (Maluf and Erktmann, 2000; Zang et al., 2000; Zhong et al., 2001; Kamer and Rinkevich, 2002). Thus, the total score for 100 comets ranges from 0 (all undamaged) to 400 (all maximally damage). The percentage of damaged cells was calculated and the results analyzed with the χ^2 test. The arbitrary unit (AU) was used to express the extent of DNA damage and was calculated as follows:

$$AU = \sum_{i=0}^{4} n_i i, \quad (2)$$

where n_i is number of cells in damage degree i (0, 1, 2, 3, 4). Statistical comparisons of DNA damage grade between the control and exposed groups were performed by Krusk–Wallis test.

3. Results and discussion

3.1. Acute toxicity

LC_{50} values of imidacloprid on the tadpoles at various exposure times and the 95% confidence intervals calculated by TSK software are shown in Tables 1 and 2. No death occurred after 48 h exposure for *Rana limnocharis* at levels lower than 30 $mg\,l^{-1}$ and for *Rana N. Hallowell* at levels less than 101.2 $mg\,l^{-1}$. However, tadpoles were all dead after 24 h exposure at the imidacloprid levels of 566.8 $mg\,l^{-1}$ for *Rana limnocharis* and 341.7 $mg\,l^{-1}$ for *Rana N. Hallowell*. Calculated LC_{50}-48 h values of imidacloprid were 165 $mg\,l^{-1}$ for tadpoles of

Table 1
The acute toxicity of imidacloprid to the tadpoles of *Rana limnocharis*

Concentration ($mg\,l^{-1}$)	Number of dead tadpoles (*Rana limnocharis*)			
	24 h	48 h	72 h	96 h
16.7	0	0	0	0
30.0	0	0	0	1
54.0	0	1	1	3
97.2	1	3	5	6
174.9	3	4	7	8
314.9	6	8	9	10
566.8	10	10	10	10
0.64% NaCl solution	0	0	0	0
LC_{50} ($mg\,l^{-1}$)	235	165	116	82
95% confidence limit	205–269	141–193	100–135	70–96

Table 2
The acute toxicity of imidacloprid to the tadpoles of *Rana N. Hallowell*

Concentration ($mg\,l^{-1}$)	Number of dead tadpoles of *Rana nigronaculata Hallowell*			
	24 h	48 h	72 h	96 h
30.0	0	0	0	0
45.0	0	0	1	1
67.5	0	0	1	1
101.2	0	0	1	3
151.8	0	2	5	6
227.8	1	4	6	8
341.7	10	10	10	10
0.64% NaCl solution	0	0	0	0
LC_{50} ($mg\,l^{-1}$)	268	219	177	129
95% confident limit	226–318	153–313	160–200	115–145

Rana limnocharis and 219 $mg\,l^{-1}$ for tadpoles of *Rana N. Hallowell*, respectively. As for RH-5849, since its solubility is less than 400 $mg\,l^{-1}$ in distilled water, a higher dosage than 400 $mg\,l^{-1}$ might not increase the concentration levels in the solutions. No tadpoles were dead after the 96-h exposure even at the highest dosages of RH-5849 (742.8 and 480 $mg\,l^{-1}$ respectively for the two kinds of tadpoles). Imidacloprid and RH-5849 are usually prepared for spraying in the crop fields in solutions of about 100–150 and 200–350 $mg\,l^{-1}$, respectively. The actual levels of imidacloprid and RH-5849 that tadpoles are exposed to in the agricultural ecosystems may be much lower than the prepared concentrations. Our study indicated that the acute toxicity of the pesticides on the two kinds of tadpoles was very low from the end-point of lethality. In comparison, both Luo et al. (1999) and Zang et al. (2000) also reported that their acute toxicity to earthworms was very low. The acute toxicity test, however, is only the first step to evaluate the potential risk of the pesticides to tadpoles; analyzing the sublethal effects of the pesticides on tadpoles will help us better understand their effects in the aquatic and agricultural environment.

3.2. Micronucleus test

Jaylet et al. (1986) first adapted the MN test to amphibians. Many MN tests on amphibians have been proven to be suitable for evaluating mutagens and genotoxic agents (Van Hummelen et al., 1989; Chen and Xia, 1993; Zoll-Moreux and Ferrier, 1999). Chen and Xia (1993) used MN test in erythrocytes of tadpoles, *Rana N. Hallowell*, to evaluate mutagens and genotoxic agents in aquatic environment and pointed out that for a successful test the background MN frequency in test organisms should be low and stable, and that a seven-day exposure period would be better for the MN test. In the present study a seven-day exposure period was also adopted. The background MN frequency in the present study was less than 2‰ (Table 3), just in the same level as the study by Chen and Xia (1993).

MN frequencies by the two pesticides in erythrocytes of the tadpoles of *Rana N. Hallowell* are shown in Table 3. There were no significant differences between the negative control and the groups treated with 2 $mg\,l^{-1}$ of imidacloprid and 2.5 $mg\,l^{-1}$ of RH-5849, respectively. But there were significant differences between the negative control and groups treated with 8 $mg\,l^{-1}$ ($p < 0.05$) and 32 $mg\,l^{-1}$ ($p < 0.01$) of imidacloprid and 40 $mg\,l^{-1}$ ($p < 0.01$) of RH-5849, respectively. MN test revealed ability of imidacloprid and RH-5849 to induce numerical or structural chromosomal damage at levels with no acute toxicity being observed.

3.3. Comet assay

Singh and Stephen (1997) suggested that counting 50 cells in one slide was sufficient to detect signals of chemically induced damages in comet assay. We counted 100 cells in each slide to ensure statistical power. The number of cells in each damage grade, the percentage of damaged cells and the DNA damage score of each group are shown in Table 4. In the same group erythrocytes were distributed in different DNA damage degrees, indicating that comet assay was able to detect the intercellular differences in DNA damages of heterogeneous mixture of cells (Ostling and Johnson, 1987). The distributions of the damage grades in all of the pesticide-treated groups were significantly different from the control ($p < 0.01$). DNA damage scores expressed as AU increased as the exposure concentrations of the two

Table 3
Micronucleus frequencies in erythrocytes of the tadpoles (*Rana N. Hallowell*) with different levels of imidacloprid and RH-5849

Pesticides	Dosage ($mg\,l^{-1}$)	Number of animals observed	Number of cells observed	Micronucleus frequencies (‰)
Imidacloprid	2	7	14149	1.7 ± 0.5
	8	7	14163	2.4 ± 0.3*
	32	7	14112	3.8 ± 0.8**
RH-5849	2.5	7	14079	1.6 ± 0.6
	10	7	14128	1.7 ± 0.5
	40	7	14136	3.6 ± 1.1**
Negative control	0.64% NaCl solution	7	14380	1.5 ± 0.6
Positive control	CP (30 $\mu g\,l^{-1}$)	7	14151	8.3 ± 1.5**

*$p < 0.05$, **$p < 0.01$ (significant differences between negative control and exposed groups by Dunnett's *t*-test).

Table 4
Number of frog (*Rana N. Hallowell*) erythrocytes in each damage degree and DNA damage scores in the control and treated groups

Chemicals	Exposure dosage ($mg\,l^{-1}$)	Number of cells in each damage grade (mean ± SD)					Damage percentage (%)	AU[a]
		0	1	2	3	4		
Imidacloprid	0.05	76 ± 12	24 ± 9	0	0	0	24*	24
	0.1	23 ± 6	67 ± 13	10 ± 3	0	0	77*	86
	0.2	13 ± 6	61 ± 12	22 ± 5	4 ± 1	0	87*	117
	0.5	8 ± 1	18 ± 5	41 ± 13	31 ± 9	2 ± 1	92*	202
RH-5948	5	76 ± 15	24 ± 7	0	0	0	24*	25
	25	33 ± 5	56 ± 15	11 ± 3	0	0	67*	79
	50	15 ± 3	38 ± 4	36 ± 8	11 ± 4	0	85*	144
	100	5 ± 1	10 ± 1	41 ± 11	41 ± 9	2 ± 1	95*	224
Tween-80	0.5%	96 ± 9	4 ± 1	0	0	0	4	4
Mitomycin C	10 $\mu g\,l^{-1}$	0	10 ± 2	39 ± 4	42 ± 7	10 ± 4	100*	251

*$p < 0.01$ (significant difference compared with control by χ^2 test).
[a] DNA damage score expressed as arbitrary units.

pesticides increased, and the dose–effect relationship (refers to relationship between concentration and AU) was observed on erythrocytes of *Rana N. Hallowell* frogs for both imidacloprid (coefficient $r^2 = 0.92$) and RH-5849 (coefficient $r^2 = 0.98$), as reported by Zang et al. (2000) on the coelomocytes of earthworm, *Eisenia fetida*.

The comet assay in the present study found that DNA damage occurred in erythrocytes of frogs in vitro at levels from 0.05 to 0.5 $mg\,l^{-1}$ for imidacloprid, and from 5 to 100 $mg\,l^{-1}$ for RH-5849 (Table 4). Therefore, the two pesticides, if inefficiently diluted or degraded after applied in the field, might reach levels that pose genotoxicity to frogs. On the other hand, since frogs are important natural enemies of many agricultural pests, they might expose to the residual pesticides in pests via food chains, as imidacloprid residues can be present in vegetables, crops and fruits, and also in pests (e.g. Fernandez-Alba et al., 1996; Zhang and Shi, 1997; Dai et al., 2002). So the potential adverse effects of imidacloprid on frogs, direct or indirect, cannot be neglected considering its genotoxicity. For RH-5849, few data are available about its residues in soil, water, food products and pests. It is still hard to evaluate its genotoxicity to frogs, and more investigations are needed considering the exposure of frogs to RH-5849.

Mitchelmore and Chipman (1998) recommended that DNA strand breaks, particularly as measured by the comet assay, act as a biomarker of genotoxicity in fish and other aquatic species. But they also emphasized that this approach should be combined with the use of other biomarker. Due to the different conditions between the MN test and comet assay in the present study, it is difficult to make a comparison between the results of comet assay and MN test. But as for the lowest observable adverse effect level (LOAELs) only, the comet assay is more sensitive than the MN test. The LOAELs were 0.05 $mg\,l^{-1}$ for imidacloprid and 5 $mg\,l^{-1}$ for RH-5849 in the comet assay (Table 4), and the LOAELs in the MN tests were 8 $mg\,l^{-1}$ for imidacloprid and 40 $mg\,l^{-1}$ for RH-5849 (Table 3). This difference was partly due to

variations in the type of DNA alterations they detect. MN test detects chromosome breakage after repair while the comet assay systems detect DNA single/double strand breaks and alkali-labile sites if repair is not allowed to occur (Kassie et al., 2000). Also target cells are different. The target cells in the MN test were the erythrocytes of tadpoles in vivo, while in the comet assay were freshly isolated erythrocytes of frogs in vitro. Nonetheless, both MN test and comet assay confirmed the DNA-damaging property of the two pesticides from different end-points.

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