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Mechanisms of Imidacloprid-Induced Alteration of Hypothalamic-Pituitary-Adrenal (HPA) Axis after Subchronic Exposure in Male Rats

Alya Annabi*, Ines El-Bini Dhouib, Houssem Dkhili, Yassine Bdiri, Ines Rejeb, Najoua Gharbi, Saloua El-Fazâa, Mohamed Montassar Lasram
Department of Biology, Faculty of Sciences, Laboratory of Aggression Physiology and Endocrine Metabolic Studies, Tunis, Tunisia.

***Correspondence:** alya_annabi@yahoo.fr

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Abstract
Imidacloprid (IMI) is known to target the nicotinic acetylcholine receptors (nAChRs) in insects, and potentially in mammals. However, IMI toxicity on mammalian tissues has not been adequately evaluated. The aim of the present study was to examine whether IMI induced functional impairment in hypthalamic-pituitary-adrenal (HPA) axis tissues. An oral exposure of 40 mg IMI/kg for 28 days in male rats caused a significant increase in malondialdehyde (MDA) level. The antioxidant catalase, superoxide dismutase, and glutathione S-transferase showed various alterations following administration, but a significantly depleted thiol (SH) groups was only recorded in hypothalamic tissues. The increase in the relative weight of adrenal glands and the increased adrenal cholesterol and plasma adrenocorticotropic hormone (ACTH) levels are indicative of general adaptation syndrome. The hypothalamic and pituitary acetylcholinesterase activity and calcium level were significantly increased, highlighting the alteration of cholinergic transmission. In conclusion, the findings obtained show that chronic exposure to IMI may alter biochemical processes of HPA axis.

Keywords: Imidacloprid; HPA axis; Oxidative stress; Cholinergic transmission; Rat.

1. INTRODUCTION

Pesticides are a broad group of heterogeneous chemicals that have a significant public health benefit by increasing food production productivity and decreasing food-borne and vector-borne diseases. However, they are found to affect nontarget organism, including humans, depending on the agent and the exposure (Duzguner and Erdogan, 2010).

Imidacloprid (IMI; N-{1-[(6-chloro-3-pyridyl)methyl]-4,5-dihydroimidazol-2-yl}nitramide), a chloronicotynl neonicotinoid compound, is a member of a new class of systemic insecticide and is the most important representative of this group. It was introduced into commercial use only in the last decade (Shadnia and Moghaddam, 2008) and is increasingly used around the world to control insects and pests and for seed treatment. Despite its use in agriculture, it is also used in veterinary medicines for flea control in cats and dogs (Duzguner and Erdogan, 2010). Global increasing trend for use of IMI is due to its low soil persistence and high insecticidal activity at very low application rates (Council Directive 91/414/EEC) (Kapoor *et al.*, 2014).

Insecticidal activity is attributed to stimulation of postsynaptic nicotinic acetylcholine (ACh) receptors (nAChRs) in insects, with low toxicity to mammals due to IMI's relatively low activity for nAChR subtypes in vertebrates and poor penetration of the blood–brain barrier (Seifert, 2014). Indeed, its selective toxicity results from its high affinity to insects' nAChR compared to mammals (Tomizawa and Casida, 2003; Zhang *et al.*, 2000).

It is known that different classes of pesticides induce oxidative stress, which may contribute to the toxicity of these xenobiotics. Indeed, experimental studies have demonstrated that IMI can induce hepatotoxicity and nephrotoxicity at a dose much lower than LD_{50} in mice (Arfat *et al.*, 2014).

Duzguner and Erdogan (2012) have shown that both acute and chronic IMI exposure cause oxidative stress and inflammation by altering antioxidant systems and inducing pro-inflammatory cytokine production in the liver and central nervous system of nontarget organisms.

Furthermore, *in vivo* and *in vitro* studies (John *et al.*, 2001; Singh *et al.*, 2006; Thapar *et al.*, 2002) indicate that antioxidant enzymes are altered under the influence of pesticides. Thus, oxidative stress and DNA damage have been proposed as the link between pesticide exposure and human pathologies such as cancer, neurological diseases, and endocrine perturbations.

On the other hand, according to *in vivo* toxicology studies, the order of endocrine organ toxicity by frequency of reported effects is: adrenal gland > testis > thyroid > ovary > pancreas > pituitary gland > parathyroid glands, with the adrenal cortex, rather than the medulla, being the most frequent site of toxicity within the adrenal gland (Colby and Longhurst, 1992; Harvey *et al.*, 2007). The high content of cytochrome P-450 enzymes in the adrenal cortex together with its remarkable tendency to accumulate hydrophobic substances, probably contributes to the extraordinary vulnerability of this endocrine gland to a high number of xenobiotics (Harvey *et al.*, 2007; Hinson and Raven, 2006).

Indeed, the functional role of the adrenal gland is very important in health, metabolism, development, and endocrine and immune systems activity (Rosol *et al.*, 2013).

Since IMI is now being considered for replacement of other existing pesticides, therefore the relative risk and benefits of this insecticide must be compared to the existing pesticides. In fact, IMI continues to be a human health concern due to its worldwide use and documented occupational and environmental exposure (I-Jeng *et al.*, 2010; Proença *et al.*, 2005; Wen *et al.*, 2001).

There have not been any studies on the effects of IMI on oxidative stress in the hypothalamic-pituitary-adrenal (HPA) axis tissues. Therefore, the aim of the present study was to examine whether IMI induced oxidative damage in HPA tissues.

2. MATERIALS AND METHODS

2.1. Chemicals

The commercial product of IMI (Nuprid 200SL, 200 g/l) used in this study was purchased from the local market in Tunis, Tunisia. All other chemicals were purchased from Sigma–Aldrich Co. (Germany).

2.2. Animals

Adult male Wistar rats weighing 120 ± 20 g were procured from the Tunisian Society of Pharmaceutical Industries and housed two per clean plastic cage and allowed to acclimatize in the laboratory environment. Animals were maintained in a mass air-displacement room with a 12-h light:12-h dark cycle at 24 ± 2°C with a relative humidity of 50 ± 10%. Balanced food and drinking water were given to the animals ad libitum. Animal experiments were carried out under strict compliance with the Guidelines for Ethical Control and Supervision in the Care and Use of Animals.

2.3. Determination of Optimum Dose

The choice of IMI dose was based on previous tests starting with very high doses. The chosen dose of 40 mg IMI/kg of body weight (b.w.) corresponds to an acceptable dose that did not cause any sign of toxicity until the end of the experimental period. The used dose of IMI is calculated directly from commercial grade and corresponds to 1/10th DL_{50}, since DL_{50} ranged from 424 to 475 mg/kg/b.w. in rats (Duzguner and Erdogan, 2010).

2.4. Experimental Design

A total of 24 animals were randomized into two groups of 12 rats each and were treated as below for 28 consecutive days. IMI or a vehicle (corn oil) was administered in the morning (between 09:00 and 10:00 h) to nonfasted rats.

The control group (CTR) received 1 ml of corn oil. The IMI-treated group (IMI) received a daily intragastric intubation of 40 mg/kg of IMI dissolved in a total volume of 1 ml of corn oil.

At the end of the experimental period, fasted animals were decapitated without preliminary anesthesia. HPA axis tissues were removed and homogenized in appropriate buffers for various estimations.

2.5. Adrenocorticotropic Hormone (ACTH) Determination

For the determination of the plasma ACTH concentration, we used the ACTH (Rat) ELISA kit provided by Phoenix Pharmaceuticals Inc., Germany. This kit is based on a sandwich ELISA and can be used for the determination of both rat and human ACTH (analytical sensitivity 0.22 pg/ml, intra-assay and inter-assay coefficients of variation ≤ 7.1%).

2.6. Assessment of Oxidative Stress

Lipid peroxidation was measured in terms of malondialdehyde (MDA) production by the method of Buege and Aust (1978). Total thiols content (SH) was measured by the method of Miao-Lin (1994). Catalase (CAT) activity was assayed by the method as described by Aebi (1984). The activity of superoxide dismutase (SOD) was estimated according to Misra and Fridovich (1972). Glutathione S-transferase (GST) activity was assayed as per the method described by Habig *et al.* (1974). The protein content of HPA tissues, supernatants, was estimated by the method of Biuret (Gornall *et al.*, 1949) using bovine serum albumin (BSA) as standard.

2.7. Acetylcholinesterase (AChE) Activity

AChE activity was assayed in hypothalamic and pituitary homogenates by the method of Ellman, using acetylthiocholine iodide (ATCi) as a substrate. The rate of hydrolysis of acetylthiocholine was measured at 405 nm by the reaction of thiocholine with dithiobisnitrobenzoic acid (DTNB) to give the yellow 5-thio-2-nitrobenzoate anion, which was measured with spectrophotometer. The enzyme activity was expressed as nmole of substrate hydrolyzed/min/mg protein (Ellman *et al.*, 1961).

2.8. Total Cholesterol

Adrenal total cholesterol concentration was measured by the commercial kit provided by Randox (Randox Laboratories, United Kingdom).

2.9. Calcium Level

Hypothalamic, pituitary, and adrenal calcium (Ca^{2+}) were measured by spectrophotometer according to the method of Stern and Lewis (1957). Results are expressed in mg/g tissue.

2.10. Statistical Analysis

All results were expressed as mean ± standard deviation. Comparisons between the groups were performed by one-way ANOVA followed by Student's *t*-test. Differences were considered significant at $p < 0.05$.

3. RESULTS

Death was not observed in any of the experimental groups during the experimental period. Also, no clinical signs of IMI poisoning (salivation, lacrimation, diarrhea, convulsion, paralysis) were observed among treated rats.

3.1. Adrenal Gland-Related Parameters

The values of absolute and relative adrenal weights are presented in Figure 1. There was only a significant ($p < 0.01$) increase in relative adrenal weight of IMI-treated animals when compared to controls.

Figure 1 also shows that IMI administration increased significantly ($p < 0.01$) adrenal cholesterol and plasma ACTH levels in treated rats when compared to controls.

3.2. Oxidative Stress Parameters

The results in Table 1 show that the level of lipid peroxidation, as indicated by MDA formation, was markedly increased by 34.78, 34.55, and 271.4%, respectively, in hypothalamic, pituitary ($p < 0.05$), and adrenal ($p < 0.01$) tissues when IMI-treated animals were compared to controls.

Figure 1: Effect of exposure to imidacloprid on plasma ACTH, adrenal cholesterol levels, and adrenal absolute and relative weights in male rats orally exposed to 40 mg IMI/kg for 28 days.

CTR: Control group; IMI: Imidacloprid-treated group; ACTH: Adrenocorticotropic hormone; b.w.: body weight. Values are mean ± standard deviation for groups of $n = 12$ rats. Statistically significant differences are indicated *$p < 0.05$; **$p < 0.01$ compared with CTR.

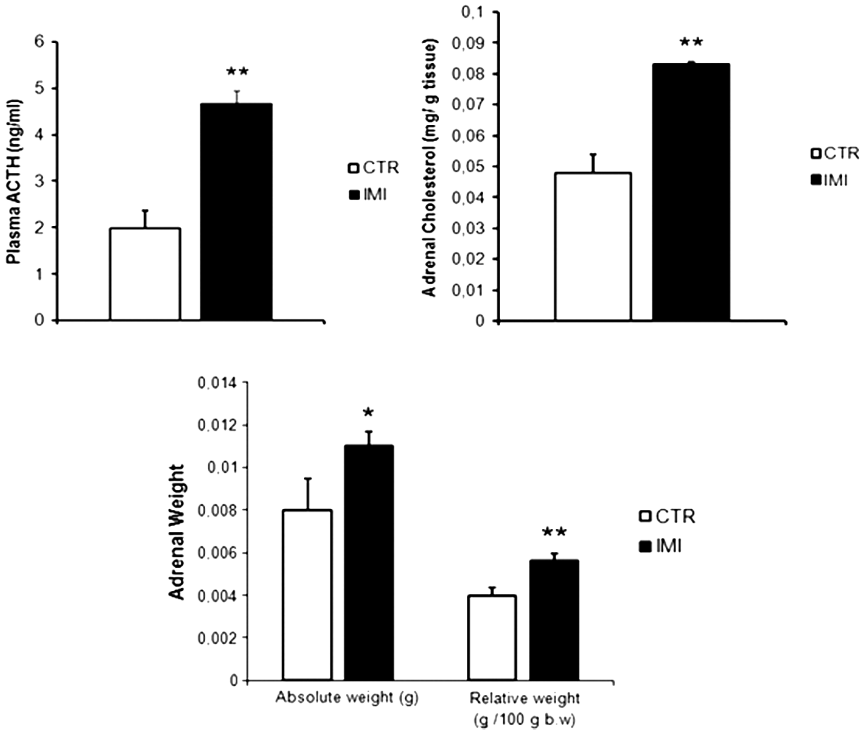


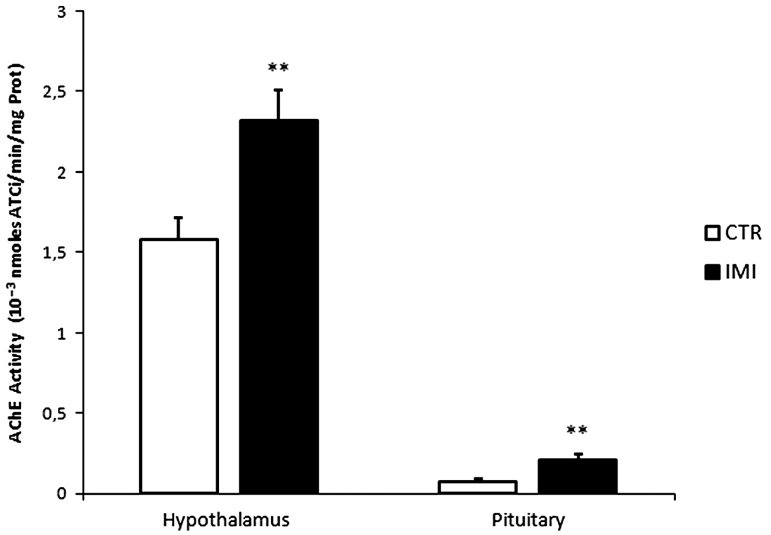
Table 1: Malondialdehyde, thiols, total proteins levels, and antioxidant enzymes activities in hypothalamic, pituitary, and adrenal tissues of male rats orally exposed to 40 mg IMI/kg for 28 days.

	MDA (A)	SOD (B)	CAT (C)	SH (D)	GST (E)	Proteins (F)
Hypothalamus						
CTR	0.23 ± 0.05	4.71 ± 0.82	0.23 ± 0.03	0.026 ± 0.004	0.0112 ± 0.0007	14.86 ± 1.24
IMI	0.31 ± 0.09[a]	3.28 ± 0.58	0.14 ± 0.02[a]	0.010 ± 0.001[b]	0.0147 ± 0.001[a]	10.04 ± 1.46[a]
Pituitary						
CTR	0.55 ± 0.08	1.06 ± 0.2	0.064 ± 0.01	0.023 ± 0.001	0.0193 ± 0.002	4.84 ± 0.67
IMI	0.74 ± 0.08[a]	2.1 ± 0.17[b]	0.136 ± 0.01[b]	0.023 ± 0.002	0.0152 ± 0.0003	6.64 ± 0.35[a]
Adrenal						
CTR	0.07 ± 0.001	1.02 ± 0.06	0.155 ± 0.03	0.021 ± 0.006	0.216 ± 0.02	5.25 ± 0.71
IMI	0.26 ± 0.04[b]	7.51 ± 2.38[b]	0.460 ± 0.14[a]	0.021 ± 0.003	0.078 ± 0.02[b]	7.3 ± 1.82[a]

A = nmoles/mg proteins; B = nmoles/min/mg proteins; C = nmoles of H_2O_2 consumed/min/mg proteins; D = mM; E = nmoles of CDNB-GSH conjugate formed/min/mg protein; F = mg/ml. Values are mean ± standard deviation for groups of $n = 8$-12 rats. Statistically significant differences are indicated [a]$p < 0.05$; [b]$p < 0.01$ compared with CTR.

Figure 2: Acetylcholinesterase activity in the hypothalamic and pituitary tissues of male rats orally exposed to 40 mg IMI/kg for 28 days.

CTR: Control group; IMI: Imidacloprid-treated group; AChE: Acetylcholinesterase; ATCi: Acetylthiocholine iodide. Values are mean ± standard deviation for groups of $n = 12$ rats. Statistically significant differences are indicated **$p < 0.01$ compared with CTR.



SOD activity was also increased by 98.11 and 636.3%, respectively, in pituitary and adrenal ($p < 0.01$) tissues, though unchanged in hypothalamus. However, hypothalamic CAT activity was decreased ($p < 0.05$) by 39.13% and showed a marked increase in both pituitary ($p < 0.01$) and adrenal ($p < 0.05$) tissues by 112.5 and 196.77%, respectively. Furthermore, oral administration of IMI to rats for 28 days resulted in perturbation of GST activity in only hypothalamic and adrenal tissues when compared to controls. Indeed, an increase by 31.25% and a decrease by 63.89% were respectively recorded.

The total SH level showed a significant decrease ($p < 0.05$) by IMI exposure only in the hypothalamic tissue (61.54%) in comparison with controls.

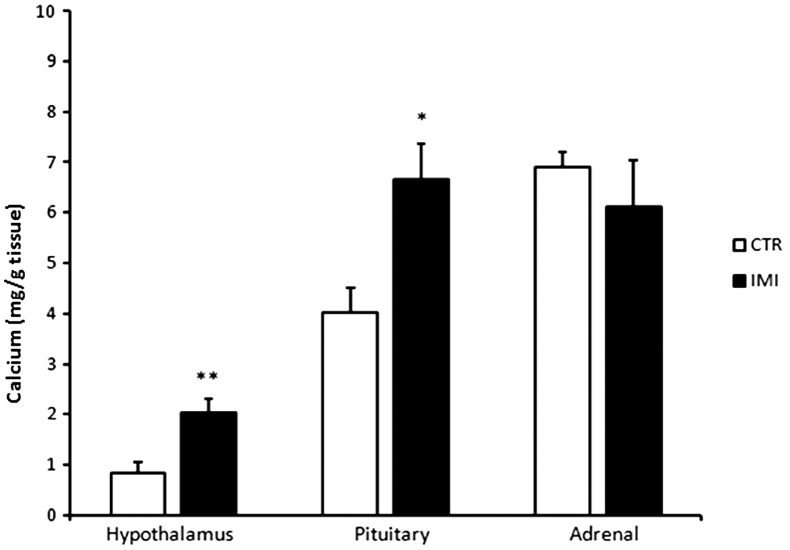
The data indicated also a marked decrease ($p < 0.05$) in hypothalamic protein level by 32.44%; yet a net increase ($p < 0.05$) in pituitary (35.79%) and adrenal (39.05%) protein levels was recorded when IMI group was compared to controls.

3.3. AChE Activity

The activity of AChE in hypothalamic and pituitary tissues following IMI treatment for 28 days is presented in Figure 2. The results show a significant ($p < 0.01$) increase in the enzyme activity by 46.84 and 197.18%, respectively, when compared to controls.

Figure 3: Calcium level in hypothalamus, pituitary, and adrenal glands of male rats orally exposed to 40 mg IMI/kg for 28 days.

CTR: Control group; IMI: Imidacloprid-treated group. Values are mean ± standard deviation for groups of $n = 12$ rats. Statistically significant differences are indicated $*p < 0.05$; $**p < 0.01$ compared with CTR.



3.4. Calcium Level in HPA Axis Tissues

Figure 3 illustrates the changes produced by IMI exposure on total calcium level. Results show a significant increase in hypothalamic ($p < 0.01$) and pituitary ($p < 0.05$) calcium level when no change was recorded in adrenal calcium level in IMI-treated rats compared with the corresponding control values.

4. DISCUSSION

Pesticides are designed to interfere with living species and are necessarily toxic. Despite the increased attention to the ecotoxicological effects of neonicotinoids, there remains a lack of sufficient information on their toxicodynamics and, in particular, their negative effects in mammals. Neonicotinoids, such as IMI, are characterized by their high potency against sucking insects and many other pests, combined with their relatively low mammalian toxicity (Colby and Longhurst, 1992).

Physiologic disorders are usually associated with modulation of HPA axis activity that is a vital regulator of homeostasis in vertebrates (Johren *et al.*, 2007), controlling the primary neuroendocrine response of the body to a challenge from the environment.

Stress-induced activation of the HPA axis is associated with the release of corticotrophin releasing hormone (CRH) and vasopressin (VP), the principal regulators of anterior pituitary ACTH secretion into the hypophyseal portal system (Bornstein and Chrousos, 1999). In hierarchical response, ACTH acts at the adrenal cortex (as the end organ of the stress system) to stimulate corticosterone secretion, the major glucocorticoid in rats (De Kloet *et al.*, 1998). Thus, HPA axis reacts with changes that involve stress and the body develops an "adaptive" response, initially described as the "general adaptation syndrome" (Selye, 1936).

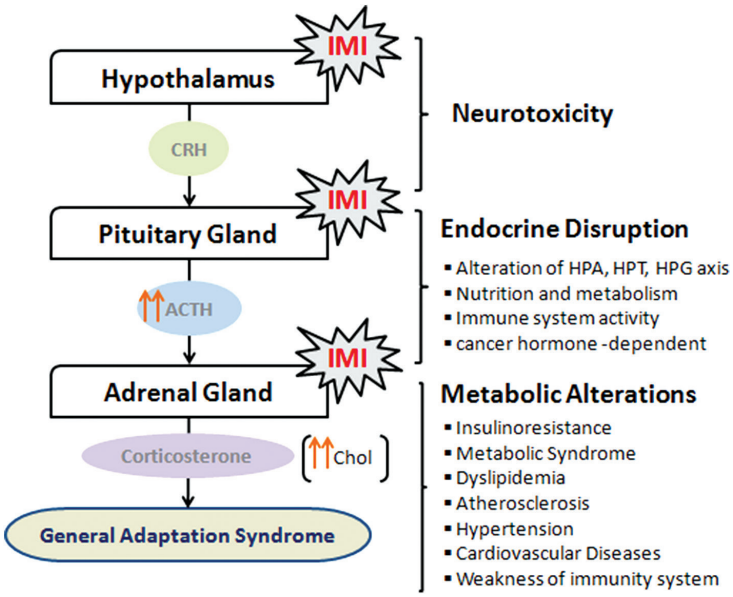
Studies investigating hormonal consequences to xenobiotics showed that organophosphorus insecticide acephate increased plasma ACTH concentration after acute exposure for 1 h to 500 mg/kg in male rats (Spassova *et al.*, 2000). Cadmium exposure during adulthood also increased plasma levels of ACTH with doses lower than 50 ppm in male rats treated for 30 days (Lafuente *et al.*, 2003).

In our study, the results showed an increase in serum ACTH concentration as an immediate consequence to IMI exposure. This may lead to the release of corticosterone following mobilization of cholesterol from lipid stores to the inner mitochondrial membrane, where it is converted to pregnenolone. Indeed, the increase in the relative weight of adrenal glands and the increased adrenal cholesterol level noted in this study are indicative of the involvement of the HPA axis in rats treated with IMI. The purpose of this activation is the release of corticosteroids, including corticosterone, from the adrenal cortex to ensure adaptive response to stress (Figure 4).

Toxicological studies of IMI are limited, but they have shown mild pathological changes in the brain, kidney, and liver of exposed rats at high doses (Bhardwaj *et al.*, 2010).

Figure 4: Imidacloprid exposure affects the hypothalamic-pituitary-adrenal axis at different levels. These alterations could be related to many diseases, such as neurological, endocrine, and metabolic pathologies.

IMI: Imidacloprid; CRH: Corticotropin releasing hormone; ACTH: Adrenocorticotropic hormone; Chol: Cholesterol; HPA: Hypothalamic-pituitary-adrenal; HPT: Hypothalamic-pituitary-thyroid; HPG: Hypothalamic-pituitary-gonadal.



The data of the present study indicate that IMI induces toxicological effects by disturbing oxidative balance in male rats exposed to 40 mg IMI/kg for 28 days. Indeed, the most common group of indices used to assess oxidative stress is that of peroxidation products of lipids, usually polyunsaturated fatty acids, which are susceptible to attack by free radicals (Dotan *et al.*, 2004). MDA is the most used end product of lipid peroxidation. Atessahin *et al.* (2005) have reported that cypermetrin leads to lipid peroxidation in brain, kidney, and blood. Lasram *et al.* (2014) showed that lipid peroxidation is increased in the liver of malathion-exposed rats. The significant increase of MDA, the index of lipid peroxidation, in HPA tissues following IMI treatment is an important evidence of lipid peroxidation in the present study.

On the other hand, cells have different mechanisms to alleviate oxidative stress and repair damaged macromolecules. The main defense is by enzymatic and nonenzymatic antioxidants, which have been shown to scavenge free radicals and reactive oxygen species (ROS).

The antioxidant enzymes CAT, SOD, and GST and the nonenzymatic antioxidant thiols SH have been shown to be significantly affected by IMI exposure in our study.

The hypothalamus has been shown to regulate some physiological processes, such as cardiovascular system, thermoregulatory responses, and defensive–aggressive and ingestive behaviors. Furthermore, of fundamental importance in the function of hypothalamus is its intimate relationship with the pituitary gland for neuronal control of endocrine secretion (Paxinos, 2004). Taking into account these functions, toxic effects in these brain areas can have very important consequences at different physiological levels. Thus, oxidative stress and lipid peroxidation have been shown to be mechanisms of IMI toxic activity on HPA axis.

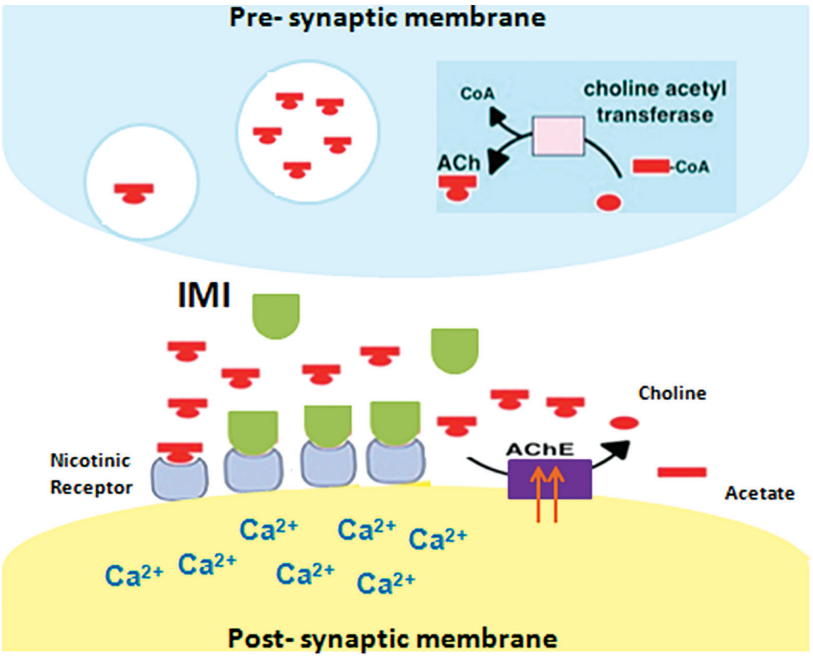
Worldwide use of neonicotinoids, including IMI, and their selective affinity toward insects' neuronal nAChRs suggested that these compounds may be safe for mammalian nervous system. There have been a few studies of neonicotinoids-induced toxicity in the nervous systems of mammals, and these studies were conducted with only a few of the neonicotinoids, such as IMI, thiamethoxam, and clothianidin. Thus, it has been reported that neonicotinoids alter behavioral and biochemical processes related to rats' cholinergic systems (Rodrigues *et al.*, 2010). Indeed, our results show that 28 days of oral exposure to IMI (40 mg IMI/kg b.w.) in male rats has produced significant increase in hypothalamic and pituitary AChE activity, which belongs to a family of enzymatic proteins distributed in cholinergic neurons.

During neurotransmission, ACh is released from the nerve into the synaptic cleft and binds to nAChRs on the postsynaptic membrane, relaying the signal from the nerve. AChE, which is also located on the postsynaptic membrane, terminates the signal transmission by hydrolyzing ACh.

Increased AChE activity in our study is in line with reports on increased AChE activity following stress, as observed in other cellular systems as well as in *in vivo* work (Hartl *et al.*, 2011). Furthermore, the present experiment

Figure 5: Imidacloprid–acetylcholine competition on nicotinic receptors.

During neurotransmission, acetylcholine (ACh) is released from the presynaptic nerve into the synaptic cleft and binds to nicotinic receptors situated on the postsynaptic nerve membrane. Acetylcholinesterase (AChE), which is also located on the postsynaptic membrane, terminates the signal transmission by hydrolyzing ACh into choline and acetate. Imidacloprid (IMI) acts as an agonist at the nicotinic receptors and competes with Ach, which leads to its accumulation in the synaptic cleft. Then, AChE increases its activity as an adaptive response to eliminate ACh. The sustained IMI fixation on nicotinic receptors is responsible for the hyperstimulation of these receptors, which leads to a significant increase of intracellular calcium (Ca^{2+}) level.



demonstrates that IMI causes oxidative stress in the hypothalamus and pituitary and the major consequence is the generation of ROS. It was thus noted that ROS can also directly enhance AChE activity as previously shown by the action of beta-amyloid (Melo *et al.*, 2003) and of tert-butylhydroperoxide (Hartl *et al.*, 2011) generating ROS.

However, this alone cannot explain the increase of AChE activity. We thus hypothesized the ligand-receptor competition as a mechanism that can explain this result. Since IMI acts as an agonist at the nAChRs, ACh accumulates at the synaptic cleft. Then, AChE increases its activity as an adaptive response in order to hydrolyze ACh into choline.

On the other hand, the sustained agonist (IMI) fixation on nAChRs appears to be responsible for the hyperstimulation of these receptors. Indeed, exposure to IMI causes significant elevation of calcium (Ca^{2+}) level in hypothalamic and pituitary tissues but not in adrenal tissues. The study undertaken by Rodrigues *et al.* (2010) shows that thiomethoxam (a neonicotinoid pesticide) has agonist activity at mammalian nAChRs even at micromolar concentrations, and the peak of Ca^{2+} influxes and the proportions of neurons that were excited did not depend on the dose. Rather, it exhibited an all-or-none response. Indeed, the nAChR-dependent increase of intracellular Ca^{2+} is mainly mediated by Ca^{2+} entry through nAChRs; however, the involvement of other calcium channels is also feasible. Thus, the nAChR-mediated Ca^{2+} influx activates voltage-dependent calcium channels (VDCCs), and the Ca^{2+} uptake via VDCC augments the primary Ca^{2+} signals generated by nAChRs, which may underlie the all-or-none response.

It is also known that nAChRs can undergo desensitization, a reversible reduction in response during sustained agonist application, which has been proposed to be important in controlling synaptic efficacy, responses to cholinergic agents, and certain nAChR-related disease states (Giniatullin *et al.*, 2005). Although the role of desensitization in the effects of nAChRs remains unclear, it has been proposed that desensitization can modulate the cholinergic activity of nAChRs (Bhardwaj *et al.*, 2010), and chronic exposure to agonists can inhibit the normal actions of ACh at nAChRs via desensitization (Dwyer *et al.*, 2009). Thus, it can be hypothesized that exposure to IMI causes initial stimulation followed by fatigue of the agonized neurons and ultimately interferes with the transmission of neuronal impulses (Shadnia and Moghaddam, 2008) (Figure 5). Indeed, ACh-mediated innervation acting through nAChRs regulates processes such as transmitter release, cell excitability, and neuronal integration, which are crucial for network operations and influence physiological functions (Hogg and Bertrand, 2004). Furthermore, it is becoming evident that the perturbation of nicotinic ACh neurotransmission can lead to various diseases during development, adulthood, and aging (Gotti *et al.*, 2006).

5. CONCLUSION

In conclusion, the findings obtained in the present study show that chronic exposure to IMI, which is generally accepted as being a less toxic compound in comparison with other insecticides, may affect mammalian biochemical processes to a greater extent than previously believed. Thus, IMI disrupts the regulatory mechanism of the HPA axis by altering the main hormone, ACTH, involved in this regulation and develops the "general adaptation syndrome" by affecting adrenal weight and composition. Furthermore, IMI induces oxidative stress and alters cholinergic transmission by increasing AChE activity and hypothalamic and pituitary Ca^{2+} levels.

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