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Imidacloprid and/or Esfenvalerate Induce Apoptosis and Disrupt Thyroid Hormones in Neonatal Rats

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Abstract: The well-known insecticides imidacloprid and esfenvalirate are neurotoxic agents that work on acetylcholine receptors and ion channel of nerve axons, respectively. Beside that they are suspected to produce hormonal disruption and apoptosis. In the present work, the impact of these insecticides in produce DNA damage, stimulate apoptosis and disrupt thyroid hormones were investigated in neonatal rats. Twenty one days-old neonatal rats were orally intubated with serial low doses of imidacloprid and/or esfenvalerate daily for consecutive seven days. At the end of the treatment, blood samples were withdrawn and animals were sacrificed, the brain and the liver were removed and quickly frozen. Total tetra-iodothyronine (T_4) and tri-iodothyronine (T_3) levels were determined in plasma and caspase-3 activity was determined in brain and liver. DNA damage was also investigated in brain and liver using agarose gel electrophoresis. Results indicated that imidacloprid at high and medium doses produced significant decrease in plasma T_3whereas; esfenvalerate produced significant decrease in plasma T_4 and T_3at high and medium doses. In addition, treatment with low doses of imidacloprid and esfenvalerate produced significant decrease in both plasma T_4 and T_3. In contrast, the activity of caspase-3 increased dramatically in both brain and liver tissue at all dose levels after independent and combined intoxications with imidacloprid and esfenvalerate. Agarose gel electrophoresis showed that the increase in caspase-3 activity in brain and liver tissues in treated groups was accompanied with increase in the DNA fragmentation. These results indicated that both imidacloprid and/or esfenvalerate had impact on thyroid hormones in neonatal rats. These effects were accompanied with initiation of apoptosis through activation of caspase-3 and producing DNA fragmentation. It is suggested that these effects expected to have an impact on the brain function in the weanling and adult stages and this suggestion may be target to further research work.

Key words: Imidacloprid · Esfenvalerate · Thyroid hormones · DNA fragmentation · Caspase-3 · Apoptosis · Neonates

INTRODUCTION

The neonicotinoid and pyrethroid insecticides are extensively used for insect control in agriculture and household either separately or as a mixture [1]. Neonicotinoids act on the central nervous system as nicotinic acetylcholine receptors (nAChRs) agonists [2].Whereas, pyrethroids exert its action by interact with the voltage-sensitive sodium channel in both insects and mammals [3]. Many members of neonicotinoid insecticide are suspected to interrupt the hormonal balance [1, 4]. Imidacloprid, a chloronicotinyl neonicotinoid caused reduction in the levels of T_3, T_4 and TSH andthis reduction revealed disruption of the pituitary–thyroid axis [5] or may due to disruption of nuclear thyroid hormones [6]. Also, esfenvalerate, a type II pyrethroid, induced decrease in the serum level of thyroid hormones [7], in the activity of hepatic type I iodothyronine 5'-monodeiodinase [8] and it's disruptive effects on the binding of thyroid hormones to their receptors are rarely studied [9]. Regardless the important roles of apoptosis during maturation of the nervous system [10], chronic neurodegenerative diseases are characterized by progressive, irreversible neuronal cell loss [11], which was

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suggested to be occurred in part as a result of apoptosis or alternative pathways of neuronal death [12]. Since, neurons have minimal regenerative potential [13], it is suggested that exposure to neonicotinoid and pyrethroid insecticides in the early life stage may have impact on brain function in the late stages. Few evidences have showed that neonicotinoids may induce apoptosis and DNA damage in reproductive organs and this may due in part to oxidative stress [4]. Also, neonicotinoid such as thiamethoxam which is not mutagenic either *in vitro* or *in vivo* produced liver toxicity and cell necrosis and apoptosis after 10 weeks treatment [14]. Although, neonicotinoids are neurotoxin, there is no research work deal with their ability to produce apoptosis or damage DNA in nerve tissues especially in the weanling period. Regarding to pyrethroids, there is a body of evident indicated that pyrethroids initiate apoptosis in reproductive and endocrine tissues [15, 16]. However, the molecular pathways leading to pyerithroids-induced apoptosis have not been completely established [17].

The present study aims to evaluate the possible adverse effects of imidacloprid and/or esfenvalerate on thyroid hormones and DNA (and the correlation between them if there any) on pre-mature rats.

MATERIALS AND METHODS

Pesticides and Chemicals Used: Imidacloprid technical grade (96% active ingredient) and esfenvalerate technical grade (98% active ingredient) were gifted from Pesticides Analysis Department, Central Agricultural Pesticides Lab., Dokki, Egypt. All chemicals were purchased from Sigma Chemical Company (USA), DNA ladder was obtained from Bioron (Germany) and thyroid hormones kits were obtained from Siemens Healthcare Diagnostic Inc. (USA).

Experimental Protocol

Animals and Housing: The experiment was performed according to the guidance for care and use of laboratory animals [18]. During this experiment, forty pregnant Sprague-Dawley rats were withdrawn from the breeding colony of the Mammalian Toxicology Department, Central Agricultural Pesticides Lab. and housed in individual cages. The cages were kept in air conditioned room at a temperature of 22±2°C and a relative humidity of ~ 55% and normal light/dark cycle. Animals were fed on a well-balanced chow which obtained from Animals Food Manufactory, Ministry of Agriculture, Embaba, Giza, Egypt and tap water *ad libitum*. Immediately after delivery, pups were weighted, counted, sexed and checked for anomalies and then breasted feeding from their dams. After lactation period (at 21stday postnatal), a total of forty male pups were weaned from their dams in which everyone from different dams to avoid the siblings and arranged in eight groups each comprised five animals [19] to achieve the following experiment.

Treatment Procedure: The animals were daily intubated (by oral rout) for consecutive seven days with the corresponding doses (1/100, 1/50 and 1/25 LD_{50}) of each tested pesticides. In this respect, control group was received corn oil at one μL/g body weight, imidacloprid (IM) groups were received 0.529, 1.058 and 2.116 mg/kg, esfenvalerate (ES) were received 0.142, 0.284 and 0.568 mg/kg and a mixture group was received a combination of the two pesticides at low doses (0.529 and 0.142 mg/kg of IM and ES, respectively).

Sampling: After intubation period, blood was withdrawn from retro-orbital plexus according to the method of Schalm [20] and plasma samples were separated and kept at -80°C until used for thyroid hormones determination. Neonates were decapitated by cervical dislocation; brain and liver were quickly frozen at -80°C and saved till the preparation for caspase-3 and DNA fragmentation assay.

Biochemical Assays

Determination of Thyroid Hormones Levels: Total tetra-iodothyronine (T_4) and tri-iodothyronine (T_3) levels in plasma were determined by Coat-A-Count radioimmunoassay kits according to manufacturer's procedures using the method of Hollander and Shenkman [21].

Caspase-3 Assay: Activity of caspase-3 was determined according to the method of Coelho *et al.* [22]. Briefly, brain and liver were individually homogenized in the extraction buffer (25 mM/L HEPES buffer, pH 7.4, containing 5 mM/L EDTA, 2 mM/L dithiothreitol and 0.1% CHAPS). The homogenate was centrifuged at 20,000g for 30 minutes. The supernatant was diluted with the assay buffer (50 mM/L HEPES, 10 mM/L dithiothreitol,1.0 mM/L EDTA, 100 mM/L NaCl, 0.1% CHAPS and 10% glycerol, pH 7.4) and incubated at 37°C with 200 mM/L caspase-3 substrate (Ac-DEVD-pNA). Standard was run through the test using a known concentration of p-nitroaniline and cleavage of the substrate was monitored at 405nm. Specific activity was expressed in pmol of the product, nitroaniline, per minute per mg protein. A standard and quantitative assay for determining protein content in tissue fraction was done based on the method of Bradford [23] with bovine serum albumin as standard.

DNA Gel Electrophoresis: Brain and liver DNA were isolated according to the method of Sambrook *et al.* [24]. Equal amounts of DNA samples were subjected to 1.0% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light. Gel was photographed and the resulting images were analyzed using (Gel-pro ANALYZER version 3.0 media Cypernetic-pro Plus software).

Statistical Analysis: A standard computer program SPSS for Windows, release 21.0 (IBM SPSS Inc, USA) was used for data entry and analysis [25]. Mean values and standard error of the mean (mean±S.E) were calculated for each tested group on the basis of values obtained from five individual rats. One-way analysis of variance (ANOVA) followed by Turkey's honestly significant difference (HSD) test with significant level of 0.05 was used for statistical analysis of data.

RESULTS

Thyroid Hormones: Intoxication of the neonatal rats with esfenvalerate at medium and high doses induced significantly decreases in the concentrations of the plasma T_4 by 23.06 (P<0.05) and 28.28% (P<0.01), respectively beside in a mixture group by 20.01% (P<0.05) as compared with control one. Also, there was a significant different (P<0.05) when low dose of imidacloprid compared with high one of esfenvalerate (Table 1). Statistical analysis also revealed that concentrations of T_3 significantly decreased by 10.61 (P<0.05) and 14.39% (P<0.01) in imidacloprid groups at medium and high doses, respectively as compared with control. Similarly, there were a declines by 17.00 (P<0.005) and 21.89% (P<0.001) in esfenvalerate groups at medium and high doses, respectively beside that there was a significant decrease by 21.70% (P<0.005) in mixture group as compared with control. There were significant differences also when low dose of imidacloprid compared within high one (P<0.05) and within a mixture (P<0.005) group (Table 1).

Caspase-3: As shown in Table 1, the brain activity of caspase-3 was significantly increased by 33.48 (P<0.05), 67.89 (P<0.005) and 89.44% (P<0.0001) in imidacloprid at low, medium and high doses, respectively, as well as in esfenvalerate at low, medium and high doses by 58.71 (P<0.05), 91.74 (P<0.001) and 138.53% (P<0.0001), respectively beside that there was a significant elevation by 82.56% (P<0.05) in a mixture group as compared with control. Statistical analysis also revealed that there were significant differences between low dose of imidacloprid within medium (P<0.05), within high (P<0.005) and within a mixture (P<0.05) groups. Also, there was a significant different between high dose of esfenvalerate within low (P<0.005) and within a mixture (P<0.05) groups. The same effective pattern was observed in the liver caspase-3 activity where there were significant increases in the enzyme activity by 33.5 (P<0.01), 43.95 (P<0.005), 27.51 (P<0.05), 47.31 (P<0.005), 81.20 (P<0.001) and 37.58% (P<0.01) in IM.M, IM.H, ES.L, ES.M, ES.H and IM.L/ES.L groups, respectively as compared with control. There were significant differences also between low dose of imidacloprid within high one (P<0.005) and when low dose of esfenvalerate compared within medium (P<0.05) and within high dose (P<0.01) groups (Table 1).

Table 1: Effects of oral intoxication with imidacloprid and/or esfenvalerate for consecutive seven days on the plasma thyroid hormones levels and caspase-3 specific activity of neonatal rats

Parameters	Control	IM.L 0.529 mg/kg	IM.M 1.058 mg/kg	IM.H 2.116 mg/kg	ES.L 0.142 mg/kg	ES.M 0.284 mg/kg	ES.H 0.568 mg/kg	IM.L/ES.L
Plasma T_4 (µg/dl)	4.93±0.22	4.57±0.28^{g}	4.24±0.17$^{N.S}$	4.22±0.20$^{N.S}$	4.00±0.09$^{N.S}$	3.79±0.30^{a}	3.53±0.19^{a,b}	3.94±0.05^{a}
Plasma T_3 (ng/dl)	109.99±1.89	103.72±1.38^{d,f,h}	98.31±1.07^{a,g,h}	94.15±1.40^{a,b}	97.22±2.25$^{N.S}$	91.29±1.42^{a,b}	85.90±1.37^{a,c}	86.11±2.02^{a,b,c}
Brain caspase-3 (Pmol/min/mg protein)	80.74±3.73	107.77±3.8^{a,c,d,h}	135.55±4.19^{a,b}	152.96±4.67^{a,b}	128.14±6.34^{a,g}	154.81±4.52^{a}	192.59±7.38^{a,e}	147.40±6.32^{a,b}
Liver caspase-3 (Pmol/min/mg protein)	110.37±4.16	128.51±3.02^{d,f}	147.40±4.16^{a}	158.88±3.12^{a,b}	140.74±3.15^{a,f,g}	162.59±2.57^{a,b,e}	200.00±7.70^{a,e,h}	151.85±5.03^{a,g}

Each value represents the mean of five animals ±SE. Total tetra-iodothyronine (T_4) levels in plasma expressed as µg/dl, total tri-iodothyronine (T_3) levels expressed as ng/dl and specific activity of caspase-3 in brain and liver was expressed as Pico moles of p-nitroaniline which formed per minute per milligram protein (Pmol/min/mg protein). One-way analysis of variance (ANOVA) followed by Turkey's honestly significant difference (HSD) test, N.S is non-significant (P value> 0.05), while the p≤ 0.05 level was set as statistically significant different. (a) significant compared to control, (b) significant compared to imidacloprid low dose (IM.L), (c) significant compared to imidacloprid medium dose (IM.M), (d) significant compared to imidacloprid high dose (IM.H), (e) significant compared to esfenvalerate low dose (ES.L), (f) significant compared to esfenvalerate medium dose (ES.M), (g) significant compared to esfenvalerate high dose (ES.H), and (h) significant compared to mixture of IM.L and ES.L (IM.L/ES.L).

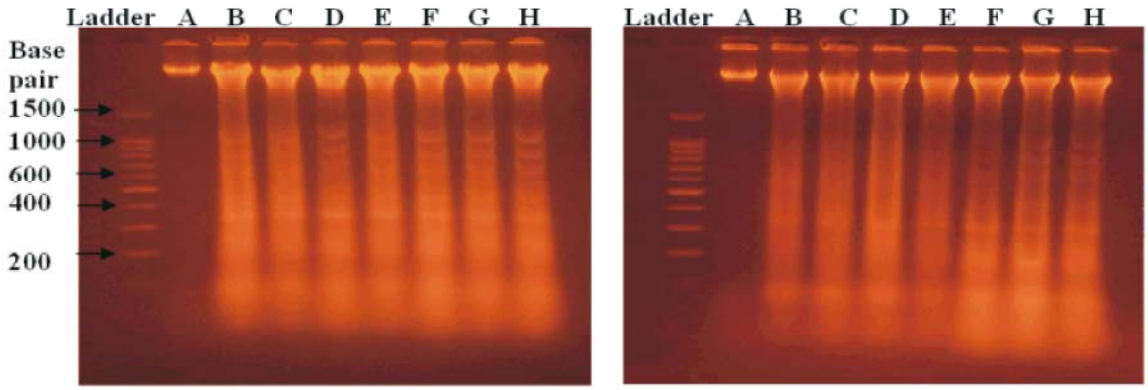

Fig. 1: DNA agarose gel electrophoresis of neonatal brain (left) and liver (right) after oral intoxication with imidacloprid and esfenvalerate individually or their combination for consecutive seven days. Lane A is control group, lane B is imidacloprid low dose group, lane C is imidacloprid medium dose group, lane D is imidacloprid high dose group, lane E is esfenvalerate low dose group, lane F is esfenvalerate medium dose group, lane G is esfenvalerate high dose group and lane H is a mixture of imidacloprid and esfenvalerate at low dose.

DNA Fragmentation: In order to evaluate the nature of apoptotic bodies, we analyzed the DNA by agarose gel electrophoresis. Apoptotic cells usually contain fragmented DNA, which can be used as a criterion for apoptosis. The DNA isolated from the brain and liver of neonatal rats exposed to imidacloprid and esfenvalerate individually or their combination showed degradation into oligonucleotide fragments, forming a clear laddering pattern of apoptosis when separated by electrophoresis (Fig. 1). A dose response was observed for the two tested pesticides, where exposure increasing resulted in an increase in brain and liver DNA fragmentation. The DNA fragmentation was less obvious in the liver and esfenvalerate was a potent effect.

DISCUSSION

Hormonal disruption and DNA damage represent challenges regarding pesticides risk management and pesticides used [26]. In addition, sensitivity of neonates to pesticides represents another challenge [27]. Evidences from animal [28] and epidemiological studies [29] indicated that many pesticides have the capability of disrupting hormonal balance [30] and interact with genetic materials in both animals and human. This includes the two major groups of insecticides pyrethroids [31] and neonicotinoids [5]. Our results revealed that there were significant decreased in plasma T_3 and T_4 levels after esfenvalerate intoxication at medium and high doses beside with a mixture as well as the plasma T_3 reduced after imidacloprid intoxication at medium and high doses. Unlike pyrethroids, the toxicological studies of neonicotinoids on thyroids takes a little care of the researches and the mechanisms by which neonicotinoid (in general) and imidacloprid decrease thyroid hormone is unknown [32]. However, based on the evidence abstracted from the cholinergic receptor inhibition properties of imidacloprid [33] and the observed effect on hypothalamic–pituitary–thyroid (HPT) axis which resulting in disruption of HPT axis that pronounced as decrease in plasma T_4 and T_3 [5] in addition to disrupting TRs via H-bond formation like that of T_3 [6], the decrease in T_3 which observed in our study may be attributed to the antagonistic effect of imidacloprid on thyroid receptors.

The mechanism by which esfenvalerate may produce the disruption in thyroid hormones is still unclear, but there are evidences indicated that pyrethroids in general may interact with thyroid through receptor binding showing antagonistic effects on thyroid receptors and might disrupt the function of multiple nuclear hormone receptors and thus have the potentials to affect the endocrine systems [9]. On the other hand, disruption of thyroid by pyrethroid may base on their direct effect on hepatic type I iodothyronine5'-monodeiodinase (5'D-I) [8], the enzyme responsible for T_4/T_3 conversion [34]. A literature review revealed that the effects of the neonicotinoids and pyrethroids mixture on thyroid hormones have not yet been investigated [1]. Therefore, the present study is the first one on the two insecticides mixture. The results of the present study indicated that the combined effect of the two tested pesticides did not show any synergistic effect upon the T_3 or T_4 and that imidacloprid and esfenvalerate may be affect directly on

pituitary and induced an additive effect [35]. Since, disruption of thyroid hormones during early developmental period delayed the nervous system maturation [27]; the consequence of the decease thyroid hormone observed in the present study will has an impact on the brain function in the adulthood. Although, apoptosis is important for the regulation of normal physiological function throughout life, excessive apoptosis contributes to pathological cell death observed in pesticides induced neurodegeneration [13]. In fact, there are two main apoptotic pathways and both of them differ in how they are initiated; the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway [36]. Caspase-3 serves as the convergence point between two pathways and a key factor in both pathways toward apoptosis [37]. It is activated by either caspases-8 of the extrinsic or caspase-9 of the intrinsic pathways [38]. So, DNA gel electrophoresis used to detect the DNA fragments that occur in apoptosis and caspase-3 activity determined as an endpoint marker of apoptosis [39].

Results obtained in the present study indicated that independent and combined treatments with imidacloprid and esfenvalerate increased the activity of caspase-3 at dose dependent manner and DNA gel electrophoresis showed the same result. Esfenvalerate is more effective than imidacloprid and brain was a potent when compared with liver at the same experimental conditions. These differences may be attributed to the vulnerability of immature brain of neonatal and the less capacity of the neonate's detoxification system, as well as the high sensitivity of the target site at the early stage of life [40]. There are many suggestions that were set to explain this situation and a major one is based on the free radical theory. Since, the two tested pesticides are considered as oxidative stress inducer [41, 42], which plays a major role in apoptosis induction [43], It is suggested that independent and combined treatments with imidacloprid and esfenvalerate may be induce the intrinsic pathway of apoptosis includes oxidative stress *via* indirect caspase-3 activation. Oxidative stress induce *via* producing reactive oxygen species which are highly reactive with DNA and may produce damage, such as single- and double-strand DNA breaks and apoptosis [44] that occurs by up regulation of pre apoptotic and down regulation of anti-apoptotic proteins [45]. Pre apoptotic proteins are critical for inducing permeabilization of the outer mitochondrial membrane [46] which causes loss of mitochondrial trans-membrane potential [47]. This led to release of cytochrome C to the cytosol and result in the formation of a massive complex known as apoptosome [48]. Apoptosome can trigger the activation of initiator caspase-9, which in turn activates effector caspase-3 [49]. Caspase-3 initiates apoptosis by releasing caspase-activated deoxyribonuclease which in turn triggers rapid DNA fragmentation and responsible for destruction of structural and specific proteins that leads to DNA damage and apoptotic cell death [39].

Finally, it can be concluded that independent and combined exposure to these kinds of pesticides during early stage of live even at low doses produce disruption in thyroid hormones and these effects were accompanied with initiation of apoptosis and expected to affect the brain maturation and function. Further studies could be extended to evaluate the neurotoxic effect of these pesticides to understand their mode of action and the mechanisms by which they can induce apoptosis in hand and to determine their endocrine disruptors' properties in another hand, especially in the early stages of life because of the importance of thyroid hormones during growth and development. Therefore, children must be avoided completely from come in contact with these neurotoxic agents.

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