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Assessing the genotoxicity of imidacloprid and RH-5849 in human peripheral blood lymphocytes in vitro with comet assay and cytogenetic tests

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Abstract

A combined approach employing comet assay and micronucleus (MN) and sister chromatid exchanges (SCE) tests was utilized to assess the genotoxicity of two pesticides, imidacloprid [1-(6-chloro-3-pyridylmethyl)-*N*-nitro-imidazolidin-2-ylideneamine] and RH-5849 [2′-benzoyl-1′-tert-butylbenzoylhydrazine], on human peripheral blood lymphocytes in vitro. No significant difference in the frequencies of MN and SCE from the negative groups ($P>0.05$) was observed at low dose levels (i.e., 0.05 mg/L for imidacloprid and 5 mg/L for RH-5849). As the concentrations of imidacloprid and RH-5849 were increased to 0.1 and 25 mg/L, respectively, significant effects to the frequencies of MN and SCE ($P<0.05$) were achieved relative to those of the negative controls. MN and SCE frequencies increased similarly in a dose-related manner with both pesticides. With the comet assay, however, the distribution of DNA damage grades in all the pesticide-treated groups was significantly different from those in the control ($P<0.01$). DNA damage scores increased with the exposure levels of both pesticides, and linear dose–effect relationships were observed for both imidacloprid ($r^2=0.98$) and RH-5849 ($r^2=0.92$). The cytogenetic techniques and comet assay revealed potential adverse effects of both imidacloprid and RH-5849 in human peripheral blood lymphocytes in vitro. Combination of the comet assay and cytogenetic tests appears commendable to assess the potential risks of human exposure to the pesticides.
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Keywords: Comet assay; Micronucleus; Sister chromatid exchanges; Lymphocyte; Imidacloprid; RH-5849

1. Introduction

Biomonitoring of human beings exposed to potential mutagens/carcinogens is a useful method to detect early stage genetic diseases or cancers. It also allows the identification of risk factors when effective control measures may still be implemented. Human biomonitoring can be performed using different genetic markers (Bradley et al., 1979; Heddle et al., 1983; Ostling and Johanson, 1984; Evans, 1988; Tice et al., 1990; Tucker and Preston, 1996; Kassie et al., 2000; Majer et al., 2001). Cytogenetic markers, such as micronucleus (MN) and sister chromatid exchanges (SCE), are among the most extensively used indicators of early biological effects associated with DNA-damaging agents. Micronuclei are acentric fragments or complete chromosomes that fail to attach to the mitotic spindle during cytokinesis and are excluded from the nuclei. Different mechanisms may be involved in the formation of micronuclei, including chromosome breakage (clastogenesis) and spindle disruption (aneugenesis) (Heddle et al., 1983; Evans, 1988; Majer et al., 2001). SCE analysis on peripheral blood lymphocytes, one of the

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most popular methods in toxicology and human biomonitoring, is a well-established technique for evaluating human exposure to toxic agents. The mean value of SCE per cell, based on an adequate number of metaphases scored per subject, has been the recommended index to measure the extent of cytogenetic damage (Bonassi et al., 1999). MN and SCE tests are superior to other cytogenetic assays in several aspects, including quickness and ease of analyses (Bradley et al., 1979; Heddle et al., 1983; Evans, 1988; Tucker and Preston, 1996; Majer et al., 2001).

In the past few years, the comet assay, first introduced by Ostling and Johanson (1984) and later modified independently by Singh et al. (1988) and Olive (1989), has been utilized as an alternative technique for monitoring human exposure to genotoxic hazards (Maluf and Erdtmann, 2000; Garaj-Vrhovac and Zeljezic, 2000). It is a relatively simple, inexpensive, rapid, and sensitive method for detecting DNA damage (strand breaks and alkali-labile sites) induced by a variety of genotoxic agents and intercellular differences of the damage with a small number of individual cells (Ostling and Johanson, 1984, 1987; Tice et al., 1990). It can also be used for in vitro and in vivo studies to evaluate DNA damage and repair. The comet assay is based on electrophoresis of cells embedded and lysed in agarose on a microscope slide. The underlying mechanism is via organization of DNA in large supercoiled structures that can stretch out during electrophoresis when relaxed by strand breaks, forming a tail-like configuration. Several reviews of the methodology and applications of the comet assay are available (Fairbairn et al., 1995; Anderson et al., 1998; Rojas et al., 1999; Kassie et al., 2000). In addition, the comet assay has been increasingly combined with cytogenetic techniques to assess the potential effects of mutagens/carcinogens (Betti et al., 1994; Hartmann et al., 1995, 1998a, b; Goethem et al., 1997; Vroz and Petras, 1997; Sram et al., 1998; Maluf and Erdtmann, 2000).

Genotoxic risks of human exposure to pesticides remain a worldwide concern. This study was initiated to combine MN and SCE tests and comet assay to assess the genotoxicity of two new pesticides, imidacloprid and RH-5849 (Fig. 1), in human peripheral blood lymphocytes in vitro from different endpoints. Imidacloprid and RH-5849 have been used in China since 1992 (Sun et al., 1995), but their genotoxicity in human beings has yet to be fully investigated. RH-5849 is an entomological growth-regulating agent (Wing, 1988), while imidacloprid directly affects the central nervous system (Nagata et al., 1997). The toxicity of these two pesticides in earthworms and amphibians, and their physical-chemical behavior in soils has been studied in detail (Cox et al., 1996; Luo et al., 1999; Zang et al., 2000; Feng et al., 2004). The objective of this study was to gain a comprehensive understanding of the effects and potential risks of imidacloprid and RH-5849 on human beings. The efficacy of the three test methods for pesticide genotoxicity assessment was also evaluated.

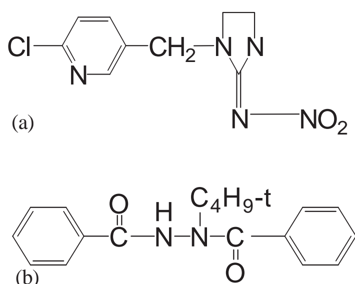

Fig. 1. The molecular structures of (a) imidacloprid and (b) RH-5849.

2. Materials and methods

2.1. Test samples and chemicals

Human peripheral blood samples, collected from healthy volunteers 23–30 years old with no smoking history, were provided by the Nanjing Central Blood Bank (Nanjing, Jiangsu, China). Imidacloprid and RH-5849 were obtained from the Jiangsu Institute of Pesticides (Nanjing, Jiangsu, China) and were of >95% purity. Cyclophosphamide (CP) was purchased from the 12th Pharmaceutical Factory of Shanghai (Shanghai, China). All other chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA). To prepare test solutions, imidacloprid was dissolved in distilled water while RH-5849 was dissolved in Tween 80 and then diluted with distilled water.

2.2. Lymphocyte cultures

Blood samples in heparinized tubes were transferred to the laboratory within a few minutes of collection. A 0.3-mL sample of whole heparinized blood was incubated in 4.7 mL RPMI 1640 medium (including 20% heat-inactivated fetal calf serum, 2% phytohemagglutinin (PHA), 100 IU/mL of penicillin, 100 μg of streptomycin, and 2 mM of L-glutamin) at 37 °C under 5% CO_2 atmosphere for the MN and SCE tests.

2.3. Micronucleus test

The procedure was described in detail previously (Kong et al., 1995, 1998) and only a brief description is presented here. After 24 h of PHA stimulation, imidacloprid and RH-5849 were added separately into 5-mL cultures to obtain final concentrations of 0.05, 0.1, and 0.5 mg/L for imidacloprid and 5, 25, and 100 mg/L for RH-5849. CP at 30 μg/L and 1% Tween 80 (final concentration of Tween 80 for RH-5849 tests) were used

as positive and negative controls, respectively. Three replicates were prepared for each dose group. All cultures continued to incubate for another 48 h. Upon completion of 72-h incubation, lymphocytes were harvested and stained for MN analysis. Three slides were prepared from each culture. For each slide, at least 1000 cells were observed with an optical microscope (Model XSP-4C; Shanghai Shangguang Microscope, Shanghai, China). The number of micronuclei was used to calculate MN‰:

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

Statistical differences between controlled and treated groups were determined with Dunnett's t test.

2.4. Sister chromatid exchanges test

SCE analysis was performed using the method of Perry and Wolff (1974) with minor modifications (Kong et al., 1995). After 24-h PHA stimulation, the pesticides and 5-bromodexyribouridine were added separately into 5-mL cultures to yield final concentrations of 0.05, 0.1, and 0.5 mg/L for imidacloprid, 5, 25, and 100 mg/L for RH-5849, and 10 μg/L for 5-bromodexyribouridine in all test solutions. CP of 30 μg/L was used as a positive control and 1% Tween 80 solution as a negative control. Each dose group was prepared in three replicates. All cultures continued to incubate for another 48 h at 37 °C in the dark, and metaphases were blocked during the last 4 h with colchicine (10^{-7} mg/L). Three slides were prepared from each culture. All slides were fixed and stained as described by Perry and Wolff (1974). For each slide, the frequency of SCE was determined in at least 50 well-differentiated second metaphase cells with an optical microscope (Model XSP-4C; Shanghai Shangguang Microscope). The frequency of SCE for each cell was counted and used to calculate the mean frequency of SCE among all the cells,

$$\text{Mean frequency} = \frac{\sum_{i=1}^{N} n_i}{N}, \quad (2)$$

where n_i is the frequency of SCE for cell i and N is the total number of cells. Statistical differences between controlled and treated groups were determined with Dunnett's t test.

2.5. Comet assay

Lymphocytes were separated by centrifugation over lymphocyte separation medium at 1000 rpm for 15 min (Singh and Stephens, 1997). After the plasma layer was removed and saved, the buffy coat was carefully removed and the cells were washed with RPMI 1640 (600 rpm for 15 min). The viability of lymphocytes after the isolation was higher than 96% as examined by trypan blue exclusion.

Lymphocytes obtained from about 100 μL of blood sample were suspended with 0.8 mL of medium RPMI 1640 in Eppendorf tubes. Solutions of imidacloprid, RH-5849, H_2O_2 (positive control), and Tween 80 (negative control) were added (100 μL each) to the lymphocytes suspension, and final concentrations were 0.05, 0.1, 0.2, and 0.5 mg/L for imidacloprid, 5, 25, 50, and 100 mg/L for RH-5849, 10 mM for H_2O_2, and 1% for Tween 80. The treatment was carried out at 37 °C under 5% CO_2 atmosphere in the dark. Three replicates were prepared for each dose group. After 1 h the cells were settled down with 10-min spinning (3000 rpm) and washed twice with phosphate-buffered saline at 4 °C to minimize possible damage repair. Samples were immediately placed on ice for comet assay. The viability of peripheral blood lymphocytes after the treatment was higher than 90% as examined by trypan blue exclusion.

DNA breaks and alkali-labile sites were analyzed using the method of Singh et al. (1988) with minor modifications (Zhong et al., 2001). Approximately 1500 lymphocytes mixed with 0.5% low-melting-point agarose in phosphate-buffered saline were sandwiched with 0.5% normal agarose on the slide. Once the top layer had solidified, the slide was gently immersed in a chilled lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Trizma, and 1% sarkosyl with addition of 10% dimethyl sulfoxide and 1% Triton X-100 prior to use; pH 10) at 4 °C for at least 1 h. The slide was then immersed into chilled fresh electrophoresis buffer (300 mM NaOH and 1 mM Na_2EDTA; pH 13) for 25 min at 0 °C. Electrophoresis was conducted at 0–2 °C for 50 min at 25 V and 100 mA. All the above steps were taken under red light or in the dark to prevent DNA damage. The slide was neutralized three times (5 min each) with a buffer solution (0.4 M Tris–HCl; pH 7.5) at 4 °C before being stained with ethidium bromide.

DNA analysis was performed immediately upon staining. For evaluation of DNA damage, 100 cells per slide (Singh and Stephens, 1997) were analyzed at 400 × magnification with a TMD-EF fluorescent microscope (Nikon, Japan). Cells were assessed visually and scores from 0 (undamaged) to 4 (maximally damaged) were assigned according to the tail intensity (size and shape) (Maluf and Erdtmann, 2000; Zang et al., 2000; Zhong et al., 2001). The percentage (%) of damaged cells was calculated and the results were analyzed with the χ^2 test (Zhong et al., 2001). An arbitrary unit (AU) used to express the extent of DNA damage is defined as

$$\text{AU} = \sum_{i=0}^{4} n_i \times i, \quad (3)$$

where n_i is number of cells with damage degree i (0, 1, 2, 3, or 4). Statistical comparison of DNA damage in the

controlled and exposed groups was performed using the Krusk–Wallis test (Zang et al., 2000; Zhong et al., 2001).

3. Results

3.1. Micronucleus test

The MN‰ values at 0.05 mg/L of imidacloprid and 5 mg/L of RH-5849 were not significantly different ($P>0.05$) from that of the negative control but were significantly different ($P<0.05$) from that of the negative control at the concentrations of 0.1 and 25 mg/L for imidacloprid and RH-5849, respectively (Fig. 2). Interestingly, the MN frequencies in human peripheral blood lymphocytes in vitro increased with increasing concentrations of both imidacloprid and RH-5849 in the same manner as those in erythrocytes of tadpoles, *Rana nigronaculata* Hallowell, in vivo (Feng et al., 2004). The present study also found that CP could directly increase the frequency of MN occurrence in human peripheral blood lymphocytes in vitro; however, other researchers have expressed different views (Vroz and Petras, 1997).

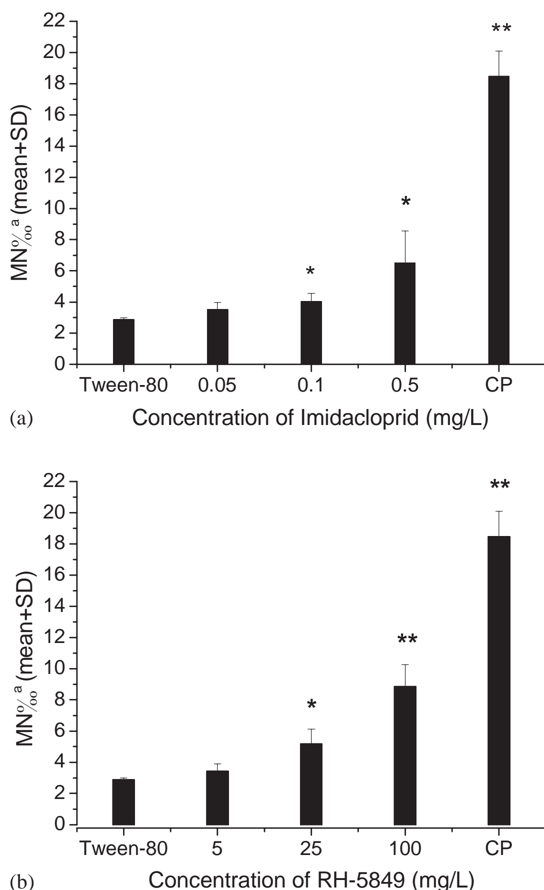

Fig. 2. Effects of imidacloprid (a) and RH-5849 (b) on the frequencies of micronucleus (MN) in human peripheral blood lymphocytes. [a]Per 1000 cell micronucleated lymphocytes; *$P<0.05$, **$P<0.01$ (significant differences between control and treated cultures by Dunnett's *t* test).

3.2. Sister chromatid exchanges test

At the lowest dose levels of 0.05 mg/L for imidacloprid and 5 mg/L for RH-5849, the SCE values (per cell) were not significantly different from that with the negative control (Table 1). As the concentrations of imidacloprid and RH-5849 were increased to 0.1 and 25 mg/L, respectively, the difference between the treated and the control cultures became significant ($P<0.05$). In addition, the frequencies of SCE occurrence increased with increasing dose levels in the same manner for both pesticides. The largest frequency of SCE occurrence was caused by CP (Table 1), indicating that proliferating human peripheral blood lymphocytes had the metabolic ability to activate CP. Similar results were also obtained by Kong et al. (1995).

3.3. Comet assay

Singh and Stephens (1997) suggested that counting 50 cells in a slide was sufficient to detect a significant level of DNA damage caused by chemicals in comet assay. In the present study, 100 cells in a slide were counted to enhance the power of statistical analysis. Table 2 lists the number of cells in each degree of DNA damage and DNA damage scores in control and treated groups. Even at the lowest dose levels significant differences between the negative control and the treated groups with regard to the percentage of damaged cells were observed ($P<0.01$). The percentage of damaged cells, degree of DNA damage, and DNA damage scores expressed in AU all increased with increasing dose levels of both pesticides. The dose–AU relationship of both imidacloprid ($r^2 = 0.98$) and RH-5849 ($r^2 = 0.94$) observed in the present study for human peripheral blood lymphocytes was also observed by Feng et al. (2004) for erythrocytes of *R. nigronaculata* Hallowell frogs and by Zang et al. (2000) for coelomocytes of eathworm, *Eisenia fetida*. In addition, lymphocytes were widely distributed among different degrees of DNA damage within the same group (Table 2), indicating that the comet assay was able to detect the intercellular differences of DNA damage in heterogeneous cell mixtures (Ostling and Johanson, 1984, 1987; Tice et al., 1990).

4. Discussion

Numerous studies employing the comet assay for comparative analyses of DNA strand breaks and other endpoints of genotoxicity have been conducted in the

Table 1
Effects of imicacloprid and RH-5849 on SCEs in human peripheral blood lymphocytes

Test materials	Concentrations (mg/L)	Number of observed metaphases	SCE/cell[a] (mean ± SD)
Imidacloprid	0.05	150	4.9 ± 1.2
	0.1	153	5.4 ± 1.4[b]
	0.5	156	6.6 ± 1.6[c]
RH-5849	5	156	4.3 ± 1.5
	25	152	6.2 ± 1.4[b]
	100	151	7.1 ± 1.6[c]
Tween 80	1%	150	4.2 ± 1.0
Cyclophosphamide	30 (μg/L)	158	27.3 ± 3.1[c]

[a]Sister chromatid exchanges per cell.
[b]$P<0.05$ (the difference between the treated and the control cultures by Dunnett's t test).
[c]$P<0.01$ (the difference between the treated and the control cultures by Dunnett's t test).

Table 2
Number of cells in each degree of DNA damage and the DNA damage scores (in arbitrary unit, AU) in control and treated groups

Test chemicals	Dose level (mg/L)	Number of cells in each damage degree (mean ± SD)					Damage (%)	AU
		0	1	2	3	4		
Imidacloprid	0.05	76.7 ± 8.5	10.3 ± 2.3	12.9 ± 3.5	0.0 ± 0.0	0.0 ± 0.0	23.3[a]	36.20
	0.1	40.9 ± 1.6	49.3 ± 0.85	9.7 ± 1.03	0.0 ± 0.0	0.0 ± 0.0	59.0[a]	68.78
	0.2	29.5 ± 7.4	45.6 ± 16.8	24.9 ± 13.4	0.0 ± 0.0	0.0 ± 0.0	70.5[a]	95.38
	0.5	10.3 ± 5.2	25.3 ± 8.3	35.1 ± 15.2	22.6 ± 10.7	6.7 ± 3.4	89.7[a]	190.12
RH-5849	5	50.0 ± 24.0	47.1 ± 22.7	2.8 ± 1.2	0.0 ± 0.0	0.0 ± 0.0	49.9[a]	52.73
	25	25.8 ± 12.2	70.1 ± 10.2	4.1 ± 4.1	0.0 ± 0.0	0.0 ± 0.0	74.2[a]	78.32
	50	19.9 ± 8.5	66.7 ± 8.7	12.8 ± 12.8	0.7 ± 0.5	0.0 ± 0.0	80.1[a]	94.19
	100	2.8 ± 1.3	26.6 ± 12.7	16.4 ± 16.4	45.1 ± 16.4	10.8 ± 7.0	97.2[a]	237.7
Blank		95.5 ± 13.2	4.5 ± 2.3	0 ± 0	0 ± 0	0 ± 0	4.5	4.5
Tween 80	1%	89.6 ± 8.3	10.4 ± 5.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	10.4	10.4
H_2O_2	10 mM	0.0 ± 0.0	0.0 ± 0.0	1.8 ± 0.3	7.6 ± 1.3	91.6 ± 2.4	100.0[a]	392.8

[a]The difference between the treated group and the control was significant ($P<0.01$) as determined by χ^2 test.

past few years (Singh et al., 1988; Olive, 1989; Betti et al., 1994; Hartmann et al., 1995, 1998a, b; Goethem et al., 1997; Vroz and Petras, 1997; Sram et al., 1998; Maluf and Erdtmann, 2000). The sensitivity of the comet assay was frequently compared with that of cytogenetic techniques, but conflicting results were more than often reported. For example, some researchers indicated that comet assay was a more sensitive method than cytogenetic techniques (Betti et al., 1994; Goethem et al., 1997; Hartmann et al., 1998a; Maluf and Erdtmann, 2000). Conversely, other researchers suggested that the MN (Sram et al., 1998) or SCE (Hartmann et al., 1995) test was more sensitive than the comet assay. Particularly, Hartmann et al. (1995) reported that comet assay was 100 times less sensitive than the SCE test with respect to the detection of CP-induced damage in human lymphocyte cultures. In light of these results, H_2O_2 and CP were used as positive controls in the present study for the comet assay and MN or SCE tests, respectively. Our results indicated that the comet assay was sensitive in the detection of H_2O_2-induced DNA damage (Table 2), while the MN or SCE test was sensitive to CP-induced damage (Fig. 2 and Table 1).

This study indicates that the comet assay was more sensitive than both the MN and the SCE tests, while the MN and SCE tests had similar sensitivity in relation to the lowest observable adverse effect levels. For example, the lowest observable adverse effect levels were 0.05 and 5 mg/L for imidacloprid and RH-5849, respectively, for the comet assay (Table 2) and 0.1 and 25 mg/L for imidacloprid and RH-5849, respectively, for the MN/SCE tests (Fig. 2 and Table 1). In addition, the comet assay and cytogenetic tests revealed different dose levels at which the percentage of damaged cells was significantly different from that of the control groups. With the comet assay, treated groups even at the lowest dose levels had the numbers of damaged cells significantly different ($P<0.01$) from those induced by the control groups (0.05 and 5 mg/L for imidacloprid and H-5849,

respectively; Table 2). On the other hand, treated groups with the MN and SCE tests produced results significantly different ($P<0.05$) from those of the control groups at relatively higher dose levels, i.e., 0.1 and 25 mg/L for imidacloprid and H-5849, respectively (Fig. 2 and Table 1). This sensitivity difference between the comet assay and the cytogenetic tests could be attributed to the varying types of DNA alterations that the comet assay and cytogenetic tests target. Comet assay systems detect DNA single/double-strand breaks and alkali-labile sites, while MN and SCE tests identify fixed mutations that persist for at least one mitotic cycle. The type of target cells and the time duration between exposure and analysis may have also contributed to the difference. The cytogenetic test data were acquired exclusively with proliferation-stimulated lymphocytes exposed for 48 h, while those with the comet assay were obtained based on the responses of freshly isolated lymphocytes exposed for 1 h. Furthermore, DNA repair occurred with both the MN and the SCE tests, but it was not observed with the comet assay.

Although the comet assay is generally considered a more sensitive method than cytogenetic tests for assessing DNA damage by genotoxic agents (Betti et al., 1994; Goethem et al., 1997; Hartmann et al., 1998a; Maluf and Erdtmann, 2000), different mechanisms of detection are involved with the MN, SCE, and comet assays. The MN test detects chromosome breakages after repair and chromosome loss events, while the SCE test is a measure of DNA repair response to genome damage. Furthermore, the comet measurement is indicative only of initial genome damage if repair is not allowed to occur (Kassie et al., 2000). Therefore, many researchers have combined the comet assay with in vitro cytogenetic tests on genotoxins to assess lowest efficient doses (Betti et al., 1994; Hartmann et al., 1995, 1998a, b; Goethem et al., 1997; Vroz and Petras, 1997; Sram et al., 1998; Maluf and Erdtmann, 2000). It may also be interesting to investigate the correlation between the DNA breakages measured in individual cells by the comet assay and the frequency of MN and SCE occurrence found under the same experimental conditions. Such a comparison would allow us to estimate the amount of DNA breakages translated into chromosome and/or genome mutations. Although the comet assay and MN/SCE tests were not performed under the same experimental conditions in the present study, a certain degree of DNA damage caused by the two pesticides at low doses (e.g., imidacloprid at 0.05 mg/L and RH-5849 at 5 mg/L) with the comet assay were not translated into chromosome and/or genome mutations, as indicated by the results from the comet assay and cytogenetic tests (Tables 1 and 2 and Fig. 2). On the other hand, as the dose level increased, so did the degree of DNA damage in both the comet assay and the cytogenetic tests, signaling the possibility that DNA damage might be translated into chromosome and/or genome mutations. These results suggest that there might be a threshold for the mutation of lymphocytes caused by imidacloprid and RH-5849; i.e., lymphocytes had certain DNA-repairing capability. As a result, a combined approach employing comet assay and cytogenetic tests may be useful for investigating both the direct DNA strand breakages and the chromosome and/or genome mutations induced by genotoxic agents (Betti et al., 1994; Hartmann et al., 1995, 1998a, b; Goethem et al., 1997; Vroz and Petras, 1997; Sram et al., 1998; Maluf and Erdtmann, 2000).

The potential risk of pesticide residues is strongly reliant upon their ability to cause adverse health effects and the degree of human exposure. For application in crop fields to control pests, imidacloprid and RH-5849 are usually prepared as water solutions of about 100–150 and 200–350 mg/L, respectively (Sun et al., 1995). As the first commercial synthetic neonicotinoid, imidacloprid is the most important insecticide to control plant pests because of its lasting effects on insects. Its residues may stay in vegetables, crops, fruits, soils, and waters upon application in the field (e.g., Fernandez-Alba et al., 1996; Zhang and Shi, 1997; Dai et al., 2002). Sun et al. (1995) reported that imidacloprid could be absorbed by plant roots and transferred to other organs. These results unveiled the potential human exposure to imidacloprid via food chains. Compared to imidacloprid, even fewer data on the distribution of RH-5849 residues in soil, water, and produce and on its metabolic mechanism in biological species are available. As an agonist at the nicotinic acetylcholine receptor imidacloprid has shown low toxicity for warm-blooded mammals (e.g., Elbert et al., 1998), but it could cause DNA damage or genome mutation in lymphocytes at the dose levels of 0.05–0.5 mg/L (Tables 1 and 2). Finally, because of its genotoxicity, RH-5849 residues in soil, water, and agricultural products need to be evaluated thoroughly to mitigate its potentially negative effects.

In conclusion, the present study obtained evidence for the genotoxicity of imidacloprid and RH-5849 in human peripheral blood lymphocytes in vitro. This study also demonstrated the utility of combining the comet assay and cytogenetic tests to investigate both direct DNA strand breakages and chromosome and/or genome mutations induced by imidacloprid and RH-5849. The combined approach is potentially able to detect the fraction of DNA damage that would lead to fixed mutations.

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References

Anderson, D., Yu, T.W., McGregor, D.B., 1998. Comet assay responses as indicators of carcinogen exposure. Mutagenesis 13, 539–555.

Betti, C., Davini, T., Giannessi, L., Loprieno, N., Barale, R., 1994. Microgel electrophoresis assay (comet test) and SCE analysis in human lymphocytes from 100 normal subjects. Mutat. Res. 307, 323–333.

Bonassi, S., Fontana, V., Ceppi, M., Barale, R., Biggeri, A., 1999. Analysis of correlated data in human biomonitoring studies-The case of high sister chromatid exchange frequency cells. Mutat. Res. 438, 13–21.

Bradley, M.O., Hsu, I.C., Harris, C.C., 1979. Relationship between sister chromatid exchange and mutagenicity, toxicity and DNA damage. Nature 282, 318–320.

Cox, L., Koskinen, W.C., Yen, P.Y., 1996. Sorption–desorption of imidacloprid and its metabolites in soils. J. Agric. Food Chem. 45, 1468–1472.

Dai, H., Li, Y.J., Zhang, Y., 2002. Determination of imidacloprid in rice by solid phase extraction-HPLC. J. Instrum. Anal. 21, 70–72 (in Chinese).

Elbert, A., Nauen, R., Leicht, W., 1998. Imidacloprid, a novel chloronicotinyl insecticide: biological activity and agricultural importance. In: Ishaaya, I., Degheele, D. (Eds.), Insecticides with Novel Modes of Action. Springer, Berlin, Germany, pp. 50–73.

Evans, H.J., 1988. Mutation cytogenetics: past, present and future. Mutat. Res. 204, 355–363.

Fairbairn, D.W., Olive, P.L., O'Neill, K.L., 1995. The comet assay: a comprehensive review. Mutat. Res. 339, 37–59.

Feng, S.L., Kong, Z.M., Wang, X.M., Zhao, L.R., Peng, P.A., 2004. Acute toxicity and genotoxicity of two novel pesticides on amphibian, *Rana N. Hallowell*. Chemosphere 56, 457–463.

Fernandez-Alba, A.R., Valverde, A., Aguera, A., Contreras, M., Chiron, S., 1996. Determination of imidacloprid in vegetables by high-performance liquid chromatography with diode-array detection. J. Chromatogr. A 721, 97–105.

Garaj-Vrhovac, V., Zeljezic, D., 2000. Evaluation of DNA damage in workers occupationally exposed to pesticides using single-cell gel electrophoresis (SCGE) assay: pesticides genotoxicity revealed by comet assay. Mutat. Res. 469, 279–285.

Goethem, F.V., Lison, D., Kirsch-Volders, M., 1997. Comparative evaluation of the in vitro micronucleus test and the alkaline single cell gel electophoresis assay for the detection of DNA damaging agents: genotoxic effects of cobalt powder, tungsten carbide and cobalt-tungsten carbide. Mutat. Res. 392, 31–43.

Hartmann, A., Herkommer, K., Gluck, M., Speit, G., 1995. DNA-damaging effect of cyclophosphamide on human blood cells in vivo and in vitro studied with the single-cell gel test (comet assay). Environ. Mol. Mutagen. 25, 180–187.

Hartmann, A., Pfuhler, S., Dennog, C., Germdnik, D., Pilger, A., Speit, G., 1998a. Exercise-induced DNA effects in human leukocytes are not accompanied by increased formation of 8-hydroxy-2-deoxyguanosine or induction of micronuclei. Free Radical Biol. Med. 24, 245–251.

Hartmann, A., Fender, H., Speit, G., 1998b. Comparative biomonitoring study of workers at a waste disposal site using cytogenetic test and the comet (single-cell gel) assay. Environ. Mol. Mutagen. 32, 17–24.

Heddle, J.A., Hite, M., Kirkhart, B., Mavornin, K., MacGregor, J.T., Newell, G.W., Salamone, M.F., 1983. The induction of micronuclei as a measure of genotoxicity: a report of the US Environmental Protection Agency Gen-Tox Program. Mutat. Res. 123, 61–118.

Kassie, F., Parzefall, W., Knasmuller, S., 2000. Single cell gel electrophoresis assay: a new technique for human biomonitoring studies. Mutat. Res. 463, 13–31.

Kong, Z.M., Liu, Z.T., Ding, B.Y., 1995. Study on the antimutagenic effect of pine needle extract. Mutat. Res. 347, 101–104.

Kong, Z.M., Zang, Y., Wu, Q.L., 1998. Monitoring the genotoxicity of Lake Taihu, using two kinds of micronucleus tests. Environ. Pollut. 99, 279–283.

Luo, Y., Zang, Y., Zhong, Y., Kong, Z.M., 1999. Toxicological study of two novel pesticides on earthworm *Eisenia foetida*. Chemosphere 39, 2347–2356.

Majer, B.J., Laky, B., Knasmuller, S., Kassie, F., 2001. Use of the micronucleus assay with exfoliated epithelial cells as a biomarker for monitoring individuals at elevated risk of genetic damage and in chemoprevention trials. Mutat. Res. 489, 147–172.

Maluf, S.W., Erdtmann, B., 2000. Follow-up study of the genetic damage in lymphocytes of pharmacists and nurses handling antineoplastic drugs evaluated by cytokinesis-block micronuclei analysis and single cell electrophoresis assay. Mutat. Res. 471, 21–27.

Nagata, K., Iwanaga, Y., Shono, T., Narahashi, T., 1997. Modulation of the neuronal nicotinic acetylcholine receptor channel by imidacloprid and cartap. Pestic. Biochem. Physiol. 59, 119–128.

Olive, P.L., 1989. Cell proliferation as a requirement for development of the contact effect in Chinese hamster V79 spheroids. Radiat. Res. 117, 79–92.

Ostling, O., Johanson, K.J., 1984. Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. Biochem. Biophys. Res. Commun. 123, 291–298.

Ostling, O., Johanson, K.J., 1987. Bleomycin in contrast to gamma radiation induces extreme variation of DNA strand breakage from cell to cell. Int. J. Radat. Biol. 52, 683–691.

Perry, P., Wolff, S., 1974. New Giemsa method for the differential staining of sister chromatids. Nature 251, 156–158.

Rojas, E., Lopez, MC., Valverde, M., 1999. Single cell gel electrophoresis assay: methodology and applications. J. Chromatogr. B 722, 225–254.

Singh, N.P., Stephens, R.E., 1997. Microgel electrophoresis: sensitivity, mechanisms, and DNA electrostretching. Mutat. Res. 383, 167–175.

Singh, N.P., McCoy, M.T., Tice, R.R., Schnider, E.L., 1988. A simple technique for quantification of low levels of DNA damage in individual cells. Exp. Cell Res. 175, 184–191.

Sram, R.J., Podrazilova, K., Dejmek, J., Mraekova, G., Pileik, T., 1998. Single-cell gel electrophoresis assay: sensitivity of peripheral white blood cells in human population studies. Mutagenesis 13, 99–103.

Sun, J.Z., Fang, J.C., Du, Z.W., Xia, L.R., 1995. Imidacloprid, an super-efficacious, all-purpose and assimilabe insecticide. Plant Protection 21, 44–45 (in Chinese).

Tice, R.R., Andrews, P.W., Singh, N.P., 1990. The single cell gel assay: a sensitive technique for evaluating intercellular differences in DNA damage and repair. In: Sutherland, B.M.W.A. (Ed.), DNA Damage and Repair in Human Tissues. Plenum, New York, NY, pp. 291–301.

Tucker, J.D., Preston, R.J., 1996. Chromsome aberrations, micronuclei, aneuploidy, sister chromatid exchanges, and cancer risk assessment. Mutat. Res. 365, 147–159.

Vroz, M., Petras, M.L., 1997. Comparison of alkaline single cell gel (comet) and peripheral blood micronucleus assays in detecting

DNA damage caused by direct and indirect acting mutagens. Mutat. Res. 381, 31–40.
Wing, K.D., 1988. RH 5849, a nonsteroidal ecodysone agonist: effects on a *Drosophila* cell line. Science 241, 467–469.
Zang, Y., Zhong, Y., Luo, Y., Kong, Z.M., 2000. Genotoxicity of two novel pesticides for the earthworm, *Eisenia fetida*. Environ. Pollut. 108, 271–278.
Zhang, J., Shi, J., 1997. A dynamic study on imidacloprid residues in apples. Agro-environ Protect. 16, 247–251 (in Chinese).
Zhong, Y., Feng, S.L., Luo, Y., Zhang, G.D., Kong, Z.M., 2001. Evaluating the genotoxicity of surface water of Yangzhong City using the *Vicia faba* micronucleus test and the comet assay. Bull. Environ. Contam. Toxicol. 67, 217–224.