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Short Title: Inhibition of δ ALA-D by neonicotinoids and reactivation by antioxidants

Abstract

Currently, the neonicotinoid represent class of insecticides most used worldwide, and its precursor, imidacloprid, the most widely marketed. They are classified as agents of low toxicity, however there are recent reports of neonicotinoid poisoning and studies *in vitro* and *in vivo* demonstrate their toxicity as well as potential oxidizing effect. In this line, the aim of this study was to evaluate the effect of imidacloprid on the activity of hepatic δ ALA-D and the protective effect of potential antioxidants against this potential effect. Furthermore, we investigated the presence of metals in the constitution of this pesticide. We observed that the activity of hepatic δ ALA-D was significantly inhibited in the presence of imidacloprid at all concentrations tested (2, 5, 10, 20 and 40 mM) and this inhibition was dose-dependent. IC_{50} value obtained was 20.06±0.17 mM and used to evaluate the restoration of the enzymatic activity. This inhibition was completely restored by addition of DTT and partly by $ZnCl_2$, demonstrating that the inhibition occurs by oxidation of thiol groups and by displacement of the Zn (II), which can be explained by the presence of metals found also in the constitution of pesticides, which are probably contributing with the insecticide toxicity. The main endogenous antioxidant GSH (1000μM) had the best antioxidant (67%) against to the effect of imidacloprid, followed by curcumin (5μM) and resveratrol (5μM/10μM) (65 % and 61% respectively). Knowing that inhibition of the enzyme δ ALA-D results in accumulation of its substrate neurotoxic δ-ALA, further studies are needed to investigate the possible neurotoxicity induced by neonicotinoids and usability of these antioxidants as antidotes for poisoning cases neonicotinoids.

Keywords: Neonicotinoids; inhibition; δ ALA-D activity, toxicity, pesticides.

Short Abstract

Was investigated the effect of neonicotinoids on the δ ALA-D hepatic activity and this pesticide was able to inhibit the enzymatic activity. This inhibition was completely reversed by DTT and partially by $ZnCl_2$, demonstrating that occurred displacement of zinc, possibly related with metals found in its constitution. Antioxidants were able to partially reverse this inhibition. δ-ALA, enzyme substrate, is neurotoxic therefore, further studies are needed to investigate the possible neurotoxicity induced by neonicotinoids and the protective effects of antioxidants tested.

1. Introduction

The widespread use of pesticides in agriculture and health programs resulted in an increase of environmental pollution and health risks for non-target organisms, culminating in cases of acute and chronic poisoning (Abdollahi et al. 2004). Neonicoitinoides are currently the most important chemical class of insecticides marketed worldwide since the synthesis of pyrethroids. The structures of neonicotinoids resemble nicotine and act on their site of action upon the nicotinic acetylcholine receptor (nAChR), (El-Gendy et al. 2010); these compounds are classified as N-nitroguanidines (imidacloprid, thiamethoxam, dinotefuran, and clothianidin) and N-cyano-aminides (acetamiprid and thiacloprid) (Calderón-Segura et al. 2012). In 1991, Bayer CropScience has launched this new class into the market by its precursor imidacloprid [IMI, 1 - (6-cloro-3-piridilmetil)-N-nitroimidazolidin-2- ilidenoamina (Jeschke et al. 2010). Since then, the widespread development of neonicotinoids for protection of modern cultures reflects the importance of this chemical class. This new class is used for crop protection against piercing–sucking insects of cereals, vegetables, tea and cotton, and for flea control in cats and dogs (Duzguner and Erdogan 2012). Neonicotinoid pesticides represent 17% of all processed insecticides on the global market, and the class precursor imidacloprid is the most commercialized, representing 41.5% of sales (Calderón-Segura et al. 2012; Jeschke et al. 2010).

Recent results demonstrate that the biotransformation of pesticides generates reactive oxygen species and nitrogen, and that these free radicals are associated with the toxicity induced by these pesticides as they may trigger lipid peroxidation and oxidative stress (El-Gendy et al. 2010). Oxidative stress is characterized by an unbalance between the production of free radicals and antioxidant defenses. Increased production of reactive oxygen species (ROS) may result from pathological conditions and action of xenobiotics such as pesticides inducing tissue damage in several organs such as the heart, brain, kidney and liver (Dwivedi, Das, and Khanna 1998; Yu et al. 2008; El-Gendy et al. 2010). Some studies have shown that neonicotinoids induce toxicity by several mechanisms, including changes in markers of oxidative stress.(Duzguner and Erdogan 2012)

Taking into account that δ-aminolevulinate dehydratase (δ ALA-D) or porphobilinogen synthase is a metalloenzyme with thiol groups (-SH) and requires zinc ions for its activity (Jaffe 2000), this enzyme can be inhibited by substances that compete with zinc, or by substances that oxidize -SH groups. Therefore, the δ ALA-D activity can be oxidized by different soft electrophiles and metals that compete with Zn (II) in its active site. Recent studies have shown that this enzyme is a good molecular sensor for oxidative stress situations; indeed, numerous tests have shown a negative correlation between enzyme activity and the occurrence of oxidative stress (Rocha et al. 2012).

In vertebrates, liver is the main organ involved in detoxification of xenobiotics, presenting high metabolic rates and high concentrations of enzymes from the endogenous antioxidant system (Hinderer and Menzer 1976). The aim of this study was to evaluate the activity of hepatic δ ALA-D toward the presence of imidacloprid *in vitro*. Furthermore, we evaluated the protective effect of antioxidant agents such as resveratrol, curcumin, ascorbic acid and reduced glutathione (GSH) against the toxic effects caused by this pesticide. Additionally, the presence of metals not declared in the

composition of imidacloprid was analyzed, which could interfere with the enzymatic activity of δ ALA–D or promote toxicity.

2. Materials and Methods

2.1 Chemicals

Imidacloprid [1-(6-chloro-3-pyridin-3-methyl) N-nitroimidazolidin-2-ylidenamine], 70% technical grade was obtained from Bayer CropScience (Evidence®). δ-aminolevulinic acid (δ-ALA), dithiotreitol (DTT), ascorbic acid (L-3-ketothreohexuronic acid lactone), L-glutatione redeuced (GSH), resveratrol, curcumin, Bradford reagent and bovine serum albumin were purchased from Sigma (St. Louis, MO, USA). Zinc chloride ($ZnCl_2$) was purchased from Proquimios (Bangu, RJ, Brazil). All chemicals and solvents used were of analytical reagent grade quality and were used as received. Twice-deionized water was used.

2.2 Animals

The study was conducted using male adult Wistar rats weighing 270 ± 60 g, aged 6–8 weeks obtained from Fundação Estadual de Produção e Pesquisa em Saúde (FEPPS), maintained at 22±2.8ºC under a 12/12 h light/dark cycle, receiving standard food and water *ad libitum*. The animals were looked after in accordance with the ‘‘Guiding Principles in the Care and uses of Animals’’ (Olfert, Cross, and McWilliam 1993) and were approved by the local Ethics Committee of Universidade Federal do Rio Grande do Sul, N^{o}. 18427.

2.3 Tissue preparation

Rats (n=4) were sacrificed with an overdose of ketamine and xylazine anesthesia. Each liver sample was divided into equal parts of 1 g and stored at -80 °C until homogenization. Liver samples were homogenized with 50 mM Tris–Cl, pH 7.4 (1/10, w/v) and kept on ice. Homogenates were centrifuged at 2000 rpm for 10 min to yield a low-speed supernatant (S1) fraction. Freshly prepared S1 was used for δ ALA-D assays to obtain IC50 values and for enzyme activity reversibility tests.

2.4 Protein

Protein levels from supernatants were determined according to Bradford, (Bradford 1976) using bovine serum albumin as standard.

2.5 δ ALA-D activity

δ ALA-D activity was assayed by the method of Sassa (1982) (Sassa 1981) with some modifications. After a pre-incubation period, enzymatic reaction was initiated by adding the substrate (δ ALA) in the medium and incubating for 1 h at 37°C. The incubation was stopped by adding trichloroacetic acid solution (10% TCA) with 10 mM $HgCl_2$. PBG, which is formed within a fixed time, is mixed with modified Ehrlich’s reagent, and the color developed is measured photometrically (555 nm) against a blank. Results were reported as nmol PBG/mg protein/h. All experiments were performed 4 times, and 1 g of liver from each rat as used.

2.6 Inhibitory effect of imidacloprid to δ ALA-D activity and IC_{50} determination.

The effect of imidacloprid on the liver δ ALA-D activity was determined in the presence of different concentrations of pesticide (2 - 40 mM). The freshly prepared S1

was pre-incubated at 37 °C for 10 min with imidacloprid and after this time, the substrate (δ-ALA) was mixed to start the reaction. After data evaluation the IC_{50} value was determined. This concentration was utilized to study the protective effect of resveratrol, curcumin, ascorbic acid and reduced glutathione.

2.7 Effect of dithiothreitol (DTT) and zinc chloride ($ZnCl_2$)

Since δ ALA-D activity may be inhibited by compounds that oxidize the –SH groups or which remove Zn (II) from the enzyme structure. We studied the possible mechanism of imidacloprid toxicity. Thus, we verified the effect of DTT (3 mM) and zinc chloride ($ZnCl_2$) (100 mM) in restore δ ALA-D inhibition caused by imidacloprid (IC_{50}). For that, imidacloprid was pre-incubated with freshly prepared S1 of liver tissue containing DTT or $ZnCl_2$ (S1) for 10 min at 37 °C. After this time the reaction was started by the addition of substrate (δ-ALA).

2.8 Effect of resveratrol, curcumin, ascorbic acid and reduced glutathione on liver δ ALA-D activity in the presence of imidaclorprid

The protective effects of resveratrol, curcumin, ascorbic acid and GSH were studied in the presence of imidacloprid (IC_{50}).The freshly prepared (S1) hepatic tissue was pre-incubated at 37° C for 10 min with imidacloprid plus resveratrol or curcumin (0.001, 0.1, 1, 5, 10, 100 and 1000 μM), and imidacloprid plus ascorbic acid or GSH (10, 100 and 1000 μM).

2.9 Quantification of metals in imidacloprid

The metals As, Al, Cd, Co, Cr, Cu, Pb, Ni, Mn, Hg and Sr were quantified by ICP-MS (NexION 300X, PerkinElmer). For the measurement of metals, 1.0 ml of 65% nitric acid PA (redistilled) was added to 100 mg of pesticide in sterile polypropylene tube. The mixture was digested by heating at 95 °C for 4 h. The extracts were cooled at room temperature and the volume was made up to 10.0 ml with ultrapure water.

Calibration was performed using standard solutions of 1.0 $mg.L^{-1}$ (Perkin Elmer 29 and Merck Titrisol) and acidified with bidistilled nitric acid. Calibration curve concentrations ranged from 10 mg L^{-1} to 100 mg L^{-1}, and the internal standard used was Rh at a concentration of 10 mg L^{-1} for calibration. The limit of detection (LOD) was calculated using the formula LOD= 3x (SD/S) and limit of quantification (LOQ) was determined by the formula LOQ= 10x (SD/S), where SD represents the standard deviations of the readings of 10 blanks and S is the sensitivity of the analytical curve (slope). The metal concentrations in pesticide were expressed in mg L^{-1}.

2.10 Statistical analysis

Data were expressed as mean ± S.D. Statistical analysis was performed by one-way ANOVA and Bonferroni pos-test. Values of $p<0.05$ were considered statistically significant. The IC_{50} value was reported as geometric means accompanied by their 95% confidence limits. The IC_{50} value was determined by linear regression from individual experiments using GraphPad software (GraphPad software, San Diego, CA, USA).

3 Results

3.1 Inhibitory effect of imidacloprid at δ ALA-D activity and determination of IC_{50}

Hepatic δ ALA-D activity was significantly inhibited by imidacloprid at the concentrations 2, 5, 10, 20 and 40 mM (Fig. 1) and thi inhibition was dose-dependent.

IC_{50} value was 20.06±0.17 mM. Therefore, potential protective effect of antioxidants curcumin, resveratrol, acid ascorbic and GSH were performed utilizing IC_{50} value of imidacloprid.

3.2 Effect of dithiothreitol (DTT) and zinc chloride ($ZnCl_2$)

The inhibitory effect of imidacloprid (20 mM) on hepatic δ ALA-D activity was completely (100%) restored by the addition of DTT (3 mM) and partially restored (75%) by $ZnCl_2$ (100 mM) when compared to the initial enzyme activity (Fig. 2).

3.3 Effect of resveratrol, curcumin, ascorbic acid and reduced glutathione on liver δ ALA-D activity in the presence of imidacloprid

Results showed that resveratrol at 0.1, 1, 5, and 10 μM restored the enzyme activity inhibited by imidacloprid (20 mM) at 54%, 59%, 61%, 61% and 58%, respectively, when compared to the initial enzyme activity. However, resveratrol at 100 μM did not restore the inhibitory effect of imidacloprid (20 mM) on δ ALA-D activity, and at 1000 μM had an inhibitory effect on enzymatic activity, which decreased to 29% when compared to the baseline activity (Fig. 3).

Curcumin at 0.001, 0.1, 1, 5 and 10 μM restored the enzyme activity inhibited by imidacloprid (20 mM) at 55 %, 55 %, 63 %, 65 % and 58%, respectively, when compared to the initial enzyme activity, while at 100 and 1000 μM was not able to restore enzyme activity (Fig. 4).

We verified that ascorbic acid treatment at 10, 100 and 1000 μM was not able to restore the enzymatic inhibition (Fig. 5), and reduced glutathione at 100 and 1000 μM partially restored the inhibition to 53 % and 67 % (Fig. 6).

3.4 Determination of metals in imidacloprid

The concentrations of metals analyzed in commercial imidacloprid are shown in Table 1.

4. Discussion

Currently, neonicotinoids are classified by the EPA system as toxicity class II and/or class III agents, because they block a specific neuron pathway that is more abundant in insects than warm blooded animals, so the toxicity of these insecticides is more selective to insects than mammals (El-Gendy et al. 2010). However, since these insecticides affect insects by interfering with nAChRs, this suggests that these receptors may also be a target in mammals. Moreover, there are reports of neonicotinoid intoxications, in which clinical manifestations of acute intoxications included nausea, vomiting, drowsiness, disorientation, dizziness, oral and gastroesophageal erosions, hemorrhagic gastritis, productive cough, fever, leukocytosis, muscle weakness, hypothermia and convulsions (Mohamed et al. 2009; Imamura et al. 2010; David, George, and Peter 2007). Furthermore, some studies *in vitro* and *in vivo* demonstrated the toxicity of neonicotinoids. Calderón - Segura et al (Calderón-Segura et al. 2012) demonstrated that commercial neonicotinoid formulations, directly induced DNA damage reduced the viability of human lymphocytes and caused cell death. Aydin Birsen (Aydin 2011) demonstrated that treatment with neonicotinoid thiacloprid results in increased NO levels in rat polymorphonuclear leukocytes and plasma. El-Gendy et al (El-Gendy et al. 2010) showed that imidacloprid treatment to rats induced a marked increase in the hepatic lipid peroxidation. Moreover another study found changes in liver enzymes (LDH and AST), in oxidative stress markers (MDA, SOD, CAT and

GSH), induction of pro-inflammatory cytokines such as TNF-a, IL-1b, IL-6, IL-12 and IFN-c and NO levels increased in the brain and liver of imidacloprid-exposed rats (Duzguner and Erdogan 2012).

In the present study, we demonstrated for the first time that the neonicotinoid imidacloprid is able to inhibit the activity of δ ALA-D in liver tissue. This enzyme has thiol groups (-SH) in their active sites, which are essentially involved in the coordination of Zn (II) ions, and the proximity between these groups renders the enzyme easily oxidizable (Markham et al. 1993) (Farina et al. 2002). The enzyme substrate aminolevulinic acid (δ-ALA) is a pro-oxidant compound, therefore the δ ALA-D inhibition by toxic agents, such as demonstrated to imidacloprid the increased circulating δ-ALA level, which is a weak gamma-aminobutyric acid (GABA) agonist, is responsible to decrease GABA release by presynaptic inhibition and may cause neurotoxicity (Needleman 2004). Additionally, other experimental studies have shown that δ-ALA possibly presents other central nervous system effects such as induction of free radical formation, effects on the uptake and release of glutamate, inhibition of Na^+,K^+,-ATPase activity and seizures induced by glutamatergic mechanisms (Emanuelli et al. 2001). Patients affected by disorders characterized by increased levels of aminolevulinic acid, such as occurs in porphyrias, present acute attacks characterized by neurological manifestations, including seizures and psychiatric manifestations such as hysteria, anxiety and depression (Emanuelli et al. 2001).

Furthermore, knowing that δ ALA-D is directly involved in the synthesis of grouping tetrapyrroles, such as heme, the enzymatic inhibition caused by the addition of imidacloprid may interrupt or interfere with the synthesis of heme groups resulting in damage to the cell metabolism, and may also cause damage to the health of non-target organisms exposed to pesticides, by the induction of neurotoxicity and producing an increase in oxidative effects due to the pro-oxidant activity of the accumulated enzyme substrate δ ALA.

Recent studies have shown that the enzymatic activity of δ ALA-D can be used as a biomarker of pro-oxidant situations because it is extremely susceptible to oxidizing agents and must be in the reduced state to catalyze the substrate formation (Rocha et al. 2012). Several studies have shown a negative correlation between the activity of δ ALA-D and the occurrence of oxidative stress. Previous studies have shown that δ ALA-D activity was inhibited in hemodialysis patients (Valentini et al. 2008) (Roehrs et al. 2009), patients after bone marrow transplantation (Gonçalves et al. 2009) and in patients with diabetes (Fernandez-Cuartero et al. 1999). Furthermore, the activity of δ ALA-D was inhibited after exposure to other pro-oxidant situations such as hyperoxygenation (Rocha et al. 2011) and after exposure to paints, which contain a broad mixture of solvents (Moro et al. 2010). Therefore, based on previous studies which demonstrate that the enzyme is a good marker for pro-oxidant conditions and oxidative stress, we can infer that imidacloprid is a compound capable of causing oxidative stress since it was able to significantly inhibit δ ALA-D enzymatic activity.

In this line, was possible to demonstrate that the enzymatic inhibition caused by the imidacloprid was restored by the addition of dithiothreitol (DTT), which is a dithiol that possesses the ability of protecting δ ALA-D against inhibition by sulfhydryl oxidizing agents (Folmer et al. 2005). On the other hand, we verified that $ZnCl_2$ was partially able to restore δ ALA-D activity, thus we propose that the mechanism involved in the inhibitory effect of imidacloprid on δ ALA-D activity is also by zinc displacement of the enzyme structure. Metals found in the composition of the pesticide may be contributing to the direct oxidation of thiol groups -SH or interacting,

corroborating the displacement of Zn (II). Study has shown strong reactivity *in vitro* of some metals such as Pb , Hg , Cd, As and Al with –SH, causing inhibition of enzyme activity (Rocha et al. 2012), these metals were found in the constitution of imidacloprid.

We used curcumin and resveratrol as potential antioxidants to reverse the inhibition of δ ALA-D caused by imidacloprid. Curcumin (diferuoylmethane) is the most active component of turmeric, an agent derived from dried rhizomes of the plant turmeric (Curcuma longa L.), is one of the most recently studied chemopreventive compound. Resveratol (3, 5, 4 ' - tri - hydroxystilbeno) is a phytoalexin found in grapes and in foods such as peanuts, blueberries, and red wines, and along with curcumin is a polyphenolic compound. As shown in the results, both curcumin and resveratrol were effective to partially restore enzyme activity, in other words, they are able to restore enzyme activity due to their antioxidant activity, protecting the enzyme from oxidative effects of imidacloprid. On the other hand, the highest concentrations of resveratrol tested (100 and 1000 μM) were unable to restore enzyme activity. In addition, the concentration 1000 μM caused an increase in enzymatic inhibition caused by imidacloprid, demonstrating a pro-oxidant effect of resveratrol, this was not observed with the curcumin. Our findings corroborate some reports in the literature where both were recognized as potential antioxidants, because they present the ability to eliminate free radicals and raise antioxidant mechanisms (Sebastià et al. 2011; El-Azab et al. 2011; Eybl, Kotyzova, and Koutensky 2006); et al studies have shown that in high concentrations these antioxidants may present pro–oxidant effects (Stocco et al. 2012).

Moreover, we also used GSH and ascorbic acid to test its influence on enzyme activity inhibited by the pesticide. Ascorbic acid, better known as vitamin C, is widely known due to its antioxidant properties. Ascorbic acid is an excellent source of electrons and thus able to neutralize free radicals by donating these electrons, and because of its solubility in water, vitamin C promotes an antioxidant effects both inside and outside the cell and preventing free radical damage (Bendich 1990; Bindhumol, Chitra, and Mathur 2003). Ascorbic acid may scavenge peroxyl radicals and inhibit cytotoxicity induced by oxidants (Yen, Duh, and Tsai 2002). El-Gendy et al (El-Gendy et al. 2010) demonstrated that ascorbic acid promoted protective effects on the toxicity induced by imidacloprid in rats as observed by some markers of oxidative stress such as MDA, GSH , CAT , SOD and GPx. In our study, ascorbic acid was not able to restore enzyme activity inhibited by imidacloprid. However El-Gendy et al (El-Gendy et al. 2010) also showed that pre-treatment with ascorbic acid is most effective when compared to post-treatment; in this study, imidacloprid and ascorbic acid are incubated simultaneously, then probably would need a pre- incubation with ascorbic acid to obtain the protective effects of this antioxidant.

Reduced glutathione (GSH) is a non-protein thiol group widely distributed in animal tissues, and is closely linked to antioxidant cell response against the toxic effects of reactive oxygen species (Meister 1983). In the present study, GSH demonstrated its antioxidant effect when it was able to partially reverse the enzymatic activity inhibited by imidacloprid in the two highest concentrations tested (100 and 1000 μM). Other studies showed the benefits of GSH treatment, such as resulting in a net reduction in the oxidant burden at the alveolar epithelial surface in the idiopathic pulmonary fibrosis (Borok et al. 1991), attenuation of reactive oxygen species generation and decreased antioxidant defenses (Liu, Wang, and Mori 1994), attenuation of lipid peroxidation (Brzezińska-Ślebodzińska et al. 1995) and prevention of neurotoxicity induced by treatment with oxaliplatin without reducing the clinical activity of oxaliplatin (Cascinu et al. 2002).

In view of our data, this study supports the evidence that imidacloprid, an insecticide widely used and classified as being of low toxicity, induces oxidative damage since it significantly inhibited the enzymatic activity of δ ALA-D which is used as a marker of pro-oxidant situations. Moreover, the enzymatic inhibition of δ ALA-D could contribute to accumulation of its enzymatic substrate δ-ALA that may rapidly oxidize to generate ROS such as superoxide ions, hydroxyl radicals and hydroxyl peroxides generating a vicious pro-oxidant cycle (Rocha et al. 2012) and could promote neurotoxic effects (Needleman 2004). The inhibition was reversed completely after treatment with the reducing agent DTT and the partial restoration by the use of $ZnCl_2$ indicating that the enzyme inhibition does not occur only by oxidation of thiol groups, but also by Zn (II) displacement, which is possibly related to the presence of various metals in their constitution. Considering our results together with previous findings in the literature, it could be stated that this neonicotinoid, despite its classification of low toxicity, should be investigated carefully because it may cause serious damages to non-target organisms, especially in chronic exposure. Hepato-protective effects was observed in imidacloprid-induced liver toxicity after the use GSH at 1000 μM followed by curcumin at 5 μM, appeared as the second most powerful antioxidant and resveratrol at 5 and 10 μM concentrations. Therefore, it could be suggested that these compounds could contribute to prevention of inhibition caused by imidacloprid on δ ALA-D activity.

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Conflict of interest

The authors declared no conflicts of interest.

References

Abdollahi, M., A. Ranjbar, S. Shadnia, S. Nikfar, and A. Rezaie. 2004. Pesticides and oxidative stress: a review. *Med Sci Monit* 10 (6):RA141-7.

Aydin, Birsen. 2011. Effects of thiacloprid, deltamethrin and their combination on oxidative stress in lymphoid organs, polymorphonuclear leukocytes and plasma of rats. *Pesticide Biochemistry and Physiology* 100 (2):165-171.

Bendich, Adrianne. 1990. Antioxidant micronutrients and immune responses. *Annals of the New York Academy of Sciences* 587.

Bindhumol, V, KC Chitra, and PP Mathur. 2003. Bisphenol A induces reactive oxygen species generation in the liver of male rats. *Toxicology* 188 (2):117-124.

Borok, Zea, R Buhl, RC Hubbard, KJ Holroyd, JH Roum, DB Czerski, RG Crystal, GJ Grimes, AD Bokser, and AM Cantin. 1991. Effect of glutathione aerosol on oxidant-antioxidant imbalance in idiopathic pulmonary fibrosis. *The Lancet* 338 (8761):215-216.

Bradford, Marion M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry* 72 (1):248-254.

Brzezińska-Ślebodzińska, E, AB Ślebodziński, B Pietras, and G Wieczorek. 1995. Antioxidant effect of vitamin E and glutathione on lipid peroxidation in boar semen plasma. *Biological trace element research* 47 (1-3):69-74.

Calderón-Segura, María Elena, Sandra Gómez-Arroyo, Rafael Villalobos-Pietrini, Carmen Martínez-Valenzuela, Yolanda Carbajal-López, María del Carmen Calderón-Ezquerro, Josefina Cortés-Eslava, Rocío García-Martínez, Diana Flores-Ramírez, and María Isabel Rodríguez-Romero. 2012. Evaluation of Genotoxic and Cytotoxic Effects in Human Peripheral Blood Lymphocytes Exposed In Vitro to Neonicotinoid Insecticides News. *Journal of toxicology* 2012.

Cascinu, Stefano, Vincenzo Catalano, Luigi Cordella, Roberto Labianca, Paolo Giordani, Anna Maria Baldelli, Giordano D Beretta, Emilio Ubiali, and Giuseppina Catalano. 2002. Neuroprotective effect of reduced glutathione on oxaliplatin-based chemotherapy in advanced colorectal cancer: a randomized, double-blind, placebo-controlled trial. *Journal of Clinical Oncology* 20 (16):3478-3483.

David, Deepu, Ige Abraham George, and John Victor Peter. 2007. Toxicology of the newer neonicotinoid insecticides: Imidacloprid poisoning in a human*. *Clinical toxicology* 45 (5):485-486.

Duzguner, Vesile, and Suat Erdogan. 2012. Chronic exposure to imidacloprid induces inflammation and oxidative stress in the liver & central nervous system of rats. *Pesticide Biochemistry and Physiology*.

Dwivedi, PD, Mukul Das, and SK Khanna. 1998. Role of Cytochrome< i> P</i>-450 in Quinalphos Toxicity: Effect on Hepatic and Brain Antioxidant Enzymes in RatsITRC Communication No. 1965. *Food and Chemical Toxicology* 36 (5):437-444.

El-Azab, Mona, Hailemichael Hishe, Yasser Moustafa, and El-Sayed El-Awady. 2011. Anti-angiogenic effect of resveratrol or curcumin in Ehrlich ascites carcinoma-bearing mice. *European journal of pharmacology* 652 (1):7-14.

El-Gendy, Kawther S, Nagat M Aly, Fatma H Mahmoud, Anter Kenawy, and Abdel Khalek H El-Sebae. 2010. The role of vitamin C as antioxidant in protection of oxidative stress induced by imidacloprid. *Food and Chemical Toxicology* 48 (1):215-221.

Emanuelli, Tatiana, Fernanda W Pagel, Letícia B Alves, Andrea Regner, and Diogo O Souza. 2001. Inhibition of adenylate cyclase activity by 5-aminolevulinic acid in rat and human brain. *Neurochemistry international* 38 (3):213-218.

Eybl, Vladislav, Dana Kotyzova, and Jaroslav Koutensky. 2006. Comparative study of natural antioxidants–curcumin, resveratrol and melatonin–in cadmium-induced oxidative damage in mice. *Toxicology* 225 (2):150-156.

Farina, M, NBV Barbosa, CW Nogueira, V Folmer, G Zeni, LH Andrade, AL Braga, and JBT Rocha. 2002. Reaction of diphenyl diselenide with hydrogen peroxide and inhibition of delta-aminolevulinate dehydratase from rat liver and cucumber leaves. *Brazilian journal of medical and biological research* 35 (6):623-631.

Fernandez-Cuartero, B, JL Rebollar, A Batlle, and R Enriquez de Salamanca. 1999. Delta aminolevulinate dehydratase (ALA-D) activity in human and experimental

diabetes mellitus. *The International Journal of Biochemistry & Cell Biology* 31 (3):479-488.

Folmer, Vanderlei, Rodrigo Cordeiro Bolzan, Marcelo Farina, Gilson Zeni, Cristina Wayne Nogueira, Tatiana Emanuelli, and João Batista Teixeira Rocha. 2005. Mechanism of delta-aminolevulinate dehydratase inhibition by phenyl selenoacetylene involves its conversion to diphenyl diselenide. *Toxicology* 206 (3):403-411.

Gonçalves, Thissiane L, Dalila M Benvegnú, Gabriela Bonfanti, Andressa V Frediani, and João BT Rocha. 2009. δ-ALA-D activity is a reliable marker for oxidative stress in bone marrow transplant patients. *BMC cancer* 9 (1):138.

Hinderer, Robert K, and Robert E Menzer. 1976. Comparative enzyme activities and cytochrome P-450 levels of some rat tissues with respect to their metabolism of several pesticides. *Pesticide Biochemistry and Physiology* 6 (2):148-160.

Imamura, Tomonori, Youichi Yanagawa, Kahoko Nishikawa, Naoto Matsumoto, and Toshihisa Sakamoto. 2010. Two cases of acute poisoning with acetamiprid in humans. *Clinical toxicology* 48 (8):851-853.

Jaffe, Eileen K. 2000. The porphobilinogen synthase family of metalloenzymes. *Acta Crystallographica Section D: Biological Crystallography* 56 (2):115-128.

Jeschke, Peter, Ralf Nauen, Michael Schindler, and Alfred Elbert. 2010. Overview of the status and global strategy for neonicotinoids. *Journal of Agricultural and Food Chemistry* 59 (7):2897-2908.

Liu, Jiankang, Xiaoyan Wang, and Akitane Mori. 1994. Immobilization stress-induced antioxidant defense changes in rat plasma: effect of treatment with reduced glutathione. *International Journal of Biochemistry* 26 (4):511-517.

Markham, George D, Cynthia B Myers, Kenneth A Harris, Marina Volin, and Eileen K Jaffe. 1993. Spatial proximity and sequence localization of the reactive sulfhydryls of porphobilinogen synthase. *Protein Science* 2 (1):71-79.

Meister, Alton. 1983. Selective modification of glutathione metabolism. *Science* 220 (4596):472-477.

Mohamed, Fahim, Indika Gawarammana, Thomas A Robertson, Michael S Roberts, Chathura Palangasinghe, Shukry Zawahir, Shaluka Jayamanne, Jaganathan Kandasamy, Michael Eddleston, and Nick A Buckley. 2009. Acute human self-poisoning with imidacloprid compound: a neonicotinoid insecticide. *PLoS ONE* 4 (4):e5127.

Moro, Angela M, Mariele Charão, Natália Brucker, Rachel Bulcão, Fernando Freitas, Gilian Guerreiro, Marília Baierle, Sabrina Nascimento, Fernanda Waechter, and Vânia Hirakata. 2010. Effects of low-level exposure to xenobiotics present in paints on oxidative stress in workers. *Science of the Total Environment* 408 (20):4461-4467.

Needleman, Herbert. 2004. Lead poisoning. *Annu. Rev. Med.* 55:209-222.

Olfert, ED, BM Cross, and AA McWilliam. 1993. Canadian Council on Animal Care—guide to the care and use of experimental animals, vol. 1. *Brada Printing Services, Ottawa, ON*.

Rocha, J. B., N. M. Heinzmann Bulow, E. F. Correa, C. Scholze, C. W. Nogueira, and N. B. Barbosa. 2011. Dexmedetomidine protects blood delta-aminolevulinate dehydratase from inactivation caused by hyperoxygenation in total intravenous anesthesia. *Hum Exp Toxicol* 30 (4):289-95.

Rocha, Joao BT, Rogerio A Saraiva, Solange C Garcia, Fernanda S Gravina, and Cristina W Nogueira. 2012. Aminolevulinate dehydratase (δ-ALA-D) as marker

protein of intoxication with metals and other pro-oxidant situations. *Toxicology Research* 1 (2):85-102.

Roehrs, Miguel, Juliana Valentini, Rachel Bulcão, José Cláudio Moreira, Hanz Biesalski, Renata P Limberger, Tilman Grune, and Solange Cristina Garcia. 2009. The plasma retinol levels as pro-oxidant/oxidant agents in haemodialysis patients. *Nephrology Dialysis Transplantation* 24 (7):2212-2218.

Sassa, S. 1981. Delta-aminolevulinic acid dehydratase assay. *Enzyme* 28 (2-3):133-145.

Sebastià, Natividad, Alegria Montoro, Amparo Montoro, Miguel Almonacid, Juan Ignacio Villaescusa, José Cervera, Esperanza Such, Ma Angeles Silla, and Jose Miguel Soriano. 2011. Assessment in vitro of radioprotective efficacy of curcumin and resveratrol. *Radiation Measurements* 46 (9):962-966.

Stocco, Bianca, Karina Toledo, Mirian Salvador, Michele Paulo, Natália Koyama, and Maria Regina Torqueti Toloi. 2012. Dose-dependent effect of resveratrol on bladder cancer cells: chemoprevention and oxidative stress. *Maturitas* 72 (1):72-78.

Valentini, Juliana, Denise Grotto, Clóvis Paniz, Miguel Roehrs, Geni Burg, and Solange C Garcia. 2008. The influence of the hemodialysis treatment time under oxidative stress biomarkers in chronic renal failure patients. *Biomedicine & Pharmacotherapy* 62 (6):378-382.

Yen, Gow-Chin, Pin-Der Duh, and Hui-Ling Tsai. 2002. Antioxidant and pro-oxidant properties of ascorbic acid and gallic acid. *Food Chemistry* 79 (3):307-313.

Yu, Fu, Ziren Wang, Bao Ju, Yongqiang Wang, Jing Wang, and Decheng Bai. 2008. Apoptotic effect of organophosphorus insecticide chlorpyrifos on mouse retina< i> in vivo</i> via oxidative stress and protection of combination of vitamins C and E. *Experimental and Toxicologic Pathology* 59 (6):415-423.

Table 1. Concentration of metals presents in imidacloprid

Metals	Concentration ($mg.L^{-1}$)
Aluminum	8.43
Arsenic	0.28
Cadmium	0.02
Cobalt	0.05
Copper	2.98
Chromium	2.81
Lead	0.11
Manganese	77.62
Mercury	0.11
Nickel	0.51
Strontium	3.27

Figure Captions

Figure 1. Effect of imidacloprid on δ ALA-D activity from rat liver. Data are expressed as mean ± SD, (n=4). δ ALA-D activity of control (100%) was of 13,57±0.17 (mean ± SD) nmol of porphobilinogen per mg protein per hour. (*) Denoted P<0.05 as compared with the control, considering 100 % (One-way ANOVA/ Bonferroni).

Figure 2. Effect of dithiothreitol (DTT) and $ZnCl_2$ as restoring agents δ ALA-D inhibition caused by imidacloprid (IC_{50}=20 mM). Data are expressed as mean ± SD, (n=4). (*) Denoted P<0.05 as compared with the control (100 %) (One-way ANOVA/Bonferroni). (#) Denoted P<0.05 as compared with the imidacloprid (20 mM) (One-way ANOVA/Bonferroni). The positive and negative signs mean added or not to the assay.

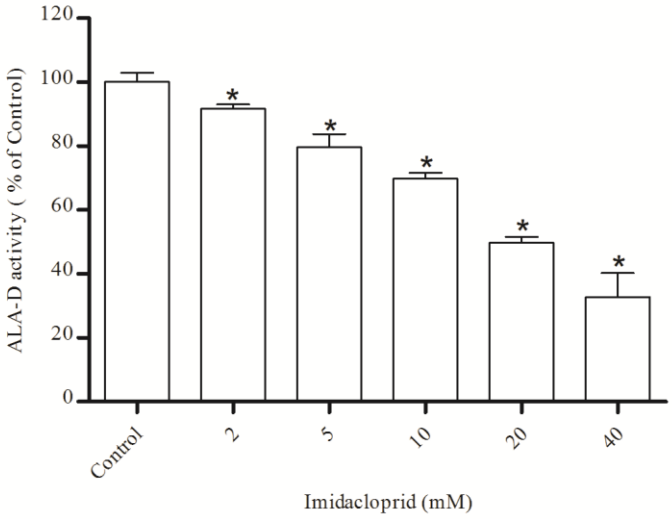
Figure 3. Effect of resveratrol (0.001, 0.1, 1, 5, 10, 100 and 1000 µM) as restoring agent for δ -ALA-D inhibition caused by imidacloprid (IC_{50}=20 mM). Data are expressed as mean ± SD, (n=4). (*) Denoted P<0.05 as compared with the control (100 %) (one-way ANOVA/Bonferroni). (#) Denoted P<0.05 as compared with the imidacloprid (20 mM) (One-way ANOVA/Bonferroni).The positive and negative signs mean added or not to the assay.

Figure 4. Effect of curcumin (0.001, 0.1, 1, 5, 10, 100 and 1000 µM) as restoring agent for δ -ALA-D inhibition caused by imidacloprid (IC_{50} = 20 mM). Data are expressed as mean ± SD, (n=4). (*) Denoted P<0.05 as compared with the control (100 %) (one-way ANOVA/Bonferroni). (#) Denoted P<0.05 as compared with the imidacloprid (20 mM) (One-way ANOVA/Bonferroni).The positive and negative signs mean added or not to the assay.

Figure 5. Effect of acid ascorbic (10, 100 and 1000 µM) as restoring agent for δ ALA-D inhibition caused by imidacloprid (IC_{50}=20 mM). Data are expressed as mean ± SD, (n=4). (*) Denoted P<0.05 as compared with the control (100 %) (One-way ANOVA/Bonferroni).The positive and negative signs mean added or not to the assay.

Figure 6. Effect of reduced glutathione (10, 100 and 1000 µM) as restoring agent for δ ALA-D inhibition caused by imidacloprid (IC_{50}=20 mM). Data are expressed as mean ± SD, (n=4). (*) Denoted P<0.05 as compared with the control (100 %) (One-way ANOVA/Bonferroni). (#) Denoted P<0.05 as compared with the imidacloprid (20 mM). The positive and negative signs mean added or not to the assay.

Figure 1


120
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ALA-D activity (% of Control)
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Control
2
5
10
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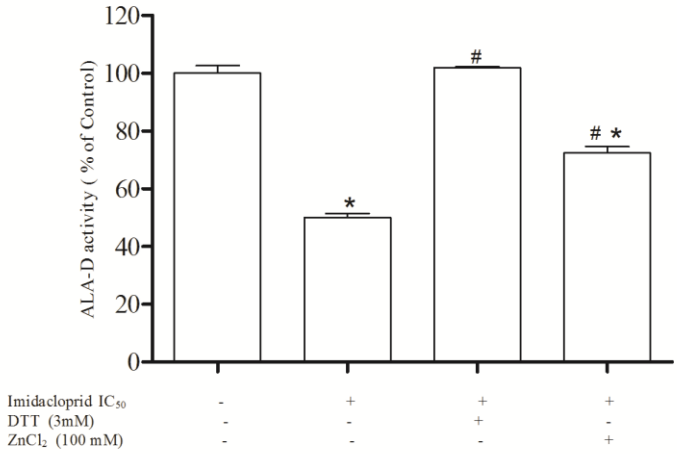
40
Imidacloprid (mM)

Figure 2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20
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ALA-D activity (% of Control)
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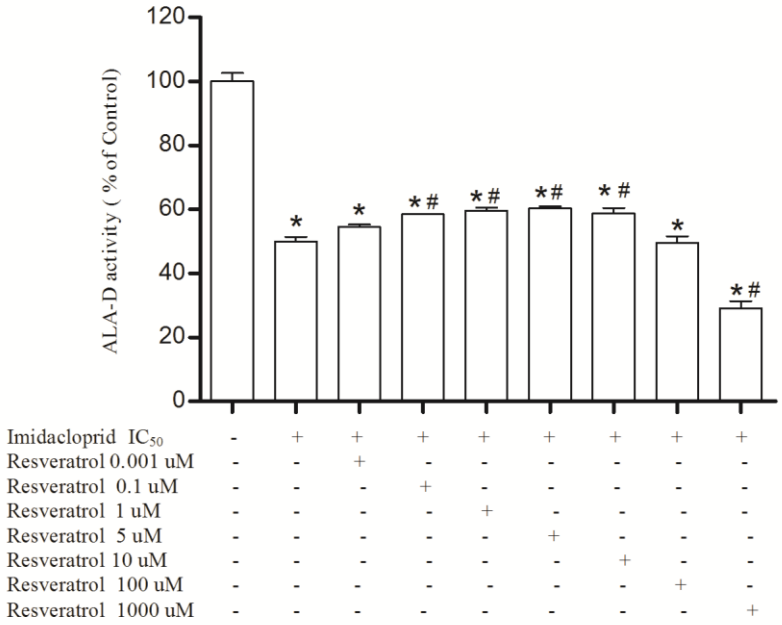
Imidacloprid IC50 - + + +
DTT (3mM) - - + -
ZnCl2 (100 mM) - - - +

Figure 3


ALA-D activity (% of Control)
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Imidacloprid IC50 - + + + + + + + +
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Resveratrol 0.1 uM - - - + - - - - -
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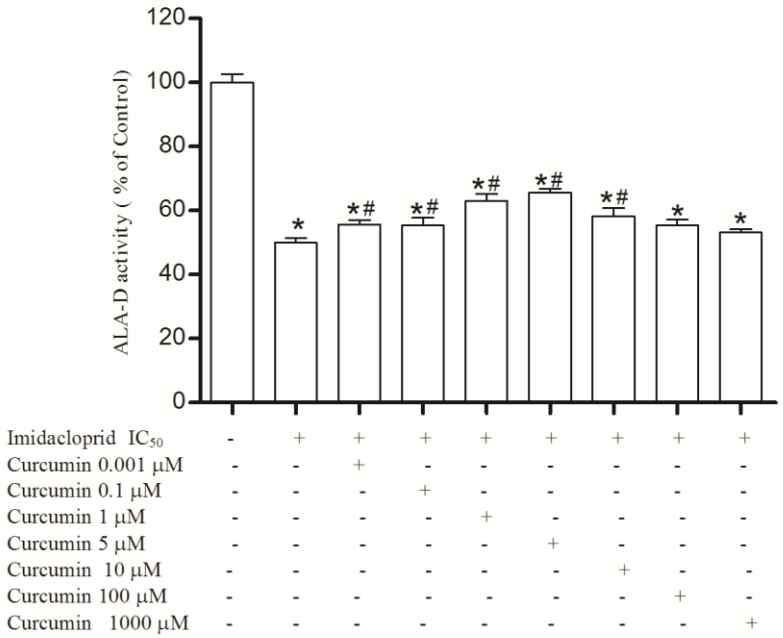
Resveratrol 5 uM - - - - - + - - -
Resveratrol 10 uM - - - - - - + - -
Resveratrol 100 uM - - - - - - - + -
Resveratrol 1000 uM - - - - - - - - +

Figure 4


ALA-D activity (% of Control)
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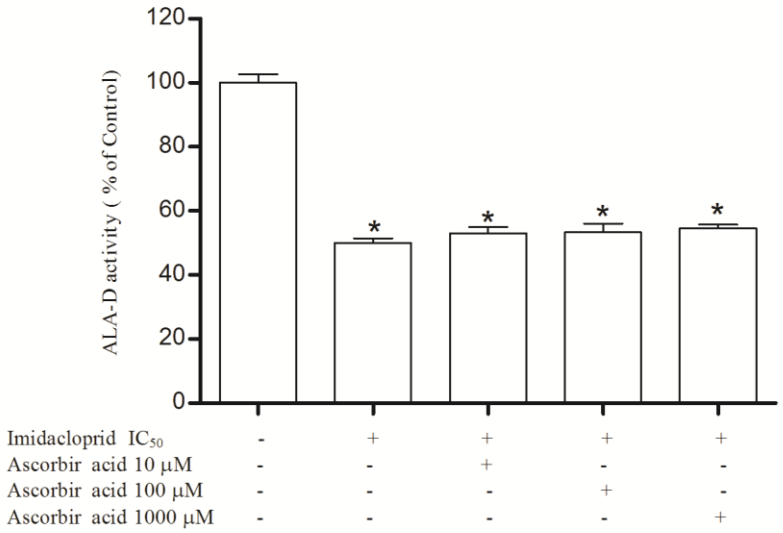
Imidacloprid IC50 - + + + + + + + +
Curcumin 0.001 µM - - + - - - - - -
Curcumin 0.1 µM - - - + - - - - -
Curcumin 1 µM - - - - + - - - -
Curcumin 5 µM - - - - - + - - -
Curcumin 10 µM - - - - - - + - -
Curcumin 100 µM - - - - - - - + -
Curcumin 1000 µM - - - - - - - - +

Figure 5


ALA-D activity (% of Control)
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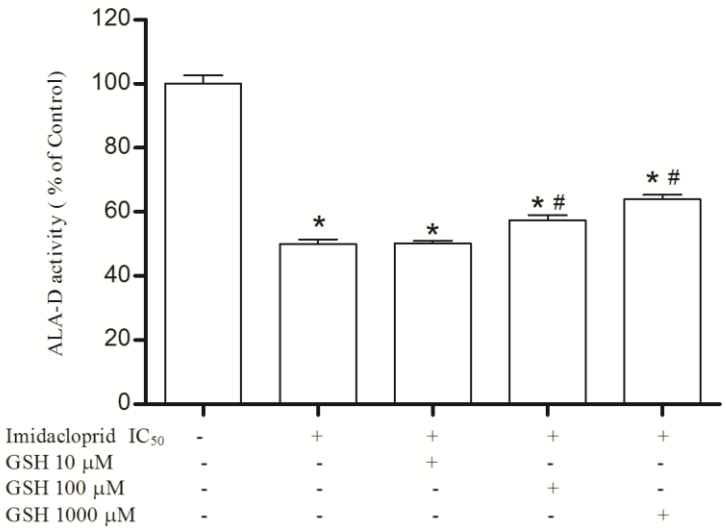
Imidacloprid IC50 - + + + +
Ascorbir acid 10 µM - - + - -
Ascorbir acid 100 µM - - - + -
Ascorbir acid 1000 µM - - - - +

Figure 6


ALA-D activity (% of Control)
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Imidacloprid IC50 - + + + +
GSH 10 µM - - + - -
GSH 100 µM - - - + -
GSH 1000 µM - - - - +

Author Guidelines

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Book
Magnusson B, Kligman AM. 1970. *Allergic Contact Dermatitis in the Guinea Pig: Identification of Contact Allergens.* Charles C. Thomas: Springfield

Chapter in Book
Luthy J, Benn M. 1980. Cyanoepithioalkanes: some chemical and toxicological studies. In *Natural Sulfur Compounds: Novel Biochemical and Structural Aspects* , Cavillini D, Gaull GE, Zappia V (eds). Plenum Press: New York; 381-389

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Review hypothesis or theoretical papers are also welcomed. These should be brief analyses of topical subjects, new proposed mechanisms or balanced discussion of controversies/debates in toxicological science and be approximately 3500 words.

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