RESEARCH ARTICLE

Single or combined exposure to chlorpyrifos and cypermethrin provoke oxidative stress and downregulation in monoamine oxidase and acetylcholinesterase gene expression of the rat's brain

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Abstract

The extensive uses of organophosphates and pyrethroids have made it necessary to investigate the neurotoxicity of their combination as they may implicate in the neurodegenerative syndromes. Monoamine oxidase-A (MAO-A) and acetylcholinesterase (AChE) gene expression in the rat brain were evaluated after independent and combined intoxications with chlorpyrifos and cypermethrin. Twenty-four mature male rats were equally distributed into four groups. The first one was kept as a control group, whereas the second, third and fourth were orally gavage with chlorpyrifos (16.324 mg/kg), cypermethrin (25.089 mg/kg) and their combination (9.254 mg/kg), respectively, for 4 weeks. As compared to the control group, intoxications with chlorpyrifos and/or cypermethrin revealed significant ($P < 0.05$) declines in the levels of brain neurotransmitters (dopamine and serotonin) plus the enzymatic activities of MAO-A, AChE and sodium-potassium adenosine triphosphatase. The mRNA genes expression of MAO-A and AChE have also confirmed the enzymatic actions. Moreover, the oxidative injury recorded as the levels of malondialdehyde and nitric oxide markedly increased ($P < 0.01$), while the total thiol content reduced and the histopathological outcomes have confirmed these impacts. In conclusion, chlorpyrifos and cypermethrin revealed antagonistic inhibitions on the brain MAO-A and AChE gene regulation through neurotransmission deteriorations and oxidative damage, which could describe their contributions in the neuropathological progressions.

Keywords Chlorpyrifos · Cypermethrin · Monoamine oxidase · Acetylcholinesterase · Gene expression

Introduction

Neurotoxicity arises via exposure to natural or artificial neurotoxic substances as pesticides, which alters the standard structure and function of the nervous system (Singh et al. 2014). Pesticides induce disorder through different mechanisms of action as several of epidemiological and trial studies have revealed that there is an association between exposure to pesticides, organophosphates and pyrethroids, and the progress of neurodegenerative syndromes (Mohammadi et al. 2019).

Chlorpyrifos (O,O-diethyl-O-3,5,6-trichloro-2-pyridyl phosphorothioate), an organophosphate insecticide, plays a crucial role in the pest control throughout a wide range of crops, fruits and vegetables (Kopjar et al. 2018). It is a well-known acetylcholinesterase (AChE) inhibitor, which primes to the buildup of acetylcholine, consequences of stimulus of excessive postsynaptic receptors and subsequent signs of toxicity (Mehta et al. 2009).

Cypermethrin ((RS)-α-cyano-3-phenoxybenzyl(1RS)cis-trans-3-(2,2-dichloro-vinyl)-2,2-dimethylcyclopropanecarboxylate) is a type II pyrethroid insecticide and used in the commercial agricultural applications. It has a lipophilic nature, accumulates in the brain and is involved in the pathological process of numerous neural disorders (Mohammadi et al. 2019).

The neurotoxicity of cypermethrin mediated primarily by the modulation of sodium channels, which results from

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staying open of the voltage-dependent sodium channels, consequence as a long-lasting prolongation of the standard transient in sodium permeability (Raszewski et al. 2015). The voltage-gated calcium channels, potassium channels, gamma-aminobutyric acid receptors, glutamate receptors, acetylcholine receptors, adenosine triphosphatases, and AChE were also influenced by cypermethrin (Singh et al. 2012).

Since chlorpyrifos and cypermethrin alter acetylcholine, which is widespread throughout the brain as occasioned from AChE inhibition, other neurotransmitters can be changed by these toxicants (Soreq and Seidman 2001). Dopamine and serotonin are the most crucial monoamine neurotransmitters in the pathophysiology of mental disorders and mechanisms of action of many psychotropic drugs. The biosynthesis and catabolism of monoamines are playing essential roles in their availability. The amino acids L-tyrosine and L-tryptophan are utilised for biosynthesis of dopamine and serotonin; however, the main enzymes involved in the catabolism are monoamine oxidase and catechol-O-methyltransferase (Fišar 2012).

Monoamine oxidase (MAO) is a flavin-containing amine oxidoreductase enzyme, and all mammals have two distinctive, MAO-A and MAO-B, proteins which encode by separate genes (Fišar et al. 2011). Both MAO enzymes found with high level in the neurons and distribution of these enzymes in the nervous system reflects their primary functions (Billett 2004). Studies show that the activity of MAO declined due to cypermethrin toxicity (Hussien et al. 2013), and chlorpyrifos may interfere with a dopaminergic pathway through the inhibition of MAO gene and protein expressions in vitro (Xu et al. 2012). Several other mechanisms also contribute to the toxic action of cypermethrin (Mohammadi et al. 2019) and chlorpyrifos (Ibrahim et al. 2019) including the creation of reactive oxygen species, which encourages oxidative damage and disturbances in the antioxidant body defence systems.

The trend of using cypermethrin and chlorpyrifos as a mixture is recently established as essential tools to overcome the resistance to these insecticides. Consequently, the establishment of new patterns of toxicity and environmental pollution are encouraged where the combined formulations provide different interactions between the active ingredients and induce prolonged-lasting residual impacts (Khan et al. 2013). Science chlorpyrifos and cypermethrin have different modes of action; their mixture has commonly been in practice against a variety of pests and increases the toxicity of co-exposure as chlorpyrifos can prolong the action of cypermethrin when used in a mixture (Latuszyńska et al. 2001). However, the combined impact among the different classes of pesticides is more complex to expect and recognize (Lydy et al. 2004); the mixture may increase the rapidity of action as in the case of virus vectors, which suggests the responses of antagonism or synergistic between the active agents. It helps in the downregulation of the metabolic detoxification enzymes which results in the blocking of hydrolysis and excretion (Latuszyńska et al. 2001).

Meanwhile, there is little evidence about the interaction between the organophosphates and pyrethroids and its impacts on the brain monoamine neurotransmitters and their metabolising enzymes. As the commercial formulation of chlorpyrifos and cypermethrin mixture has some second products or impurities which are unknown, study of the toxicity of this mixture is very essential for risk assessment. Here, we investigate whether the commercial products of chlorpyrifos and/or cypermethrin would influence the dopamine and serotonin levels, including underlying mechanisms, such as gene and protein expressions of MAO-A and AChE besides the correlation between these effects and oxidative damage.

Materials and methods

Pesticides used

The marketable products as emulsifiable concentrate (EC) formulations of cypermethrin (Nasr thrin super 10% EC), chlorpyrifos (Fosfolid 48% EC), and their mixture (chlorpyrifos 50% and cypermethrin 5%; Atifos super 55% EC) were obtained from Pesticides Analysis Department, Central Agricultural Pesticides Laboratory, Dokki, Egypt. Other chemicals were procured from Sigma Chemicals Company (USA).

Experimental procedures

Animals housing

A total of 89 mature male albino rats (*Rattus norvegicus*) aged 10–12 weeks were supplied by the Egyptian Organization of Biological Products and Vaccine. They were randomly housed at Mammalian Toxicology Department, Central Agricultural Pesticides Laboratory, Agricultural Research Center and monitored daily for abnormal symptoms for 2 weeks. The animals were accommodated in plastic cages with grill stainless steel covers in an air-conditioned room at a temperature of $23 \pm 2\ ^\circ C$, a relative humidity of 55% (55–70%) and a normal light/dark cycle. They were fed on a well-balanced nutrient that was obtained from Animals Food Manufactory of the Agriculture Ministry, Embaba, Giza, Egypt, and fresh tap water ad libitum. The experimental work was performed according to the guidelines for care and use of laboratory animals and the present study was accepted by the Ethics Committee of Cairo University (CU/I/F/2/18).

Study scheme

Experiment (I): acute lethal dose study

For each individual pesticide (Fig. 1), a total of 20 rats were divided into four groups of five animals each and used for the

estimation of the acute oral median lethal dose (LD$_{50}$). Accordingly, four serial doses were prepared for each insecticide where the doses of 88.88, 133.33, 200 and 300 mg/kg were used for chlorpyrifos with 1.5 as increment factor as well as the doses of 148.148, 222.22, 333.33 and 500 mg/kg were used for cypermethrin. Furthermore, the doses of 59.25, 88.88, 133.33 and 200 mg/kg were used for LD$_{50}$ assessment of cypermethrin and chlorpyrifos (mixture). The animals orally received the corresponding doses and a parallel of the control (5 rats) was run using a plain vehicle (distilled water). Mortality was recorded and LD$_{50}$ values were calculated by the moving average method using the special tables of Weil (1952).

Experiment (II): sub-acute study

Animals (24) were arbitrarily allocated into four experimental groups, six animals per each, as follows: the first one was saved as a control group where animals received distilled water. Rats in a second group were orally intubated by chlorpyrifos at a dose of 16.324 mg/kg body weight. In the third group, cypermethrin was administered at a dose of 25.089 mg/kg body weight. The fourth group was administrated a mixture of chlorpyrifos and cypermethrin at a dose of 9.254 mg/kg body weight. All animals have received their corresponding doses (equivalent to 1/10 LD$_{50}$) in 1 ml distilled water/kg body weight by gastric intubation for 4 weeks (Fig. 1).

Samples

By the end of the experimental period, the animals were anaesthetised and sacrificed, and the brain from each animal was rapidly removed. Then, they were washed with ice-cold normal saline, and the tissues were dried and fixed with 10% formol saline for histopathological examination. Other brain tissues were kept frozen at −80 °C for biochemical analysis and quantification of MAO-A and AChE gene expression by real-time polymerase chain reaction (RT-PCR).

Tissue preparation

The brain tissues were homogenised individually in precooled 50.0 mM potassium phosphate buffer, pH 7.5 containing 1.0 mM ethylene diamine tetra-acetic using a potter type homogeniser with a Teflon piston. The Sigma Laboratory Centrifuge (3K30, Germany) was used for centrifugation of the homogenate at 10,000×g for 15 min at 4 °C, and the collected supernatant stored at −80 °C for further use. The protein level was measured colourimetrically by Bradford (1976) method with bovine serum albumin as the standard using Jasco-V-630 UV/Vis bandwidth (Japan).

Neurochemicals analysis

Dopamine (OKEH02560) and serotonin (OKEH02558) levels were measured in the brain tissue by enzyme-linked immunosorbent assay kits (Aviva Systems Biology, San Diego, USA) according to the manufacturer's protocols. Lipid peroxidation biomarker, malondialdehyde (MDA), was measured spectrophotometrically according to the method of Uchiyama and Mihara (1978), while the techniques of Ridnour et al. (2000) and Sedlak and Lindsay (1968) were used for quantification of the nitric oxide (NO) and total thiol concentrations,
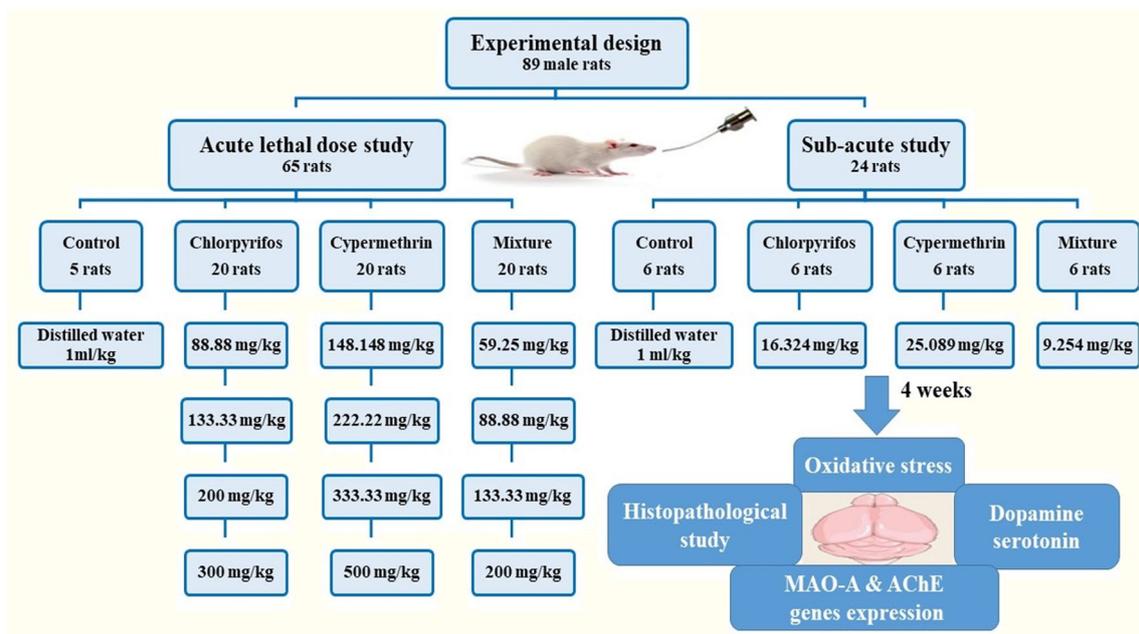

Fig. 1 A schematic diagram of the experimental design of the whole study

respectively. The activity of sodium-potassium adenosine triphosphatase (Na$^+$/K$^+$ ATPase) was measured by the method of de Souza Wyse et al. (2000) and the modified incubation system proposed by Raza et al. (2011). A spectrophotometric assay of Huang et al. (2016) was used to examine the activity of MOA-A, while the activity of AChE was tested by Ellman et al. (1961) procedure.

Quantification of AChE and MAO-A gene expression

The instruction protocol of QIAamp RNeasy Mini kit (Qiagen, GmbH) was used for extraction of RNA from the brain tissue. The final concentration and purity of RNA were measured by Nanodrop 8000 spectrophotometer (Thermo Scientific, USA) and the obtained A260/A280 ratios between 1.8 and 2.1 in all the samples. A single phase of RT-PCR kit (Qiagen, GmbH) was applied in a 25 μl reaction mixture containing 12.5 μl of the 2x QuantiTect SYBR Green PCR Master Mix, 0.25 μl of RevertAid Reverse Transcriptase (200 U/μL) (Thermo Fisher), 0.5 μl of each primer (Table 1) with 20 pmol final concentration (Metabion, GmbH), 8.25 μl of RNase-free water and 3 μl of RNA template. Expression was normalised to ß-actin as an internal housekeeping gene, and a negative control involved in each set of experiments.

The reaction was started by reverse transcription at 50 °C for 30 min then primary denaturation at 95 °C for 5 min, succeed by 40 cycles of the subsequent steps: 15 s at 94 °C (denaturation), 30 s at 61 °C (annealing) for AChE (Kazi and Oommen 2012), 56 °C for MAO-A (Kumar et al. 2013), 60 °C for β-actin (Banni et al. 2010) and 30 s at 72 °C (extension). The specificity of the PCR products was verified by the dissociation stage and quantitative analysis performed by the Stratagene MX3005P software. The variations of the mRNA gene expression on the different samples were estimated according to the "ΔΔCt" method (Yuan et al. 2006).

Histopathological assessment

The fixative specimens of the brain were embedded in paraffin in a hot air oven, and the paraffin beeswax tissue blocks were prepared. The gotten tissue slices were collected on glass slides, deparaffinized and stained by haematoxylin and eosin stains for examination (Downie 1990). A light microscope with a digital camera was used for imaging and investigation (Olympus, Japan).

Statistical analysis

Data entry and analysis were achieved by GraphPad Prism software (GraphPad version 7.02, Inc. CA, USA). Data were initially submitted to the exploratory analysis of normality (Shapiro-Wilk test) and homogeneity of variances (Levene's test). Data were presented (6 animals/group) as the mean ± standard error (S.E), and analysis of variance (ANOVA) followed by Tukey's multiple comparisons post hoc test was used for multiple comparisons between different groups. The grade of statistical significance was set at probability $P < 0.05$. The relationship between the quantitative variables was testified by 2-tailed Pearson correlation coefficient, and joint action analysis was done by the scheme Mansour et al. (2008).

Results

The acute oral median lethal dose

The estimated oral LD$_{50}$ of cypermethrin was 250.89 mg/kg body weight with (194.17–324.9) as a confidence interval, while chlorpyrifos was recorded 163.24 mg/kg body weight with (123.28–213.308) as a confidence interval, and 92.54 mg/kg body weight with (75.88–112.87) as a confidence interval for their mixture.

Signs of toxicity

There was no death observed in the experimental animals throughout the experiment of the sub-acute study. The apparent signs of intoxication were observed within the third after the pesticide administration. These symptoms include hyperirritability, decreased feed intake, diarrhoea and salivation in the cypermethrin-intoxicated group, while the rats suffered from tremor, convulsion and salivation in the chlorpyrifos group. There were more effects in a mixture group like convulsion, salivation, twisting movement and shivering in the body.

Table 1 Set of primer sequences with accession number for each gene used in real-time quantitative PCR

Gene	Accession number	Sequence 5′-3′	Amplicon size (bp)
β-actin	V01217	F-TCCTCCTGAGCGCAAGTACTCT	116
		R-GCTCAGTAACAGTCCGCCTA GAA	
MOA-A	NM_001270458.1	F-TGCATGGTGTATTACAAGGA	238
		R-CTTGAGATCCCAGAACTTTG	
AChE	NW_001084677	F-CCAATGACCCTCGAGACTCTAA	253
		R-GGTCGAACTGGTTCTTCCAG	

Effects of cypermethrin and/or chlorpyrifos on the body weights

The mean of body weight in the rats intoxicated with cypermethrin and chlorpyrifos was significantly ($P < 0.05$) lessened by 15.97% and 14.08%, respectively, as compared with the control group, but there was a non-significant decrease (1.88%) in the combined group. Although there was a significant difference between a mixture group when compared with the groups of cypermethrin and chlorpyrifos, no significant change was recorded between the cypermethrin and chlorpyrifos groups (Table 2).

Effects of cypermethrin and/or chlorpyrifos on the level of neurotransmitters

The concentration of dopamine significantly decreased by 28.21, 48.80 and 67.80% in the brain after intoxications with chlorpyrifos ($P < 0.05$), cypermethrin ($P < 0.01$) and their combination ($P < 0.001$), respectively, as compared with control. Moreover, there was a significant decline in serotonin level in the groups of chlorpyrifos ($P < 0.05$), cypermethrin ($P < 0.01$) and their mixture ($P < 0.001$) by 13.56, 28.71 and 51.35%, separately as compared with control. Additionally, a mixed group was recorded a significant reduction ($P < 0.01$) in dopamine and serotonin concentrations when compared

Table 2 The impacts of chlorpyrifos (16.324 mg/kg), cypermethrin (25.089 mg/kg) and their mixture (9.254 mg/kg) on the body weights and different neurochemical parameters

	Control	Chlorpyrifos	Cypermethrin	Mixture
Body weights	255.66 ± 3.24 [a]	219.66 ± 7.75 [b]	214.83 ± 7.12 [b]	250.83 ± 7.90 [a]
Dopamine ng/g tissue	169.55 ± 4.29 [a]	121.71 ± 4.36 [b]	86.81 ± 5.01 [c]	54.58 ± 3.11 [d]
Serotonin ng/g tissue	223.34 ± 3.11 [a]	193.04 ± 4.72 [b]	159.20 ± 4.45 [c]	108.63 ± 4.48 [d]
MDA nmol/g tissue	44.80 ± 3.85 [a]	77.18 ± 3.91 [b]	105.43 ± 5.03 [c]	142.08 ± 4.33 [d]
NO umol /g tissue	59.47 ± 5.31 [a]	88.08 ± 3.65 [b]	142.89 ± 3.80 [c]	172.19 ± 4.65 [d]
Total thiol uM/mg protein	80.27 ± 6.70 [a]	44.39 ± 4.14 [b]	23.25 ± 2.58 [b,c]	14.31 ± 2.63 [c,d]
Na$^+$ K$^+$ ATPase uM/min/mg protein	69.62 ± 2.24 [a]	57.57 ± 2.64 [a,b]	37.32 ± 2.65 [c]	24.07 ± 2.65 [c,d]
MOA-A uM/min/mg protein	119.34 ± 3.86 [a]	92.29 ± 5.24 [b]	72.73 ± 3.31 [b,c]	56.46 ± 4.61 [c,d]
AChE uM/min/mg protein	152.41 ± 3.70 [a]	71.70 ± 4.33 [b]	102.15 ± 4.67 [c]	53.52 ± 3.04 [b,d]

Each value represented the mean ± S.E of six animals. Means with different letters indicated the variations between the groups within the same row using Turkey's honestly significant difference ($p < 0.05$) test

with individual intoxication of chlorpyrifos and cypermethrin and the last was a potent one (Table 2).

Effects of cypermethrin and/or chlorpyrifos on the oxidant and antioxidant levels

The levels of MDA (72.25, 135.30 and 217.09%) and NO (48.09, 140.24 and 189.51%) markedly increased in the brain after intubation with chlorpyrifos ($P < 0.01$), cypermethrin ($P < 0.001$) and their combination ($P < 0.0001$), respectively, as compared with the control. In contrast, there were significant reductions in the concentration of total thiol by 44.69 ($P < 0.01$), 71.03 ($P < 0.01$) and 82.18% ($P < 0.001$) among the same groups as compared with the control one. The contents of MDA, NO and total thiol recorded significant differences ($P < 0.05$) when a mixture group was compared with chlorpyrifos and cypermethrin with an exception in case of cypermethrin among the total thiol (Table 2).

Effects of cypermethrin and/or chlorpyrifos on the activity of Na$^+$/K$^+$ ATPase

The enzymatic activity of Na$^+$/K$^+$ ATPase significantly ($P < 0.01$) declined by 46.38 and 65.41% in the groups of cypermethrin and mixture, respectively, as compared with the control one. However, intoxication with chlorpyrifos alone revealed non-significant decrease (17.30%) as compared with a control group ($P \geq 0.05$); it recorded a significant difference ($P < 0.05$) with a combined one (Table 2).

Effects of cypermethrin and/or chlorpyrifos on MAO-A and AChE enzymatic activities

The enzymatic activity of MAO-A significantly decreased after intoxications with chlorpyrifos ($P < 0.05$), cypermethrin ($P < 0.01$) and their mixture ($P < 0.01$) by 22.01, 38.53 and 52.29%, respectively, as compared with control. There were significant reductions in the activity of AChE within the same groups by 52.95 ($P < 0.01$), 32.97 ($P < 0.05$) and 64.88% ($P < 0.01$) as compared with the control group. Additionally, there were marked ($P < 0.05$) declines in MAO-A and AChE enzymatic activities when a mixture group was compared with individual intoxication of chlorpyrifos and cypermethrin. Intoxication with cypermethrin revealed a strong inhibitor to MAO-A than chlorpyrifos and vice versa in case of AChE (Table 2).

Correlations analysis

The 2-tailed Pearson correlations among the different examined biochemical parameters showed that there were noteworthy positive correlations ($P < 0.01$) between the brain MAO-A within dopamine, serotonin, total thiol,

Na^+/K^+ ATPase and AChE with correlation coefficients (r) of 0.97, 0.93, 0.91, 0.90 and 0.76, respectively. In contrast, there were negative correlations between MAO-A in comparisons with MDA and NO ($r = -0.93$ and -0.92), respectively. Likewise, there were significant positive correlations ($P < 0.01$) between AChE with dopamine, serotonin, total thiol and Na^+/K^+ ATPase ($r = 0.79, 0.75, 0.80$ and 0.69, respectively); however, MDA and NO recorded negative correlations ($r = -0.76$ and 0.68, respectively).

Joint action analysis

The investigation of joint action revealed that a mixture (chlorpyrifos/cypermethrin) group has different types of interaction among the measurable biochemical markers. Mostly, the antagonistic interactions were observed in dopamine, total thiol, MAO-A and AChE, while serotonin scored a potentiation effect as well as MDA, NO and Na^+/K^+ ATPase recorded the additive effects (Table 3).

Effects of cypermethrin and/or chlorpyrifos on MAO-A and AChE gene expression

The fold changes of MAO-A mRNA gene expression (0.6445-, 0.2615- and 0.0891-fold) markedly declined after intoxications with chlorpyrifos ($P < 0.05$), cypermethrin ($P < 0.01$) and their mixture ($P < 0.001$), respectively, as compared with a control group. There were significant downregulations in the mRNA gene expression of AChE within the same groups by 0.369- ($P < 0.01$), 0.684- ($P < 0.05$) and 0.160-fold ($P < 0.001$) as compared with the control. Furthermore, the mixture group scored a significant downregulation in the mRNA gene expression of AChE as compared with individual intoxications of chlorpyrifos and cypermethrin ($P < 0.05$); however, this pattern recorded in chlorpyrifos only in case of MAO-A (Fig. 2).

Table 3 Joint action analysis of different studied parameters with a mixture of chlorpyrifos and cypermethrin insecticides

Parameters	Effect	I.I	Joint action
Dopamine	Decrease	1.07	Antagonism
Serotonin	Decrease	0.94	Potentiation
MDA	Increase	1.02	Additive
NO	Increase	1.00	Additive
Total thiol	Decrease	1.39	Antagonism
$Na^+ K^+$ ATPase	Decrease	0.98	Additive
MOA-A	Decrease	1.06	Antagonism
AChE	Decrease	1.18	Antagonism

Interaction index (I.I) $= 1 \pm 0.05$ for additive effect. In cases of positive effect on the baseline values (increase) > 1 for potentiation, or < 1 for antagonism. In cases of negative effect on the baseline values, < 1 for potentiation, or > 1 for antagonism
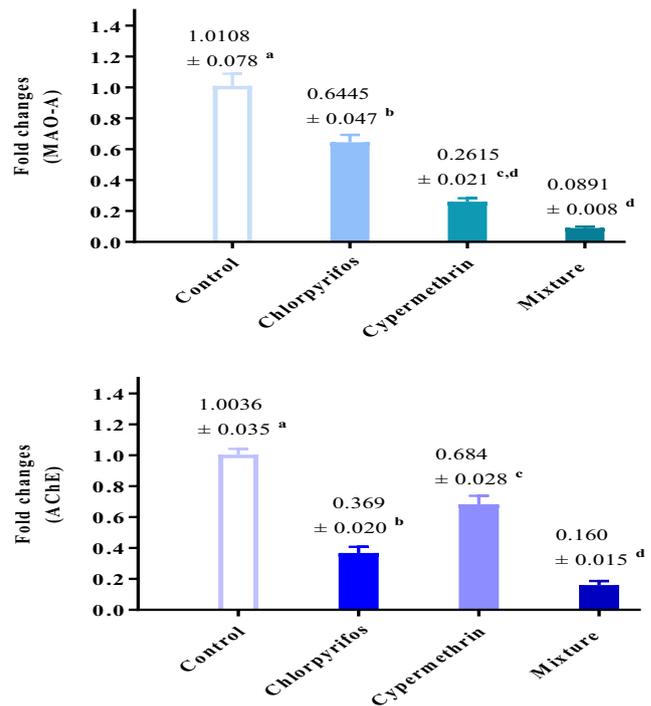

Fig. 2 The impacts of chlorpyrifos (16.324 mg/kg), cypermethrin (25.089 mg/kg) and their mixture (9.254 mg/kg) on mRNA gene expression of MAO-A and AChE. Each value represented the mean ± S.E of six animals. Means with different letters indicated the variations between the groups within the same column using Turkey's honestly significant difference ($p < 0.05$) test

Histopathological finding

There was no histopathological alteration in the control group as the cerebral cortex showed normal molecular layer (ML) with glial cells and pyramidal layer (PL) (Fig. 3a). Degeneration and nuclear pyknosis were detected in the brain after intoxication with chlorpyrifos. Most of PL cells have an irregular shape and darkly stained nuclei (arrow) with pericellular halo (Fig. 3b). A marked retraction in GL with vacuolations and eosinophilic (degenerating) neurons with dark stain were observed in the cypermethrin group (Fig. 3c). In a mixture group (Fig. 3d), some of the degenerated PLs appeared irregular and shrunken with pyknotic nuclei (arrow) associated with oedema in the covering meninges, and focal haemorrhage of meninges (arrowhead).

Discussion

Over the past decade, the extensive usage of organophosphates and pyrethroids as a combination in agriculture and for public health applications has led to extreme effects in many non-target species including human and made the toxicity assessment of these pesticides, alone or in a mixture, very vital. This knowledge encourages us to study

Fig. 3 Photomicrographs of the brain section stained with H&E for the histopathological study. Control (**a**); the cerebral cortex showed normal molecular layer (ML) with glial cells (GL), and pyramidal layer (PL). Chlorpyrifos (**b**); PL cells have an irregular shape and darkly stained nuclei (arrow). Cypermethrin (**c**); marked retraction in GL, vacuolations and eosinophilic neurons with darkly stained (arrow). Mixture (**d**); an irregular degenerated PL, shrunken with pyknotic nuclei (arrow) and focal haemorrhage of meninges (arrowhead). Scale bar 250 μm
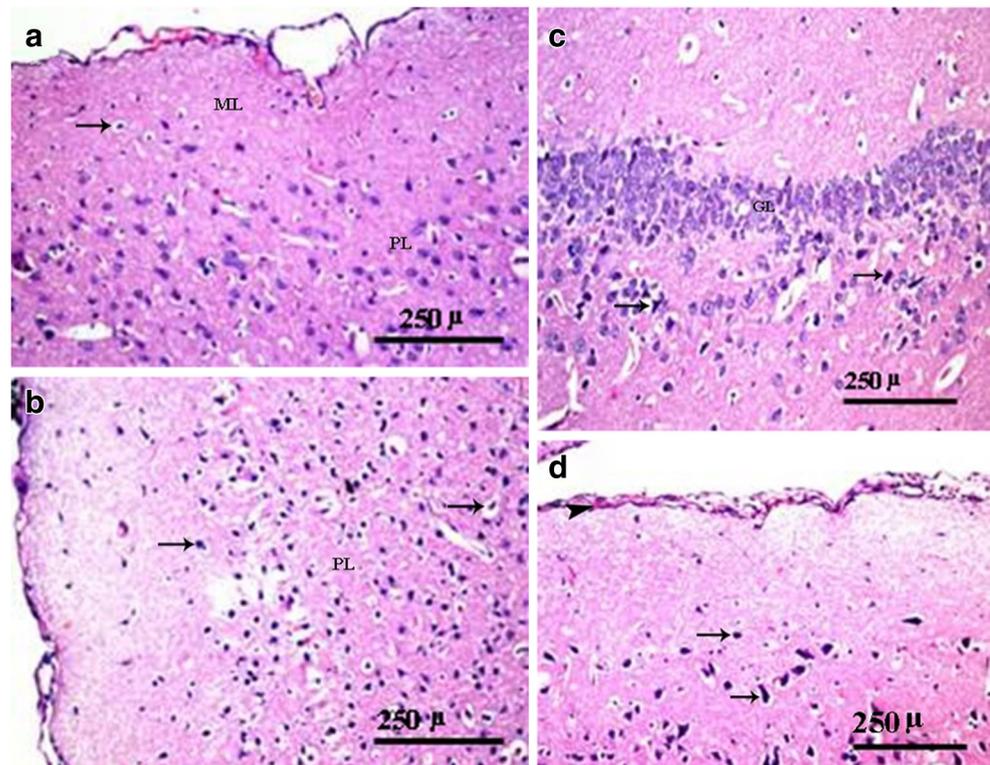
the efficiency of independent and combined intoxications with chlorpyrifos and cypermethrin in the levels of brain monoamines with mechanistic attention of MAO-A and AChE mRNA gene regulation besides the correlation between these effects and oxidative damage.

In the assessment and evaluation of the toxic characteristics of a chemical substance, the estimation of acute oral toxicity is usually an initial step (WHO 2009). It is traditionally a stage in the establishment of dosage regimen in other studies by providing the initial information on the mode of toxic action and health hazards of this chemical (Perkins and Garcia-Reyero 2013). Although acute oral median lethal dose values of chlorpyrifos and cypermethrin for rats are well known, there is still much controversy concerning the value of their combined. Our results revealed that the estimated oral LD_{50} of cypermethrin, chlorpyrifos and their mixture were 250.89, 163.24 and 92.54 mg/kg body weight, respectively. This means that the commercial formulation of the mixture becomes more toxic and highly hazardous according to the classification of WHO (2009) which may result from the complex mixture of the components like surfactant and organic solvent rather than the active ingredient.

Symptoms of toxicity were observed in all intoxicated animals following the oral administration that may be due to the rapid and complete absorption of these pesticides. The neurotoxic signs suggested the involvement of the central nervous system dysfunction which results from the accumulation of chlorpyrifos or its metabolites in the brain following the direct

exposure (Mehta et al. 2009). Cypermethrin, on another hand, can cross the blood–brain barrier and exert their effects on dopaminergic and cholinergic systems (Mohammadi et al. 2019) that induce the behavioural alterations. The less in the body weight due to intoxications with chlorpyrifos and cypermethrin may be attributed to the neurotoxic effects of these pesticides that endanger animal, lessen food intake and result in body weight loss (Kapoor et al. 2010). So a portion of body weight that declined in the current study may be attributed to starvation or malnutrition which is an indication of the adverse effects of these insecticides (Aroonvilairat et al. 2018), may serve as growth rate index in animals and may be optional for risk assessment of the neurotoxic pesticides.

Notably, despite the reduction of body weight resulted from the single exposure to chlorpyrifos and cypermethrin, the mixture group showed no significant decline. The possible explanation for this result may be attributed to the opposite stress of chlorpyrifos and cypermethrin on the craving. However, cypermethrin decrease the blood glucose level (Ince et al. 2012), and chlorpyrifos evoke the levels of glucose through increasing the resistance of insulin and leptin hormone (Peris-Sampedro et al. 2015) which is responsible for the control of food intake and appetite. In this issue, the higher amounts of body fat due to the increase in leptin levels indicate that chlorpyrifos could rise the adiposity. Consequently, adipose tissue is a possible target for chlorpyrifos (Peris-Sampedro et al. 2015), which is highly lipophilic in nature and responsible for recovering the decrease in the body weight caused by cypermethrin.

Dopamine and serotonin are catecholaminergic neurotransmitters and play a fundamental role in controlling the body posture, and cognitive and emotional behaviours. Dopaminergic and serotonergic neurotransmissions are more vulnerable to the adverse factors as environmental toxins where the disorder of their functions results in human neurodegenerative diseases (Chen et al. 2011). Our findings exhibited that the oral gavage of chlorpyrifos and/or cypermethrin (1/10 LD$_{50}$) for 28 days revealed significant decreases in the brain dopamine and serotonin levels and these influences might reflect with their neurotoxic effects.

Remarkably, organophosphates and pyrethroids are commonly insoluble in water; so a variety of solvents is consumed in their formulation products and used in conjunction with potentiators. Where pesticides are used in combinations, there is a potential for interactions not only between the active ingredient of pesticides but also between pesticides, solvents and potentiators. So, it suggested that the vehicles of commercial formulation as an emulsifiable concentrate or another ingredient in the formulation might have been responsible for neurotoxicity (Axelrad et al. 2002). The solvents may have numeral ways to increase the toxicity of chlorpyrifos and/or cypermethrin as they may support the passage of these insecticides through the cell membrane, thus permitting higher toxicity action on a nerve cell. Alternatively, the impact of these pesticide metabolism by the nerve cells is a part of the enriched toxicity in the presence of solvents as the induction of cytochrome P450 family may increase by acetone itself and/or after the metabolism of ethanol to acetone (Wickramasinghe 1987). These signs suggest that exposure to multiple formulations of organophosphates and/or pyrethroids may enhance the neurotoxicity of these insecticides at the cellular level by a direct mechanism (Axelrad et al. 2002).

The harmful impact of chlorpyrifos on the brain content of dopamine may occur via a variety of biological and pathological mechanisms. In this topic, chlorpyrifos intoxication leads to activation of acetylcholine receptor after the release of acetylcholine from striatal cholinergic interneurons which interacts with dopamine signalling at several planes which enclosed the pre-synaptic regulation of neurotransmitter release and postsynaptic effects (Torres-Altoro et al. 2011). Alternatively, the decline of dopamine level may result from the neurotoxic insult of pyrethroids by increasing the susceptibility of dopaminergic neurons which lead to degradation of dopamine as a consequence to increase the levels of the dopamine transporter (Elwan et al. 2006). So cypermethrin may modulate the dopaminergic neurotransmission through a catabolic pathway. As the tested pesticides may be affected in the dopaminergic system via totally different mechanisms, an antagonism interaction scored in their mixture.

Our results suggested that the effects of chlorpyrifos and cypermethrin on serotonin levels may result as a response to

interactions between several types of neurons which express different ion channels and receptors. Accordingly, the decrease in serotonin level may attribute to the activation of autoreceptors by pyrethroid which leads to the decline in the synthesis and/or release of serotonin in the striatum and frontal cortex (Casanovas et al. 1997). The increase in the release of gamma-aminobutyric acid due to cypermethrin intoxication is another possible mechanism involved in the decline of serotonin. This hypothesis is maintained by prior results as allethrin and deltamethrin indirectly inhibited glutamate release in the hippocampus by increasing the release of gamma-aminobutyric acid via acting on sodium channels of interneurons, which suppress the release of serotonin from the terminals of a serotonergic nerve (Hossain et al. 2013). Also, the decrease in serotonin level after chlorpyrifos intoxication may result as a response to its impact on the uptake of serotonin via calcium channel inhibition (Meijer et al. 2015). So the potentiation effect observed as the sodium and calcium channels may be involved in the modulation of serotonergic neurotransmission by chlorpyrifos and cypermethrin insecticides.

The current work indicated that the deteriorations in dopaminergic and serotonergic neurotransmissions accomplished with downregulation in the neurotransmitter metabolising enzymes (MAO-A and AChE) and the mRNA gene expression have also confirmed the enzymatic activities. Our results suggested that the inhibitions of MAO-A and AChE after chlorpyrifos and/or cypermethrin treatments confirmed its effects on the pathway of neurotransmissions metabolism.

The mechanism by which organophosphate insecticides induced this decline in the activity of MAO-A is still completely unclear. However, Xu et al. (2012) proposed that the effect of chlorpyrifos on dopaminergic neuron may mediate by the inhibition in the gene and protein expressions of MAO through interfering of chlorpyrifos with a dopaminergic pathway. Otherwise, direct competitive inhibition is a possible mechanism involved in the interaction between cypermethrin and MAO-A. In this respect, it was stated that the two pyrethroids namely permethrin (type I pyrethroid) and cyhalothrin (type II pyrethroid) competitively inhibited MAO-A by altering the Michaelis–Menten constant and the maximum velocity. The values of inhibitor constant indicated that cyhalothrin is a more MAO inhibitor than permethrin (Rao and Rao 1993).

Indeed, AChE plays a vital role in cell communication, and the inhibition of this enzyme is the main target for the toxicity of chlorpyrifos. In mammals, chlorpyrifos is quickly absorbed and broadly distributed with low probable accumulation (Arnold et al. 2015). The liver is the main organ for the metabolism of chlorpyrifos to its oxidised form, chlorpyrifos-oxon, which binds to enzyme throughout a robust covalent bond by phosphorylation of the enzyme serine residue causing irreversible inhibition of AChE. This block leads to continuous stimulation of the parasympathetic system and

accumulation of acetylcholine in the synaptic cleft and subsequent activation of cholinergic muscarinic and nicotinic receptors (Mehta et al. 2009).

The downregulation of AChE by cypermethrin may result as a direct action of this insecticide in the enzyme active site or an indirect effect through the nicotinic acetylcholine receptors. Consequently, cypermethrin may disturb the permeability of neuron membrane and interfere with the conduction of nerve impulses that therefore increase the release of the neurotransmitter acetylcholine (Mohammadi et al. 2019). Also, the findings of Singh et al. (2014) showed that the inhibition of AChE might occur through the interaction between cypermethrin and the anionic substrate binding site of the enzyme. Another possible communication (pi-pi interaction) may occur between diphenyl ether of cypermethrin and side chain of amino acids in AChE active site and/or cypermethrin may interact with serine residue of the enzyme through the trans-esterification process. Lastly, due to the high lipophilicity of pyrethroids as cypermethrin, it can bind at the active site of AChE through the hydrophobic surface of AChE, resulting in the decline of enzyme activity (Rao and Jagannatha Rao 1995).

Our results proposed that the inhibitions in the gene and protein expressions of brain AChE and MAO-A could characterise the mode of action through which chlorpyrifos and/or cypermethrin may further contribute to pathological progressions in neurotoxicity. The degree of inhibition is varied between AChE and MAO-A as cypermethrin revealed a potent inhibitor to MAO-A than chlorpyrifos and vice versa in case of AChE. This dissimilarity revealed an antagonistic interaction within a mixture which may attribute to the differences in the interaction type between the tested pesticides and/or its metabolites with AChE and MAO-A in various organs, as well as the relative coherence between the enzymatic inhibitions and the degree of nerve innervations in these organs.

Conferring to these outcomes and based on the proposed mechanism of Corbel et al. (2006), the subsequent cascade of events may be involved in the observed antagonistic effect within the mixture of chlorpyrifos and cypermethrin. Firstly, the release of acetylcholine increased within the synaptic cleft as a response to the impact of cypermethrin on the voltage-dependent sodium channels. At the same time, chlorpyrifos produced an elevation of non-hydrolysed acetylcholine after blocking the activity of acetylcholinesterase at postsynaptic level. Finally, the accumulative effects of both chlorpyrifos and cypermethrin on acetylcholine in the synaptic cleft created an imbalance in neurotransmission pattern via the negative feedback mechanism.

Subsequently, the steady status of disabilities in the brain dopamine, serotonin and neurotransmitter metabolising enzymes (MAO-A and AChE) are associated with decay in the activity of Na^+/K^+ ATPase. In this issue, there was a significant decrease in Na^+/K^+ ATPase activity after intoxications with cypermethrin and combination; however, chlorpyrifos scored non-significant decline. These influences may result from the disturbance in neurotransmitters and their metabolising enzymes as there were notable positive correlations between the brain MAO-A with AChE, dopamine, serotonin and Na^+/K^+ ATPase.

It is not sure whether the impact of chlorpyrifos on ATPase is direct or not as it induced non-significant reduction, which may be attributed to the interaction of chlorpyrifos with the dephosphorylation process (Ajilore et al. 2018). Also, the disturbance in the energetic mitochondrial system due to the movement of cypermethrin within the mitochondria after its access through the meninges may be involved in the decline of Na^+/K^+ ATPase activity by disturbing the energy creating a metabolic pathway or interrelates with the enzyme directly (Singh et al. 2009). As the enzyme activity may be affected by the two tested pesticides via the energetic route, an additive effect scored within their mixture.

Notably, inactivation of ATPase leads to partial membrane depolarisation, which permits the excessive entry of cations inside cells. The mechanism of inactivation under such conditions also involves the disruption of phospholipid or direct damage of the enzyme that associated with alteration of fluidity or other membrane properties (Hossain and Richardson 2011). Therefore, chlorpyrifos and/or cypermethrin exerts their neurotoxic effects through voltage-dependent ion channels and integral protein of the enzymatic ATPase in the cell membrane (Hussien et al. 2013). Consequently, they may affect cell membrane via their strong affinity with membrane lipids, which cause the inhibition in membrane-bound of ATPase activity. These grades suggested that the impairment of ATPase could be one of the underlying biochemical mechanism that leads to brain dysfunction as a consequence of the enhancement of oxidative damage.

The oxidative injury was scored in the current study after independent and combined intoxications with chlorpyrifos and cypermethrin as the levels of MDA and NO markedly increased, while the concentration of the total thiol significantly reduced and the histopathological findings have confirmed these impacts. These results proposed that the oxidative stress may be involved in the neurotoxic effects of the tested insecticides as there were markedly positive correlations between MAO-A within dopamine, serotonin, total thiol, Na^+/K^+ ATPase and AChE, while MDA and NO scored negative one.

Recently, chlorpyrifos (Ghahremani et al. 2018) and cypermethrin (Kanbur et al. 2016) produced oxidative stress by disturbing the redox status in the wide variety of organs, especially the brain. Oxidative damage principally occurs through the creation of reactive oxygen species (ROS) that subsequently reacts with biological molecules and leads to loss of cellular membranes. The oxygen, hydroxyl and hydroperoxyl radicals are the primary sources of ROS, which were mostly produced by

mitochondrial electron transport chain in which the molecular oxygen interacts with NO to build peroxynitrite (Farooqui and Farooqui 2009). The peroxidation of the lipids results after the attack by these molecules where lipid peroxyl radicals and hydroperoxides are the main products of lipid peroxidation as well as MDA is a secondary one (Yin et al. 2011). As both chlorpyrifos and cypermethrin possibly overexpressed MDA and NO to produce the free radical, an additive effect was observed in a concoction.

The decline in the total thiol contents may be attributed to its consumption through ROS generation as it has scored negative correlations with MDA and NO. This reduction may be resulted from the oxidation of cellular thiol groups by chlorpyrifos (Ghahremani et al. 2018), which lead to the decline of the cellular reduction potential. The fall of total thiol after intoxication with cypermethrin (Mohammadi et al. 2019) may be attributed to its contrary role to free radicals which lead to utilisation of glutathione as it has a compensatory mechanism of the antioxidants to combat the oxidative injury. The antagonistic action was documented in a combined group as chlorpyrifos decreased the total thiols via the oxidation response, which is fully diverse from cypermethrin.

Our results hypothesise that the initiation of oxidative stress is perhaps the essential mechanism by which this kind of pesticides exert their cellular action. So, the elevations of the brain MDA and NO levels may be due to oxidative degradation in the lipid mainly polyunsaturated fatty acids and utilisation of thiol groups for the exclusion of excessive free radicals. These records promote variation in the membrane assembly and function as the brain has a high vulnerability to oxidative insult, a large amount of polyunsaturated fatty acids and relatively low antioxidant system.

Our results anticipated that oxidative injury is associated with histopathological alterations, including degeneration, nuclear pyknosis, vacuolations, eosinophilic neurons, oedema and focal haemorrhage. These impacts may result after the damage of cell membrane proteins via oxidative stress, which ultimately results in the destruction of its fluidity and decline in the antioxidant ability of the brain cells. This hypothesis could be confirmed by the findings of Latuszyńska et al. (2003) who reported that chlorpyrifos and cypermethrin induced many histopathological alterations including accumulation of the cytoplasm in neurocytes accompanied with pyknosis in the cerebral cortex, pyknosis in cerebellum area with oedema, haemorrhage in the covering meninges and formation of eosinophilic plagues. We suggest that these pesticides have a lipophilic nature (Afoke and Igho 2015) which simplify their absorption and crossing of the blood–brain barrier into the central nervous system which could result in nervous tissue damage as impairment of neural conduction followed by degeneration of neurocytes via oxidative injury.

Conclusion

From the present finding, we have concluded that independent and combined intoxications with chlorpyrifos and cypermethrin revealed significant disabilities in the catecholamines and neurotransmitter metabolising enzymes via a vulnerability of the brain tissue to oxidative damage. The antagonistic inhibitions of the brain MAO-A and AChE mRNA gene regulation could characterise the mode of action through which these insecticides may further contribute to pathological progressions in neurotoxicity, and more research could be conducted to cover this issue.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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