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Effect of fipronil on brain and muscle ultrastructure of *Nilaparvata lugens* (Stål) (Homoptera: Delphacidae)

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ABSTRACT

The ultrastructure of *Nilaparvata lugens* brain cells was damaged by treatment at different fipronil concentrations. The cell showed swollen mitochondria and vacuolization, but no mitochondrial cristae. Rough endoplasmic reticulum (RER) fragmentation and degranulation were seen. The dilatation of endoplasmic reticulum cisterns was very prominent, and the predominant lamellar RERs were arranged chaotically. The Golgi apparatus demonstrated obvious changes in configuration, as dilated with closed cisternae and atypical vesicles. The mitochondria mainly showed large vacuolization in muscles. Nuclear degeneration and condensation and increased numbers of large hydropic vacuoles and lysosomes were observed. It was concluded that the effect on cellular components was fipronil-specific. Changes in cellular ultrastructure seem to be an appropriate ecotoxicological indicator of the insecticide's efficacy.

1. Introduction

The rice planthopper, *Nilaparvata lugens* (Stål) (Homoptera: Delphacidae), is an important pest of rice in Asian countries (Vontas et al., 2000). The major control measure for *N. lugens* is the increased application of chemicals, which are known to cause resistance and control problems. Resurgence of insect pests after pesticide application is a well-documented phenomenon. Fipronil is the first of the phenylpyrazole insecticides used commercially and has a broad spectrum of activity against the diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) (Sayyed and Wright, 2004; Li et al., 2006); the rice planthopper, *N. lugens*, the rice stemborer, *Tryporyza incerfulas* (Walker); *Chilo suppressalis* (Walker) (Li et al., 2007); and other rice insect pests in most rice-growing areas (Cao et al., 2004). Its mode of action involves disruption of chloride ion flow at the GABA-gated chloride ionophores of the central nervous system (Cole et al., 1993; Grant et al., 1998). Since 1997, fipronil has been used for *N. lugens* control and has shown excellent effects on the insect pest. However, concerns for fipronil effects on rice health and rice planthopper resurgence have been raised because of the resistance problem.

There are a few articles reporting ultrastructural effects of pesticides on insect tissues (Marana et al., 1997; Sorour and Larink, 2001; Medina et al., 2004; Saito et al., 2006), which can provide clues to the mechanisms of insecticidal action. Changes in cellular ultrastructure seem to be an appropriate ecotoxicological indicator of the effects of insecticides, due to the presence of many potential target sites for insecticides in cells.

In this study, ultrastructural changes in organelles of brain and muscle cells in the head segment of adult *N. lugens*, treated with a sublethal dose of fipronil, were observed with transmission electron microscopy (TEM). It was shown that the cells were sensitive to fipronil, and their ultrastructure was severely altered by the insecticide.

2. Materials and methods

2.1. Fipronil treatment

In this study, fipronil (Bayer CropScience Hangzhou Co., Ltd., Hangzhou, China, 97.6%) was used for all the bioassays. Fipronil was diluted to three actual measured concentrations (10.3, 23.4, and 38.2 mg/L) with acetone and distilled water.

2.2. Analytical methods

A reverse phase liquid chromatographic (RPLC) analysis was performed under the following conditions (Rivas et al., 2006):

Column: Diamond C18; 5 μm; 4.6 × 250 mm^2 I.D. (Tecknokroma, Barcelona, Spain). Mobile phase: methanol:water (80:20 v/v). Flow rate: 1.0 ml/min.

Abbreviations: ER, endoplasmic reticulum; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum

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Temperature: room temperature (26 °C). Injected volume: 2 μl. Wavelength: 280 nm.

Three prepared nominal fipronil concentrations were used and checked analytically in this experiment. Furthermore, these prepared solutions were left for 7 days and the actual concentrations were checked again.

2.3. Insects

Adult planthoppers collected from the rice fields of Qingyuan City, Guangdong Province, were reared on susceptible rice variety TN1 at 20–32 °C in a culture room. Adults were sprayed with different fipronil concentrations (10.3, 23.4, and 38.2 mg/L) using a Houtou model sprayer. Tap water was used as a control. At 7 days after treatment, treated adults were randomly collected for bioassay. Adults were killed with a scalpel to cut off the head and dissected. Brain tissue samples from the control and treated adults were quickly removed, immediately fixed in 2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2), washed in 0.1 M phosphate buffer, and then postfixed in 1% osmium tetroxide. After dehydration in a graded series of ethyl alcohol, the specimens were embedded in spur, sectioned, and stained with uranyl acetate and lead citrate. The stained sections of the specimens were observed and photographed under an electron microscope.

3. Results

3.1. RPLC analysis

A chromatogram of fipronil standard solution (97.6%) is shown in Fig. 1a. Actual measured concentrations of fipronil at $t=0$ and $t=7$ days vs. nominal concentrations are presented in Table 1. For 37.5 mg/L, the nominal fipronil concentration was 37.5 mg/L and the measured concentration was 38.2 mg/L (Fig. 1b). Errors in preparation cause the increase of the concentration. After 7 days, the measured value was 24.9 mg/L (Fig. 1c). Fipronil degraded with a half-life of 9.7 days. For 25 mg/L, the nominal concentration was 25 mg/L and the measured concentration was 23.4 mg/L. At 7 days, the measured value was 22.5 mg/L. For 12.5 mg/L, the nominal concentration was 12.5 mg/L and the measured concentration was 10.3 mg/L. After 7 days, the measured value was 10.3 mg/L. Based on these results of RPLC, it was suggested that the nominal value was different from the actual value and high fipronil concentrations degraded faster than low fipronil concentrations.

3.2. Ultrastructural analysis

3.2.1. Protocerebrum and tritocerebrum mitochondria

Very closely packed cristae and a dense material appearance with distinct outer and inner membranes were shown in mitochondria in the brains of control planthoppers (Fig. 2a). However, after 7 days of treatment with 10.3 mg/L fipronil, the cells showed swollen mitochondria with vacuolization and appeared to be enlarged (Fig. 2b and c). At 7 days after treatment with 24.9 mg/L fipronil, the mitochondria exhibited large vacuolization and no cristae (Fig. 2d). During 7 days of treatments with 10.3 mg/L fipronil to tritocerebrum, the mitochondria demonstrated progressive swelling with loss of dense material and showed the formation of

Table 1
Actual measured concentrations of fipronil at $t=0$ and $t=7$ days vs. nominal concentrations.

Nominal concentration (mg/L)	Actual measured concentration (mg/L)	
	$t=0$ days	$t=7$ days
12.5	10.3	10.3
25	23.4	22.5
37.5	38.2	24.9

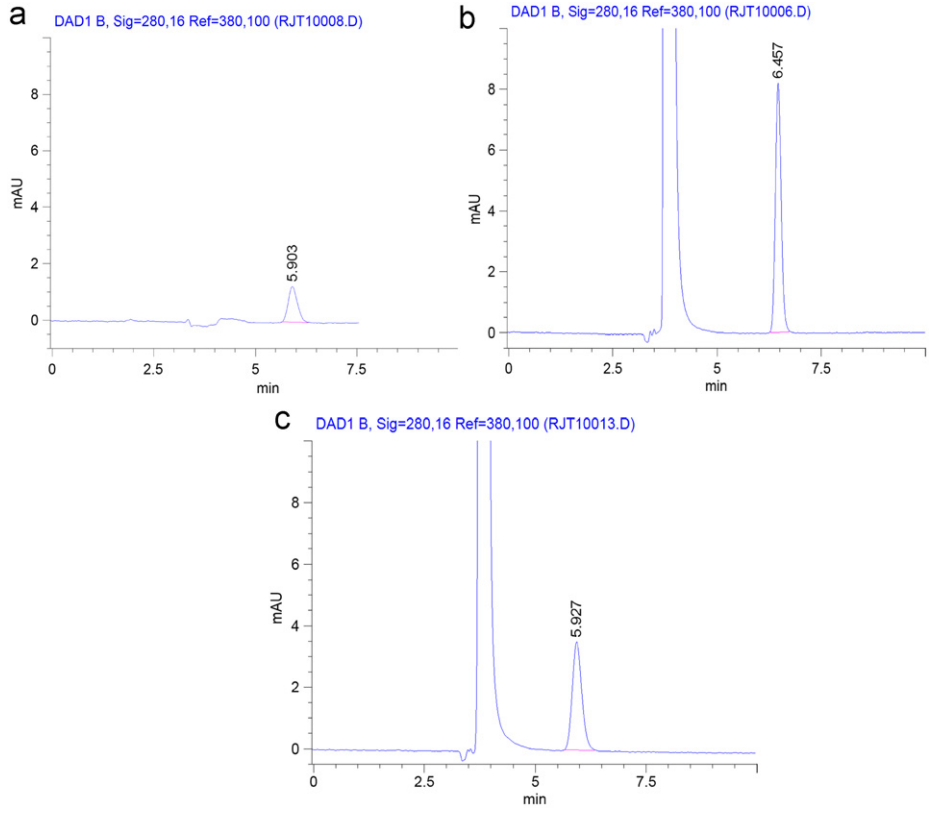

Fig. 1. (a) Chromatogram of fipronil standard solution (97.6%) obtained by RPLC. Peak at retention time 5.903 min = fipronil 97.6% standard. (b) Chromatogram of fipronil actual exposure concentration (38.2 mg/L). Peak at retention time 6.457 min = fipronil 38.2 mg/L. (c) Chromatogram of fipronil actual exposure concentration (24.9 mg/L at 7 days). Peak at retention time 5.927 min = fipronil 24.9 mg/L.

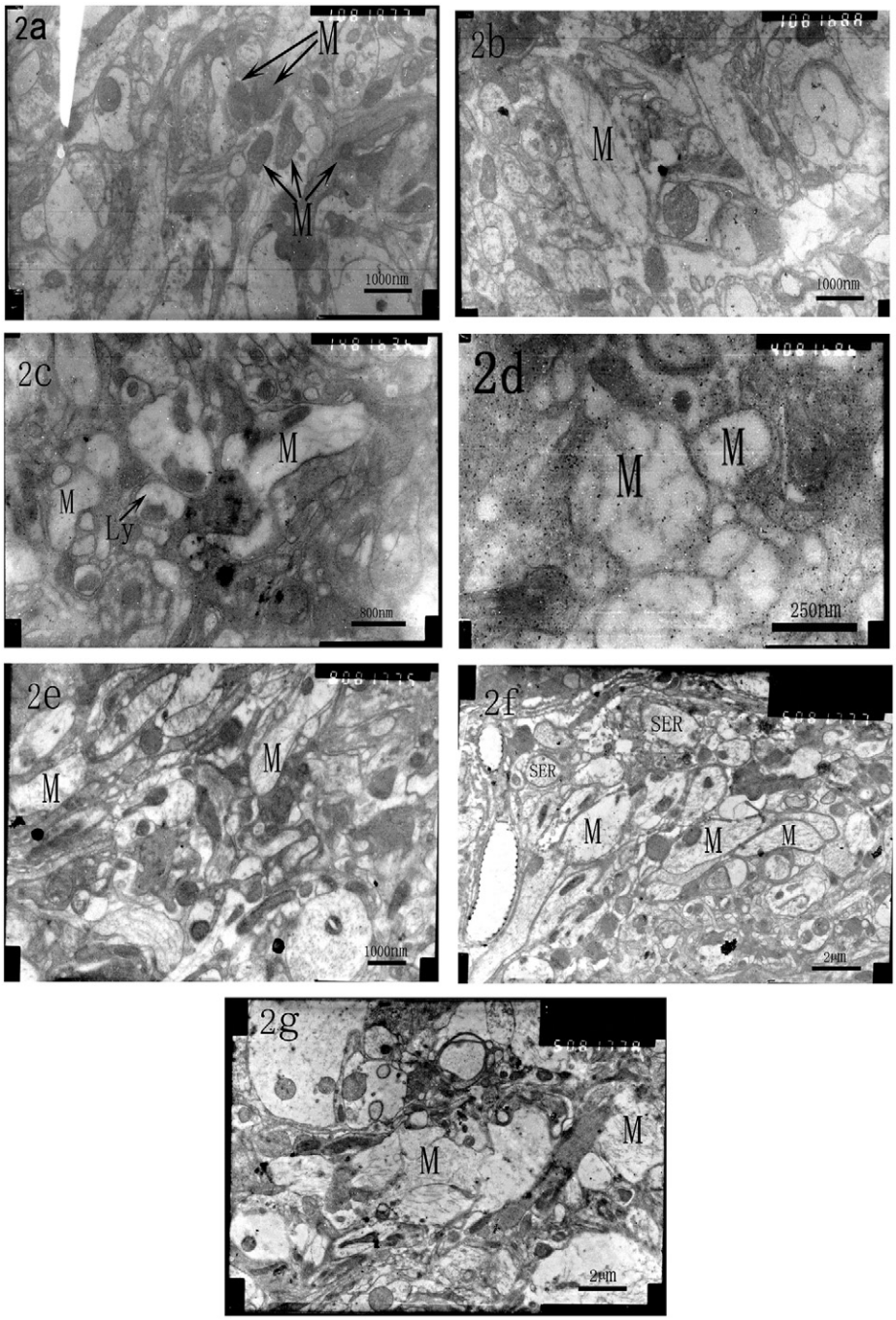

Fig. 2. Transmission electron micrographs of brain mitochondria from *N. lugens* after spraying with different fipronil concentrations. (a) Tap water and 7 days (10,000 ×). Protocerebrum: (b) 10.3 mg/L and 7 days (10,000 ×), (c) 22.5 mg/L and 7 days (14,000 ×), (d) 24.9 mg/L and 7 days (40,000 ×). Tritocerebrum: (e) 10.3 mg/L and 7 days (8,000 ×), (f) 22.5 mg/L and 7 days (5,000 ×), (g) 24.9 mg/L and 7 days (5,000 ×). Characteristic features include mitochondria (M), lysosomes (Ly), and smooth endoplasmic reticulum (SER).

vacuoles (Fig. 2e). As fipronil toxicity increased, the mitochondria showed even greater swelling (Fig. 2f). Extensive disruption of the mitochondrial membrane, distortion, loss of dense material within the mitochondria, and large vacuolization were evident (Fig. 2g). In muscles, the mitochondria were intact in the control group (Fig. 3a and b). But the appearance of one or three large vacuoles was exhibited in the treatment group (Fig. 3c and d).

3.2.2. Nuclei

The brain cells in controls contained large elliptical nuclei with prominent nucleoli and heterochromatins in the pale nucleoplasm. Furthermore, the outer and inner leaves of the nuclear envelopes were close to each other (Fig. 4a and e). However, distortion of brain nuclei was a common abnormality after treatment with different fipronil concentrations. After 10.3 mg/L fipronil treatment for 7 days, the perinuclear cistern between the inner and outer membranes of the nuclear envelope became more distended (Fig. 4b). After 22.5 mg/L fipronil treatment for 7 days, the cells showed nuclear swelling and distortion and disruption of some parts of the nuclear membrane, indicating hydropic degeneration of the cells (Fig. 4c). In addition, chromatin condensation (clumping) in the nucleus and an increased number of nucleoli were also observed (Fig. 4d).

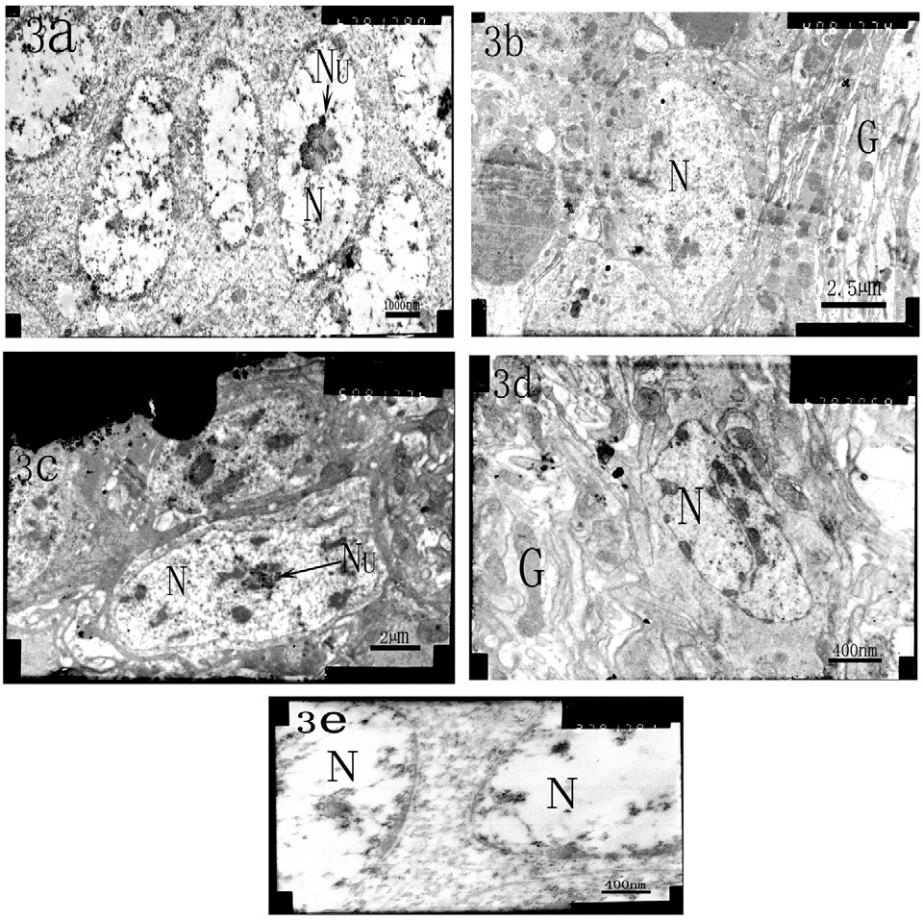

Fig. 3. Transmission electron micrographs of muscle from *N. lugens* after spraying with different fipronil concentrations: (a) tap water and 7 days (20,000 ×); (b) tap water and 7 days (14,000 ×); (c) 10.3 mg/L and 7 days (20,000 ×); (d) 22.5 mg/L and 7 days (14,000 ×). Characteristic features include mitochondria (M).

3.2.3. *Lysosomes and Golgi apparatus*

The brain cells in controls contained some lysosomes. However, the fipronil-treated cells showed an increased number of electron-dense autophagelysosomes (Figs. 5a–c and 2c), vacuolar structure of secondary lysosomes (Fig. 5d), a large number of various sized hydropic vacuoles, and dark granules (Fig. 5e). Furthermore, lysosomes were sequestering damaged organelles, e.g., mitochondria (Fig. 5b). The Golgi apparatus demonstrated obvious changes in configuration, as they dilated with closed cisternae and atypical vesicles (Fig. 4b and d).

3.2.4. *RER, SER, and ribosomes*

In controls, the cytoplasm of brain cells was filled with well-developed rough endoplasmic reticulum (RER) in lamellar or tubular arrangement and the lamellar RERs were arranged in parallel rows (Fig. 6a). In addition, most of the ribosomes were attached to the membrane of the endoplasmic reticulum (ER). In contrast, the intracisternal space of RERs in the cytoplasm of the fipronil-treated insect brain was almost flattened, with little distension (Fig. 6b). Dilation of the cisterns of the smooth endoplasmic reticulum (SER) was prominent and a marked increase in the number of SER was observed in treated specimens (Figs. 2f and 6b). The ribosomes were chipped away and RER fragmentation was seen. The predominant lamellar RERs were arranged chaotically and not in parallel rows (Fig. 6c and d).

4. Discussion

The actual exposure concentrations are checked analytically because the exposure doses are based on the active ingredient. The reasons for the concentration change include losses of compound and errors in preparation of the samples. Fipronil could be degraded to four metabolisms in the environment (Zhou et al., 2004) and degradation is the main reason.

In the studied tissues of contaminated *N. lugens*, massive swelling of the mitochondria was observed. Mitochondria, after the use of fipronil, were found to be extremely vulnerable to pesticide intruding into the cell. Any pesticide interfering with normal mitochondrion operation would produce a change in physiological processes within the cell (Jun et al., 1999). Substances that disrupt mitochondrial energetics could cause mitochondria to swell (Cheville, 1994). Mitochondrial swelling was observed in the brain cells after treatment with fipronil. The mitochondrial dysfunction might result in depletion of ATP as an energy source and subsequent loss of cellular integrity (Saito et al., 2006). Therefore, mitochondria may be the most prominent sites of fipronil cytotoxicity in cells and a primary target in fipronil-initiated cytotoxicity. Dilation and fragmentation of RER were also observed. Dilation was considered as the typical ultrastructural change of cell injury. Moreover, the proliferation and dilation of endoplasmic reticulum (ER) indicated stimulation of defense and regenerative processes linked to detoxification and could be classified as adaptation. These results might help

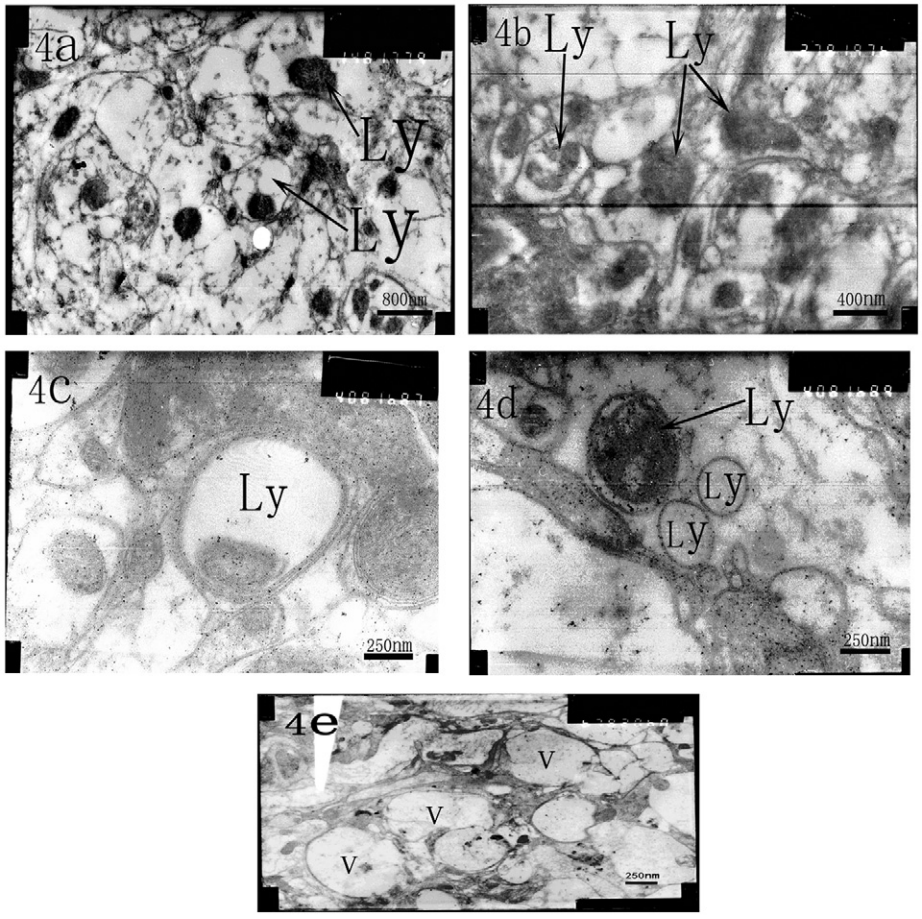

Fig. 4. Transmission electron micrographs of brain nuclei from *N. lugens* after spraying with different fipronil concentrations. (a, e) Tap water and 7 days (6,700 ×); (b) 10.3 mg/L and 7 days (4,000 ×); (c) 22.5 mg/L and 7 days (5,000 ×); (d) 24.9 mg/L and 7 days (27,000 ×). N, nucleus; Nu, nucleolus; G, Golgi apparatus.

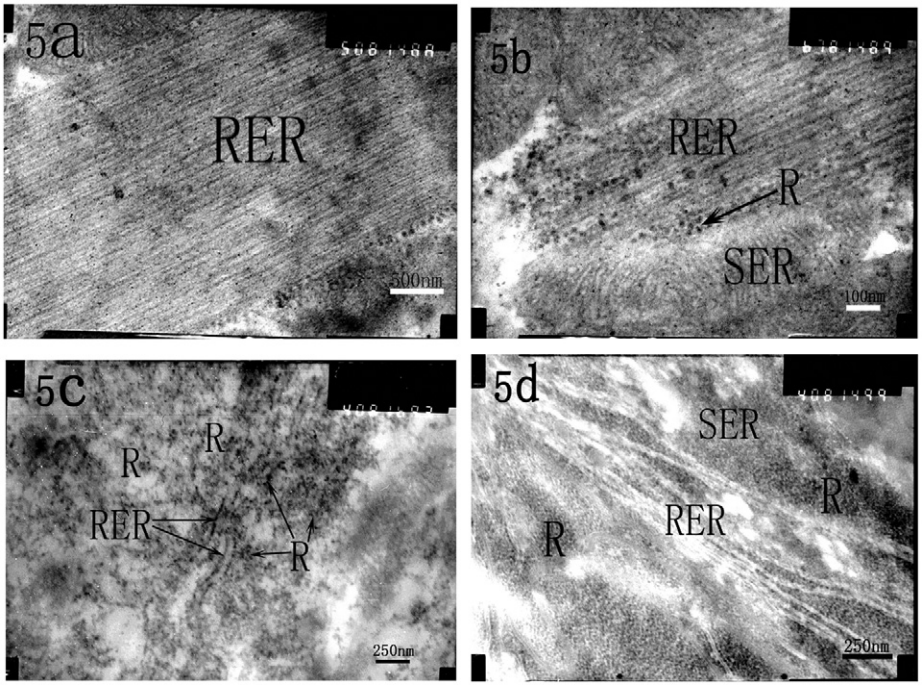

Fig. 5. Transmission electron micrographs of brain lysosomes from *N. lugens* after spraying with different fipronil concentrations. (a) 10.3 mg/L and 7 days (14,000 ×); (b) 22.5 mg/L and 7 days (27,000 ×); (c) 24.9 mg/L and 7 days (40,000 ×); (d) 24.9 mg/L and 7 days (40,000 ×). Vacuoles: (e) 24.9 mg/L and 7 days (40,000 ×). Ly, Lysosomes; V, vacuole.

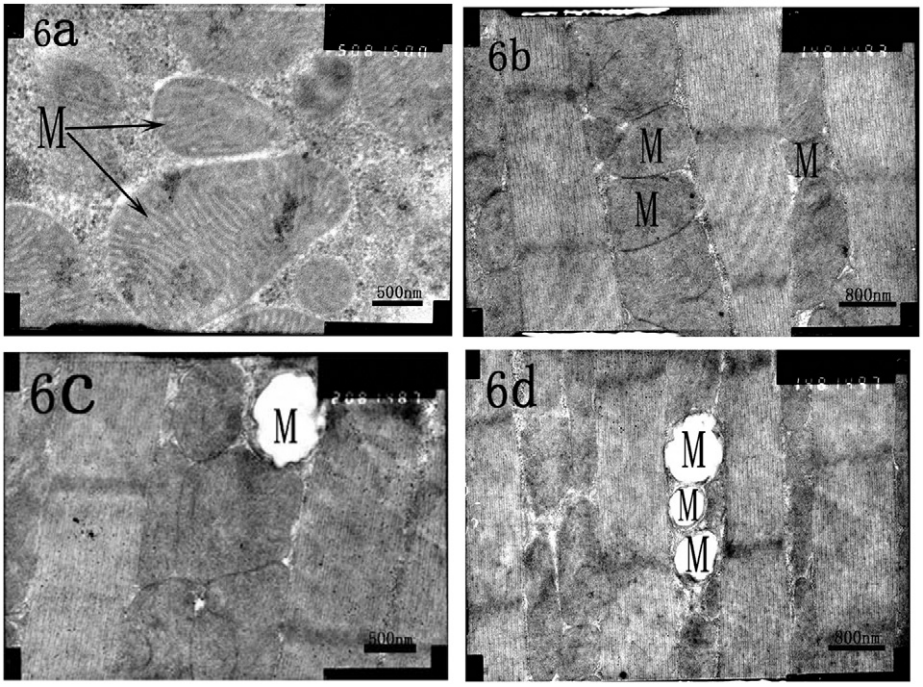

Fig. 6. Transmission electron micrographs of brain ER and ribosomes from *N. lugens* after spraying with different fipronil concentrations. (a) Tap water and 7 days (20,000 ×); (b) 10.3 mg/L and 7 days (67,000 ×); (c) 22.5 mg/L and 7 d (40,000 ×); (d) 24.9 mg/L and 7 d (40,000 ×). RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum; R, ribosomes.

in gaining an understanding of the underlying toxicological process. However, the chain of reactions by which fipronil induced alterations in the ultrastructure of the cells needs further scrutiny.

Degenerative modifications in the volume and shape of nuclei were considered early signs of increased metabolic activity and acute cell injury (Braunbeck et al., 1990). Degenerative changes in the nucleus, such as swelling with the disappearance of the nucleolus, have been found in acute cell injury. Changes in the nucleus, such as heterochromatin condensation and marginalization, which were found in the present study, indicate that the organelle is affected in a major way by fipronil treatment. Generally, it is believed that heterochromatin condensation and marginalization can be progressive inactivation of the nuclear component. These processes affect esterase gene expression and regulation. Insecticide resistance in *N. lugens* was based on expression and amplification of a carboxylesterase gene (Small and Hemingway, 2000; Vontas et al., 2000). It was confirmed that the gene was amplified three- to seven-fold in the genomes of resistant compared to susceptible planthoppers. Higher expression levels for carboxylesterase mRNA were five- to eleven-fold in the body homogenates of resistant planthopper females than in those of susceptibles (Vontas et al., 2000). The results in our experiment show that fipronil treatment enhances esterase and P450 monooxygenase activities of *N. lugens*.

Pesticide entails proliferation of lysosomes accompanied by augmentation of other lysosomal elements (autophagosomes): lysosomes were able to accumulate a wide range of pesticides and to sequester damaged organelles as a mechanism of cellular repair (Cheville, 1994). For example, some damaged mitochondrial proteins could be removed through lysosomal autophagy. Increases in the numbers of large vacuoles and lysosomes were seen in our experiments, and this vacuolation was characteristic of the treated brains. It was suggested that the formation of autophagolysosomes could be an indication of increased turnover of cellular components following cell degeneration and was induced by fipronil.

One of the first targets of lysosome enzymes could be actin or other cytoskeletal-related molecules (Malagoli et al., 2006), the degradation of which would result in the blocking of cell trafficking and ultimately in cell death (Medina et al., 2004). It was believed that the underlying mechanism of resistance to fipronil was based on elevation of esterase activity (Tang et al., 2010) and the most important target of lysosome enzymes in planthoppers was esterase (EST) (Karp 1999). It was suggested that lysosomes are involved in the fipronil-causing damage and seem to be the first subcellular component damaged by fipronil, indicating that lysosomes serve as the target of fipronil in the brain cells of planthoppers.

The ultrastructural alterations of the Golgi apparatus were ascribed to fipronil treatment. A variety of changes, including hypertrophy and dilatation, had been reported in pathological tissues of the Golgi apparatus (Saito et al. 2006). Marked destruction, reduction of the number of Golgi stacks, and the disappearance of recognizable Golgi apparatus elements had been noted in brain cells subjected to fipronil treatment (Cheville, 1994). The transformation of the Golgi apparatus into a cluster of vesicles was described by brain cells after benomyl treatment (Sorour and Larink, 2001). In our experiment, dilation and atypical vesicles were found, indicating that the Golgi apparatus was sensitive to fipronil treatment. The results may well serve as a tool to localize the intracellular sites of the toxic action of fipronil.

5. Conclusion

In conclusion, fipronil treatment caused significant pathological changes. Mitochondrial swelling, disappearance of cristae, and vacuoles were observed. Dilation and atypical vesicles were found in the Golgi apparatus. RERs showed obvious changes, including dilation, fragmentation, and degranulation. Fipronil caused proliferation of lysosomes accompanied by augmentation of autophagosomes. Degenerative changes in the nuclei were observed, including swelling, distortion, disappearance of the nucleolus, heterochromatin condensation, and nucleolus increase.

These results indicated that they were sensitive organelles and might serve as the primary targets of the toxic action of fipronil.

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