Effects of Sublethal Doses of Imidacloprid in Malpighian Tubules of Africanized *Apis mellifera* (Hymenoptera, Apidae)

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KEY WORDS cytotoxicity; honeybees; neonicotinoid; excretory system; cell death

ABSTRACT In Brazil, imidacloprid is a widely used insecticide on agriculture and can harm bees, which are important pollinators. The active ingredient imidacloprid has action on the nervous system of the insects. However, little has been studied about the actions of the insecticide on nontarget organs of insects, such as the Malpighian tubules that make up the excretory and osmoregulatory system. Hence, in this study, we evaluated the effects of chronic exposure to sublethal doses of imidacloprid in Malpighian tubules of Africanized *Apis mellifera*. In the tubules of treated bees, we found an increase in the number of cells with picnotic nuclei, the lost of part of the cell into the lumen, and a homogenization of coloring cytoplasm. Furthermore, we observed the presence of cytoplasmic vacuolization. We confirmed the increased occurrence of picnotic nuclei by using the Feulgan reaction, which showed the chromatin compaction was more intense in the tubules of bees exposed to the insecticide. We observed an intensification of the staining of the nucleus with Xylidine Ponceau, further verifying the cytoplasmic negative regions that may indicate autophagic activity. Additionally, immunocytochemistry experiments showed TUNEL positive nuclei in exposed bees, implicating increased cell apoptosis after chronic imidacloprid exposure. In conclusion, our results indicate that very low concentrations of imidacloprid lead to cytotoxic activity in the Malpighian tubules of exposed bees at all tested times for exposure and imply that this insecticide can alter honey bee physiology. *Microsc. Res. Tech. 00:000–000, 2013*.

INTRODUCTION

The indiscriminate use of pesticides not only submits pollinators to situations of severe stress but may also cause strong economic damage that is evidenced by a constant decrease of bee density proximal to agricultural fields in various parts of the world. One of the most widely used insecticides is imidacloprid, which acts by inhibiting the action of nicotinic acetylcholine receptors located in many regions of the brain of the bee (Bicker, 1999; Brazil, 2011). However, the insecticide comes in contact with other organs during the course of the metabolism of its compounds. One of these organs is the Malpighian tubules, which are responsible for the excretion of substances in the body.

The excretory system comprises, in most types of insects, a variable number of Malpighian tubules. These play an important role in the detoxification process by actively functioning to dispose of substances that are not metabolized and are in excess in the organism (Cruz-Landim, 2009).

Previous studies have used behavioral and biochemical analysis of bees as an additional tool for interpreting the level of toxicity of some insecticides (Bernadou et al., 2009, Decourtye et al., 2005). Several reports using these methods show the effects of insecticides on proboscis' extension response (Bernadou et al., 2009, Decourtye et al., 2005, El Hassani et al., 2005), orientation in complex mazes (Decourtye et al., 2008), survival (Alioune et al., 2009) and enzymes as biomarkers (Badiou-Bénéteau et al., 2012). However, morphological data of the organs from bees treated with imidacloprid has not been previously examined. Therefore, we evaluated the effects of a diet containing sublethal doses of imidacloprid in workers of Africanized *Apis mellifera* through analysis of the Malpighian tubules using morphological, histochemical, and immunocytochemical techniques.

MATERIAL AND METHODS

Chemicals

The analytical standard imidacloprid (92.5% of purity) was obtained from *Bayer CropScience* (Brazil). Sodium chloride (NaCl), sodium phosphate dibasic (Na_2HPO_4), potassium phosphate monobasic (KH_2PO_4), paraformaldehyde, ethanol, hematoxylin, eosin, xylidine ponceau, periodic-acid, Schiff's reagent and chloride acid (HCl), xylene and polylysine were

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obtained from Sigma Aldrich (Brazil). TUNEL reaction Kit (ISCDDK *in situ* Cell Death Detection Kit) was obtained from Roche Molecular Biochemicals (USA). The historesin embedding kit was purchased from Leica Microsystems (Germany) and the paraffin—Histosec from Merck (Brazil).

Honeybee Collection

Newly emerged adult bees of Africanized *A. mellifera* were collected directly from the combs in the Apiary of the Institute of Biosciences of Rio Claro Campus. We verified the health and physiological status of the colony, according to the guidelines for testing chemicals in bees of OECD (Organization for Economic Cooperation and Development, 1998).

Honeybee Intoxication Assay

Bees were placed in disposable cages (10 bees per cage) previously lined with filter paper. Three replicates were made for each dose and control groups. The experiments were conducted in an BOD chamber with temperature of 32°C ± 1°C and a relative humidity of 70%. The experimental and control groups received the same water supply, consisting of cotton soaked in water and food. There were two control groups: one without solvent, fed only with the diet (sucrose solution in water—50% w/v), and the second control group with solvent, fed with the diet containing 1% acetone (solvent used to dissolve the pesticide).

The sublethal doses used in this study were calculated using the LD_{50} of 80.9 ηg of imidacloprid/bee obtained by Rossi et al., 2012 (personal communication). For the experimental groups, we prepared contaminated diet from a stock solution in which the imidacloprid was dissolved using acetone and mixed with sucrose solution. From this solution, we made three dilutions yielding concentrations $LD_{50/100}$ (0.809 ηg/bee), $LD_{50/50}$ (40.4 ηg/bee) and $LD_{50/10}$ (8.09 ηg/bee). The periods of exposure to the insecticide were 1, 3, 5, 7, and 10 days for each group, and for the assay, six bees were collected per group.

Morphology and Histochemistry Analysis

To obtain the Malpighian tubules, dissection was carried out on bees in Petri dishes containing saline solution (20 mM of Na_2HPO_4/KH_2PO_4, pH 7.4, 130 mM of NaCl) under a Zeiss stereomicroscope with the aid of dissecting forceps and microscissors. Malpighian tubules of honeybees present three distinct regions known as distal, medial and proximal. After the dissection of bees the medial region was isolated and fixed in 4% paraformaldehyde in phosphate buffered saline (0.1 M, pH 7.4). Subsequently, the material was dehydrated in a series of ethanol solutions in concentrations of: 15, 30, 50, 70, 85, 90, 95, and 100% on ice (each lasting 2 h) (Silva-Zacarim et al., 2012). After dehydration, the material was incubated in embedding resin for 3 days, embedded in historesin. Sections of 7 μm were made using Leica Microtome (Germany).

Some sections of Malpighian tubules were stained with Hematoxylin-Eosin (HE) for morphological analysis and others were subjected to Xylidine Ponceau to detect total protein (Junqueira and Junqueira, 1983)

TABLE 1. Results of morphology and immunocytochemical analysis performed on the Malpighian tubules of A. mellifera *exposed or not exposed to imidacloprid*

		Morphological analysis				Tunnel reaction
		Cytoplasmic vacuolization	Pyknotic nuclei	Loss of part of the cell	Homogenization of staining of the cytoplasm	Nuclei positive
Treatment	Days					
Control without solvent	1	−	−	−	−	−
	3	−	−	−	−	−
	5	−	−	−	−	−
	7	−	−	−	−	−
	10	−	−	−	−	−
Control with solvent	1	−	−	−	−	−
	3	−	−	−	−	−
	5	−	−	−	−	−
	7	−	−	−	−	−
	10	−	−	−	−	−
$LD_{50}/100$	1	−	+	+++	−	+
	3	+	+	+	+	−
	5	++	++	++	++	−
	7	−	++	++	++	+
	10	−	+++	++	++	+
$LD_{50}/50$	1	+++	++	+	−	+
	3	+	++	++	−	+
	5	++	+	+++	++	+
	7	−	+	++	++	−
	10	−	++	++	++	+
$LD_{50}/10$	1	+	+	++	−	−
	3	+	++	+	−	−
	5	+	++	+	+	+
	7	−	+++	++	++	+
	10	−	++	++	++	+

(+) Presence of alteration; (++) Alteration moderately present; (+++) Alteration extremely present; (−) alteration absence.

and to the Feulgen reaction (counter-stained with Fast Green) to determine the level of chromatin compaction (Feulgen and Rossenbeck, 1924).

Finally, the sections were examined under a light microscopy (Olympus BX51—Olympus America) and the photomicrographs obtained by a digital camera

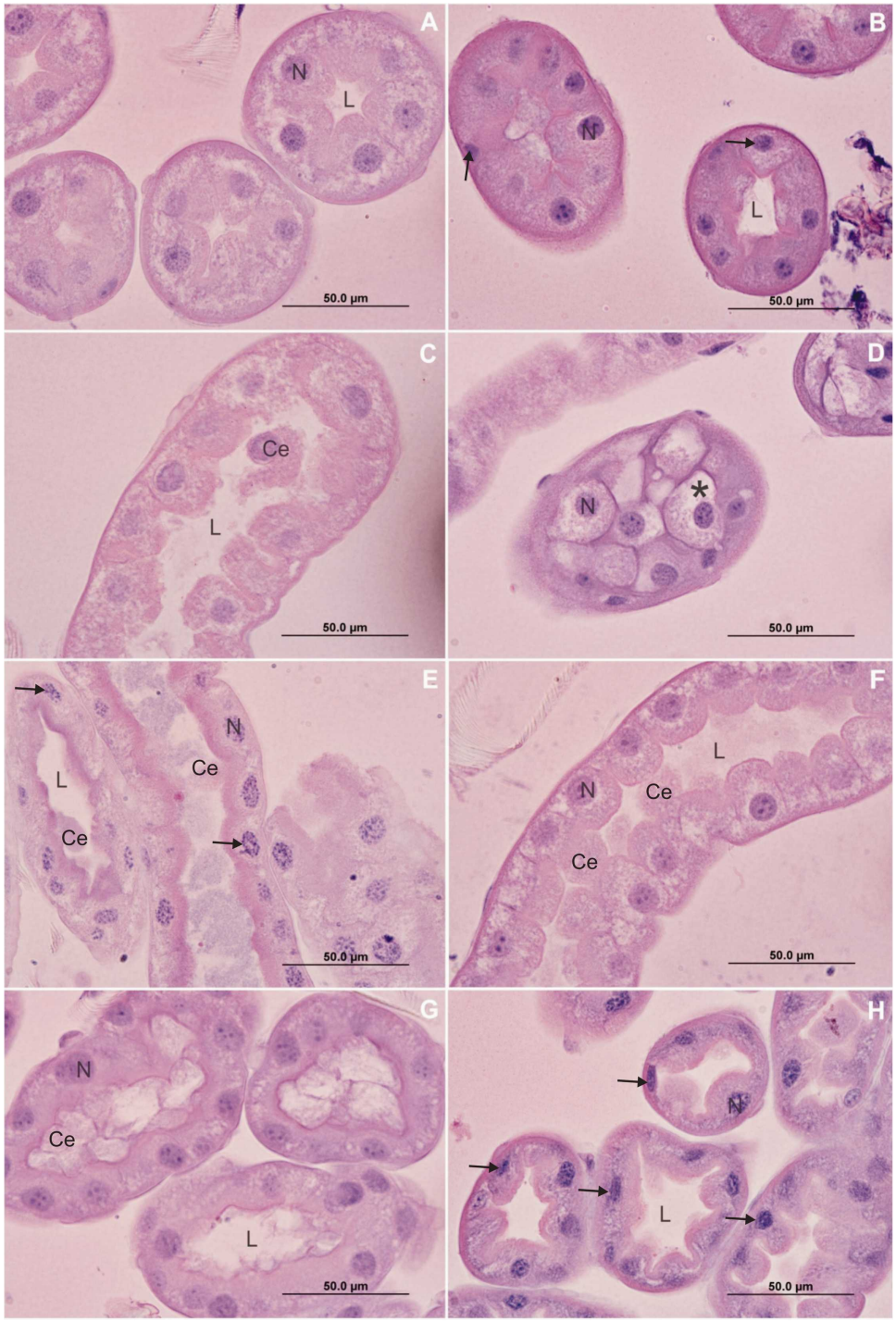

Fig. 1. Histological sections of the Malpighian tubules stained with HE of honeybees exposed or not exposed to imidacloprid. **A**: Malpighian tubule of a bee from the control group without solvent after 3 days, showing typical morphology of this organ. **B**: Malpighian tubule of a bee from $LD_{50}/100$ group after 3 days. Note homogenization in the staining of the cytoplasm and the presence of pyknotic nuclei (arrow). **C**: Malpighian tubule of a bee exposed $LD_{50}/10$ for 5 days. Verification of the presence of a part of cell disposed in the lumen (Ce). **D**: Malpighian tubule of a bee exposed to $LD_{50}/50$ for 1 day, note the vacuolation (*). **E–G**: Malpighian tubules of bees exposed to $LD_{50}/100$ for 1, and 7 days and from group treated with $LD_{50}/50$ for 10 days. Note the lost of part of the cell into the lumen (Ce) and in E presence of pyknotic nuclei (arrow). **H**: Malpighian tubule of bees exposed to $LD_{50}/10$ for 7 days. Note the presence of pyknotic nuclei (arrow). L = lumen, N = nuclei. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(Olympus DP-71) and a Dell computer, linked to the microscopy. For image acquisition was used the DP Controller software.

Immunocytochemical Analysis for Detection of DNA Fragmentation by Endonucleases (TUNEL Reaction—Terminal Deoxynucleotidyl Transferase—Mediated Biotinylated UTP Nick End Labeling)

Malpighian tubules were dissected and fixed as described above. After dehydration in an ethanol series, organs were diaphanized in xylene and embedded in paraffin (Histosec, Merck).

Sections of 8 μm thickness were transferred to histological slides previously cleaned and treated with polylysine. The Histosec wax was removed in several xylene baths before the sections were rehydrated and then submitted to a TUNEL reaction (ISCDDK *in situ* Cell Death Detection Kit) according to the manufacturer's instructions. Negative and positive controls were made for the TUNEL reaction. After incubation, the slides were examined for detection of the fluorescein-labeled 3′-OH ends of fragmented DNA and photographed on a fluorescence microscope Olympus BX-51 (Olympus America), using a wavelength of 450–500 nm.

RESULTS

H–E Stain

The results obtained for the imidacloprid-exposed and controls groups are summarized in Table 1. The Malpighian tubules of the control group without solvent showed typical morphology. The basal portions of the cytoplasm were slightly stained, indicating the presence of basal labyrinth. The cells presented large nuclei with many nucleoli. These basic characteristics appear in all groups to a greater or lesser extent (Fig. 1A). The Malpighian tubules of the bees in the solvent control group showed morphology similar to the control group without solvent.

In Malpighian tubules of bees exposed to $LD_{50}/100$, $LD_{50}/50$ and $LD_{50}/10$, we observed a homogeneous staining of the apical and basal cytoplasm, suggesting a reduction of the basal labyrinth (Fig. 1B). In the $LD_{50}/100$ group, these characteristics were observed in bees exposed for 3, 5, 7, and 10 days. In the other treated groups, the homogenization of apical and basal cytoplasmic staining was observed in individuals collected after 5 days of exposure to concentrations of $LD_{50}/50$ e and $LD_{50}/10$.

The vacuolization of the cytoplasm (Fig. 1D) was present in the tubules of the $LD_{50}/100$ group in bees after exposure for 3 and 5 days. In the $LD_{50}/50$ and $LD_{50}/10$ groups, the vacuolization was observed earlier, occurring after only 1 day and persisting until 5 days of exposure.

The most evident features of the exposed groups were the increased frequency and intensity of pyknotic nuclei (Fig. 1H) and the lost of part of the cell into the lumen (Figs. 1C and 1E–1G), which was present in all exposed groups at all ages analyzed.

Feulgen Reaction

The results obtained with histochemical analysis are summarized in Table 2. In the Feulgen reaction test, the Malpighian tubules of both the control groups with and without solvent showed cells with weakly

TABLE 2. Results of Fuelgen reaction and Xylidine Ponceau technique performed in the Malpighian tubules of A. mellifera *exposed or not exposed to imidacloprid*

		Fuelgen reaction	Xylidine Ponceau		
		Chromatin compaction	Region of the cytoplasm with negative reaction	Staining of the nuclei	Uniform staining of basal and apical cytoplasm
Treatment	Days				
Control without solvent	1	−	−	+	−
	3	−	−	+	−
	5	−	−	+	−
	7	−	−	+	+
	10	−	−	+	+
Control with solvent	1	−	−	+	−
	3	−	−	+	−
	5	−	−	+	−
	7	−	−	++	+
	10	−	−	++	+
$LD_{50}/100$	1	+	+	+	+
	3	+	+	+	+
	5	+	+	+	+
	7	+	+	++	+
	10	++	+	++	+
$LD_{50}/50$	1	+	−	++	+
	3	++	+	+	+
	5	+	+	+	+
	7	++	+	++	+
	10	+	+	++	+
$LD_{50}/10$	1	+	+	++	+
	3	++	+	+	+
	5	+	+	+	+
	7	+++	+	++	+
	10	++	+	++	+

(+) Presence of alteration; (++) Alteration moderately present; (+++) Alteration extremely present; (−) alteration absence.

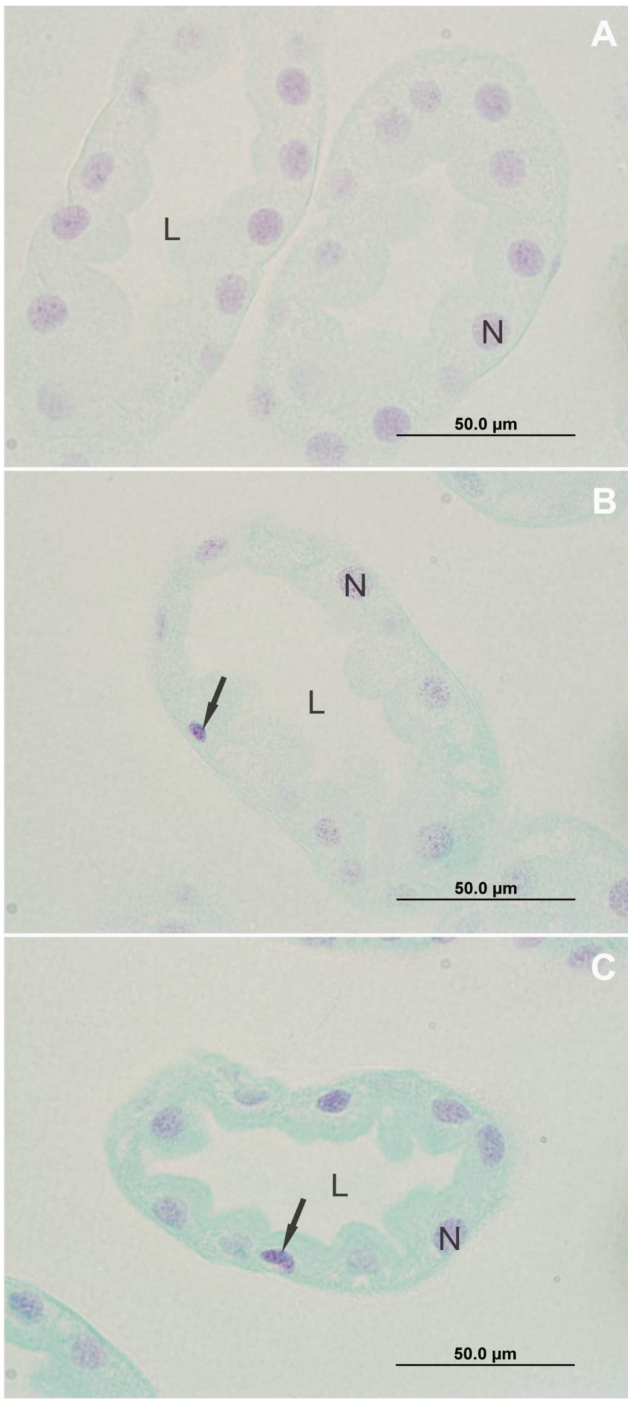

Fig. 2. Histological sections of *Apis mellifera* bee Malpighian tubules subjected to the Feulgen reaction from both exposed and control groups. **A**: Malpighian tubules of a bee from solvent control group after 1 day with slightly stained nuclei showing decondensed chromatin uniformly distributed. **B** and **C**: Malpighian tubules of bees exposed to $LD_{50}/10$ on days 3 and 7, respectively, presenting histochemical alterations. Note the presence of strongly stained nuclei with pyknosis (arrow). L = lumen, N = nuclei. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

stained nuclei at all timepoints indicating the presence of decondensed chromatin (Fig. 2A). Tubules of the bees in the solvent control group showed the characteristics. Bees from all groups exposed to insecticide at all timepoints, presented more strongly stained nuclei, indicating a higher level of chromatin compaction (Figs. 2B and 2C). These results further support our previous findings of increased pyknotic nuclei revealed by HE staining.

Xylidine Ponceau Technique

The Malpighian tubules stained with Xylidine Ponceau of both control groups showed a distinct staining in the basal and apical portions of cells until 5 days. The basal region was negative to this technique, most likely due to the basal labyrinth, while the apical portion was positive, indicating the presence of cytoplasmic proteins (Fig. 3A). The nuclei and nucleoli of the control groups were positive due to the marking of nuclear proteins (histones and others) and nucleolar. The tubules of bees exposed to insecticide at all timepoints had cytoplasm that was stained homogeneously with Xylidine (Fig. 3B), indicating the loss of basal labyrinth. Some cells presented a cytoplasm with negative regions to Xylidine staining (Figs. 3C and 3D), which may correspond to vacuolated regions stained by HE. The increase in the intensity of nuclei staining sometimes occurs under volume reducing conditions (Fig. 3C).

Immunocytochemical Analysis

Nuclei positive for TUNEL reactions were observed only in cells from the Malpighian tubules of bees exposed to insecticide (Fig. 4). For the group exposed to $LD_{50}/100$, positive results were observed in bees from the 1, 7, and 10 days exposure groups. For the $LD_{50}/50$ group, positive results were observed in bees at 1, 3, 5, and 10 days of exposure. For the $LD_{50}/10$ group, we observed positive nuclei reactions in all groups after the 5th day of exposure.

DISCUSSION

Morphological, histochemical, and immunocytochemical alterations were observed in Malpighian tubules of bees from exposed groups compared to control groups. Moreover, comparing the groups exposed to each other, we could not observe significant difference in cytotoxicity, showing that even at very low doses the imidacloprid can alter the physiology of honeybees. The differences found between the treatments and exposure times mainly revealed alterations of intensity in the observed results.

Comparing the tubules of cells from bees exposed to $LD_{50}/100$, $LD_{50}/50$, or $LD_{50}/10$, we found an increase in the amount of pyknotic nuclei, the lost of parts of the cell into the lumen, the homogenization of the cytoplasmic staining and vacuolated cells.

Increased homogeneous staining of the cytoplasm, as observed by analysis with Xylidine Ponceau, suggests the loss of basal membrane invagination of these cells when compared to the controls. These structures promote greater contact with the hemolymph and thus enhance the uptake of substances from it (Cruz-Landim, 1998). Therefore, this reduction or loss of the basal labyrinth may indicate a tissue degeneration, which can compromise the reabsorption of water and ions and excretion. These functions are essential to

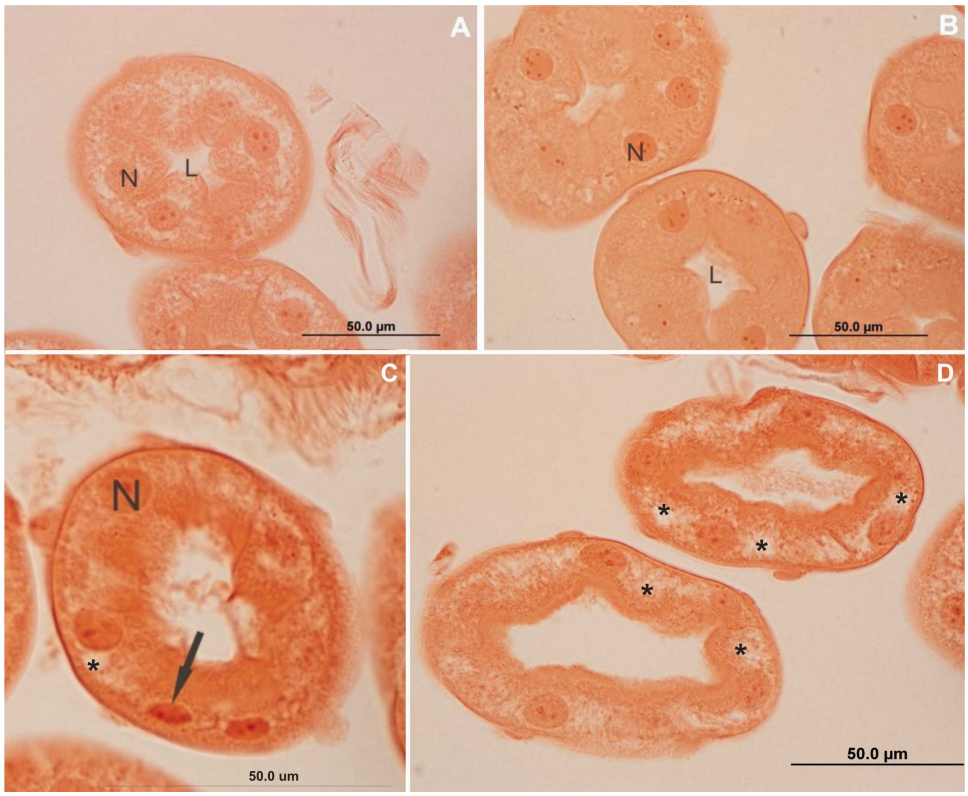

Fig. 3. Hitological sections of the Malpighian tubules of *Apis mellifera* bees subjected to reaction with Xylidine Ponceau from control and insecticide exposed groups. **A**: Malpighian tubule of bees from the control group without solvent after 3 days showing the negative basal Xylidine staining and the weakly positive staining of the apical region of the cytoplasm. **B–D**: Malpighian tubules of bees from the LD_{50}/50 group from 10 and 1 day, and LD_{50}/10 group after 1 day, respectively. Note the histochemical changes, the uniform cytoplasmic staining, the presence of strongly stained nuclei (arrow), and the negative region of cytoplasm to Xylidine Ponceau reaction (*). L = lumen, N = nuclei. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

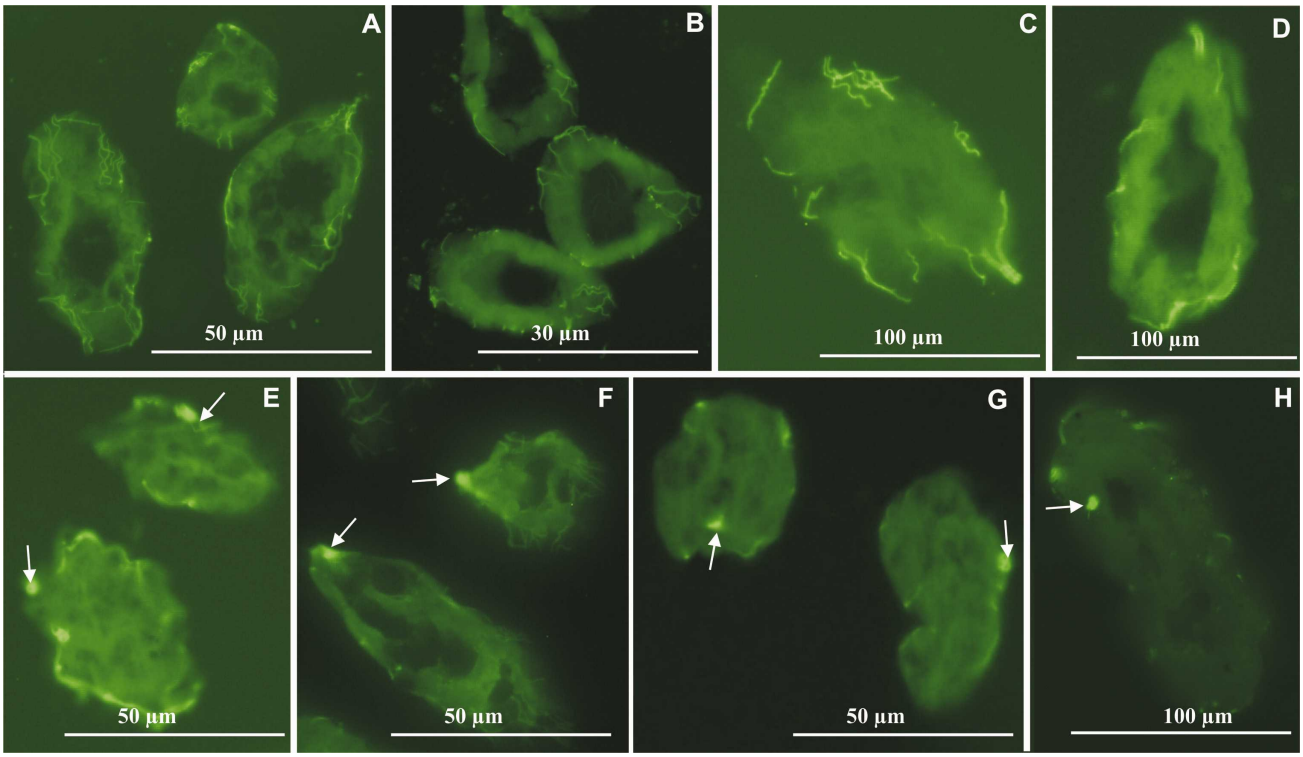

Fig. 4. Histological sections of the Malpighian tubules of honeybees subjected to a Tunel reaction in control or imidacloprid exposed groups. **A–D**: The Malpighian tubule of a bee from the control groups, featuring no positive nuclei to the reaction. A: control group without solvent after 3 days. B: control group with solvent after 5 days. C: control group without solvent after 7 days. D: control group without solvent after 10 days. **E–H**: Malpighian tubules of exposed bees showing the presence of positive nuclei (arrow). E: LD_{50}/100 group after 7 days. F: LD_{50}/50 group after 1 day. F: LD_{50}/100 group after 10 days. G: LD_{50}/10 group after 7 days. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

normal body metabolism (Cruz-Landim, 1998) and show the cytotoxic effect of the insecticide.

The loss of part of the cell to the lumen of the Malphigian tubules was observed in the groups exposed to the insecticide. This characteristic was not observed in control groups and can be considered an indication of the cytotoxicity of imidacloprid for the organ.

The vacuolization of these cells from bees exposed to insecticide, as evidenced by the negative region of the cytoplasm to Xylidine Ponceau. This alteration was observed mainly in the early days of the exposure of bees to insecticide, and in the final period of exposure this characteristic was not observed. It may indicate the presence of autophagic vacuoles suggesting autophagic activity (Clarke, 1990). This indicates that the cell initially underwent an autophagic phase, which is a normal physiological process to have a turnover (recycling) of many proteins and organelles cells (Yoshimori, 2004).

The presence of pyknotic nuclei observed by HE staining, confirmed by the Feulgen reaction, was more intense in bees exposed to the insecticide. Chromatin compaction in the nucleus can result in low transcriptional activity, suggesting the final phase of the apoptotic process of cells undergoing cell death (Häcker, 2000; Silva-Zacarim et al., 2008; Wyllie, 1981).

The autophagic phase may not necessarily culminate in cell death (Lockshin and Zakeri, 2004) but if the stress situation prevails as in the present study, the cell can be completely degenerated by this process or trigger the apoptotic process of cell death (Lockshin and Zakeri, 2004).

The obtained results suggest that exposure to sublethal doses of imidacloprid induces cell death in the Malpighian tubules, which was confirmed by TUNEL assay. Although programmed cell death is related to the reorganization of tissue during metamorphosis (Gregorc et al., 2004), we found that this type of cell death may also occur in organs of insects exposed to toxic substances.

In conclusion, sublethal doses of imidacloprid can cause morphological, histochemical, and immunocytochemical alterations on the Malpighian tubules of bees. Such changes could undermine the process of excretion, the primary function of this structure, and could affect the entire physiology of bees. Toxic substances, when they are not completely eliminated, can cause intoxication of the insect and lead to impaired behavior or even death of the bee.

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