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RESEARCH ARTICLE

Acute in vivo toxicity of neonicotinoid pesticide residues in common Egyptian fruits

***Shaimaa A. ElShebiney[a], Marwa El-Araby[b], Amr M. Khattab[c]**

[a]Narcotics, Ergogenics and Poisons Department, Medical Research and Clinical Studies Institute, National Research Centre, Giza, Egypt, Affiliation ID: 60014618.
[b]Pathology Department, Medical Research Division, National Research Centre, Giza, Egypt
[c]Forensic Medicine and Clinical Toxicology Department, Faculty of Medicine, Cairo University, Cairo, Egypt

*shaimaaelshebiney@gmail.com

ABSTRACT

Pesticide residues in food is a major health hazard. Contamination of pesticide residues in common Egyptian fruits was studied. Samples of peach and cantaloupe (500g each) from five different local markets were examined. Fruit samples were liquid-liquid extracted. Thiamethoxam was detected in three cantaloupe samples at 0.085 mg/kg and acetamiprid in two samples at 0.24 mg/kg, whereas acetamiprid was detected in all the peach samples at 0.08 mg/kg. Detected levels were below MRL. Male albino rats were given either distilled water (0.2 ml) or acetamiprid (17 µg/kg) or thiamethoxam (6 µg/kg) orally for 6 consecutive days. Doses were calculated according to the estimated average daily intake of fruits (~100 g) and extrapolated to rats. Thereafter, oxidative state of liver and brain was evaluated besides liver function tests and assessment of histopathological features. Serum GPT was in normal range in thiamethoxam-exposed rats, while it showed a marked increase in acetamiprid-exposed rats. sGOT was elevated after exposure to both compounds, though total protein and sGSH were not affected. Oxidative insult was expressed in liver tissue through increased MDA, NO and protein carbonyl contents, although the antioxidant GSH pool was not depleted after pesticide exposure. Acetamiprid boosted the cytokine IL-1 level. Histopathological examination of liver tissue showed inflammatory degenerative changes. Acetamiprid affected the DNA integrity in blood. Brain oxidative state was not changed. These pesticides should be further studied for guiding local regulations towards safer use to avoid long term associated health hazards.

Keywords: Acetamiprid; DALY; Fruits; Pesticides; Thiamethoxam

Abbreviations

ADI: Average Daily Intake; EDI: Estimated Daily Intakes; GC-MS: Gas Chromatography-Mass Spectroscopy; GSH: Reduced Glutathione; HPLC: High Performance Liquid Chromatography; IL-1: Interleukin-1; MDA: Malondialdehyde; MRL: Maximum Recommended Level; sGOT: Serum Glutamic Oxalacetic Transaminase; sGPT: Serum Glutamic Pyruvate Transaminase; WHO: World Health Organization

Introduction

Fruits and vegetables are important components of healthy diet and there is accumulating evidence that they can aid in prevention of various diseases referring to their richness in antioxidants and vitamins. The longitudinal review study of Wassef (2004)[1] summarized the diet pattern of Egyptians and pointed out that at least one serving of fruit is included in the daily meal plan as the most frequently favored dessert. Ahmed et al.[2] estimated the average daily intake (ADI) of vegetables and fruits to be around 70-80 g/day for average 70 kg bodyweight person in Egyptian population.

The use of pesticides in agriculture to eradicate diseases of fruits and vegetables is one of the most important factors leading to increased yields and food security. However, pesticides are biologically active compounds reported to have adverse health effects on humans, ranging from short-term effects such as headaches and nausea to chronic effects like cancer, reproductive damage and endocrine disruption[3,4]. Therefore, routine monitoring of these pollutants in food items is required to prevent, control and reduce environmental pollution and hence the associated health risks[5]. The presence of residues in fruits and vegetables is a predominant route of human exposure specially in developing countries due to the lack of government inspections and consumer knowledge about this matter[6]. Monitoring programs for pesticide residues in Egypt are focused on export products excluding food commodities consumed locally which imparts health risks from such contaminants[7].

Neonicotinoids, the newest group of insecticides, have remarkable and systemic influence on protecting crops against pests[8]. The neonicotinoid pesticides include many agents such as acetamiprid, imidacloprid, thiacloprid, and thiamethoxam[9]. They act through binding to postsynaptic nicotinic acetylcholine receptors in the insect central nervous system[10]. Acetamiprid (Mospilan®) is widely used in vegetables and fruits cultivation for controlling aphids and whiteflies[11]. It disturbs the transfer of impulses in the nervous system of the pests differently from organophosphorus, carbamate and pyrethroid insecticides[10]. The frequency of incidence of residual acetamiprid reaches 5% in fruit samples[12]. Thiamethoxam (Actara®), a second-generation neonicotinoid, provides long residual control and fast elimination of sucking and chewing pests, stopping crop damage before it starts [13].

The maximum recommended level (MRL) for acetamiprid in peach is 0.2 mg/kg and in melons is 0.2 mg/kg, whereas the MRL for thiamethoxam in peaches is 0.07 mg/kg and in melons is 0.15 mg/kg according to updated EU Pesticides database 2021[14].

The current study aimed at investigating the level of the neonicotinoid residues in two commonly edible local Egyptian fruits, test their compliance to the European MRL, and investigate the *in vivo* health risks associated with the intake of these local fruits at the ADI of Egyptian population.

Material and methods

Chemicals

Standard acetamiprid and thiamethoxam were generously provided by department of Agricultural pesticides, Ministry of Agriculture, Cairo, Egypt. HPLC grade acetonitrile (Fluka, Germany), hexane and acetone (AR, Sigma, USA) were procured.

Fruit samples and extraction

Samples of peach and cantaloupe were purchased from 5 different local markets, each sample weighed about ~500g. Each sample was thoroughly washed with tap water then chopped and well homogenized using fruit mixer in 250 ml distilled water. The fruit juice was liquid extracted with acetone/hexane mixture (1:3, *v*). The organic extract was separated, evaporated to dryness on a rotary evaporator at 60°C and the residue was dissolved in 10 ml of acetonitrile (HPLC grade) and filtered through a membrane filter (0.45 µm) for injection on GC-MS.

Chromatographic analysis

Quantitative analysis of pesticides was carried out at Central Agricultural Pesticides Lab, Cairo, Egypt using an Agilent 6890 gas chromatograph equipped with an Agilent mass spectrometric detector. Conditions were set to HP-5MS (Agilent, CA) capillary column of 30 m× 0.25 mm× 0.25 µm. The injector temperature was set to 250°C in splitless mode. Helium was used as a carrier gas at a flow rate of 1ml/min. the oven temperature was programmed to 70°C (hold 3.5 min) and increased to 300°C at 50°C/min (hold 7 min). The flow rate was modified to 1ml/min at 150°C. MS was set to 70 eV in EI mode and scan rate of 0.8/sec with scan range m/z:80-300, ion source temperature at 280°C. The solvent delay was 3 min and the injection size was 1.0 µl. The library was employed in verification.

Stock solution of each pesticide standard was prepared as 1 mg/ml in acetonitrile and stored in dark vials at -20°C. A working standard solution of 1 µg/ml was prepared by appropriate dilution of the stock solution by acetonitrile, to form the calibration standards (0.125, 0.25, 0.5, 0.75 µg/ml) and stored at 4-6 °C.

Toxicity Quotient

The Toxicity Quotient (TQ) for each sample was calculated as the sum of ratios between each pesticide residue concentration and the corresponding MRLs[15,16].

TQ= C/MRL

Estimated Daily Intake

The estimated daily intakes (EDI) of the pesticides in each fruit was determined from the average daily intake of a 70 kg adult person. The calculated amount was extrapolated to rat dose according to Nair and Jacob[17]and dispensed for each rat daily for 6 days.

Experimental Design

Three sets of adult Wistar rats (140-150g) were used and accommodated in the normal

environment with ambient temperature, light and humidity. Maintained with food and water *ad libitum*. Experimental protocol followed Helsinki declaration approved by the NRC-IACUC. Rats were administered either distilled water: Control or acetamiprid solution (17 µg/kg), or thiamethoxam solution (5 µg/kg) orally for 6 consecutive days. At the end, blood was collected by heart puncture under pentobarbital anesthesia and brain and liver tissues were excised and frozen at -20°C till further assessments.

DNA fragmentation

DNA was extracted from blood using QIAGEN DNA Purification kit (Germany) according to the manufacturer's instructions. About 20 µg of DNA was electrophoretically fractionated on 1% agarose gel containing 0.4 µg/ml ethidium bromide. The bands were visualized by a UV (302 nm) transilluminator and the gel was photographed with a polaroid camera. Damaged DNA appears as smears of fragmented DNA, whereas intact DNA does not move far into the gel and appears as one intact band[18].

Oxidative insult assessment

The reduced glutathione (GSH) level in serum, brain and liver was evaluated by the spectrophotometric method of[19] Ellman's using commercial prepared kit reagents and expressed as mmol/mg protein (Biodiagnostic, Egypt). The lipid peroxidation products were assessed in brain and liver of rats using the method of Uchiyama[20] and expressed as nmol/mg protein (Biodiagnostic, Egypt). Plasma protein carbonyl content was estimated colorimetrically. Tissue protein level was estimated by the method of Biuret[21].

Liver Function Assay

Serum liver enzymes sGOT, sGPT, and total protein level were estimated using commercial kits (Biodiagnostic, Egypt).

Plasma cytokine IL-1

Interleukin-1 was determined in plasma in terms of concentration (pg/ml) by commercial ELISA kit (Elabscience, USA).

Histopathological investigation

Liver tissue was examined under light microscope. Sections were prepared according to the method of Bancroft and Gamble[22]. The fixed specimens were processed through the conventional paraffin embedding technique, sectioned at 5 µm and stained with hematoxylin and eosin for histopathological examination under light microscope.

Statistical Analysis

Data are presented as means ± SE and were analyzed by ANOVA using the GraphPad Prism v 9.0 statistical program. Testing of significance was performed by Dunnett's multiple comparison post-Hoc test.

Results

Concentration of acetamiprid and thiamethoxam in fruits

Two kinds of fruits were chosen from local Egyptian markets; a melon type (cantaloupe) and a stone fruit (peach). Peach samples showed the presence of acetamiprid within the recommended levels by average of 0.08±0.005 µg/g (<MRL) in 100% of the tested samples, while all samples were devoid of the presence of thiamethoxam. On the other hand, 60 % of the tested cantaloupe

samples showed the presence of acetamiprid at average concentration of 0.24±0.01 µg/g (>MRL), while thiamethoxam was detected in 40% of the samples at average concentration of 0.08 ±0.007 µg/g (<MRL).

Toxicity Quotient

The toxicity quotient of each sample was calculated and found to be of average 0.8±0.19 for cantaloupe samples and 0.42±0.02 for peach samples (table 1).

Table (1): Pesticide concentration and toxicity quotient in cantaloupe and peach fruits

Fruit sample	Pesticide detected (Concentration µg/g)	MRL (µg/g)	Toxicity Quotient
Cantaloupe 1	Acetamiprid (0.2503)	0.2	0.79
Cantaloupe 2	Thiamethoxam (0.08)	0.15	0.50
Cantaloupe 3	Thiamethoxam (0.08)	0.15	0.54
Cantaloupe 4	Acetamiprid (0.22)	0.2	1.11
Cantaloupe 5	Thiamethoxam (0.10)	0.15	0.66
Average	Acetamiprid (0.24±0.01) Thiamethoxam (0.08 ±0.01)		0.8±0.19
Peach 1	Acetamiprid (0.08)	0.2	0.41
Peach 2	Acetamiprid (0.08)	0.2	0.40
Peach 3	Acetamiprid (0.07)	0.2	0.36
Peach 4	Acetamiprid (0.10)	0.2	0.50
Peach 5	Acetamiprid (0.08)	0.2	0.42
Average	0.08±0.005		0.42±0.02

Toxicity quotient is calculated from the detected concentration and the EU-MRL of each pesticide

Estimated Daily Intake

The estimate of 100 grams of fruit as an ADI of fruits in Egyptian population was depicted in the EDI calculation. It was calculated to be around 17µg/kg for acetamiprid and 6 µg/kg for thiamethoxam.

Rats body weight

Body weight of rats was measured at the start of the experiment and after the last dose. Rats were of average weight 154.7±2.3 g. Rats given acetamiprid showed a reduction of the weight by 8.9±1.8g significant from the control weight, while those given thiamethoxam showed a non-significant reduction by 5.3±0.3g. Normal control weight was not significantly changed by 3.8±1.3g at the end of the week compared to the starting weight (Figure 1).

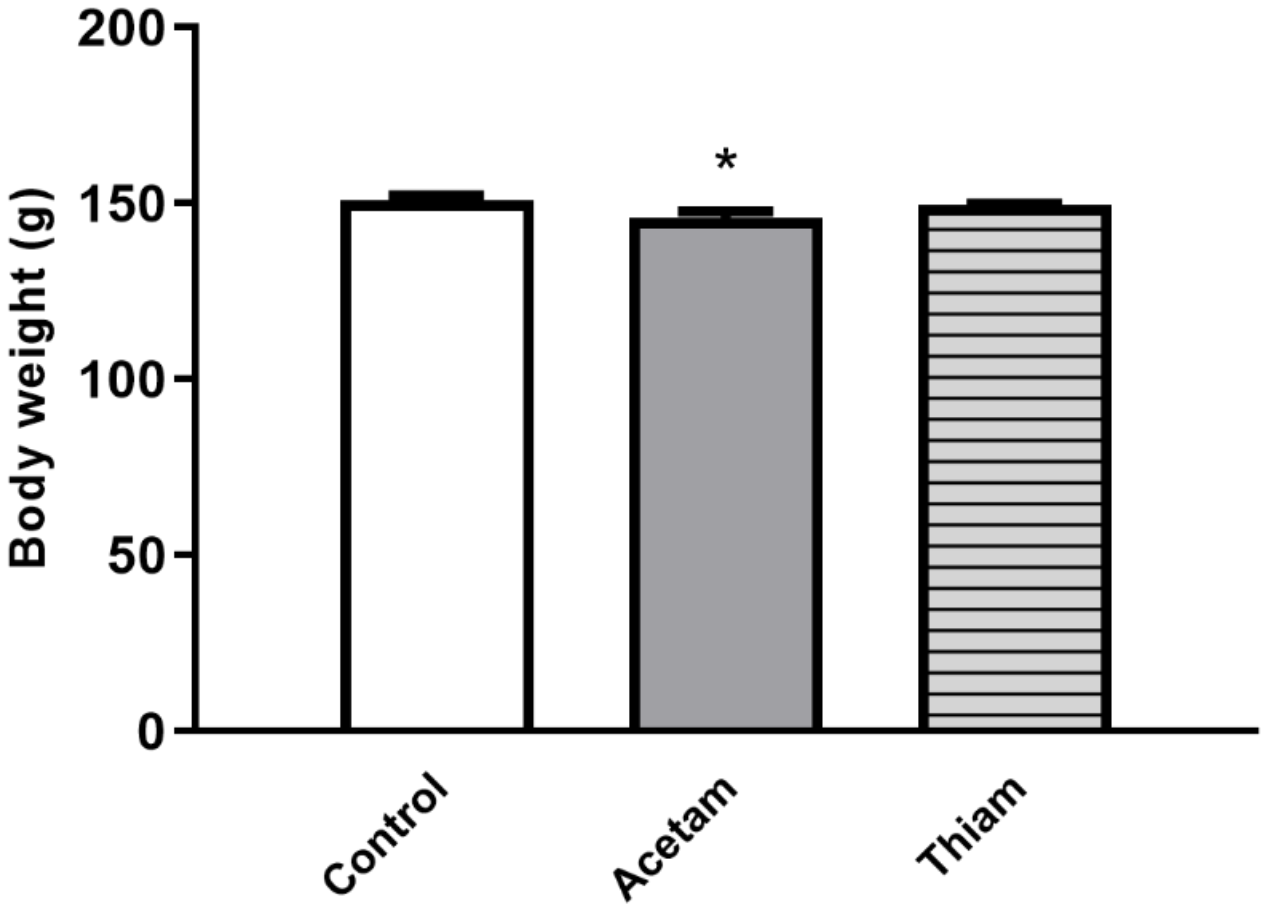

Figure 1: The effect of acetamiprid and thiamethoxam on the body weight of rats. A reduction was observed after 6 days of exposure of rats to oral acetamiprid. Data are expressed as mean± SEM (n=8). Significance was tested by Tukey's multiple comparison test after One Way ANOVA analysis. Asterisks indicate level of statistical significance (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001) compared to control group.

Effect on liver function parameters

Treatment of rats with the residual doses of thiamethoxam did not change the sGPT level from the normal range. sGPT level for control group was 31.52±2.09 IU/L and for thiamethoxam group was 27.20±1.31 IU/L. On the other hand, acetamiprid-treated rats showed increased sGPT level (47.15±4.48 IU/L, *p=0.0006*). Furthermore, serum GOT level was 28.06±1.72 IU/L in control animals. It was markedly induced by the administration of acetamiprid (40.24±4.82 IU/L, *p=0.0084)* and thiamethoxam (42.58±1.03 IU/L, *p=0.0084)* to rats. In serum, GSH level after acetamiprid or thiamethoxam exposure was at comparable level to that of control animals (*p*=0.59). Plasma total protein and protein carbonyl contents were not altered from control level (Figure 2).

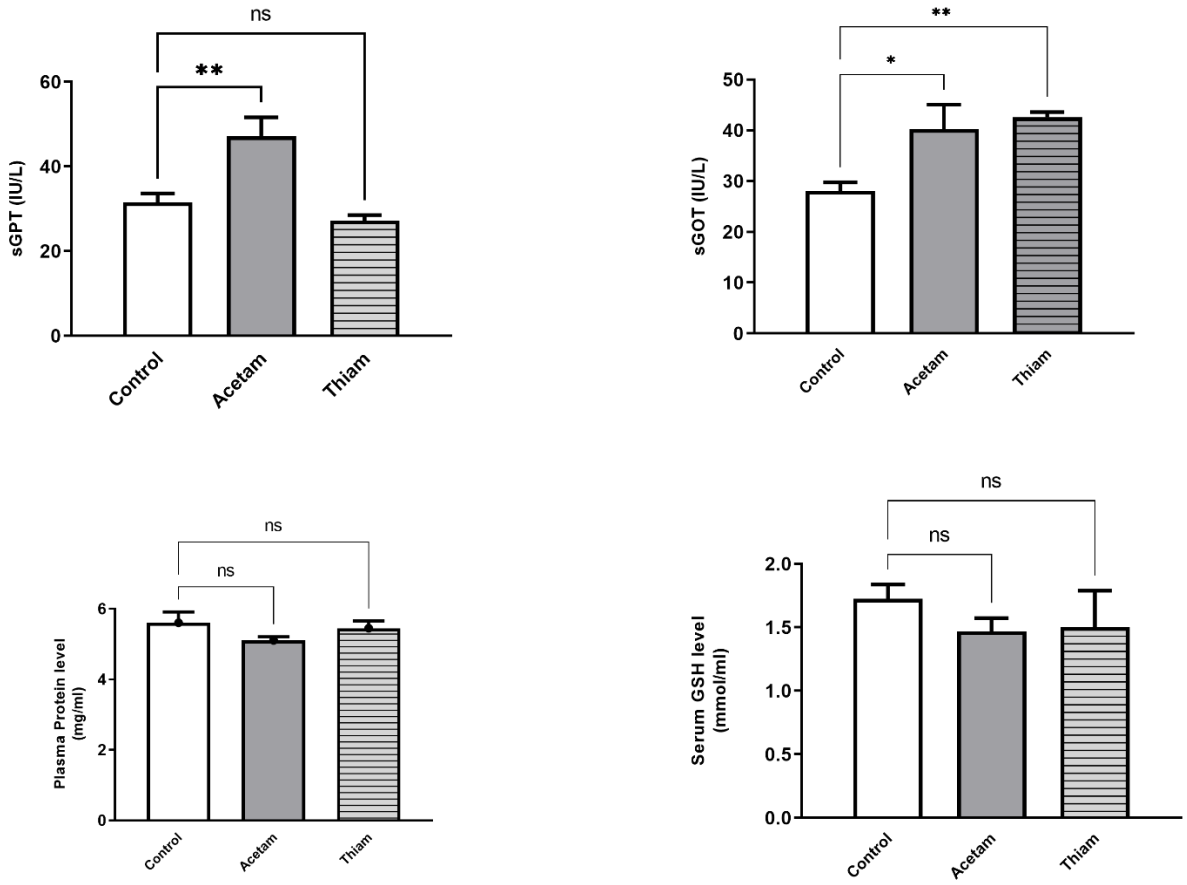

Figure 2: Effect of acetamiprid and thiamethoxam on liver functions. Data are expressed as mean± SEM (n=8). Significance was tested by Dunnett's multiple comparison test after One Way ANOVA analysis. Asterisks indicate level of statistical significance (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001) compared to control group.

Effect on oxidative biomarkers

Brain tissue showed normal oxidative state where all the tested markers were of comparable level to control levels. Likewise, the liver reservoir of the antioxidant molecule GSH was not affected by these pesticides (p=0.69). Catalase activity was normal in treated animals.

On the other hand, lipid oxidation products expressed as MDA were significantly increased in liver tissue of thiamethoxam exposed rats (~120%, *p*=0.0009) and acetamiprid-exposed rats (~70%, *p*=0.0009). Liver nitric oxide level was nearly two folds the control level in liver tissue after exposure to thiamethoxam or acetamiprid (*p*=0.0004).

Plasma protein carbonyl content as a marker of protein oxidation was markedly induced in treated rats (0.49±0.05, 0.92±0.08 and 0.93±0.07 nmol/mg protein in control, acetamiprid- and thiamethoxam-exposed rats, respectively). Results are compiled in table 2.

Table 2: Effects of acetamiprid and thiamethoxam on oxidative state of liver and brain tissues

	Control		Acetamiprid		Thiamethoxam	
	Liver	*Brain*	*Liver*	*Brain*	*Liver*	*Brain*
GSH (mM/mg protein)	0.39±0.05	0.55±0.02	0.33±0.03	0.53±0.02	0.39±0.07	0.50±0.05
MDA (nM/mg protein)	0.55±0.03	2.86±0.18	0.93±0.15*	2.62±0.21	1.24±0.07***	2.21±0.22
NO (mg/mg protein)	12.22±0.35	7.21±0.35	23.82±2.56***	7.09±0.82	22.97±1.61***	7.7±0.56
Catalase (nmol/mg protein)	10.94±0.95	3.85±0.08	8.52±0.52*	3.17±0.02	9.01±0.45	3.28±0.02*
Plasma Protein carbonyl (mg/ml)	0.49±0.05		0.92±0.08		0.94±0.07	

Data are expressed as mean± SEM (n=8). Significance was tested by Dunnett's multiple comparison test after One Way ANOVA analysis. Asterisks indicate level of statistical significance (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001) compared to control group.

Plasma cytokine IL-1

The inflammatory marker showed marked incline in response to acetamiprid exposure (45.12±1.67 vs. 35.26±1.32 pg/ml, *p*=0.0018), while thiamethoxam-exposed rats did not show significant difference form control level (40.12±1.96 pg/ml, p>0.05) (Figure 3).

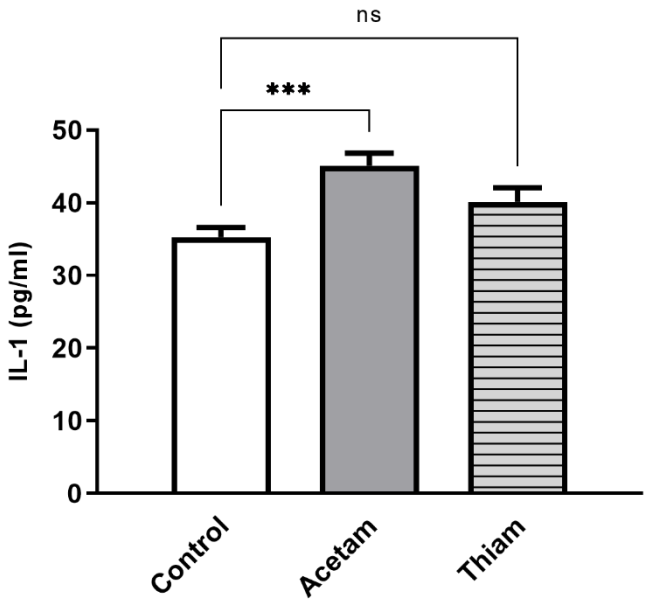

Figure 3: Plasma Interleukin-I level after exposure to acetamiprid and thiamethoxam. Data are expressed as mean± SEM (n=8). Significance was tested by Dunnett's multiple comparison test after One Way ANOVA analysis. Asterisks indicate level of statistical significance (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001) compared to control group.

Histopathological Investigation

Acetamiprid administered group showed scattered inflammatory cells surrounding portal tract and fibrosis dividing liver into lobules in a marked degenerative changes. Thiamethoxam administered group showed marked ballooning degeneration of hepatocytes as they were swollen and hydropic with scattered hyaline materials inside and central vein was dilated and surrounded with inflammatory cells, Figure 4.

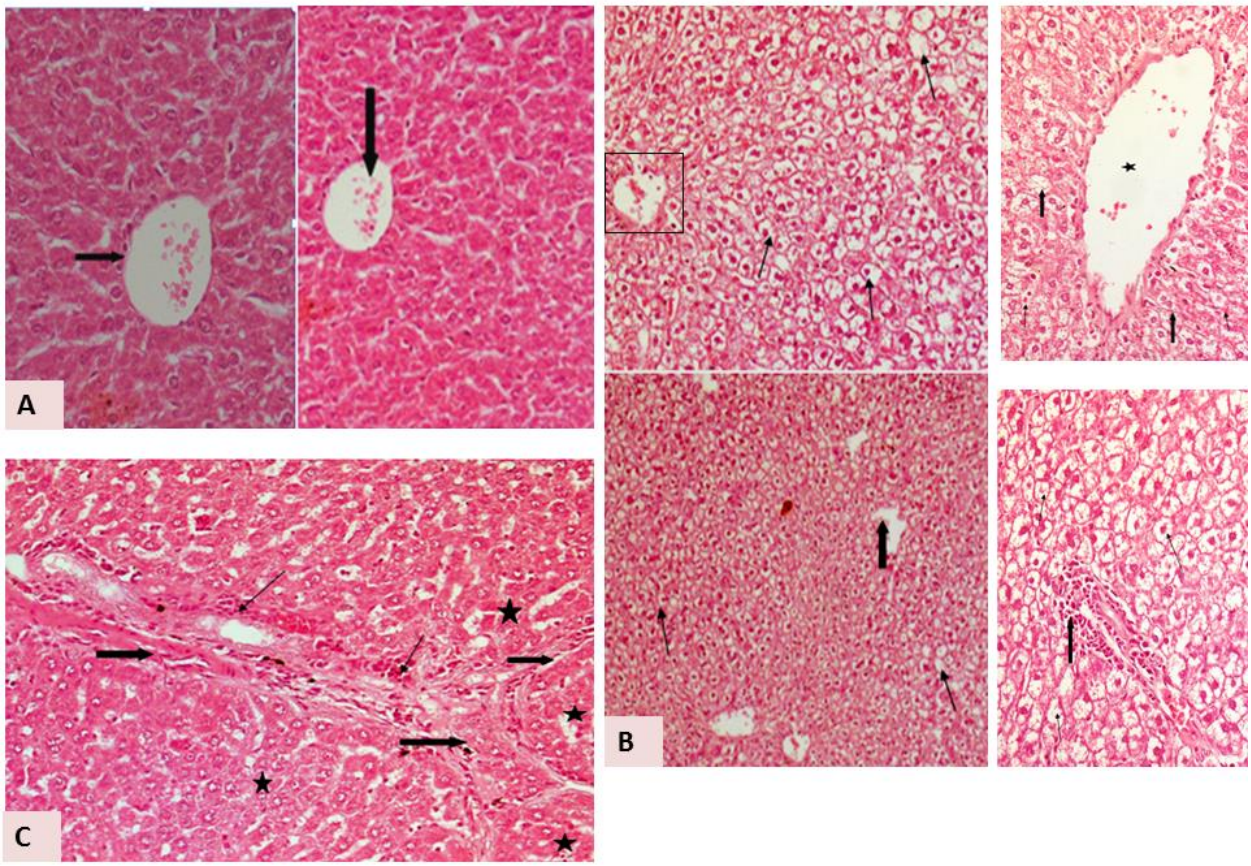

Figure 4: Examination of liver tissue of control group [A] showed normal liver tissue architecture with dilatation of central vein (thick arrow) while thiamethoxam-administered group [B] showed marked ballooning degeneration of hepatocytes cells (swollen and hydropic) (thin arrow) and dilated central vein (thick arrow) appearing irregular and surrounded with inflammatory cells upon magnification (right panel). Moreover, scattered hyaline materials inside hepatocytes cells (thin arrow) were observed. The Acetamiprid group showed scattered inflammatory cells surrounding portal tract (thin arrow) and fibrosis (thick arrow) dividing liver to lobules (star) (H&E x 100, 400).

DNA fragmentation

Agarose gel electrophoresis of DNA extracted from blood exhibited DNA fragmentation pattern after Acetamiprid administration to rats (Figure 5). On the other hand, thiamethoxam administered to rats showed distinct DNA band in comparison to normal control rats.

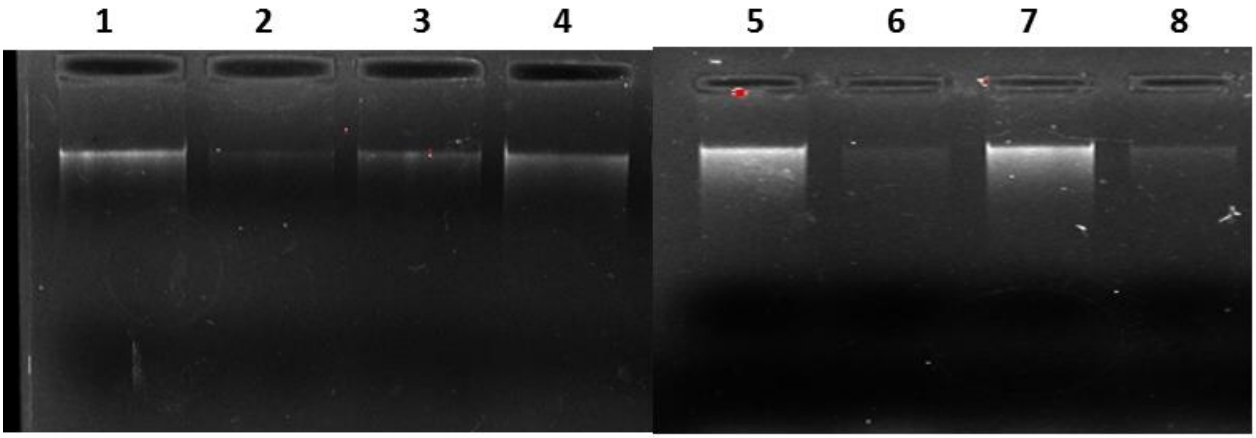

Figure 5: 1% agarose gel electrophoresis of the DNA extracted from blood of rats in control group (1 &6), Acetamiprid group (4, 5 &7) and thiamethoxam group (2, 3 &8)

Discussion

According to the World Health Organization, fruits and vegetables represent about 30% of food consumption[23]. Mediterranean region diet is widely known for higher percentage of fruits and vegetables [24]. For 2020, almost all analyzed samples were below the MRL. The European Union report on pesticide residues stated the detection of more than one pesticide in fruits where half of the analyzed samples did not exceed the MRLs[14]. Our study revealed the coincidence of the pesticide use and residual levels to the international permitted levels (MRLs). Peach samples were found to contain acetamiprid and cantaloupe samples contained thiamethoxam or acetamiprid below the MRLs. In accordance, the study of Shalaby et al., 2021[15] reported the presence of acetamiprid in around 10% of collected vegetable samples and thiamethoxam in 6% of the vegetables collected from local Egyptian markets.

The WHO classify the toxicity of acetamiprid as class II and thiamethoxam as class III in the toxicity hazard classification[25]. Since the consumer can encounter long term serious health effects due to continuous exposure to different toxins even at relatively low concentrations, life science researchers should enhance the scientific knowledge about these hazards. The present study demonstrated the possible health effects after continuous exposure at the same levels for 6 days.

Neonicotinoids, the newest group of insecticides, have remarkable and systemic influence on protecting crops against pests[8]. Several studies have demonstrated the incidence of lipid peroxidation of hepatic and

brain tissues by neonicotinoids in rats after oral administration of various doses ranging from 5 mg/kg to 40 mg/kg [26–28]. However, the present study focused on investigating the effects that may be encountered in liver and brain of rats after ingestion of the actual residual amounts of the neonicotinoid present in the average daily fruit intake, which was calculated to be around 17 µg/kg for acetamiprid and 6 µg/kg for thiamethoxam per 100g rat .

Aminotransferases are considered as a marker of liver tissue damage, where they leak during liver damage with an increased membrane permeability into the bloodstream [29]. In the current study, liver enzymes were observed to be elevated after exposure to these minute quantities of pesticides for a short time. A previous study explained that the free radicals produced by the acetamiprid bind covalently to protein and DNA leading to peroxidative degradation of lipid membranes, hence hepatic damage and necrosis, leading to inflammatory cells infiltration in the liver parenchyma and enzymes leakage into circulation[27]. Our results agree with this event cascade since acetamiprid showed fragmented DNA bands and pathological degenerative changes of hepatic tissue associated with the elevated liver enzymes and oxidative markers in addition to increased plasma pro-inflammatory marker, IL-1. Our results go in line with the reports of previous researchers[30–32].

Oxidative events such as lipid peroxidation, antioxidant molecules depletion, protein carbonylation, DNA infiltration and change, and tissue inflammation are initiated by the prevalence of free radicals[33]. The free radicals produced after exposure to acetamiprid or thiamethoxam were monitored by the content of the pro-oxidant molecules, MDA and NO and the counteracting anti-oxidant molecule, GSH and catalase enzyme. In addition, protein carbonylation is considered as a marker of pathological oxidation state in tissues[34] it was induced after the pesticide exposure. If the production of free radicals surmounts the antioxidant mechanisms and the redox state will be shifted towards oxidative stress[35].

Our results displayed a statistically significant increase in the level of MDA in the liver of rats exposed to acetamiprid, while the brain tissue was not affected. Likewise, the NO level was increased which impairs cell integrity and accelerates tissue damage and peroxidation by the production of the active peroxy nitrites. Protein carbonylation was augmented in response to the redox state. Lipid peroxidation was reported by[27,28,30] in response to acetamiprid exposure.

Previous reports showed enhanced GSH activity after acetamiprid exposure[36]. Others reported its downregulation[30,31]. In our study, GSH did not show relative change in its activity after the pesticide exposure compared to control animals, which may be due to the short time of exposure where the body is still able to tolerate the increased oxidative molecules.

Catalase is an important antioxidant enzyme that catalyzes the reduction of free radicals to water, dissolves organic hydroperoxides and can oxidize toxins to protect the tissue[35]. Acetamiprid decreased its activity in rats[30] in

agreement with our observation where liver catalase activity was suppressed. However, thiamethoxam administration did not affect its activity in liver but inhibited the activity in brain of rats.

On the contrary of our findings, the study of Khovarnagh and Seyedalipour[27] showed brain damage induced by acetamiprid, including gliosis, hyperemia, and necrosis of brain tissue which were not observed or reflected in any parameter in our study. This may be attributed to the different dose used as their study[27] included the dose of 5 mg/kg for one week while ours was 17 ug/kg for one week.

Conclusion

The current study indicated that the consumed fruits samples contained remnants of pesticides in the allowed levels. However, the hazards for disposing pathological conditions is not limited. Oxidative imbalance was imposed and liver reaction was initiated. This illustrates the necessity of awareness of consumers and farmers by these hazards and increasing the protective measurements such as vitamins and anti-oxidant intake by the general population.

Corresponding author:
Shaimaa Elshebiney
33 El-Bohouth St., Dokki, Giza, Egypt
Tel: +20233377906, +201096089508
E-mail: shaimaaelshebiney@gmail.com

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