Environ Sci Pollut Res
DOI 10.1007/s11356-017-9901-9

RESEARCH ARTICLE

Alteration of membrane integrity and respiratory function of brain mitochondria in the rats chronically exposed to a low dose of acetamiprid

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Received: 9 January 2017 / Accepted: 3 August 2017

Abstract The pesticides are used in several fields of agriculture and farms to protect crops against harmful insects and herbs. The increased and uncontrolled use of these pollutants is very hazardous for the population health. Consumption of contaminated food matrices with these pesticides could impair the cell integrity and its molecular function. The main aim of this present study was to evaluate the alteration of the integrity of mitochondrial membranes and respiratory chain potential in the brain of rats exposed during 90 days to acetamiprid (AC), organochlorine of the new generation. After oral administration of AC in rats with 3.14 mg/kg of body weight, the results of this current study showed enhance in mitochondrial oxidative stress status by significant decrease of glutathione (GSH) level, glutathione pyroxidase (GPx), and catalase (CAT) activities. On the other hand, there is an increase in the enzymatic activity of the glutathione s-transferase (GST) and superoxide dismutase (SOD); at the same time, the MDA level was also highly increased. Furthermore, evaluation results of brain mitochondrial integrity revealed a significant increase in membrane permeability and mitochondrial swelling in rats exposed chronically to AC. Instead, other results of this present work showed a significant decrease in mitochondrial respiration potent (O_2 consumption) in acetamiprid-treated rats. In conclusion, the long duration exposition of the animals to AC has led to respiratory chain dysfunction, disturbance of matrix oxidative status, and a loss of mitochondrial membranes integrity.

Keywords Acetamiprid · Brain mitochondria · Mitochondrial swelling · Membrane permeability · Oxidative stress · Rats

Responsible editor: Philippe Garrigues

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Introduction

Pesticides are used widely in agriculture to control insects all over the world with more than 10,000 commercial formulations of approximately 450 pesticidal compounds currently in use (Yousef et al. 2003). Neonicotinoid insecticides, as a replacement of organophosphates, have been used globally for the treatment of agricultural pests. The most common neoincotinoid insecticides include imidacloprid, acetamiprid, nitenpyram, and clothianidin (Tian 2016). In particular, acetamiprid is a new range of organo-chlorinated insecticides. Although, the compound belongs to the neonicotinoïdes, it has different characteristic insecticidal properties from the others in the same category of the chemical structure; it is able to alter directly the central nervous system of the insect by disturbing the receiver of acetylcholine in synapses (EFSA 2013). AC presents excellent activities against *Hemiptera*, *Thysanoptera*, and the *lepidopterans* and causes agonistic effects by binding to nicotinic acetylcholine receptors (nAchRs), resulting in abnormal excitation, paralysis, and death of pest organisms (Tian 2016). It is also applicable to fight against the parasites of plants, fruits, trees, and the tea tree (Carole and Harvé 2011). There exist various types of harmful devastating insects of the agricultural cultures and the development of resistance to insecticides in many harmful insects such as the *tinea* of cruciferous and louses became a serious problem

Published online: 10 August 2017

during the last years. Moreover, pest-destroying fight plans by this pesticide could be a harmful danger to the environment (Nawaza et al. 2015). The acute AC toxicity per oral way is approximately 200 mg/kg by the value of LD_{50} (Testud 2014; Iwasa et al. 2004). The brain is particularly vulnerable to the oxidative damage because of its richness of peroxidable fatty acid, its high-energy requests and its relatively weak antioxidant power (Carole and Harvé 2011). This irreversible molecular damage is the first cause of the neurodegenerative diseases (Adams et al. 1990). Moreover, the mitochondria are the energy centers in the cell, the form of energy in these factories is the ATP provided by the degradation of cellular fuels, and the reactions of energy in these organelles produce a physiologic quantity of free radicals (Çavas 2014). When abnormalities enhanced in mitochondria, the free-radical elements become etiologic factor in neurodegenerative diseases process, through a loss of mitochondrial function and apoptosis signaling pathway initiated by opening of mitochondrial permeability transition pores (mPTP) that leads at the end to cell death (Seaton and Mursdenf 1996). Pesticide exposure can occur directly during production process or professional use and intoxicate general population. In the first case, intoxicated users with this pesticide are addressed through professional medical services or specialized detoxification centers. However, exposure of the general population to these toxics at low doses via food matrices remains without any serious impact study. Indeed, the few studies on this subject did not provide a clearer understanding about the relationship between neonicotinoïdes, for example, and the etiology of certain diseases such as metabolic and neurodegenerative diseases, reproductive dysfunction, neurodevelopmental alterations, and cancer (Baldi et al. 2013). Many previous studies have shown that acetamiprid is neurotoxic in acute or chronic-treated animals at low doses, by disturbing locomotive activity and learning and memorizing function (El Hassani et al. 2008; Mandal et al. 2015). So in view of the lack of fundamental research on this subject, it was important to curry out some experimental studies and a review of certain risks associated to acetamiprid exposition in a living organism. The objective of the current work was to study various aspects of the brain mitochondria toxicities in rats exposed for a long duration to acetamiprid at low dose of 3.14 mg/kg, equivalent to 1/60 of LD_{50} in rats (Testud 2014; Chakroun et al. 2016).

Materials and methods

Chemicals

The pesticide used in this study is acetamiprid at the dose of 3.14 mg/kg (1/60 LD_{50}) (Testud 2014). Commercial product of acetamiprid [$C_{10}H_{11}ClN_4$] (Mospilan®20 SL (95% purity)), consisting of 200 g/l AC as active ingredient and it has been purchased from Yu Full Industry CO., Ltd., India. The pesticide was dissolved in distilled water and prepared in sufficient amount for a daily administration during 6 months. The majority of chemicals used in the present study were procured from Sigma Aldrich, Germany.

Animal treatments

Twelve male white *Wistar* rats strain, weighing 220 to 240 g, were obtained from Pasteur Institute (Algeria). Upon arrival, the rats were housed, six per cage. Animals were maintained under a daily 12-h light/dark cycle at a constant temperature (22 ± 2 °C), a relative humidity of 55 ± 10% and a free access to food and water. The rats were subjected to one period of 15 days for adaptation before the indicated treatments. In this study, the rats were divided into two groups of six rats each. All experimental assays were carried out in conformity with international guidelines for the care and use of laboratory animals. Approval number of the animal experiment (Code 01-12-2015) was given also by Algerian Pasteur Institute, Algeria. Processing is carried out by the administration of the prepared solutions by gastric gavage using a probe attached to a syringe daily for 3 months.

- Control group (T) received 0.5 ml of distilled water
- Treated group with AC, receiving 3.14 mg/kg body weight daily during 90 days

Sacrifice of the animals and brain extraction

After 6 months of exposure, rats were sacrificed by decapitation after deep ether anesthesia; brains were removed quickly and cut in two parts, the first one was maintained in −80 °C for mitochondrial isolation and the proportioning of the matrix oxidative stress parameters (GSH, GST, CAT, GPx, and MDA), and the second part was used freshly to realize the mitochondrial integrity and function essays.

Brain mitochondria isolation and matrix preparation

The different brain areas are used for the extraction of the whole mitochondrial fractions as described by the method of Clayton and Doda (2001) with slights modifications. Briefly, the tissues were washed in cold respiration buffer, pH 7.4 (50 mM Tris-HCl, 250 mM sucrose, 1 mM *d*-Ethyl Diamine Acetic Acid (EDTA), BSA 0.2%), then chopped and homogenized in three volumes of the same buffer and centrifuged at 3500 g for 10 min, then the pellet was re-centrifuged in the same conditions. Supernatants from the two centrifugations have been mixed and centrifuged at 15000 g for 20 min. The resultant pellet has been washed twice with PB buffer (50 mM Ttis-HCl, 250 mM sucrose) pH 7.4 in the same conditions; resultants mitochondrial pellets were suspended in

300 μl of PB buffer and frozen at −20 °C until its ulterior use. Mitochondrial matrix was prepared from mitochondria suspensions by freezing and defrosting with repeated homogenization in order to burst mitochondria. After centrifugation at 10,000 g for 10 min, the supernatant was considered as the source of mitochondrial GSH, GPx, GST, CAT, SOD, and MDA.

Assessment of redox status markers in brain mitochondria

GSH The dosage of glutathione in mitochondria is carried out according to the process reported by Weckbercker and Cory (1988). Briefly, 0.8 ml of brain tissue homogenate was added to 0.2 ml of a sulfosalicylic acid at 0.25% and the mixture was agitated freshly in order to eliminate proteins by centrifugation for 5 min at 1000 rpm. 0.5 ml of supernatant was added to 1 ml of Tris-HCl buffer + EDTA (0.02 M, pH 9.6). After agitation, 0.025 ml of DTNB (0.01 M) dissolved in methanol was added and the obtained mixture was incubated for 5 min at room temperature, after that, absorbance was recorded at 412 nm.

GPx Glutathione peroxidase activity was measured by the procedure of Flohe and Gunzler (1984). Briefly, supernatant obtained after centrifuging 5% brain homogenate at 1500×g during 10 min followed by 10,000×g for 30 min at 4 °C was used for GPx assay. One milliliter of reaction mixture was prepared which contained 0.3 ml of phosphate buffer (0.1 M, pH 7.4), 0.2 ml of GSH (2 mM), 0.1 ml of sodium azide (10 mM), 0.1 ml of H_2O_2 (1 mM), and 0.3 ml of brain supernatant. The reaction was terminated by addition of 0.5 ml 5% TCA after 15 min of incubation at 37 °C. Tubes were centrifuged at 1500×g for 5 min, and the supernatant was collected. 0.2 ml of phosphate buffer (0.1 M pH 7.4) and 0.7 ml of DTNB (0.4 mg/ml) was added to 0.1 ml of reaction supernatant. After mixing, absorbance was recorded at 420 nm.

GST The measurement of the activity of glutathion-S-transferase (GST) was evaluated according to the method of Habig et al. (1974). Briefly, the homogenate is centrifuged at 14,000 rpm for 30 min and the recovered supernatant will be used as a source of enzymes. The dosage consists of reacting 200 μl of the supernatant with 1.2 ml of the mixture CDNB (1 mM), GSH (5 mM) [20.26 mg CDNB, 153.65 mg GSH, 1 ml ethanol, 100 ml phosphate buffer (0.1 M, pH 6)]. Absorbance readings are performed every 15 s for 1 min at a wavelength of 340 nm against a white tube containing 200 μl of distilled water replacing the amount of supernatant.

CAT The evaluation of catalase activity in brain mitochondria was carried out according to the method of Aebi (1984). Briefly, 780 μl (100 mM) of phosphate buffer pH 7.4 and 200 μl d'H2O2 (500 mM) freshly prepared was added to 20 μl of matrix supernatant. The H_2O_2 decomposition rate was followed by monitoring absorption at 240 nm. One unit of CAT activity is defined as the amount of enzyme required to decompose 1 μmol of hydrogen peroxide in 1 min. The enzyme activity was expressed as micromole of H_2O_2 consumed/min/mg protein.

SOD The enzymatic activity of superoxide dismutase (SOD) in brain mitochondria was assessed by the method of Beauchamp and Fridovich (1971). For that, 50 μl of matrix fraction was added to a mixture composed of 2 ml of the reactive medium (sodium cyanide 10-2 M, solution of NBT at 1.76 × 10-4 M, EDTA 66 mmol, methionine 10-2 M, riboflavin 2 μmol, pH 7.8). This mixture was exposed to light of a 15 W lamp for 30 min to induce the photoreaction of riboflavin. Reduction of NBT into formozan gave a blue color. The color was measured by spectrophotometer at 560 nm. The enzymatic activity is calculated in terms of international unit per milligrams of proteins.

MDA The assessment of the MDA levels in mitochondria matrix was carried out according to the method of Esterbauer et al. (1992). One hundred twenty-five microliter of supernatant was homogenized by sonication with 50 μl of PBS, 125 μl of trichloroacetic acid-butylhydroxytoluene (TCA-BHT) in order to precipitate proteins and then centrifuged (1000 g, 10 min, and 4 °C). Then, 200 μl of supernatant was mixed with 40 μl of HCl (0.6 M) and 160 ml of TBA dissolved in Tris and then the mixture was heated at 80 °C for 10 min. The absorbance of the resultant supernatant was obtained at 530 nm.

The mitochondrial protein concentration was measured by the method of Bradford (1976) and used beef serum albumin (BSA) as a standard.

Evaluation of swelling, permeability, and mitochondrial respiration

Assessment of mitochondrial swelling was carried out according to the method of Kristal et al. (1996) and modified by Farhi (2015). Briefly, after mitochondria extraction from fresh tissues of the rats' brains at 4 °C, equal volumes were distributed in quartz cells and the absorbance is monitored spectrophotometrically at 540 nm. The decrease in absorbance indicates the increase of mitochondrial swelling.

The estimation of the mitochondrial permeability was carried out using the method of Farhi (2015) which is based on the rate of the mitochondrial size variation after the entrance of the calcium that is added to mitochondrial fresh suspension. This increase in mitochondrial size was monitored by the absorbance reading every 30 s at 540 nm wavelength during 3 min to thus allowing establishing a kinetic curve of the loss of mitochondrial membrane potential.

Mitochondrial respiration was estimated as oxygen consumption using an Oxygraph (Hansatech®, Germany) according to the method described by Henin et al. (2016).

Data analysis

The statistic analysis was carried out using software MINITAB (version 17.1). For obtained charts and histograms, the office Excel 2016 was used. The results are expressed as mean ± SEM of six rats in each group, and the differences were considered significant with $0.05 \geq p \geq 0.01$, highly significant when $0.01 \geq p \geq 0.001$, and very highly significant when $p < 0.001$ (P threshold of significance).

Results

Oxidative stress status of matrix mitochondria

The results of assessment of the different markers of matrix mitochondria redox status in brain rats after long duration acetamiprid exposure at the dose of 3.14 mg/kg body weight are shown in Table 1. GSH, such as an important antioxidant tripeptide in brain mitochondria, has shown a highly significant ($p = 0.002$) reduction in AC-treated group compared to the normal group. At the same time, the Table 1 showed a significant increase ($p = 0.024$) in the enzymatic activity of the matrix GST following a chronic exposition of the rats to acetamiprid in comparison with the control group. In addition, GPx activity, as a key antioxidant enzyme which could regulate the level of the ROS in mitochondria matrix, has been decreased with highly significant manner ($p = 0.0059$), (Table 1) in AC-treated rats comparing with the normal group. A very highly significant decrease ($p = 0.001$) appeared in brain mitochondrial CAT activity in AC-treated group in comparison to those non-AC-treated rats. On the other hand, the activity of SOD enzyme was highly significantly ($p = 0.021$) increased after 90 days of exposure to acetamiprid when compared to the control group. Levels of MDA were assessed in brain mitochondria of the whole brain in rats in order to show the impact of oxidative stress produced by acetamitrid on lipid compounds. MDA values were significantly increased ($p < 0.01$) in brain mitochondria of the AC-treated rats compared to control (Table 1).

Evaluation of swelling, permeability, and mitochondrial respiration

The toxicological results of acetamiprid on mitochondrial integrity and function parameters (swelling, permeability, respiration) are shown in Table 2. As regards the evaluation of mitochondrial swelling, the results of the present study showed a significant increase ($p \leq 0.0023$) in mitochondria swelling, which is directly proportional to the absorbance values, when the rats were exposed to AC for a long duration at the dose of 3.14 mg/kg body weight in comparison to control group.

On the other hand, the assessment of mitochondrial permeability was calculated as the change of mitochondrial size during 180 s, following the addition of calcium to mitochondrial suspension. The results of this essay are represented by a kinetic curve (Table 2) which demonstrated a significant difference between AC-treated group and normal group with significance degree of p.

Regarding the evaluation of respiratory function of brain mitochondria, the results highlighted a significant decrease in oxygen consumption rate in AC-treated rats comparing to those non-AC-exposed animals (Table 2).

Discussion

The neonicotinoids have unique physical and toxicological properties compared to earlier classes of organic insecticides. The free radicals are very reactive and can attack, if they are not destroyed, various targets such as proteins, DNA, and especially the fatty acids polyunsaturated (Sauer 2014; Pasteur 2013) and could be even more destructive for cell architecture (Rouabhi et al. 2015; Johnson and Weinberg 1993). The aim of this present study was to investigate the effect of chronic acetamiprid exposure on redox homeostasis unbalance in brain mitochondria in rats and its implication on mitochondrial membrane integrity and function. Indeed, oxidative stress is one of the main common toxicity mechanisms between many

Table 1 Variation of mitochondrial oxidative stress parameters in rats' brain under the effect of acetamiprid chronic exposure at the dose of 3.14 mg/kg body weight

	GSH nmol/mg	GST Pmol/min/mg	GPx nmol/min/mg	CAT nmol/min/mg	SOD U/mg	MDA nmol/mg
Control	35.171 ± 1.63	71.19 ± 2.50	64.80 ± 6.08	155.72 ± 2.40	12.23 ± 2.65	0.59 ± 0.03
AC	26.89 ± 3.219**	81.48 ± 6.80*	51.10 ± 8.82**	135.48 ± 1.96***	23.44 ± 3.5**	0.82 ± 0.04***

Each value is expressed as mean ± standard deviation, we use Student test. Batch Compare treated with acetamiprid compared to control group. ($p \leq 0.05$) significant (*), ($p \leq 0.01$) highly significant (**), ($p \leq 0.001$) very highly significant (***), $p > 0.05$ not significant (ns)

Table 2 Change of mitochondrial swelling, respiration, and permeability in the brain of control rats and treated after 90 days of treatment

	Mitochodrial swelling as (optic density)	Mitochondrial respiration as O_2 consumption	Mitochondrial permeability as ($\Delta OD/\Delta t$)
Control	75.45 ± 0.027	6.33 ± 1.04	0.215 ± 0.33
AC	0.33 ± 0.038**	3.16 ± 0.90***	0.295 ± 0.029***

Each value is expressed as mean ± standard deviation, we use Student test. Batch Compare treated with acetamiprid compared to control group. ($p \leq 0.05$) significant (*), ($p \leq 0.01$) highly significant (**), ($p \leq 0.001$) very highly significant (***), $p > 0.05$ not significant (ns)

pesticides and persistent substances (Lukaszewicz-Hussain 2008). In the current study, the mitochondrial matrix preparation from the brain of rats treated with acetamiprid showed significant decrease in mitochondria GSH uptake. GSH depletion can enhance oxidative stress and may increase the levels of excitotoxic molecules; both types of action can initiate a loss in mitochondrial function and its integrity susceptible to induce apoptosis signaling pathway in distinct neuronal populations (Jaswinder and Christopher 1997; Di Monte and Lavasan, 2002; Uttara et al. 2009).

In this study, because of their high reactivity and short life, the ROS have been analyzed indirectly in vivo by measuring the changes in antioxidases including GST, GPx, CAT, and SOD. Reduced activity of GPx and CAT was observed in mitochondria when acetamiprid was administered to rats. This abnormality in the rate of different antioxidants might have resulted from intense ROS generation induced by acetamiprid administration in brain mitochondria, which in turn might have caused an increase in malondialdehyde, as a result of enhanced lipid peroxidation (Silva and Gammon 2009). Indeed, reduction in the GPx activities and CAT to increase H_2O_2 and to produce radical OH° in the mitochondrion (Cory-Slechta et al. 2005; Banerjee et al. 2001). Thus, the reduction in activity of GPx is due mainly to a hydrogen peroxide overproduction and the decrease of GSH after xenobiotics intoxication (Gasmi et al. 2016; Bourbia 2013; Kebieche et al. 2009). Thus, environmental toxicants can directly attack the mitochondria inducing the generation of ROS, which can further induce the depletion of antioxidant defenses and mediate other oxido-reduction reactions that could promote mitochondrial damage and depletion of antioxidant molecular systems in the brain cell (Lakroun et al. 2015; Franco et al. 2009). The increase accumulation of ROS which exert a direct damage upon brain mitochondria (Shi et al. 2004; Assefa et al. 2005) and the lipid peroxidation which is recorded in this study, by enhancing MDA rate and impairing consequently mitochondrial metabolism and mitochondrial pore transition permeability (MPTP). However, the activity of both SOD and GST has contrary increased in mitochondria matrix in AC-treated rats comparing to the normal group. Therefore, the SOD enhancing activity could be explained by the necessity to offset superoxide overproduction in brain mitochondria AC-treated rats, at the same time; however, it should be noticed that the increase in the expression of the GST is generally observed when the cell is stressed (Di Monte et al. 1992). The results of our study are in agreement with preceding studies, which revealed that the exposure to xenobiotics belonging to the same family can induce the activity of GST (Lahouel et al. 2016; Bourbia 2013; Chen and Ahn 1998). Indeed, the results of the present study showed an increase in mitochondria swelling and membrane permeability in the acetamiprid-treated rats, probably induced by MPTP induction mediated by ROS-generation. Several authors have reported that apoptosis induced by environmental toxicants is widely associated with alterations in oxidative stress homeostasis which includes both the depletion of antioxidant defenses the increase accumulation of ROS which exert a direct damage upon brain mitochondria (Shi et al. 2004; Assefa et al. 2005; Baltazar et al. 2014). In addition, the results of the current study have revealed a significant loss in mitochondrial respiratory function in intoxicated rats by the pesticide. When antioxidant defense systems are insufficient to compensate pro-oxidant state, the excessive ROS interact with phospholipids and proteins of mitochondrial membranes, resulting the opening of permeability transition pores, dissipation of membrane potential that could damage mitochondrial ultrastructure and subsequent release of cytochrome-c. Knowing that cytochrome-c is imperative for respiratory chain reactions (RCR) in brain mitochondria, this may be the cause of a fall in oxygen consumption monitored in the present study. Outsourcing of this cytochrome from brain mitochondria to the cytosol is considered an apoptosis-inducing factor (Franco and Cidlowski, 2009; Gao et al. 2014; Morris and Berk 2015). In addition, there is a relationship between mitochondrial dysfunction which is usually mediated by overproduction of O_2^- and membrane potential collapse (Morris and Berk 2015; Lakroun et al. 2015). RCR, known as a crucial index of mitochondrial respiratory function, has reflected a fall in its oxygen consumption rate and thus the efficiency of its function.

Conclusion

At the end, we can conclude that the long duration exposure to low dose of acetamiprid generated many toxic effects on the whole brain of rats. Enzymatic and non-enzymatic defense

systems have been impaired to produce abnormalities in brain mitochondria compared to non-acetamiprid-treated rats such as an important unbalance of oxidative stress homeostasis in brain mitochondria matrix, mitochondria swelling, membrane permeability disequilibrium, and inhibition of mitochondrial respiration, indicating the loss of functional and structural integrity of brain cell and mitochondria. These alterations could likely lead to cell death and thus be the etiological factor of neurodegenerative diseases.

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