Involvement of cholinesterases in oxidative stress induced by chlorpyrifos in the brain of Japanese quail

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ABSTRACT Chlorpyrifos is a widely used organophosphate pesticide (OP). In birds and mammals OP exhibits a toxic effect via inhibition of cholinesterases [acetylcholinesterase (AChE) and butyrylcholinesterase (BChE)] and through oxidative/nitrosative stress. In this study, the influence of chlorpyrifos on cholinesterase activity, parameters of oxidative stress [malondialdehyde (MDA); glutathione (GSH); superoxide dismutase (SOD); nitrite concentration (NO_2^-); hydrogen peroxide (H_2O_2)], and inflammatory parameter [activity of myeloperoxidase (MPO)] in the brain of Japanese quail (*Coturnix japanica*) was examined. The study was conducted on a total of 60 male Japanese quails (one control and 5 experimental groups, n = 10), 3 to 4 wk old. Quails were administered by gavage chlorpyrifos (CPF) for 7 consecutive da at doses of 0.375 mg/kg BW, 0.75 mg/kg BW, 1.5 mg/kg BW, 3 mg/kg BW, and 6 mg/kg BW. Our studies have shown that all doses of CPF significantly inhibited both cholinesterases in brain: AChE from 22.74 to 37.83% and BChE from 19.53 to 61.9%, and that inhibition was dose dependent. Also, CPF has led to an increase in the concentration of MDA, GSH, NO_2^-, and H_2O_2 and activity of SOD and MPO. Overall, these results support the hypothesis that CPF causes oxidative stress and inflammatory response. This research was carried out on quails because there is hardly any or not enough data about the neurotoxic effect of CPF and especially about its influence on oxidative stress in birds. This study is highly important because we are witnessing massive avian mortality in certain countries due to pesticides.

Key words: chlorpyrifos, Japanese quail, cholinesterase, oxidative stress, brain

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INTRODUCTION

Chlorpyrifos (**CPF**) is a chlorinated organophosphate pesticide (**OP**), which is primarily used in agriculture. Due to its relative safety and persistence in recent years, it has replaced many other pesticides. With a half-life ranging from several d to mo, CPF is moderately persistent in the environment (Palma et al., 2009). CPF was the top-selling OP insecticide in Europe in 2003. In the United States, it was widely used for pest control until the US Environmental Protection Agency (**EPA**) banned it for residential use due to safety issues related to children's exposure (Veneros et al., 2008). Birds may be exposed to pesticides via ingestion of granular formulations, seeds or foliage, contaminated water, poisoned invertebrates or vertebrates, via dermal contact, and through inhalation. Studies conducted in the United States have shown that 8,877 birds died from 1980 through 2000, in 335 cases of anticholinesterase-pesticide poisoning (Fleischli et al., 2004).

Long-term low dose effects of OP exposure are linked to cancers, diabetes, and a wide range of neurological and neurobehavioral disorders, such as depression, and neurodegenerative diseases, such as Alzheimer's and Parkinson's in humans (Amani et al., 2016).

This insecticide achieves its function in mammals and birds in at least 3 ways: inhibiting acetylcholinesterase (**AChE**) activity, causing oxidative stress, and inducing functional disorder of endocrine glands (Meeker et al., 2008; Verma et al., 2009; Viswanath et al., 2010). Inhibition of AChE, which is necessary for normal transmission in nerves, leads to accumulation of acetylcholine in the synaptic cleft and overstimulation of nicotinic and muscarinic receptors (Ivanović et al., 2016). This induces hyperactivity in cholinergic pathways, which causes neurotoxicity and eventual death (Ma et al., 2013).

CPF is capable of generating free radicals, especially free oxygen radicals and other reactive oxygen species (**ROS**), such as hydrogen peroxide (**H_2O_2**), superoxide anion, and hydroxyl radical. CPF accepts

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electrons to form free radicals and then transfers them to oxygen (Hernandez et al., 2013). Oxidative stress is a condition characterized by the imbalance between free radical production and efficiency of the antioxidant defense system. When ROS exceeds the levels of antioxidant defense due to a decrease in cellular antioxidant level or due to over production of ROS, then oxidative stress occurs. The defense mechanisms against ROS and their toxic byproducts can be enzymatic, such as superoxide dismutase (**SOD**) and a nonenzymatic compound, such as glutathione (**GSH**) (Chi et al., 2017).

OP also can cause oxidative damage by forming a reactive nitrogen species, such as nitric oxide (**NO•**) and nitrogen dioxide ($\mathbf{NO_2}$). NO• plays a role in neurodegenerative diseases, acting as a neurotoxin when excessively produced (Di Meo et al., 2016).

In this study, we investigated neurotoxicity of CPF by checking the levels of AChE and butyrylcholinesterase (**BChE**) activity in brain tissue, and determining the concentration of oxidative stress parameters, such as malondialdehyde (**MDA**), GSH, SOD, $\mathbf{NO_2^-}$, and H_2O_2 concentration and the activity of myeloperoxidase (**MPO**).

MATERIALS AND METHODS

Quails

Sixty male Japanese quails *(Coturnix japanica),* 3 to 4 wk old, were purchased from a commercial source in Belgrade, Serbia. Birds were randomly assigned to groups and identified by colored rings. Ten quails were housed in one cage (dimensions 180 cm × 138 cm × 100 cm). They were kept 7 d in laboratory conditions to acclimatize, prior to the experiment, as stated in Organisation for economic co-operation and development (OECD) guidelines (OECD, 1984). They were provided with food and water ad libitum.

The study was approved by the Ethical Committee of the Institute for Biological Research "Siniša Stanković" in Belgrade and the Ministry of Agriculture and Environment in accordance to Serbian low for protection of animal welfare and EU declaration 63/2010 (01–1965; 25 October 2016).

Reagents

CPF, as well as the following reagents, were purchased from Sigma-Aldrich Inc. (St. Louis, MO): 5,5′-dithiobis(2-nitrobenzoic acid), thiobarbituric acid (**TBA**), butyrylthiocholine iodide, acetylthiocholine iodide, sulfanilic acid, α-naphthylamine, o-dianisidine, hydrogen peroxide, phenol red, horseradish peroxidase (**HRPO**), acrylamide, NN'-methylenebisacrylamide, ammonium persulfate, tris (hydroxymethyl) aminomethane, nitrotetrazolium blue chloride, riboflavin, and glycine.

Experimental Design

Sixty male Japanese quails *(Coturnix japanica*), 3 to 4 wk old, were kept in cages, 10 per group. CPF in corn oil was orally administered to animals by gavage (stomach tube) during 7 consecutive d in 5 doses: 0.375 mg/kg BW, 0.75 mg/kg BW, 1.5 mg/kg BW, 3 mg/kg BW, and 6 mg/kg BW. One group served as a control, and this group was given pure corn oil. The election of CPF doses was based on previous studies (Cairns et al., 1991; Clegg and Van Gamert, 1999; Al-Badany and Mohammad 2007).

Brain semples were taken at the end of the experiment, on the eighth day. Whole brains were frozen at –80°C. Before future analyses, brain samples were homogenized in the buffer, 50 mM Tris HCl pH 7.4, containing 1 M NaCl, 20 mM EDTA, and 1% Triton-X100. Homogenization was carried out on ice, with the Ultra Turrax homogenizer (Janke and Kunkel IKA Works GmbH & Co.KG Staufen) at 17,000 rpm. The homogenized tissue was centrifuged at 10 000 rpm for 10 min, and the supernatant was obtained (Krummer et al., 2002).

Spectrophotometry

Activity of AChE, BChE, and MPO, and concentration of MDA, GSH, and H_2O_2 were measured spectrophotometrically using a Cecil CE 2021 UV/VIS spectrophotometer.

Measuring the activity of AChE in the brain was based on the increase of yellow color caused by the 5-thio-2-nitrobenzoate (**TNB**) production, in the reaction between thiocholine and Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid) or **DTNB**) (Ellman et al., 1961) at 412 nm. Activity of BChE also was measured using Ellman's method (1961), in the presence of butirylthiocholine iodide as a substrate, at 410 nm. Assessment of the concentration MDA is based on the principle that TBA reacts with MDA from the sample, forming red colored thiobarbituric acid-reactive substances (**TBARS**), which have a maximum absorption at a wavelength of 535 nm (Stocks and Dormandy, 1971). Nitrite concentration ($\mathbf{NO_2^-}$) was evaluated using Griess (0.346 M sulfanilic acid and 2.1 mM α-naphthylamine) reagent. Absorbance was measured on a micro plate reader (Plate reader Mod. A1, Nubenco Enterprises Inc, Paramus, NJ, USA) at a wavelength of 540 nm (Guevara et al., 1998). The GSH concentration was measured at an absorbance of 412 nm by Ellman's reagent, and the intensity of newly formed TNB was proportional to the total glutathione concentration (Tietze, 1969). Activity of MPO was determined at a wavelength of 460 nm (Bradley et al., 1982), monitoring the concentration of oxidized form of o-dianisidine in reaction with H_2O_2.

Measuring the concentration of H_2O_2 was based on the oxidation of phenol red by H_2O_2 with HRPO and

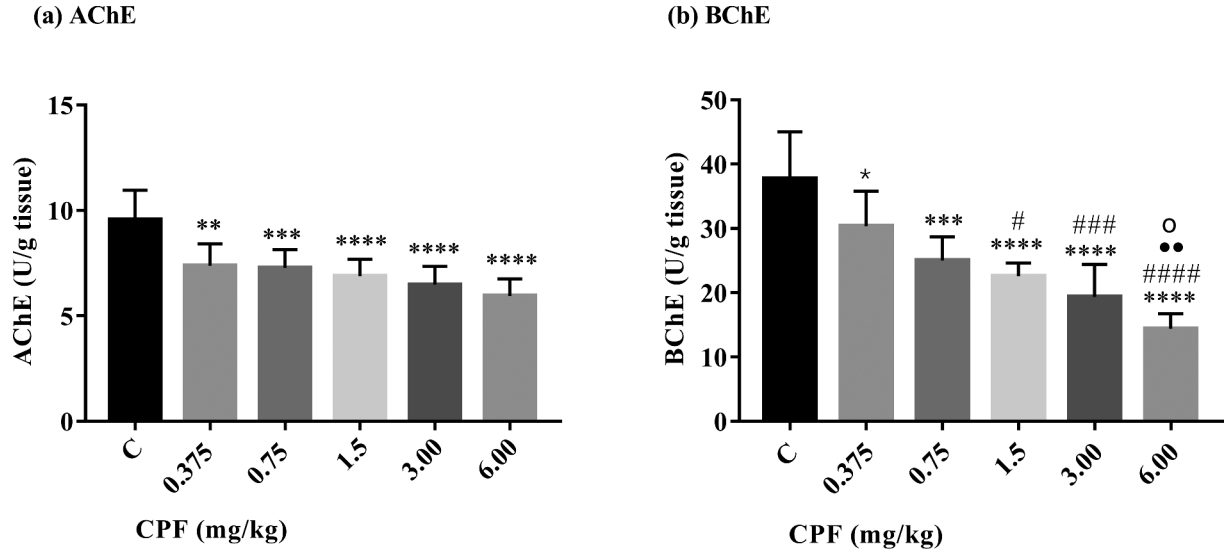

Figure 1. The activity of enzymes (mean ± standard deviation) in Japanese quails after clorpyrifos (CPF) exposure and control group. (a) Bar chart of acetylcholinesterase (AChE) activity. (b) Bar chart of butyrilcholinesterase (BChE) activity. $^{*}P < 0.05$; $^{***}P < 0.001$; $^{****}P < 0.0001$ vs. control group, $^{\#}P < 0.05$; $^{\#\#\#}P < 0.001$; $^{\#\#\#\#}P < 0.0001$ vs. 0.375 mg/kg, $^{\bullet\bullet}P < 0.01$ vs. 0.75 mg/kg, $^{\circ}P < 0.05$ vs 1.5 mg/kg.

results in the formation of a compound with absorbance at 610 nm (Pick and Keisaripick, 1980).

Electrophoresis

The activity of 2 forms of superoxide dismutase-CuZnSOD (**SOD_1**) and MnSOD (**SOD_2**) in the brain was measured by vertical 10% native polyacrylamide gel electrophoresis [**PAGE**; Hoeffer miniVe (LKB 2117, Bromma, Uppsala, Sweden)] with nitro blue tetrazolium (**NBT**) and riboflavin (Beachamp and Fridovich, 1971; Biemelt et al., 2000). Intensity of bands on the gel was analyzed by densitometry with software TotalLab TV 120, and SOD activity was expessed in units/mg proteins.

Statistical Analysis

Results were analyzed in a statistical program GraphPad Prism 7.00, using one-way ANOVA and a post-hoc Tukey test (San Diego, CA). The Tukey test was used to compare data from control and treated groups and also to compare data among treated groups. The level of statistical significance was set as $P < 0.05$, and data were expressed as mean ± standard deviation (**SD**).

RESULTS

AChE activity was inhibited by CPF administration, recording inhibitions of 22.74, 23.99, 27.96, 32.14, and 37.83% at dose levels of 0.357 mg/kg, 0.75 mg/kg, 1.5 mg/kg, 3 mg/kg, and 6 mg/kg, respectively. There was a significant difference in CPF-induced enzyme inhibition in all concentrations, especially at high doses with $P < 0.0001$ (Figure 1a).

BChE activity was affected by CPF treatment with significant inhibitions of 19.53, 33.70, 40.27, 48.70, and 61.90% at dose levels of 0.375 mg/kg ($P < 0.05$), 0.75 mg/kg ($P < 0.001$), 1.5 mg/kg ($P < 0.0001$), 3 mg/kg ($P < 0.0001$), and 6 mg/kg ($P < 0.0001$), respectively (Figure 1b).

MDA concentration in groups treated with CPF was increased by 72.24% (dose 0.75 mg/kg), 159.95% (1.5 mg/kg), and 64.24% (3 mg/kg), which was significantly higher ($P < 0.01$, $P < 0.0001$, and $P < 0.01$) than in the control group (Figure 2a). Statistically significant was the ratio between the following groups treated with CPF: 0.375 mg/kg - 1.5 mg/kg ($P < 0.0001$), 0.375 mg/kg - 3 mg/kg ($P < 0.05$), 0.75 mg/kg - 1.5 mg/kg ($P < 0.01$), 1.5 mg/kg - 3 mg/kg ($P < 0.001$), and 3 mg/kg - 6 mg/kg ($P < 0.0001$).

The level of GSH in the brain was affected only by CPF treatment in doses of 0.375 mg/kg and 3 mg/kg, and increases by 40.56% ($P < 0.001$) and 35.58% ($P < 0.01$), respectively, were recorded after treatment (Figure 2b). A notable difference ($P < 0.05$) was observed between 0.357 mg/kg - 0.75 mg/kg and 0.357 mg/kg - 1.5 mg/kg.

The total SOD and its 2 forms, SOD_1 and SOD_2, were detected in the brain by native PAGE electrophoresis (Figure 3b). The intensity of bands on the gel from Japanese quails that were treated with CPF (specimens 2 to 6) was higher than in control quails (specimen 1). Densitometry analysis confirmed that the difference was statistically significant with total SOD (Figure 3a) and SOD_1 (Figure 3c) in all doses of CPF, except for the dose of 0.357 mg/kg CPF. But in the case of SOD_2, only the doses of 0.357 mg/kg and 0.75 mg/kg CPF showed significance vs. the control group (Figure 3d). The activity of antioxidant enzyme SOD

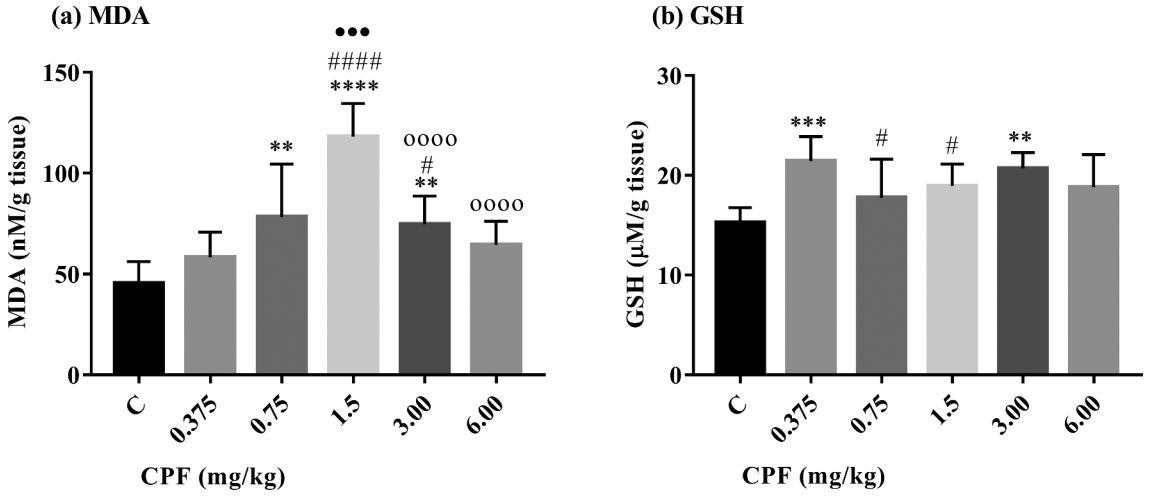

Figure 2. Concentration of MDA and level of GSH (mean ± standard deviation) in Japanese quails treated with CPF and control group. (a) Bar chart of MDA concentration (b) Bar chart of level GSH. $^{**}P < 0.01$; $^{***}P < 0.001$ vs. control group, $^{\#}P < 0.05$ vs. 0.357 mg/kg, $^{\bullet\bullet}P < 0.01$ vs. 0.75 mg/kg, $^{\circ\circ\circ}P < 0.001$, $^{\circ\circ\circ\circ}P < 0.0001$ vs 1.5 mg/kg.

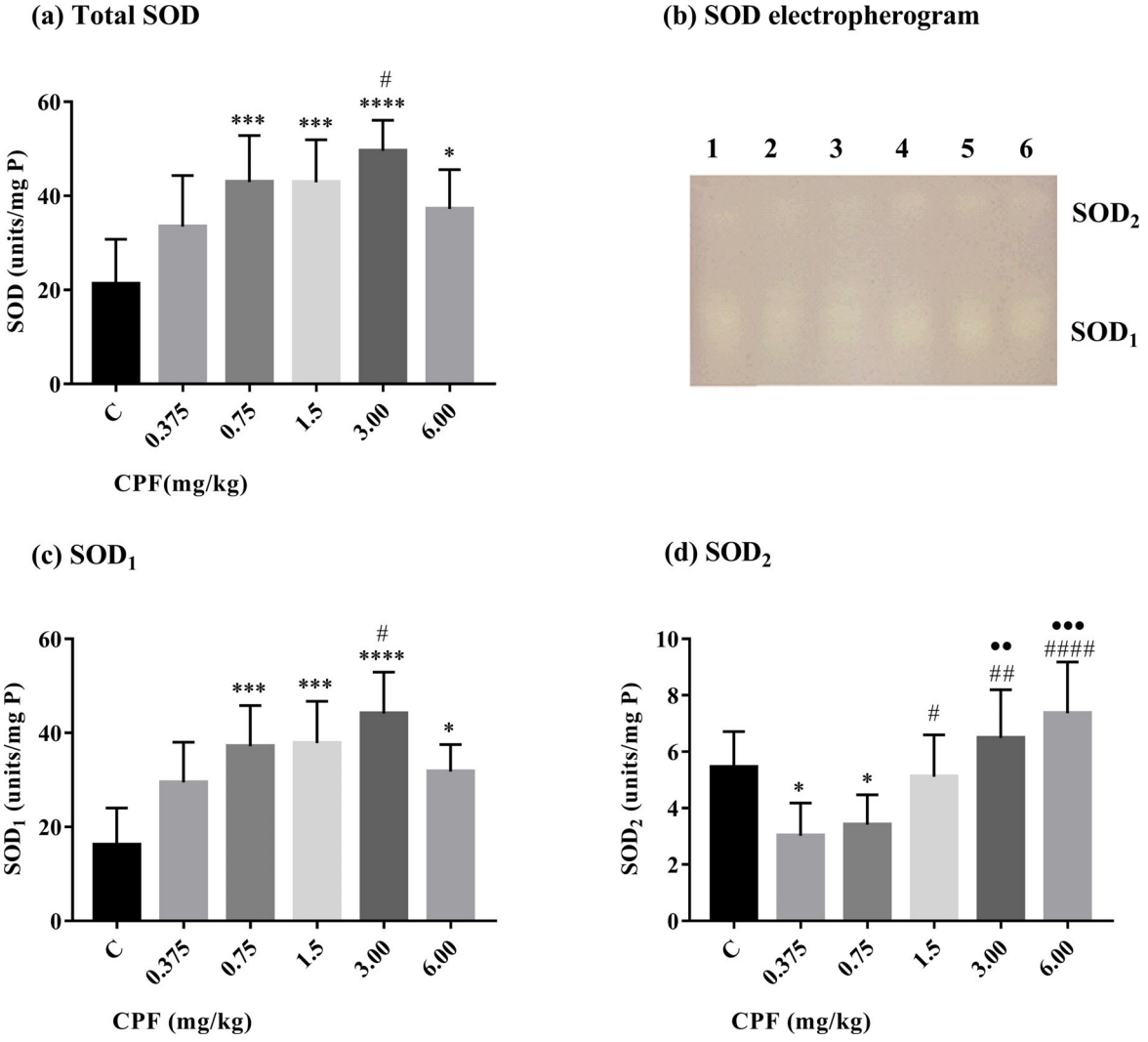

Figure 3. Activity of enzyme superoxide dismutase (mean ± standard deviation) in Japanese quails threated with CPF and in control group. (a) Bar chart of total superoxide dismutase activity, (b) SOD electropherogram on native PAGE, (c) Bar chart of CuZn superoxide dismutase activity, (d) Bar chart of Mn superoxide dismutase activity. Significant at $^{*}P < 0.05$; $^{***}P < 0.001$; $^{****}P < 0.0001$ vs. control group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#\#}P < 0.0001$ vs 0.375 mg/kg, $^{\bullet\bullet}P < 0.01$, $^{\bullet\bullet}P < 0.001$ vs. 0.75 mg/kg.

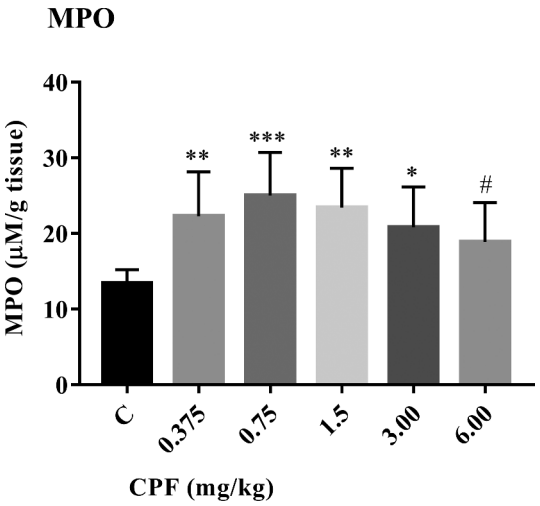

Figure 4. The activity of MPO enzyme (mean ± standard deviation) in Japanese quails treated with CPF and control group. Bar chart of MPO activity. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. control group, $^{\#}P < 0.05$ vs 0.375 mg/kg.

in quails threated with CPF significantly increased (0.75 mg/kg - 101.93%; 1.5 mg/kg - 101.65%; 3 mg/kg - 133.16%; 6 mg/kg - 75.02%) compared with the control quails. A similar increase was observed with SOD_1 (0.75 mg/kg - 129.59%; 1.5 mg/kg - 133.66%; 3 mg/kg - 172.64%; 6 mg/kg - 96.36%), while the activity of SOD_2 significantly decreased with only 2 doses of CPF (0.357 mg/kg - 44.65%, 0.75 mg/kg - 37.46%).

The concentration of H_2O_2 was significantly higher ($P < 0.0001$) than in the control group with increases of up to 21-fold at 6 mg/kg (Figure 5b). Significant differences ($P < 0.0001$, $P < 0.001$) also were observed among the experimental groups (1.5 mg/kg, 3 mg/kg, 6 mg/kg vs. 0.375 mg/kg and vs. 0.75 mg/kg).

MPO activity was significantly higher—by 65.82% (0.357 mg/kg - $P < 0.01$), 86.15% (0.75 mg/kg - $P < 0.001$), 74.46% (1.5 mg/kg - $P < 0.01$), and 55.03% (3 mg/kg - $P < 0.05$) in CPF-treated quails than in the control group quails (Figure 4). The only significant difference ($P < 0.05$) was found between doses 0.75 mg/kg and 6 mg/kg.

Quails treated with CPF had significantly higher NO_2^- concentration than those in the control group (Figure 5a), with increases of 50.25, 61.39, 76.31, and 66.28% at dose levels of 0.75 mg/kg ($P < 0.05$), 1.5 mg/kg ($P < 0.01$), 3 mg/kg ($P < 0.001$), and 6 mg/kg ($P < 0.01$), respectively.

DISCUSSION

It has been established that OP induces neurotoxicity via the inhibition of AChE, which is a key enzyme of the nervous system. There is a variety of factors that can influence the neurotoxicity of OP, such as the affinity strength between AChE and the oxon form of OP, capacity of paraoxonase to catalyze this oxon (Pond, et al., 1998), and the ability of the cytochrome P450 monooxygenase (**CYP450**) to convert OP to their corresponding oxons. The interesting finding is that mature neurons are less vulnerable to CPF toxicity than immature, even though they have a higher inhibition of AChE. The immature neurons have less ability to convert CPF to CPF-oxon, because CYP450 enzymes show less expression in these neurons (Amani et al., 2016). AChE is one of the first biomarkers of environmental medicine used for monitoring exposure to OP (Thompson et al., 1988). The AChE application has increased in the last 2 decades, especially the application of the brain AChE, as an indicator for the neurological effects of OP. Research done by Shimshoni et al. (2012) indicates that normal brain AChE activity in a wide range of birds is from 7.4 to 19.8 μmol/min/g tissue.

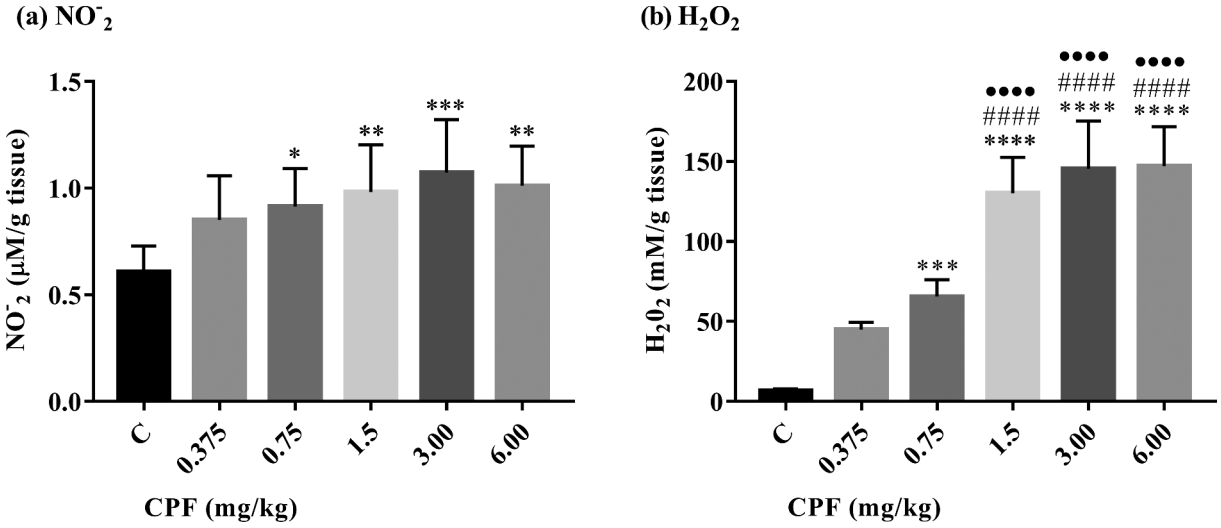

Figure 5. Concentration of nitrite (a) and hydroxgen peroxide (b) (mean ± standard deviation) in Japanese quails threated with CPF and in control group. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$ vs. control group, $^{\#\#\#\#}P < 0.0001$ vs 0.357 mg/kg, $^{\bullet\bullet\bullet\bullet}P < 0.0001$ vs 0.75 mg/kg.

There are 2 major classes of cholinesterase (**ChE**). The first is the true AChE (Layer, 1990), which is abundant in the brain, muscle, and erythrocytes membrane. The second one is BChE or pseudocholinesterase, which is synthesized in the liver but also found in the brain, lung, and kidney, and in an abundant amount in plasma (Cokugras, 2003). Interest in BChE as a detoxification enzyme has grown in recent years. BChE is able to hydrolyze a number of choline esters (acetylcholine, butyrylcholine, propionylcholine, and benzoylcholine), while AChE uses only acetylcholine as a substrate (Layer, 1990). BChE has toxicological importance, because it scavenges OP and carbamate inhibitors before they reach AChE and regulates cholinergic transmission in the absence of AChE (Cokugras, 2003).

Analysis of brain cholinesterase activity is commonly used in the diagnosis of a bird's exposure to OP. If inhibition of AChE activity is greater than 50%, that is enough to diagnose poisoning with OP as a cause of death (Shimshoni et al., 2012).

In our study, AChE and BChE activity was decreased by CPF treatment with significant difference at all dose levels. The data showed that AChE activity was inhibited significantly by CPF in the brain of mice (Maa et al., 2013), rats (Singh et al., 2013), and also in fish brain as compared to the control (Kavitha and Rao, 2008; Narra et al., 2017). Chicks who were given CPF for 7 consecutive d showed inhibition of AChE at high doses similar to quails (Al-Badrany and Mohammad, 2007).

GSH is a ubiquitous thiol tripeptide, composed of cysteine, glutamic acid, and glycine. Most of the GSH in the brain is localized in glia cells (Schulz et al., 2000). GSH is the essential detoxification substance, because of its role in maintaining intracellular redox status and antioxidant enzyme functions (Verma and Srivastava, 2001). In the cell, GSH is synthesized by γ-glutamylcysteine synthetase (**γ-GCS**) and glutathione synthetase (Zitka et al., 2012). GSH also acts as a reducing agent, eliminating the OP metabolites from the body through urine (Abel, et al., 2004). OP causes an increase in lipid peroxidation of the cell membrane. MDA is an important indicator of lipid peroxidation, causing membrane damage and contributing to loss of cellular homeostasis (Trachootham et al., 2009).

In this study, we used the GSH and MDA levels to evaluate the potential of CPF to cause oxidative stress, which also was used in other OP studies in experimental animals (Sharma, et al., 2005). Similar results were mentioned by Gabrowny et al. (2007) who found an increase in MDA and GSH levels in the rat plasma and brain tissue, during the course of treatment with CPF. Our study showed significant increase of MDA content in the brain of CPF-treated quails. An unexpected decrease of MDA at doses of 3 and 6 mg/kg CPF may occur due to elevated SOD_2 at those dose levels, which can reduce membrane lipid peroxidation (Macmillan-Crow and Cruthirds, 2001).

GSH level was affected only by doses of 0.375 mg/kg and 3 mg/kg. The increase of MDA content and GSH level in the brain and gills (Deb and Das, 2013) of CPF-treated guppy fish (*Poecila reticulate*) also has been determined.

An increase of GSH level may be explained by the increase in the activity of γ-glutamyltranspeptidase (**γ-GT**). γ-GT is a membranous astroglial ectoenzyme that catalyzes extracellular GSH, when the dipeptide cysteinylglycine is formed and hydrolyzed to cysteine and glycine. Neurons use cysteine and glycine to synthesize GSH, because they cannot take up GSH directly. Due to an increase in γ-GT activity, there is compensatory up-regulation to provide dipeptide precursors for neurons to generate more GSH (Schulz et al., 2000).

In this study, PAGE electrophoresis showed that activities of total SOD and SOD_1 were significantly increased in quails treated with CPF. The results of the study with rats showed that the activity of SOD decreased after CPF treatment (Saoudi et al., 2017). Our findings are consistent with studies conducted by Tuzmen et al. (2007), Łukaszewicz-Hussain (2011), and Abolaji et al. (2017), who report increases of SOD activity in rat brains after CPF administration. The increased SOD activity in the brain indicated that the higher production of free radicals was generated. Zinc, copper, and manganese are essential components of the oxidative system (Sohail et al., 2011). SOD is an antioxidant enzyme, scavenger for superoxide anions ($\mathbf{O_2^{\bullet -}}$), and has the ability to catalyze dismutation $O_2^{\bullet -}$ to oxygen (O_2) and H_2O_2 using NADPH or NADH (Fukai and Ushio-Fukai, 2011).

The overall mechanism by which SOD functions has been called a "Ping-Pong" mechanism, as it involves the sequential reduction and oxidation of a redox active transition metal (such as Fe^{2+}, Cu^{+}, Mn^{2+}) at the active site of the enzyme. In the Fenton-type reaction in the presence of reduced metal, SOD can convert H_2O_2 into the hydroxide ion ($\mathbf{OH^-}$) and hydroxide radical ($\mathbf{OH^{\bullet}}$), which is the most potential ROS. $OH^{\bullet}$ deactivates SOD_1 by attacking copper-binding histidines at the active site, leading to copper loss. It seems that SOD, besides dismutase, also has a peroxidase mechanism depending of the overproduction of $O_2^{\bullet -}$ or H_2O_2, respectively (Abreu and Cabelli, 2010). SOD_2 can be inactivated due to overproduction of NO. In the presence of $O_2^{\bullet -}$, NO forms the strong oxidant peroxynitrite ($\mathbf{ONOO^-}$), which induces nitration of SOD_2 tyrosine residue and causes enzyme inactivation (Macmillan-Crow and Cruthirds, 2001). The high rate of H_2O_2 production causing inactivation of SOD_1 may be crucial in explaining the slight decrease in the value of SOD_1 at the dose of 6 mg/kg CPF, while the values of SOD_2 at the same dose of CPF are not affected because SOD_2 does not exhibit inhibition by H_2O_2 (Fukai and Ushio-Fukai, 2011).

H_2O_2 has a role in intra- and extracellular toxicity by killing phagocytosed pathogens and in the extracellular destruction of other cells (Halliwell, and Aruoma.,

1991; Desagher et al., 1997). A high increase of H_2O_2 concentration after CPF treatment is as expected.

We also investigated the effect of CPF on MPO activity, since it converts H_2O_2 and chloride ions to hypochlorous acid and participants in tissue defense. MPO may oxidize CPF in vitro and transform it to corresponding oxo-analogue. Our study revealed that MPO activity in the brain was significantly increased in CPF-treated quail. The slight decrease at higher doses of CPF was probably because of high concentrations of H_2O_2, which can inhibit MPO (Kettle and Winterbourn, 1989).

In addition, the study on rats showed also that CPF increased MPO activity in rat brain (Abolaji et al., 2017). This confirms the assumption that the accumulation of H_2O_2 occurs due to CPF intoxication. There are data proving that CPF increases only the peroxidase activity of MPO, while the chlorination activity remains unaffected (Lazarević-Pašti et al., 2013). This is an important finding, because the chlorination activity is a physiological function of MPO.

Nitric oxide synthase may produce NO• from L-arginine. Nitrite, nitrate ($\mathbf{NO_3}^-$), and S-nitrosothiols (**RSNO**) are the end products of the reaction between NO• with oxygen species and biological molecules. In our study, the significant increase of NO_2^- was determined in quails treated with CPF. Also, according to the study conducted by Mehta et al. (2009), acute and chronic CPF exposure caused significant increase in the levels of nitrite in all parts of rat brain.

In conclusion, the aim of the present study was to prove intertwining between the ChE and oxidative stress. The results and effects reported in our study have proven that exposure to CPF leads to inhibition of ChE, which can cause neurotoxicity and neurodegenerative diseases. This is accompanied by ROS accumulation in brain cells, resulting in oxidative stress, and thereby the present study supports the suggestion that ChE is involved in detoxification of the CPF. Our findings may provide good insight into understanding the adverse effects of CPF. This research was carried out on quails because there is hardly any or not enough data about the neurotoxic effect of CPF and especially about its influence on oxidative stress in birds. This study is highly important because we are witnessing massive avian mortality in certain countries due to pesticides.

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