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ORIGINAL ARTICLE

Neurological alterations induced by formulated imidacloprid toxicity in Japanese quails

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Abstract

Agrochemical risk assessment that takes into account only pesticide active ingredients without the spray adjuvants will miss important toxicity outcomes detrimental to non-target species including birds. In the present study toxicity of imidacloprid (IMI) pesticide was evaluated individually and in a mixture with polyethylene glycol (PEG-600) as adjuvant against Japanese quails. Oral intubation was used to obtain concentration-mortality data. Oral intubation was used to obtain concentration-mortality data. Treatments of quails for 24 h with different doses leading to the calculation of LC_{50} values. PEG enhances the pesticide efficacy and the LD_{50} value of IMI was 17.02 mg/Kg[1], and in combination with PEG it was 15.98 mg/kg^{-1}. In the second phase of the study, the effects of a single acute dose of IMI (1/4 LD_{50}) individually or in a mixture with PEG has a potent effect on the activity of plasma AChE and brain monoamines transmitters. However, the addition of PEG-adjuvant to the selected insecticide has shown more toxic potential, more highly significant decreases in AChE activity and different changes in cortical monoamines concentration. In the present study the maximum significant inhibition of AChE activity, was recorded post 72 h exposure to IMI individually and 96 h in a mixture with PEG and exhibited −37.56% and −32.65% decreases, respectively. Moreover, the oral intubation of IMI individually or in a mixture with PEG caused a significant elevation in the quail cortical NE and 5-HT. The result also showed while the mixture of IMI + PEG induced the more potent effect in DA alterations, IMI individually was more effective in 5-HT changes. Our findings also indicated that PEG exposure induced remarkable changes in the studied monoamines level and the values were significant throughout the tested periods in DA. Moreover, the studied dose level was vigorously affected quail brain cerebral cortex histological structure. When administered individually or in a mixture with PEG, IMI disclosed neural congestion, neuronal degeneration, pyknosis and perivascular cuffing with glial cells.

Keywords PEG-600 · Imidacloprid · Histopathology · Neuroalterations · Neurotransmitters Japanese quails

Abbreviations

5-HT	Serotonin
AchE	Acetylcholinesterase
DA	Dopamine
IMI	Imidacloprid
LD_{50}	Dose that induced 50% mortlality
NE	Norepinephrine
PEG-600	Polyethylene glycol-600

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Introduction

Over the last 2 decades, a new class of insecticides, the neonicotinoids, has been the most remarkable and popular insecticides (Casida and Durkin 2013; Simon-Delso et al. 2015). When used for plant protection, about 5% of the active ingredient of neonicotinoid is possessed by crop plants (Wood and Goulson 2017) and is most instead disperses into the wider environment (Goulson 2013) affecting population of

multiple vertebrate and invertebrate species (Prosser and Hart 2005; Gibbons et al. 2015) directly and indirectly. There is the clear potential for ingestion of neonicotinoids by granivorous animals, specifically birds and mammals. There is an alarming trend between declines of local bird populations and neonicotinoids in the environment. Tennekes and Sánchez-Bayo (2011) investigated that neonicotinoids were acting indirectly on bird populations, by diminishing the plenty of their insect prey. Köhler and Triebskorn (2013) and Millot et al. (2017) have suggested that neonicotinoids may have a more important impact on bird populations, even used in small concentrations. In the present study, we focus on IMI, which has become one of the most widespread neonicotinoids insecticides and exert sub-lethal effects, ranging from neurotoxic, genotoxic (Khalil et al. 2017) and cytotoxic effects (Wankhede et al. 2017) to different types of birds.

After a widespread use of pesticides, including neonicotinoids several purpose pests have developed resistance (Jeschke et al. 2011; Szendrei et al. 2012; Alyokhin et al. 2007). These include licking pests such as planthoppers, aphids, lepidopteran species and coleopteran (Bass et al. 2015). As reported by many types of researches, the mechanisms responsible for neonicotinoids resistance include behavioural and/or physiological changes, bio-degradation, sequestration and modified penetration (Puinean et al. 2010; Bass et al. 2014). Moreover, in IMI toxicity, studies of Clements et al. (2016) and Zhang et al. (2017) have suggested that cytochrome p450 is highly evolved in their detoxification. Therefore, in order to design more sustainable pest control strategies great interest has turned to pesticide formulations. Formulations are assuming improve for pesticide storage, handling, safety, application, effectiveness against pest resistance. Till now there is a gap in risk assessments for adjuvants in pesticide formulations and most studies on agrochemical risk assessment take into consideration pesticide active ingredients only without the spray adjuvants. As reported recently, exposure to environmental levels of some of these adjuvant can affect non-target organs, including birds (Edgington et al. 2004; Stills 2005). Their effects include negative impacts and provoke severe biochemical (Wieder and Davis 1983), clinical (Bendele et al. 1999), histopathological (Ishida et al. 2006) and behavior (Rodrigue et al. 2011) toxic effects. Here, we tested the acute toxicity of IMI pesticide, and its formulation with PEG-600 on the neuronal histological structure and monoamines level on the brain of quails (*Coturnix coturnix).*

Materials and methods

Experimental birds

Healthy adult Japanese quails (*Coturnix coturnix*) (weighing about 100–120 g) were obtained from Central Animal Facility Cairo University. All animals were allowed to acclimatize to the experimental conditions for a period of 5 days. All the quails were housed in polyacrylic cages, not more than 3 animals per cage, and maintained under standard laboratory conditions (12 h of light and dark cycle, room temperature 22 ± 3 °C). Animals were given standard diet, and tap water was provided ad libitum. The institutional animal ethics in the animal care unit in the Faculty of Science, Zoology Department, Cairo University (IACUC), (CU/I/F/60/15) committee approved the experimental protocol.

Chemicals

Imidacloprid

Imidacloprid (IMI), $C_9H_{10}ClN_5O_2$ (CAS Number 138261–41-3 5, ≥98% purity, molecular weight of 255.66), was donated by Novaritis Egypt. Stock solutions with corn oil were prepared in accordance with the International Union of Pure and Applied Chemistry.

Polyethylene glycol 600 dilaurate (PEG-600)

PEG-600 was endowed by the Egyptian Company of Starch, Yeast, and Detergents in emulsified form. It is a viscous transparent liquid, of range average Molecular Weight 570–630 and diluted with corn oil.

Pesticide formulation

Before deciding the tested dose, the insecticide formulation was prepared by mixing stock solutions of 1 g IMI and 1 ml PEG in 10 ml corn oil as described (Rawi et al. 2013). After dilutions, preparation for pesticide, PEG, and pesticide-PEG was takes place.

Toxicity symptoms and the median lethal dose

An approximate LD_{50} is determined by a so-called "up and down" or the "staircase method" using two animals and increasing the doses of IMI and PEG, either individually or in a mixture (ratio 1:1). Six doses were given orally to 6 groups of quails (5 quails in each group) for the determination of LD_{50} starting from 0% mortality to 100% mortality (Randhawa 2009). The animals were observed for 2 h and then at 4th, 6th, and 24th h for any toxic signs and symptoms. After 24th h, the numbers of decreased quails in each group were counted and the percentage of mortality was calculated using the graphical method of Litchfield and Wilcoxon (1949).

Dose preparation and experimental design

After LD_{50} determinations birds were randomly divided into four groups. Group 1 served as control (Corn oil 1 ml/100 g body weight); group 2 was treated with PEG (10 ml /kg body weight); group 3 was treated with IMI (4.5 mg/kg body weight diluted in corn oil) and group 4 was treated with IMI (3.995 mg/kg) and PEG (10 ml /kg body weight) diluted with corn oil. All groups were treated once a time with the selected dose using tracheal intubation. The volume of the dose depends on the size of the animals and should not exceed 1 ml/100 g b.w. (US EPA 2012; Millot et al. 2017). At precise intervals of 1, 6, 24, 72, 96 h and, 1, 2 and 3 weeks of the oral administration, 6 animals of each group were overnight fasted, then they were sacrificed and blood samples were collected, centrifuged at 3000 rpm for 30 min and the obtained sera were used for AChE determination. At 24 and 96 h and 3 weeks, in addition to the collected blood samples, cortical brain tissue was rapidly removed and quickly divided into two parts, one was immersed in neutral buffered formalin (10%) and used for histopathological studies and the other was quickly homogenized in 0.1 M phosphate buffer (pH 7.8), using a glass-Teflon, centrifuged at 10,000 g for 60 min at 4 °C and used for the determination of monoamines.

Aetylcholinesterase (AChE) and monoamines analysis

Serum AChE activity was measured by the method of Ellman et al. (1961) as described by Gorun et al. (1978). Norepinephrine (NE), dopamine (DA) and serotonin (5-HT) contents as monoamines were measured by the method of Pagel et al. (2000) using HPLC (Perkin-Elmer).

Histopathology

At the end of the tested period, the cortical brain was dissected and histological examinations were done by the method of Humason (1972).

Statistical analysis

All values were expressed as mean ± SE. Statistical analysis was done by using SPSS 14 program. The statistical significance of differences between the two means was assessed by one way ANOVA. *P* values <0.05 were considered to be significant.

Results

Toxicity and mortality testing

The percentage of animals that died at each dose was transformed to probit using Finney's method (1952) and the percentage of dead animals for 0 and 100% were corrected before the determination of probits. After that, the calculated LD_{50} of IMI with corn oil when given orally was 17.02, mg/kg when IMI administered individually and 15.98 mg/kg when it was administered in a mixture with PEG. (Fig. 1).

Toxicity signs recorded during the experiment

Throughout the experimental periods of toxicity testing level and the neurological effects of the acute dose, the animals exhibited general weaknesses, salivation, sometimes excitation and a variable sequence of motor symptoms involving pawing and tremor. However, the addition of PEG-adjuvant to the selected insecticide has shown more toxic potential than do the insecticide alone.

Serum AChE

In the present study significant inhibition ($p \leq 0.05$) of AChE activity, was recorded at the early exposure periods and the potency is varied in the following increasing order PEG+IMI > IMI > PEG. In the animal groups treated with PEG or IMI individually, the more highly significant decreases in the enzyme activity were recorded post 72 h exposure and exhibit, −26% and-49.38 decreases, respectively. Post one and 3 weeks of exposure, the enzyme activity showed a tendency to regain normal control level and the recorded values, however, being insignificantly affected (Fig. 2).

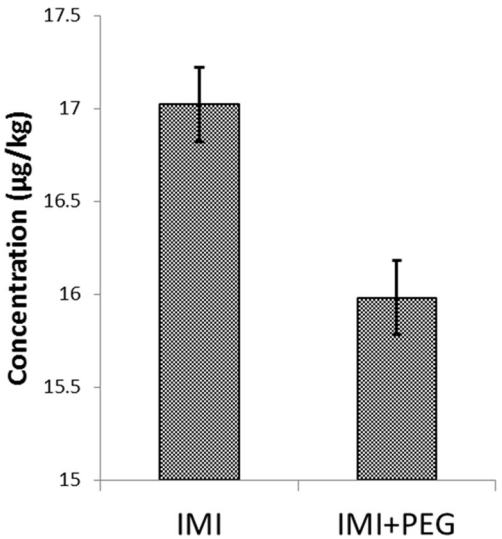

Fig. 1 Lethal dose (mean ± SD) of IMI individually and with a combination of polyethylene glycol given orally to coturnix quail

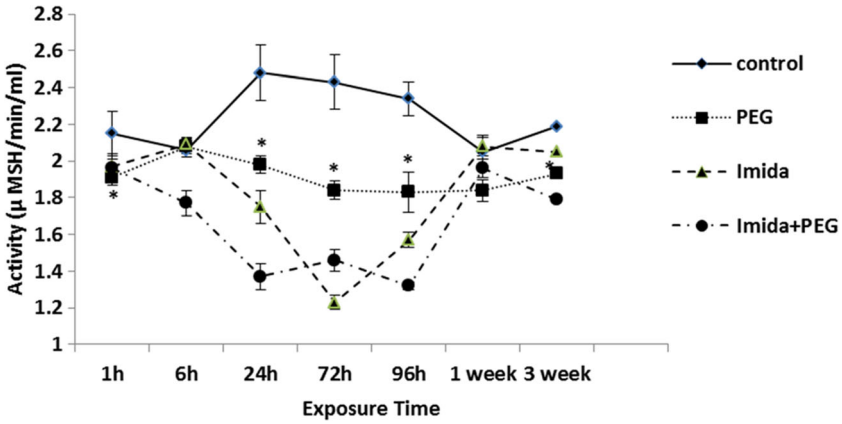

Fig. 2 Effect of IMI exposure on AChE levels in the plasma of coturnix quail. Values represent the mean ± SD of 6 indipendent samples; error bars indicate standard deviation. Statistical significance (*$P < 0.05$) was analyzed using a factorial ANOVA. PEG compared to control and IMI and IMI + PEG compared to PEG

Cortical brain monoamines

Norepinephrine

After treatment with PEG, and as compared with the control group there were no significant changes in the quail cortical epinephrine when compared with the control group ($p > 0.05$). Comparatively, the oral intubation of IMI individually or in a mixture with PEG at the tested dose levels caused a significant elevation in the quail cortical NE level. The highest increased effect ($p < 0.01$) however, was attained after 24 h exposure to the tested mixture and attained 60.76% as compared to PEG-treated group (Fig. 3).

Dopamine

The oral feeding of PEG and/or IMI to quails caused distress fluctuations in the level of DA cortical brain and the values detected being significantly affected throughout all the tested periods. Post 96 h and 3 weeks of exposure, the recorded values being significantly decreased and attained the minimum level post-exposure to PEG followed> PEG+IMI > and IMI in the increasing order (Fig. 4).

Serotonin

The results given in Fig. 5 showed significant elevations in the level of quail cortical brain 5-HT after oral intubation with IMI individually or in a mixture with PEG. Comparatively, throughout all the tested periods, the IMI-treated group exhibited more increased level and attained the maximum value post 24 h and the detected value being increased more than twice the level recorded in the PEG-treated group.

Histopathological observations

The histopathology of the cortical cerebrum of control and treated birds are given in Figs. (6 and 7). The examined sections of control brain tissue changes showed normal blood vessels, cytoplasm and nucleus in neurons (6a). In all treatment groups (PEG, IMI, and IMI + PEG) and as compared to the control group, the examined brain sections showed cerebral neuronal degeneration, pyknosis and neurophagia of some neurons due to IMI (Fig. 7a, b). In addition, relative gliosis, eosinophilic degenerated of neurons, central chromatocytolysis, demylination of nerve fibers, and focal

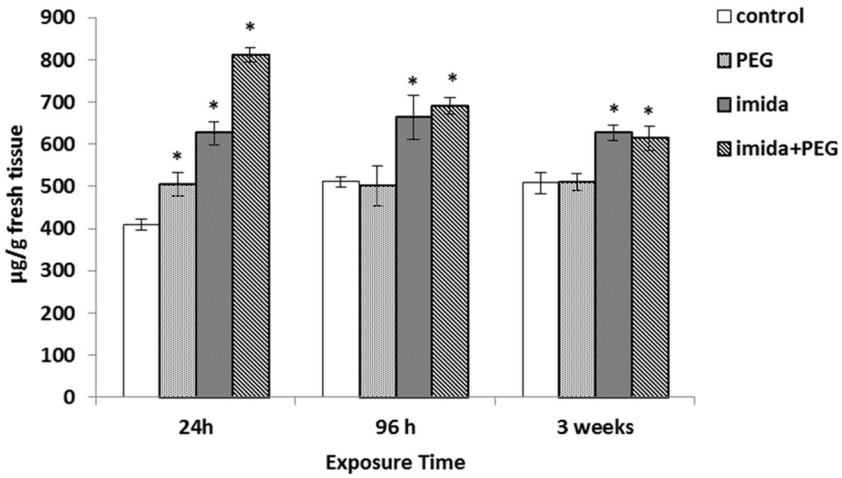

Fig. 3 Effect of PEG and IMI exposure individually or in a mixture on cortical brain NE level of coturnix quail. Values represent the mean ± SD of 6 independent samples; error bars indicate standard deviation. Statistical significance (* $P < 0.05$) was analyzed using a factorial ANOVA. PEG compared to control and IMI and IMI + PEG compared to PEG

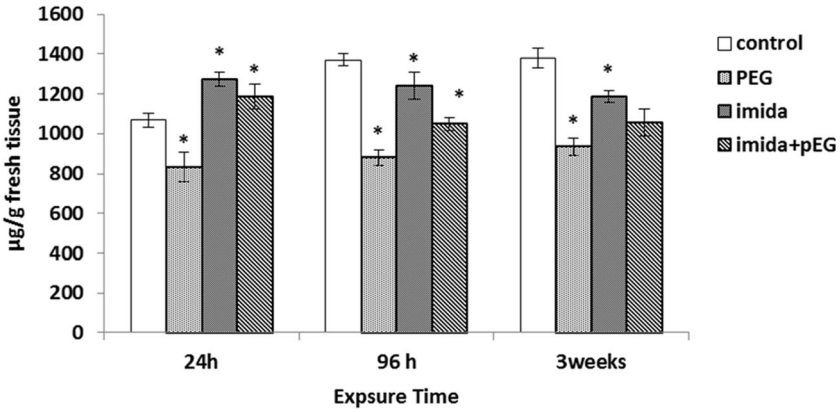

Fig. 4 Effect of PEG and IMI exposure individually or in a mixture on cortical brain DA level of coturnix quail. Values represent the mean ± SD of 6 independent samples; error bars indicate standard deviation. Statistical significance (* $P < 0.05$) was analyzed using a factorial ANOVA. PEG compared to control and IMI and IMI + PEG compared to PEG

minute hemorrhage was also seen by IMI in a mixture with PEG (Fig. 7c, d).

Discussion

To date, the toxicity that may induce by PEG individually and in a mixture with IMI doesn't studied in birds. In the present work, behavioral, biochemical, and histological changes have been recorded in Japanese quail administered with IMI and PEG individually or in a mixture. Histopathological findings of congestion in the blood vessels, necrosis, neurodegeneration, and hemorrhage were recognized in the cortical brain sections of animals treated with IMI individually or in a mixture with PEG. Further, cerebrum neuronal degeneration, pyknosis and neurophagia were observed in PEG-treated birds. Several investigators have shown that IMI (de Oliveira et al. 2010; Tufi et al. 2015; Rodriguez et al. 2016; Zhu et al. 2017) and PEG (Regulska et al. 2010; Pomierny et al. 2013) exerted adverse effects on the CNS cells and may contribute in pathogenesis of neurodegenerative disorders. As reported recently by Wankhede et al. (2017), congestion, degeneration of neurons, nuclear migration, vacuolation, oedema, haemorrhages, and gliosis were evident in the brain after treatments with IMI. In addition, results recorded by Kawthar et al. (2012) showed that PEG exhibited DNA damage, vacuolar degeneration, inflammatory cellular infiltration, necrosis and pyknosis in liver and kidney cells of mice. Moreover, it is well-established that the brain histopathological injury leads to diverse behavioral consequence (Grewal et al. 2015). In the present investigations, IMI individually or in a mixture with PEG produced toxicity involves neuronal hyper-excitability, and disorders of cognition a varying degree of mild to moderate toxic symptoms and behavioral changes and the formulated form of the tested pesticide is characterized by more recognized excitability than do the insecticide alone. Indeed, Avery et al. (1994) observed ataxia in red-winged blackbirds (*Agelaius phoeniceus*) and brown-headed cowbirds (*Molothrus ater*) that fed on IMI-treated seeds. In addition, Cox (2001) reported in-coordination and inability to fly in house sparrow exposed to IMI and the toxicity often involves neuronal hyper-excitability, and disorders of cognition. Also, as reported by Webster et al. (2007) PEG can be considered as a toxic substance and the clinical signs associated with acute exposure include jumping, tremors, and convulsions in rats. Behavioral changes produced by acute IMI have been related to neuronal cell degeneration (Lang et al. 2014; Cho et al. 2017), alterations in AChE activity (Mach et al. 2004; Bonansea et al. 2016; Rodrigues et al. 2017) and regional changes in brain monoamines levels (Koprowska et al.

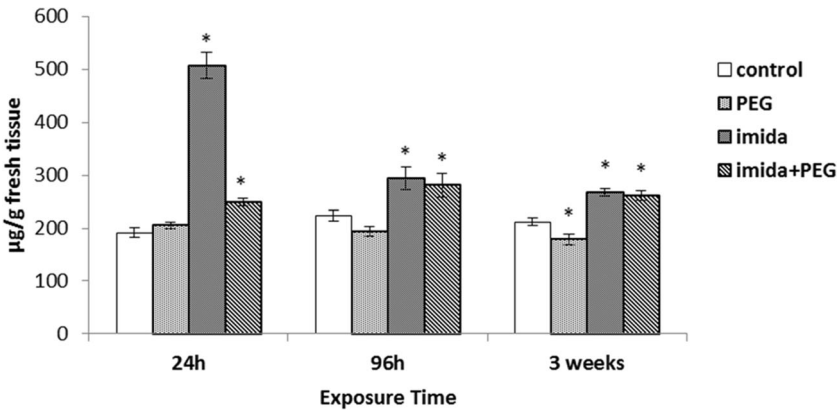

Fig. 5 Effect of PEG and IMI exposure individually or in a mixture on cortical brain 5-HT level of coturnix quail Values represent the mean ± SD of 6 indipendent samples; error bars indicate standard deviation. Statistical significance (* $P < 0.05$) was analyzed using a factorial ANOVA. PEG compared to control and IMI and IMI + PEG compared to PEG

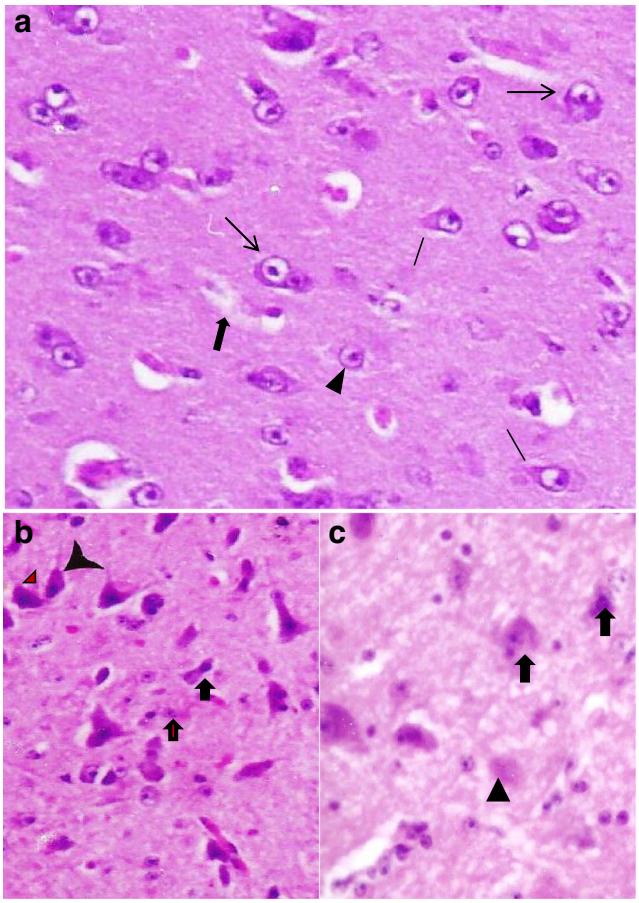

Fig. 6 Histopathological changes of IMI toxicity (96 h). **a** Normal histology of the cerebral cortex of control group. H&E. 50 μm, showing pyramidial neuron (arrows), blood vessel (solid arrow), axon (head arrow), and glial cell (lines). **b**–**c** Neuronal cells of PEG treated group showing degenerated neurons (arrows) and pyknosis of some neurons and neurophagia **c** (head arrows)

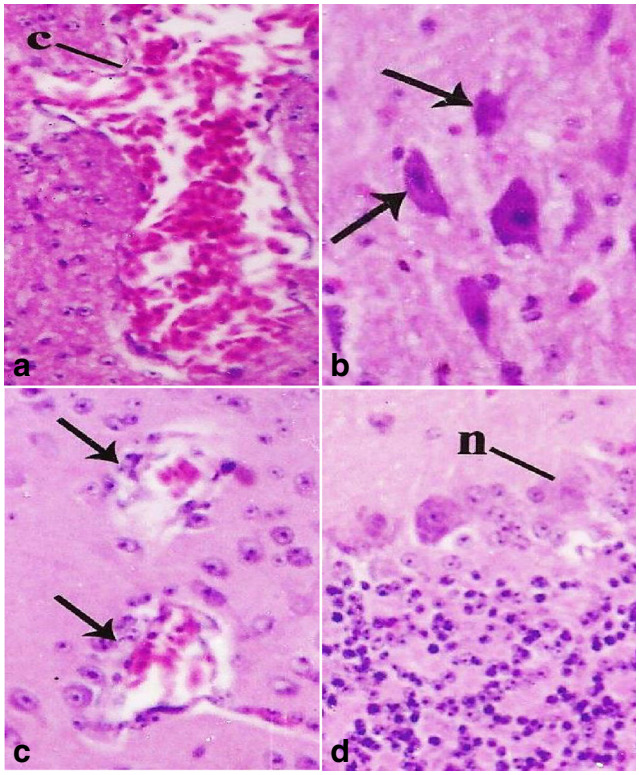

Fig. 7 Photomicrograph of quail cortical brain at the end of 96 h of exposure. **a**–**b** Cortical sections of IMI treated group showing congestion of cerebellar blood vessel (**c**), and neuronal degeneration and pyknosis of neurons (B-arrows). **c**–**d** IMI plus PEG-treated quail showing perivascular cuffing with glia cells (C-arrows) and necrosis of purkinje cells (D-n)

2004) and the more toxicity profile of the formulated form may be due to their synergistic toxicity on the neuronal cells. As indicated by several investigators both PEG (Herold et al. 1982) and IMI (Qadir et al. 2014) can account for the hypercalcemia, which can cause severe neurological symptoms. In addition, our data demonstrated that the tested mixture has, the more the inhibition effect of AChE activity in most of the tested period. As reported by Moser et al. (2006) the acute neurobehavioral effects of AChE-inhibiting pesticides are primarily due to overstimulation of the cholinergic system as well as different modes of action and different target sites. However, mortality data from this study indicated that IMI alone and with PEG generated different mortality, while the LD_{50} of the insecticide alone was 17.02 mg/Kg $^{-1}$ it was 15.98 mg/kg^{-1} in the tested binary mixture.

We further examined the impact of IMI and PEG on the cortical brain monoamines level. The current study showed that exposure to PEG and IMI individually or in a mixture induced a significant decrease in DA and significantly elevated EP and 5-HT levels. There are no reports we came across in Japanese quail about monoamines level affected by the tested compounds. Rodríguez et al. (2016) have reported analogous changes of brain neurotransmitters after pyrethroid cyfluthrin insecticides exposure in male rats. Moreover, Martínez et al. (2018) noted that glyphosate herbicide in a dose-dependent, altered central nervous system (CNS) monoaminergic neurotransmitters in a brain regional manner. Also, the results obtained by Tufi et al. (2015) and Martínez et al. (2018) proved the assumption that the pesticides exposure changes in dopamine (DA), noradrenaline (NE), and serotonin (5-HT). However, a number of evidence suggests that PEG included in unleaded gasoline vapours impaired the levels of monoamine neurotransmitters and other biochemical parameters in different brain areas and modulated several behavioural aspects in rats (Burbacher 1993; Kinawy 2009). In addition, as reported recently by Khalil et al. (2017) at specific dose levels IMI administration led to an alteration in the levels of cortisone and catecholamines and induced behavioral deficits, particularly in the animals exposed to the dose of 1.0 mg/kg. Our results about different exhibited changes for monoamines might be due to counter mechanism against different toxicity sign occurred due to exposure to IMI and/or PEG. It is commonly accepted that oxidative stress is responsible for the

brain damage and the excessive release of brain monoamines. Several investigators (Ahmad et al. 2010; Ge et al. 2015; Wang et al. 2016; Özdemir et al. 2018) have reported that IMI exposure induced oxidative damage which induces an increase in lipid peroxidation and protein oxidation. Consequently, the brain, with its rich lipid content, high energy demand, and weak antioxidant capacity become an easy target of excessive oxidative insult. The present data suggest that IMI individually or in a mixture with PEG induces neuroinflammation associated with monoamines imbalances and toxicity symptoms and PEG can increase the ability of IMI to affect significant toxicologic end points, including developmental neurotoxicity, and disruption of monoamines level.

In conclusion, there are clinical trials that have demonstrated the tolerability and the safety of PEG-600 in humans and signified the lack of central effects, but the recent study demonstrates an overview of modulation effect PEG-600 as an adjuvant on pesticides neurotoxiclogical effects on birds. The proposed mechanisms are potentiation at most of the studied parameters that could be asigned to neurotransmitters or their metabolism influence.

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