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RESEARCH ARTICLE

Carnosic acid alleviates chlorpyrifos-induced oxidative stress and inflammation in mice cerebral and ocular tissues

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Abstract

Chlorpyrifos is an organophosphate pesticide whose exposure leads to inhibition of acetylcholinesterase (AChE) enzyme and induces oxidative stress, inflammation, and neurotoxicity. The current study was designed to evaluate the efficacy of carnosic acid (CA) in ameliorating CPF-induced cytotoxicity in mice brain and eye tissues. We allocated 40 male Swiss albino mice to receive DMSO 1% solution, oral CA 60 mg/kg/day bw, CPF 12 mg/kg/day bw via gastric gavage, or CPF plus CA at 30 and 60 mg/kg/day bw. Carnosic acid was administered once/day for 14 days, while CPF was administered in the last 7 days of the experiment. Biochemical analysis showed that CPF administration was associated with significant increases in the serum concentrations of interleukin-1β, IL-6, and tumor necrosis factor-α, while it was associated with significant reductions in serum AChE levels in mice. Moreover, CPF-intoxicated mice exhibited significantly higher levels of malondialdehyde and nitric oxide in the brain and eye tissues. However, they had significantly lower levels of reduced glutathione, glutathione peroxidase, superoxide dismutase, and catalase in comparison with normal controls. Pretreatment with CA at 30 and 60 mg/kg/day bw for 14 days significantly alleviated all the aforementioned CPF-induced alterations in a dose-dependent manner; more frequent restorations of the normal control ranges were observed in the higher dose group. In conclusion, CA offers a neuroprotective effect against CPF-induced oxidative stress and inflammation and should be further studied in upcoming experimental and clinical research.

Keywords Acetylcholinesterase · Carnosic acid · Chlorpyrifos · Oxidative stress · Tumor necrosis factor · Mice

Introduction

Pesticides are chemical agents, used globally to control pests in agriculture (Ma et al. 2013). However, excessive exposure whether occupational or accidental, especially in developing countries, may lead to serious side effects. Organophosphates (OP) are the most commonly used pesticides nowadays (El-Demerdash 2011). Chlorpyrifos (CPF), [O, O-diethyl-o-(3, 5, 6-trichloro-2-pyridyl) phosphorothionate], is a broad-spectrum OP pesticide that was introduced in 1965 to control agricultural and household pests, such as flies and mosquitoes (Ma et al. 2013; Uzun and Kalender 2013).

Chlorpyrifos inhibits the activity of acetylcholinesterase (AChE), which is essential to maintain balanced neural transmission. This causes acetylcholine accumulation in the synaptic clefts and uncontrolled activation of the cholinergic pathway with neurotoxic effects (Ma et al. 2013). These effects include neurobehavioral changes, anxiety, cognitive dysfunction, memory impairment, Parkinson's disease, neuro-developmental delays, and death (Ahmed et al. 2017; Fereidounni and Dhawan 2018). Neurotoxicity is the common feature of CPF intoxication (Uzun and Kalender 2013). However, CPF induces several adverse effects in other systems as reproductive toxicity, teratogenicity, cardiotoxicity, hematotoxicity, immunological abnormalities, hepatic dysfunction, and inflammation (El-Sayed et al. 2018; Ma et al. 2013; Uzun and Kalender 2013). Further, CPF

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increases the production of reactive oxygen species (ROS) by interfering with the electron reflux of the respiratory chain components (Salama et al. 2014) and alters the activities of antioxidant enzymes as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT), leading to oxidative stress and lipid peroxidation (Uzun &Kalender 2013).

Based on the role of oxidative stress in CPF cytotoxicity, the use of antioxidants to prevent or ameliorate its toxicity is a logical approach. Carnosic acid (CA) is a natural phenolic diterpene that is present in rosemary (*Rosmarinus officinalis*) and *Salvia officinalis*. It is used in several types of food and non-food products like toothpaste and mouthwash (de Oliveira et al. 2016). Carnosic acid is used as an antioxidant, anti-carcinogenic (e.g., colonic and mammary tumors), anti-antimicrobial, anti-proliferative, anti-inflammatory, and as a chemoprotective agent against oxidative stress (González-Vallinas et al. 2015; Satoh et al. 2014; Yanagitai et al. 2012); it inhibits lipid peroxidation and prevents oxidative hemolysis of RBCs (Wu et al. 2015). Further, CA showed neuroprotective effects as it removes the pro-oxidants from the neural cells to alleviate xenobiotic- (de Oliveira et al. 2016) and ischemia-induced neuronal injuries (Hou et al. 2012).

Based on our knowledge of the published literature, data on the neuroprotective role of CA against CPF intoxication in vivo are lacking. Therefore, this study was performed to assess the protective effects of CA against CPF-induced inflammation and oxidative stress in mice cerebral and ocular tissues.

Methods

Chemicals and kits

Chlorpyrifos [O, O-diethyl-o-(3, 5, 6-trichloro-2-pyridyl) phosphorothionate] was purchased from Shanxi PUDE Pharmaceutical Company (Shanxi, China), while CA (C20H28O4) was obtained from Sigma Co. (St. Louis, MO, USA). All biochemical kits were purchased from *Biodiagnostic* Co. (Cairo, Egypt), except for interleukin-1β (IL-1β) and interleukin-6 (IL-6) kits (supplied by Glory Science Co. Ltd., Del Rio, TX, USA) and tumor necrosis factor-α (TNF-α) kits (supplied by BioSource International Inc. Camarillo, CA, USA). Other chemicals used in the present study were of analytical grade.

Animals

Forty healthy mature male Swiss albino mice (weighing 22 to 28 g; 10 to 12 weeks old) were purchased from the Egyptian Organization for Biological Products and Vaccines. The animals were housed in wire-mesh cages and fed standard laboratory diet and water ad libitum for 10 days at the animal house of the Department of Pharmacology, Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt. Mice were exposed to standard environmental conditions (12-h light-dark cycles, 25 ± 2 °C, and 60 ± 5% humidity). All animals' procedures were handled according to the standard guide of laboratory animals, and affirmed by the Ethics Review Committee at the Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt (Approval No. 201819).

Experimental design

Mice were divided randomly into five different groups (n = 8/group). Group 1 mice (controls) received DMSO 1% solution daily for 14 days; group 2 mice received oral CA only at a daily dose of 60 mg/kg bw for 14 days; group 3 mice received CPF (dissolved in DMSO 1% solution) via oral gavage at a daily dose of 12 mg/kg bw (Ma et al. 2013) during the second week of the experiment; and groups 4 and 5 received oral CPF at a dose of 12 mg/kg bw plus oral CA at a dose of 30 and 60 mg/kg bw (Shan et al. 2015; Xiang et al. 2013) for the aforementioned durations (Fig. 1).

Serum collection and tissue preparation

Blood samples were withdrawn from mice, after 24 h from the last CPF dose, under isoflurane anesthesia from the retro-orbital sinus, and then the mice in each group were sacrificed through decapitation. The brain and eyes were dissected from mice to be used for biochemical analysis and washed by distilled water and NaCl solution (0.9%), and then blotted over apiece of filter paper. The tissues were homogenized in 0.1 M potassium phosphate cold buffer (pH 7.4) and centrifuged at 2000 rpm for 30 min. The resulting supernatant was collected into sterile tubes and stored at − 80 °C in a deep freezer until used for biochemical analysis.

Assay of serum acetylcholinesterase

The collected serum samples were allowed to clot for 30 min at room temperature then centrifuged at 3000 rpm for 15 min at 25 °C and preserved at − 20 °C until used for biochemical assays. Later, the methods described by Ellman et al. were used to assess the serum activity of AChE (Ellman et al. 1961).

Assay of pro-inflammatory cytokines

The serum levels of IL-1β, IL-6, and TNF-α were measured by ELISA plates (enzyme-linked immunosorbent assay)

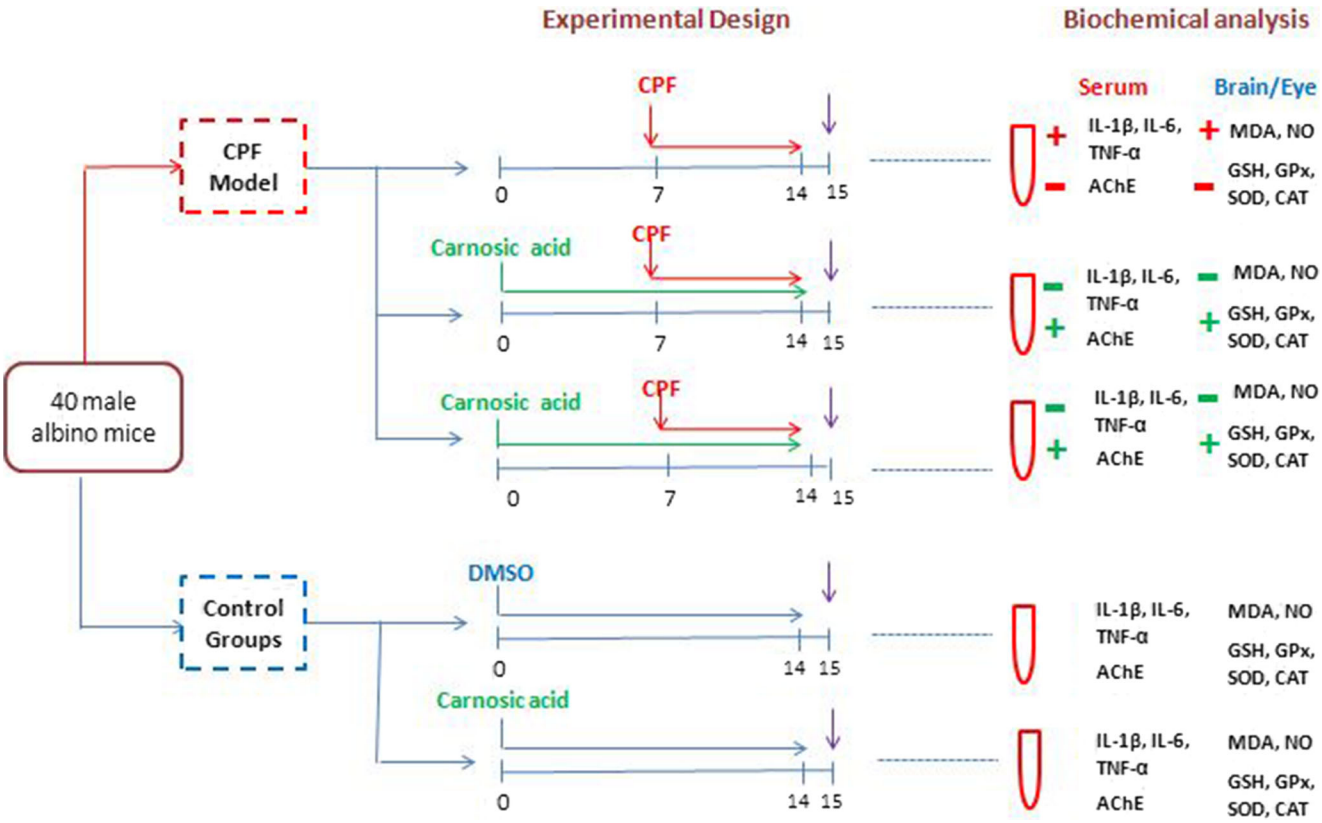

Fig. 1 Summary of the experimental design and biochemical analysis results. AChE acetylcholinesterase, CAT catalase, CPF chlorpyrifos, GSH reduced glutathione, GPx glutathione peroxidase, IL interleukin, MDA malondialdehyde, NO nitric oxide, SOD superoxide dismutase, TNF tumor necrosis factor

according to the manufacturers' protocols, and the samples were read using an ELISA reader at a wavelength of 420 nm.

Assays of lipid peroxidation, nitric oxide, and antioxidant enzymes

The levels of malondialdehyde (MDA) were assessed in the brain and ocular tissues according to Mihara and Uchiyama (1978), while nitric oxide (NO) levels in these tissues were assessed according to Green et al. (Green et al. 1982). Then, the tissue concentrations/activities of SOD, CAT, GSH, and GPx were evaluated according to methods of Nishikimi et al. (Nishikimi et al. 1972), Aebi (Aebi 1984), Beutler et al. (Beutler et al. 1963), and Paglia and Valentine (1967), respectively.

Data analysis

The statistical Package for Social Sciences (version 20 for windows) was used for statistical analyses. All data were expressed as means and standard deviations of the mean (SD). The one-way ANOVA followed by Tukey's post hoc tests was used to compare the means of different groups. A p value ≤ 0.05 was considered statistically significant.

Results

Serum pro-inflammatory cytokines

This study showed that treatment of mice with CA at 60 mg/kg/day was associated with no significant differences in terms of serum concentrations of IL-1β, IL-6, and TNF-α, compared with control mice, while administration of CPF at 12 mg/kg/day was associated with significant increases (up to 346.8%, 365.4%, and 319.8%) in serum levels of IL-1β, IL-6, and TNF-α, respectively, compared with control mice. On the other hand, pretreatment of mice with CA (at 30 and 60 mg/kg/day doses) was associated with significant reductions in serum levels of IL-1β (down to 54.1% and 35.2%, respectively), IL-6 (to 55.4% and 31.6%, respectively), and TNF-α (to 49% and 34%, respectively) from the values obtained in CPF-intoxicated mice. Interestingly, treatment of CPF-intoxicated mice with CA at the 60 mg/kg/day dose restored the values of all three parameters to normal control levels (Fig. 2).

Serum acetylcholinesterase level

Treatment with CA alone (at 60 mg/kg/day) was not associated with a significant change in serum AChE level in

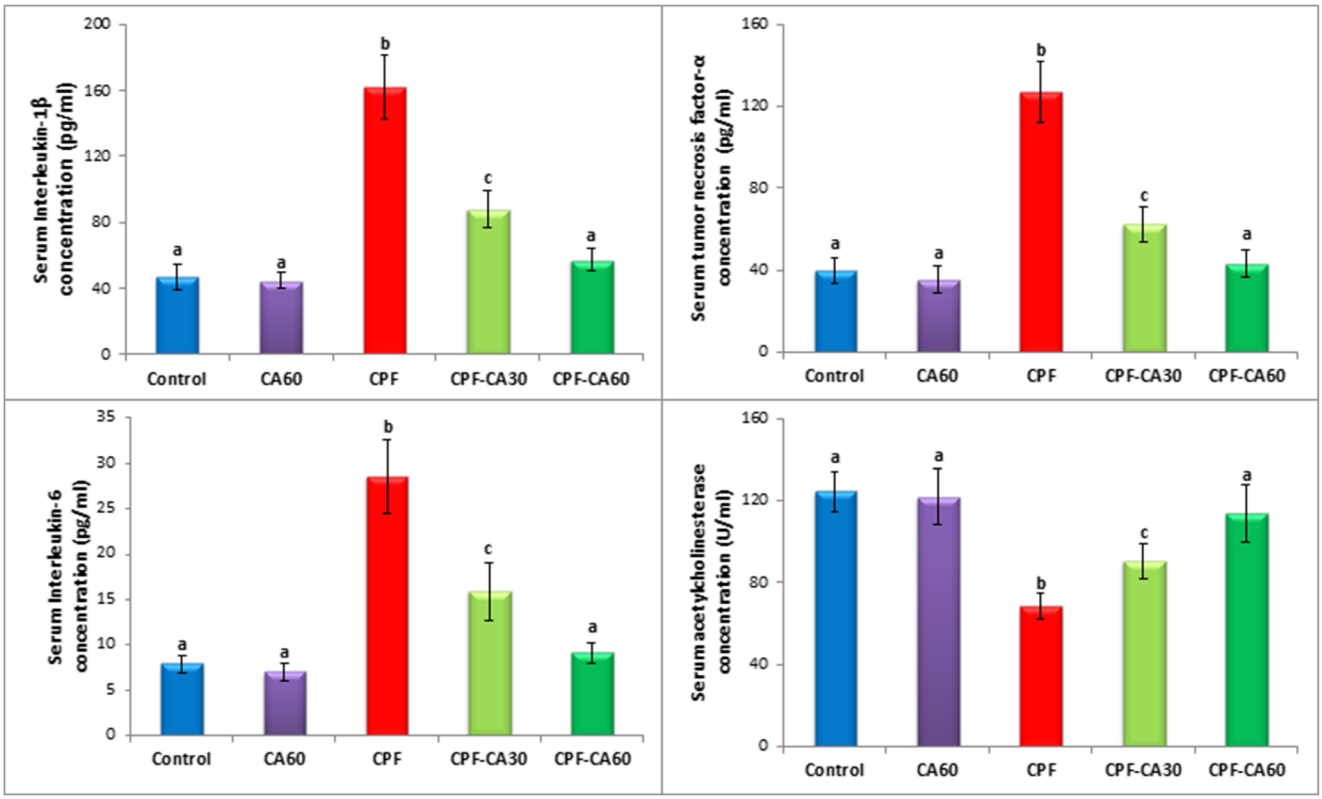

Fig. 2 The effects of carnosic acid (CA, at 30 and 60 mg/kg/day bw) against chlorpyrifos (CPF, 12 mg/kg/day bw) on the serum concentrations of interleukin 1β, -6, and tumor necrosis factor-α. The presented data are means ± SD ($n = 8$ per group). Columns with different superscripts are significantly different at $p \leq 0.05$

comparison with control mice. However, a significant reduction (down to 55.1%) was observed in this parameter after exposure to CPF at 12 mg/kg/day for 7 days. In contrast, we observed a significant increase in serum AChE in mice, treated with CA at 30 and 60 mg/kg (up to 132.3% and 166.1%, respectively) with restoration of the normal serum AChE level in the 60 mg/kg/day group.

Brain oxidant/antioxidant parameters

Our analysis highlights the safety of CA at the highest studied dose (60 mg/kg/day); no significant alterations were noticed in the mice group, treated by CA alone in terms of the cerebral tissue oxidant (MDA and NO) and antioxidant parameters (GSH, GPx, SOD, and CAT), compared with normal control mice. On the contrary, administration of CPF for 14 days was associated with significant increases in the cerebral tissue MDA (up to 205%) and NO (201.8%) levels, as well as significant decreases in the cerebral tissue GSH (down to 49.4%), GPx (44.1%), SOD (44.1%), and CAT (34.4%) concentrations/activities in comparison with normal controls.

Interestingly, pretreatment of CPF-intoxicated mice with CA at 30 and 60 mg/kg/day for 7 days was associated with significant decreases in the cerebral tissue concentrations of MDA (down to 81.1% and 54.2%, respectively) and NO (79.7% and 57.2, respectively), as well as significant increases in the cerebral tissue levels of GSH (158.3% and 196.1%, respectively), GPx (162.8% and 230.3%, respectively), SOD (149.1% and 203.7, respectively), and CAT (190.9% and 281.8%, respectively). Notably, the 60 mg/kg/dose of CA restored the normal concentration ranges of all parameters, except NO in CPF-exposed mice (Fig. 3).

Eye oxidant/antioxidant parameters

We recorded no significant differences between control mice and those treated with CA alone at 60 mg/kg/day for 14 days in terms of MDA, NO, GSH, and GPx; however, the latter group had significantly higher ocular tissue levels of the antioxidant enzymes SOD and CAT. Mice, exposed to CPF, had significantly higher ocular tissue levels of MDA (up to 228.5%) and NO (229.2%), as well as significantly lower levels of GSH (down to 43.8%), GPx (46.8%), SOD (44.6%), and CAT (31.8%) than normal controls.

However, treatment of CPF-intoxicated mice with CA at 30 and 60 mg/kg/day for 14 days was associated with significant reductions in the ocular tissue levels of MDA (down to 68.5% and 45.9%, respectively) and NO (67.4% and 48.6%, respectively), as well as significant elevations in GSH (up to 159.4% and 214.4%, respectively), GPx (157.1% and 205.5%,

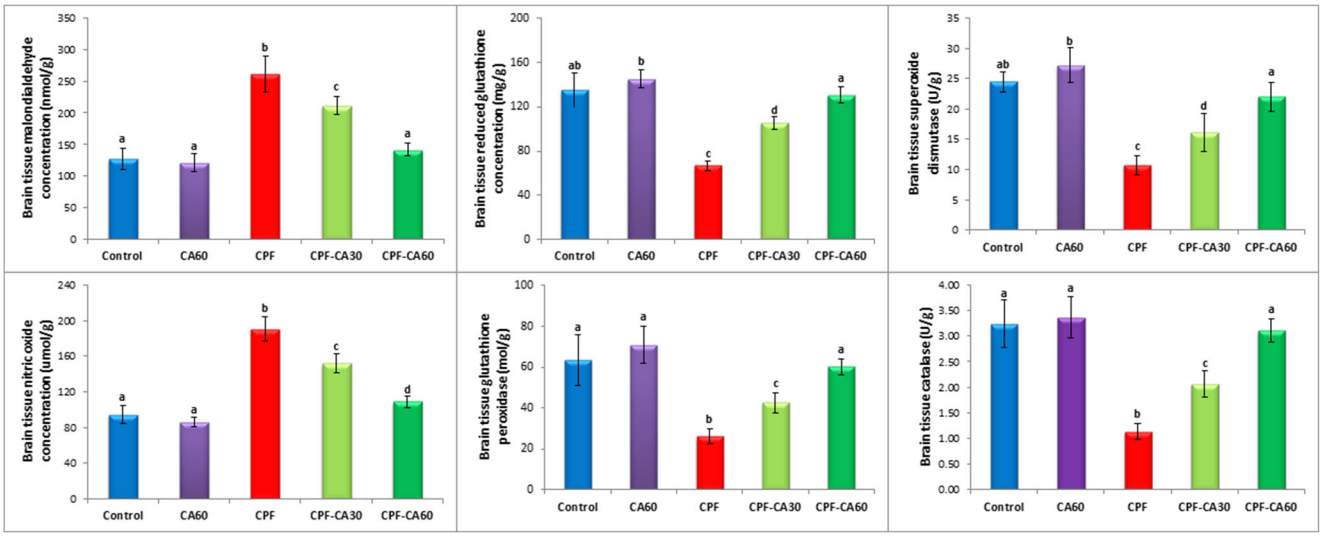

Fig. 3 The effects of carnosic acid (CA, at 30 and 60 mg/kg/day bw) against chlorpyrifos (CPF, 12 mg/kg/day bw) on the cerebral tissue concentrations of malondialdehyde (MDA), nitric oxide (NO), reduced glutathione (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT) enzymes. The presented data are means ± SD ($n = 8$ per group). Columns with different superscripts are significantly different at $p \leq 0.05$

respectively), SOD (161.7% and 202.3%, respectively), and CAT (171.4% and 285.7%, respectively) levels. The 60 mg/kg/day dose of CA restored the normal control levels for all measured ocular tissue parameters (Fig. 4).

Discussion

The current study showed the protective effects of CA against CPF-induced oxidative injuries to the mouse eye and brain tissues. Carnosic acid significantly reduced the oxidative effects of CPF as evidenced by the lowered levels of oxidants (MDA and NO) and improved non-enzymatic (GSH) and enzymatic (GPx, SOD, and CAT) antioxidant defenses in mice eye and brain tissues. Moreover, it decreased the serum levels of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) and ameliorated the CPF-induced reduction in serum AChE activity.

The oxidative stress of CPF results from deficiency in the antioxidant defense system and increase in ROS (Aly et al. 2010). Reactive oxygen species produced by CPF attack the cellular DNA, lipids, and proteins and alter the intracellular calcium and pH resulting in cell death. Malondialdehyde, the essential indicator of lipid peroxidation, indicates cell damage through phospholipids degradation (Tsikas 2017). Further,

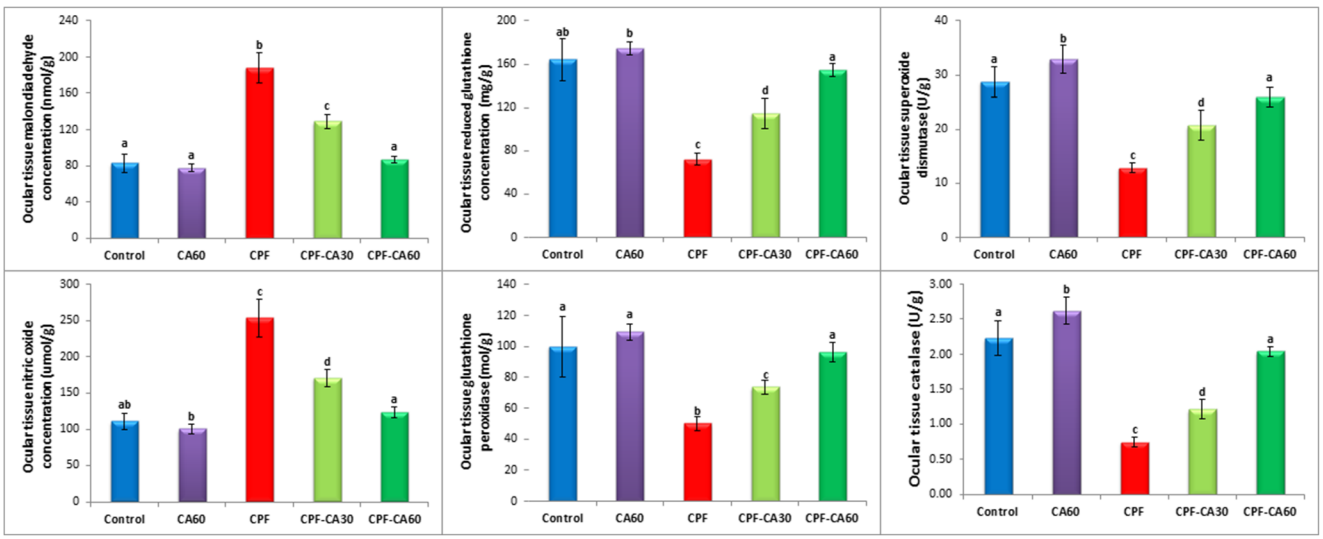

Fig. 4 The effects of carnosic acid (CA, at 30 and 60 mg/kg/day bw) against chlorpyrifos (CPF, 12 mg/kg/day bw) on the ocular tissue concentrations of malondialdehyde (MDA), nitric oxide (NO), reduced glutathione (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT) enzymes. The presented data are means ± SD ($n = 8$ per group). Columns with different superscripts are significantly different at $p \leq 0.05$

CPF has been shown to increase the expression of inducible nitric oxide synthase in the brain of common carb (Wang et al. 2013). This may explain the observed increase in NO levels after CPF exposure in the current study.

The antioxidant defense system plays an important role in prevention of oxidative stress. The reduced tissue levels of GSH were recorded previously in CPF-intoxicated mice (Goel et al. 2005; Verma et al. 2007). Reduced glutathione is essential to prevent the damage of free radicals and enhance detoxification and is an important cofactor for antioxidant enzymes like GPx, glutathione-S-transferase, and glutathione reductase (Aly et al. 2010; Hayes et al. 2005). We observed reduced levels of GPx, SOD, and CAT in CPF-intoxicated mice. These results are in agreement with Banudevi et al. (Banudevi et al. 2006) and Bindhumol et al. (Bindhumol et al. 2003). In contrast, Aly et al. (2010), Yu et al. (Yu et al. 2008) and Oncu et al. (Oncu et al. 2002) reported that SOD and CAT were increased following CPF intoxication, probably to counteract H_2O_2 and superoxide anions elevations.

In addition, our analysis detected significantly lower levels of AChE in mice intoxicated with CPF in comparison with normal controls. Ma and colleagues reported that CPF stimulates the cholinergic neurotransmission, resulting in inhibition of AChE and leading to neurotoxicity (Ma et al. 2013). This inhibition occurs after biotransformation of CPF to its oxygenated analogue, mediated by liver cytochrome P450-dependent desulfuration and resulting in dephosphorylated metabolite trichloropyridinol and diethyl phosphorus formation (Ma et al. 2013; Poet et al. 2003).

Another finding, observed in the current study, is the significantly increased levels of IL-1β, IL-6, and TNF-α after CPF intoxication. These data show that CPF creates a pro-inflammatory environment in the brain and eye tissues of mice. Similarly, Hirani and colleagues reported increased levels of IL-6 and TNF-α (in addition to MCP-1 and E-selectin) in different brain regions of CPF-intoxicated mice (Hirani et al. 2007). These findings may be explained in light of the published literature; may occur due to increasing the expression of Th1/Th2 cytokines (Duramad et al. 2006), increasing the nuclear accumulation on NF-kB (Lee et al. 2014), and induction of oxidative stress. Of note, CPF was found to induce the cytokine promoters of interferon-γ and IL-4 in Jurkat cells (Oostingh et al. 2009) and the immune organs of common carb (Wang et al. 2011). Similar mechanisms may be responsible for the observed finding in the present study.

Carnosic acid is a potent antioxidant owing to its two phenolic hydroxyl groups (Erkan et al. 2008). Previous studies have shown its ability to improve the endogenous antioxidant defenses and prevent lipid peroxidation (Wang et al. 2011; Xiang et al. 2013). Besides, CA has been shown to facilitate the nuclear translocation of the nuclear factor erythroid 2-related factor 2 (Nrf2), leading to activation of Nrf2-dependant genes that protect against oxidative stress (Guo et al. 2016). The current study showed that CA significantly ameliorated CPF-induced increases in MDA and NO levels and decreases in endogenous antioxidants in the eye and brain tissues of mice.

These results are in agreement with Guo et al. who reported that CA reduced the levels of MDA and ROS accumulation in the liver of acetaminophen-intoxicated mice (Guo et al. 2016). Similarly, Sahu et al. showed that CA enhanced the activities of GPx, CAT, and SOD in rat kidneys, exposed to toxic doses of cisplatin. However, they showed increased levels of tissue nitrite (which indicate the extent of NO) after CA treatment (Sahu et al. 2011). In another study, CA has been reported to reduce oxidative stress, activate Nrf2, and inhibit caspases actions in human neuroblastoma (SH-SY5Y) cells (de Oliveira et al. 2016).

Moreover, we found that CA pretreatment significantly reduced CPF-induced increases in serum IL-1β, IL-6, and TNF-α concentration in a dose-dependent manner. Similar findings were reported previously in vivo (Kuo et al. 2011; Xiang et al. 2013) and in cell lines (Tsai et al. 2014). These effects may be mediated by inhibiting the expression of NF-KB (Li et al. 2016), suppressing the adhesion and migration of monocytes (Yu et al. 2009), and preventing the activation of p38 MAPK and cyclooxygenase II enzymes (Hou et al. 2012). Interestingly, CA also ameliorated CPF-induced reduction in serum AChE concentrations in a dose-dependent manner. However, some previous studies have reported that CA inhibits AChE activity and hence can be used to improve memory function in Alzheimer's disease (Merad et al. 2014; Ozarowski et al. 2013; Szwajgier 2013). These studies, however, were not on toxicology models and the differences in study design and used CA doses may explain the discrepant results. Carnosic acid is considered a promising agent for neuroprotection and the current study adds to the published evidence in this regard (de Oliveira 2018).

In conclusion, CPF induces oxidative stress in the brain and eye tissues of mice and creates a systemic pro-inflammatory condition in mice. These effects were significantly ameliorated—in a dose-dependent manner—through pretreatment with CA.

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Compliance with ethical standards All animals' procedures were handled according to the standard guide of laboratory animals, and affirmed by the Ethics Review Committee at the Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt (Approval No. 201819).

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

Abbreviations *AChE,* acetylcholinesterase; *CA,* carnosic acid; *CAT,* catalase; *CPF,* chlorpyrifos; *GSH,* reduced glutathione; *GPx,* glutathione peroxidase; *IL,* interleukin; *MDA,* malondialdehyde; *NO,* nitric oxide; *SOD,* superoxide dismutase; *TNF,* tumor necrosis factor

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