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Research Article

Oxidative Stress and Hormonal Disturbance Induced by Chlorpyrifos, Diazinon and their Mixture to Male Rats: The Role of Zinc Supplementation

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Abstract

Exposure to mixtures of toxicants (e.g., pesticides) is common in real life and a subject of recent concern. The aim of the present investigation was to assess some toxicological effects in male rats following exposure to chlorpyrifos (CPF), diazinon (DIZ) and their combination (CPF+DIZ), and to evaluate the ameliorative effect of zinc co-administration. Sixty-four adult's male Wistar rats were divided into equal eight groups. Three groups were designated for chlorpyrifos (CPF), diazinon (DIZ) and the mixture (CPF+DIZ) treatments. Other three groups were designated for zinc in conjunction with the pesticides.

Two groups; one received water only (control), and the other represented positive zinc treatment. The doses either of pesticides or zinc were based on experimental and/or referenced studies. A total number of nine biochemical parameters representing anti-oxidative stress and hormonal function biomarkers were determined in rat plasma after six weeks of treatments. Generally, CPF and DIZ individually and CPF+DIZ induced significant alterations (i.e., higher or lower than control values), but alterations induced by the mixture were greater than those recorded for each of the individual insecticides. Zinc supplementation in conjunction with the tested pesticides achieved considerable ameliorative effect expressed in terms of amelioration index (AI), which was closely around 1.0 indicating maximum amelioration effect of zinc in favour of the most assessed parameters. It was concluded that zinc may therefore be useful as a powerful antioxidant against toxic damage induced by CPF, DIZ and their combination, especially in individuals who are occupationally exposed daily to low doses of such pesticides.

Keywords: Chlorpyrifos; Diazinon; Chlorpyrifos + Diazinon Mixture; Zinc; Amelioration Index

Introduction

The organophosphorus (OP) compounds represent a major class of insecticides used globally. As quantity, they amounted to 44% of the total insecticides used in Egypt during the 90's [1]. These pesticides are mainly acetylcholinesterase inhibitors affecting severely on the central or peripheral nervous system [2]. Some of these OP pesticides are endocrine disruptors and affect male fertility [3].

Exposure to OP pesticides is an important health risk issue especially for agricultural workers. Unfortunately, annual accidental poisonings and death of humans by using these pesticides are worldwide especially in developing countries [4].

Misuse of pesticides, especially in developing countries, contributes in contamination of vegetables, fruits, water, soil and different environmental components by pesticide residues. Even when pesticides are used in accordance with good agricultural practices (GAP), their residues in plants may be unavoidable [5].

This raised the concern about exposure to multi-pesticide residues and explored the fact that exposure to more than one toxic compound (e.g. pesticides) is common in real life and such exposure may occur in air, water and food. Assessment of the potential health hazard of chemical mixtures is a challenging toxicological problem, and a subject of major current concern to both the scientific and regulatory communities [6].

Both chlorpyrifos (CPF), [O, O-diethyl-O-(3,5,6-trichloro-2-pyridyl)-phosphonothioate; IUPAC], and diazinon (DIZ), [(phosphoric acid, O, O-diethyl O (2-isopropyl-6-methyl-4-pyridinyl;) phosphonothioate; IUPAC], are anticholinesterase insecticides with contact, stomach, and respiratory action. They are used widely to combat insect pests in different crops as well as in buildings and public places [7]. Therefore, their residues may be found in the same food commodities such as vegetables and fruits produced from sprayed fields. Monitoring studies conducted in different countries revealed presence of residues of both insecticides among other ones in different varieties of food commodities [8-11].

Recent studies identified reactive oxygen species (ROS) as a cause of toxic effects exerted by OP pesticides. These ROS are responsible of inducing oxidative stress in the tissues and chronic permanent damage [12].

This raised the interest of scientists to search for antioxidants which might alleviate oxidative stress caused by pesticides. Several substances including naturally occurring plant oils, vitamins, and essential mineral elements were used to alleviate toxic hazards of OP pesticides-induced oxidative stress in experimental animals. For examples, fennel (*Foeniculum vulgare* Mill.) essential oil [13], wheat germ oil and grape seed oil [14]; Vitamin E (α-tocopherol) [15]; and zinc [16,17] were used against chlorpyrifos-induced oxidative stress in rats. On the other hand, white grape seed oil [18]; Vitamin E (α-tocopherol) [19,20], and a combination of vitamin C and E [21] were used against diazinon-induced oxidative stress in rats.

It's worthy to mention that the study of the role of certain essential metals in modulating the effects of different toxicants is being one area of increasing interest. In this respect, studies have shown that zinc, an essential nutrient, can protect against oxidative damage caused by certain xenobiotics and thus may have antioxidant properties [22].

The present investigation was carried out to assess some toxicological effects in male rats following exposure to CPF, DIZ and their combination, and to evaluate the ameliorative effect of co-administration of zinc. To assess this, some biomarkers of oxidative stress [e.g. Lipid Peroxidation (LPO; MDA), Superoxide Dismutase (SOD), Catalase (CAT), Glutathione Peroxidase (GPx), Glutathione Reductase (GR), Glutathione-S-Transferase (GST), Cytochrome P450 (CYP_{450})], and hormonal disturbance biomarkers [e.g., Testosterone (T) and Tetraiodothyronine-Thyroxine (T_4)] were estimated in insecticidal treatments with and without zinc supplementation.

Materials and Methods

Chlorpyrifos was obtained from the National Company for Agrochemicals and Investment (Agrochem), Alexandria, Egypt as Pestan® (48% EC). Diazinon was procured from the Egyptian Mud Engineering and Chemicals Company (EMEC), Alexandria, Egypt as Kanzinon® (60% EC). Zinc Chloride (ZnCl2) powder (M.W. 136.29), a product of Oxford Laboratory Reagent, UK was purchased from the local market.

Reagents (Diagnostic Kits)

Diagnostic kits used in the present study were obtained from Biodiagnostic Co., Dokki, Giza, Egypt. These were oxidative/antioxidative biomarkers: Lipid peroxidation (LPO), Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx), Glutathione reductase (GR), Glutathione-s-transferase (GST), Cytochrome P_{450} (CYP_{450}), and hormonal biomarkers: Testosterone (T) and Tetraiodothyronine-Thyroxine (T_4).

Animals

Healthy male albino rats of the Wistar strain (*Rattus norvegicus*), with an average weight of 110 ± 20g, were obtained from the Animal Breeding House of the National Research Centre (NRC), Dokki, Cairo, Egypt, and maintained in clean plastic cages in the laboratory animal room (23 ± 2°C) on standard pellet diet and had free access to water in daily dark/light cycle of 12/12 hrs. Rats were acclimatized for 1 week prior to the start of experiments. The experimental work on rats was performed with the approval of the Animal Care and Experimental Committee, College of Agriculture in Damanhour, Egypt, and according to the guidance for care and use of laboratory animals [23].

Determination of Oral LD_{50} for the Tested Insecticides

Preliminary tests were carried out to determine the median lethal dose (LD_{50}) for commercial formulations of Chlorpyrifos and Diazinon on male rats. For each insecticide, three doses were prepared in water based on active ingredient, a.i., contents (e.g., 33.75, 135 and 189 mg/kg b.w. for chlorpyrifos; and 300, 350 and 400 mg/kg b.w. for Diazinon). Four rats were used for each tested dose, in addition to four rats given water only and served as control group. Dosing was performed by gavages with 0.5 ml solutions. The 24h-LD50 values were estimated according to Finney [24]. Based on the obtained LD50 values, the equivalent to the 1/10 was used in the present study (e.g., 8.77 and 40.85 mg/kg b.w from chlorpyrifos and diazinon, respectively).

Experimental Design

A total of 64 male rats were divided into eight groups (Gs), each contained eight animals. G1 (Cont.): received water free of any pesticide and served as control. G2 (Zn): administered ZnCl2 in drinking water at a concentration of 227 mg /L (as Zn) according to Goel., et al. [25]. G3 (CPF) and G4 (DIZ): were orally administered 8.7 and 40.84 mg/kg bw from chlorpyrifos and diazinon, respectively. G5 (CPF+DIZ): was given both insecticides at their respective doses singly. Groups 6, 7 and 8 were respectively administered CPF, DIZ and CPF+DIZ by oral gavages in addition to Zn in drinking water. The insecticides were given every 48h intervals; zinc solution was introduced in sufficient amounts a day after an-

other, while water in G1 was permitted ad libitum. The experimental duration was extended up to 42 days, and the doses of insecticides were adjusted weekly according to changes in body weights of the tested animals.

Biochemical Estimations

At the end of the experimental period (6 weeks), blood samples were withdrawn from the animals under ether anaesthesia by puncturing the retro orbital venous plexus with a fine sterilized glass capillary.

Blood was collected into heparinized glass tubes to separate plasma. The tubes were left for 20 min at room temperature, then centrifuged at 3000 rpm (600g) for 10 minutes using BOECO centrifuge model C-28, Germany, and kept in a deep freezer (-20°C) until analysed within one week maximum.

Enzymatic analyses were measured on Jenway 6305 UV/VIS Spectrophotometer at the specified wavelengths. Hormonal determinations were carried out by using Enzyme Linked Immuno Sorbent Assay (ELISA; GmbH model Jupiter). The analyses were carried out in accordance to the pamphlet instructions given by the manufacturers, and in light of the published methods [26-34].

Statistical analysis

All obtained data were statistically analysed using Statistical Analysis (SAS) Software Program 2000 (www.sas.com/en_sg/software/analytics/stat.html). Means were statistically compared using least significant difference (LSD) test at 0.05 and 0.01 significance levels.

Results

Acute Oral Toxicity of the Tested Insecticides

The estimated oral LD_{50} for chlorpyrifos and diazinon against the used male rats indicated that chlorpyrifos (CPF) was more toxic (87.7 mg/kg bw) than diazinon (DIZ) (408.5 mg/kg bw). These values were used to calculate the doses used in the present study (i.e. 1/10 LD_{50}).

Effect on lipid peroxidation and antioxidant enzymes

Six antioxidant enzymes and lipid peroxidation were determined in plasma of rats treated with CPF, DIZ and their mixture, either with or without zinc administration. The obtained results are presented in (Figure 1-7).

The activity of lipid peroxidation (LPO), in terms of Malondialdehyde (MDA), in plasma of control rats or those administered Zn were found 1.35 and 1.33 nmol/ml, respectively without any significant difference (Figure 1). MDA recorded high elevation ($P \leq 0.01$), accounting to 2.29, 2.29 and 2.83 nmol/ml, in the rats treated with CPF, DIZ and CPF+DIZ, respectively. Administration of Zn with CPF or DIZ limited such high elevation to some extent ($P \leq 0.05$), but failed to achieve similar result with the mixture (1.96 nmol/ml).

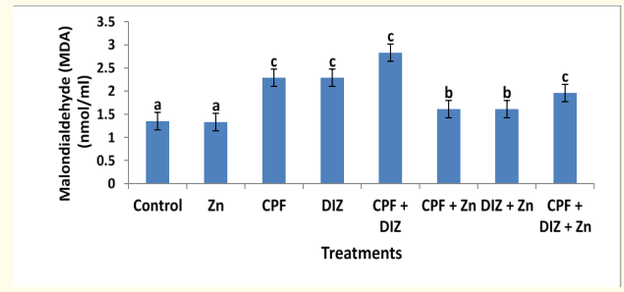

Figure 1. Effect of chlorpyrifos (CPF), diazinon (DIZ) and their mixture (CPF+DIZ), with and without zinc (Zn), on Lipid peroxidation (MDA) levels in rats.

Statistics: Bars represent the group means ± SD; n = 8. Values of similar superscript letters are not statistically different. Values of superscript "b" are significantly different than those of superscript "a" at $P \leq 0.05$; Values of superscript "c" are high significantly different than those of superscript "a" at $P \leq 0.01$.

In contrary to MDA, activity of superoxide dismutase (SOD) (Figure 2) showed high significant ($P \leq 0.01$) decline in rats treated with CPF, DIZ, CPF+DIZ, compared with that recorded for the control (104.87 µmol/mim/ml). The most declines were attributed to the mixture (74.2 µmol/mim/ml). Administration of Zn with CPF or DIZ limited such high decline to some extent ($P \leq 0.05$), but failed to achieve similar result with the mixture (91.4 µmol/mim/ml).

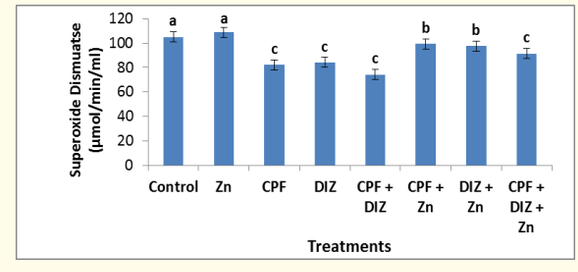

Figure 2. Effect of chlorpyrifos (CPF), diazinon (DIZ) and their mixture (CPF+DIZ), with and without zinc (Zn), on Superoxide (SOD) levels in rats.

Statistics: Bars represent the group means ± SD; n = 8. Values of similar superscript letters are not statistically different. Values of superscript "b" are significantly different than those of superscript "a" at $P \leq 0.05$; Values of superscript "c" are high significantly different than those of superscript "a" at $P \leq 0.01$.

Catalase activity in control and Zn rat groups recorded 0.48 and 0.42 µmol/mim/ml, respectively without any significant difference (Figure 3). In comparison, high significant elevations ($P \leq 0.01$) were recorded for CPF, DIZ and CPF+DIS (e.g., 0.67, 0.62 and 0.99 µmol/mim/ml, respectively). Administration of Zn with CPF or DIZ limited such high elevation to some extent ($P \leq 0.05$), but failed to achieve similar result with the mixture (0.67 µmol/mim/ml).

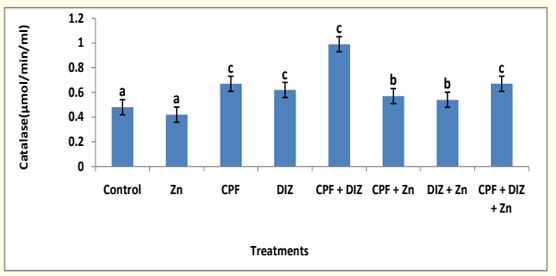

Figure 3. Effect of chlorpyrifos (CPF), diazinon (DIZ) and their mixture (CPF+DIZ), with and without zinc (Zn), on Catalase (CAT) levels in rats.

Statistics: Bars represent the group means ± SD; n = 8. Values of similar superscript letters are not statistically different. Values of superscript "b" are significantly different than those of superscript "a" at $P \leq 0.05$; Values of superscript "c" are high significantly different than those of superscript "a" at $P \leq 0.01$.

Activity of glutathione peroxidase (GPx) is presented in (Figure 4), where control result (0.87 µmol/min/ml) were significantly higher ($P \leq 0.01$) than values recorded for CPF, DIZ and CPF+DIZ (0.74, 0.75 and 0.65 µmol/min/ml, respectively). In comparison, CPF+Zn and DIZ+Zn recorded 0.87 and 0.88 µmol/min/ml, respectively; values which were insignificantly different than the control value.

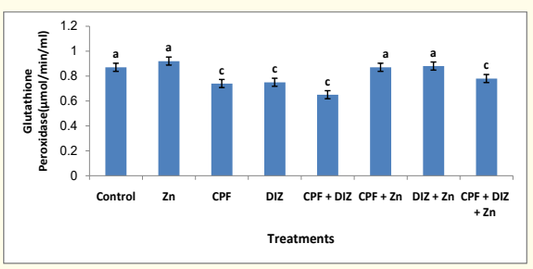

Figure 4. Effect of chlorpyrifos (CPF), diazinon (DIZ) and their mixture (CPF+DIZ), with and without zinc (Zn), on Glutathione peroxidase (GPx) levels in rats.

Statistics: Bars represent the group means ± SD; n = 8. Values of similar superscript letters are not statistically different. Values of superscript "b" are significantly different than those of superscript "a" at $P \leq 0.05$; Values of superscript "c" are high significantly different than those of superscript "a" at $P \leq 0.01$.

Administration of Zn with the mixture limited decline of GPx to some extent ($P \leq 0.05$) where the enzyme activity equalled 0.78 µmol/min/ml.

Figure 5 illustrates effect of the tested insecticides on the activity of glutathione reductase (GR). The control group recorded 85.37 nmol/min/ml, a value which was significantly higher ($P \leq 0.01$) than values recorded for CPF, DIZ, CPF+DIZ, DIZ+Zn and CPF+DIZ+Zn (65.20, 61.48, 56.17, 72.28 and 67.51 nmol/min/ml, respectively). The treatment of CPF+Zn recorded 78.31 nmol/min/ml; a value which was significantly ($P \leq 0.05$) lower than the value recorded for control.

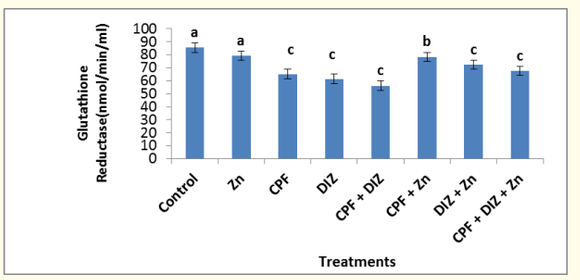

Figure 5. Effect of chlorpyrifos (CPF), diazinon (DIZ) and their mixture (CPF+DIZ), with and without zinc (Zn), Glutathione reductase (GR) levels in rats..

Statistics: Bars represent the group means ± SD; n = 8. Values of similar superscript letters are not statistically different. Values of superscript "b" are significantly different than those of superscript "a" at $P \leq 0.05$; Values of superscript "c" are high significantly different than those of superscript "a" at $P \leq 0.01$.

The effect of the tested insecticides on the activity of glutathione-s-transferase (GST) is presented in Figure 6. Control and Zn treatments recorded 1.07 and 1.13 µmol/min/ml, respectively without significant difference. Values recorded by CPF, DIZ, CPF+DIZ and CPF+DIZ+Zn were 0.79, 0.79, 0.71 and 0.85 µmol/min/ml, respectively; indicating high significant decrease ($P \leq 0.01$) compared with the control value.

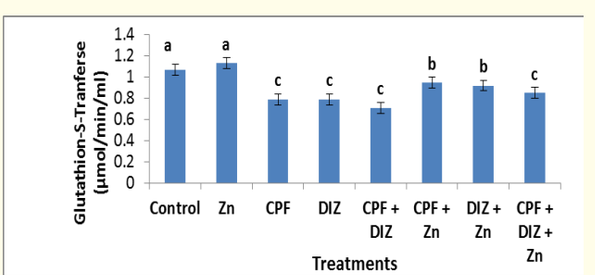

Figure 6. Effect of chlorpyrifos (CPF), diazinon (DIZ) and their mixture (CPF+DIZ), with and without zinc (Zn), on Glutathione-S-Transferase (GST) levels in rats.

Statistics: Bars represent the group means ± SD; n = 8. Values of similar superscript letters are not statistically different. Values of superscript "b" are significantly different than those of superscript "a" at $P \leq 0.05$; Values of superscript "c" are high significantly different than those of superscript "a" at $P \leq 0.01$.

Co-administration of Zn with CPF or DIZ limited decline of the enzyme to some extent ($P \leq 0.05$), where their estimated values were 0.95 and 0.92 µmol/min/ml, respectively.

Figure 7 illustrates cytochrome P450 activity in plasma of male rats treated with CPF, DIZ and CPF+DIZ, as well as Zn supplementation in other paralleled insecticide treatments. Both control and Zn treatments recorded ca. 0.136 nmol/min/ml, a value which was significantly higher ($P \leq 0.01$) than those recorded for CPF (0.036 nmol/min/ml), DIZ (0.039 nmol/min/ml), CPF+DIZ (0.029 nmol/min/ml) and CPF+DIZ+Zn (0.09 nmol/min/ml). Supplementation of Zn in conjunction with CPF and DIZ (0.112 and 0.122 nmol/min/ml) limited decline of the enzyme to some extent ($P \leq 0.05$).

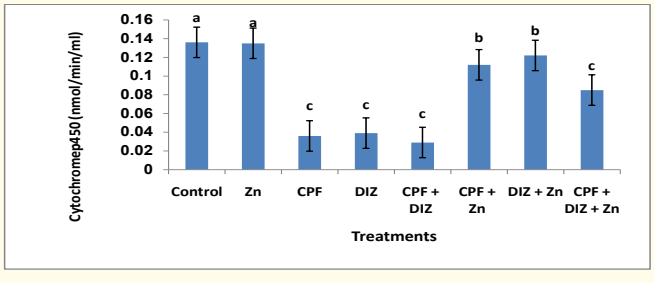

Figure 7. Effect of chlorpyrifos (CPF), diazinon (DIZ) and their mixture (CPF+DIZ), with and without zinc (Zn), Cytochrome P450 (CYP450) levels in rats.

Statistics: Bars represent the group means ± SD; n = 8. Values of similar superscript letters are not statistically different. Values of superscript "b" are significantly different than those of superscript "a" at $P \leq 0.05$; Values of superscript "c" are high significantly different than those of superscript "a" at $P \leq 0.01$.

Effect on testosterone and thyroxine hormones

Two hormones, testosterone (T) and thyroxine (T4) were determined in plasma of male rats treated with CPF, DIZ and their mixture, either with or without zinc administration. The obtained results are presented in (Figure 8 and 9).

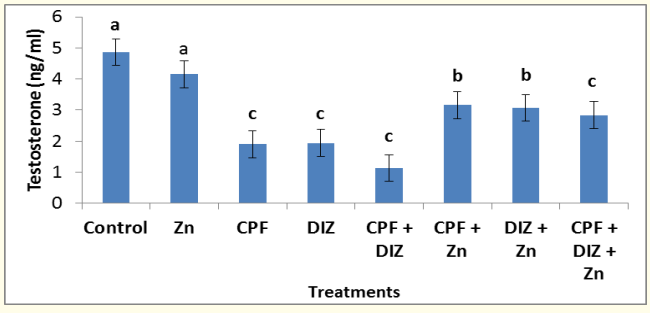

Figure 8. Effect of chlorpyrifos (CPF), diazinon (DIZ) and their mixture (CPF+DIZ), with and without zinc (Zn), on Testosterone (TE) levels in rats.

Statistics: Bars represent the group means ± SD; n = 8. Values of similar superscript letters are not statistically different. Values of superscript "b" are significantly different than those of superscript "a" at $P \leq 0.05$; Values of superscript "c" are high significantly different than those of superscript "a" at $P \leq 0.01$.

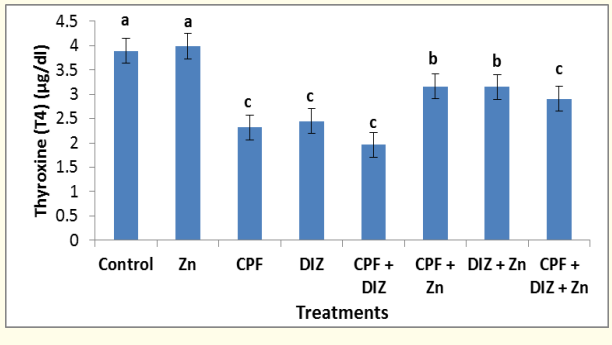

Figure 9. Effect of chlorpyrifos (CPF), diazinon (DIZ) and their mixture (CPF+DIZ), with and without zinc (Zn), on Thyroxine (T4) levels in rats.

Statistics: Bars represent the group means ± SD; n = 8. Values of similar superscript letters are not statistically different. Values of superscript "b" are significantly different than those of superscript "a" at $P \leq 0.05$; Values of superscript "c" are high significantly different than those of superscript "a" at $P \leq 0.01$.

Testosterone hormone (T) measured in plasma of control rats was found 4.86 ng/ml (Figure 8). Its level was severely declined ($P \leq 0.01$) in CPF, DIZ, CPF+DIZ and CPF+DIZ+Zn treatments, where the estimated values were 1.90, 1.94, 1.13 and 2.83 ng/ml, respectively. Co-administration of Zn with CPF or DIZ limited such decrease ($P \leq 0.05$) of the hormone to some extent, where their estimated values equalled 3.16 and 3.07 ng/ml, respectively.

The hormone, thyroxine (T4) showed similar pattern to that of testosterone where CPF, DIZ, CPF+DIZ and CPF+DIZ+Zn treatments recorded severe decline in the level of T4 compared with control (3.89 µg/dl).

Co-administration of Zn with CPF or DIZ limited such decline in the hormone activity to some extent, where their estimated values equalled 3.16 and 3.15 µg/dl, respectively; results which were still lower than the control result at $P \leq 0.05$ (Figure 9).

Discussion

The oral LD50 for technical grade chlorpyrifos in rats is 95 - 270 mg/kg (extoxnet.orst.edu/pips/chlorpyr.htm), and 300 - 400mg/kg for technical grade diazinon in rats (extoxnet.orst.edu/pips/diazinon.htm). The estimated oral LD50 for the used commercial products of CPF and DIZ to male rats were found 87.71 mg/kg (72.98 - 103.83) and 408.49 mg/kg (400.11 - 416.12), respectively. Such differences may refer to the nature of the pesticide formulation and other factors related to test conditions. On the other side, the experimentally obtained LD50 values indicate that the acute toxicity of CPF is about five times that of DIZ.

Reactive oxygen species (ROS) is a term used to designate oxygen derived free radicals (e.g., superoxide, hydroxyl radical, nitric oxide), and non-radical oxygen derivatives of high reactivity (e.g., singlet oxygen, hydrogen peroxide, peroxynitrite, hypochlorite). The human body possesses molecules, known as antioxidants that can counteract the harmful effects of these free radicals. If the generation of free radicals exceeds the protective capacity of antioxidants, this can cause a case of oxidative stress leading to chronic damage in the tissues and age-dependent diseases such as cardiovascular disease, cancer, neurodegenerative disorders, and other chronic conditions [35]. Xenobiotics, such as pesticides, enhance the formation of ROS which has been implicated in inducing oxidative stress in the tissues and chronic damage. Such damage occurs in cases of excessive formation of ROS or insufficient of protective antioxidants. Organophosphorus (OP) insecticides were reported to induce oxidative stress in cases of acute poisoning [36].

In this respect, it may be convenient to demonstrate the role of the antioxidants of relevance to the present study. Lipid peroxidation (LPO) is characterized as a chain reaction of polyunsaturated fatty acids with ROS. It produces lipid peroxides and hydrocarbon polymers which are extremely toxic to the cell.

The peroxidation of polyunsaturated fatty acids and related esters yields malondialdehyde (MDA) as an end product. Therefore, it serves as a biomarker of LPO. Treatment with hydrogen peroxide was reported to induce high increase in lipid peroxidation and enhancement of ROS generation. Quinalphos-induced testicular damage, as example, was referred to the free radicals mediated by the increase of LPO [37].

Catalase is one of the cellular defense mechanisms against cytotoxic oxygen species (H2O2). However, endogenous (H2O2) may be converted to H2O either by catalase or glutathione peroxidase, or it may generate the highly reactive free hydroxyl radical (OH•) by the fenton reaction, which is believed to be responsible mainly for oxidative damage [35].

Glutathione reductase (GR) or (Reduced glutathione, GSH) is the natural antioxidant of the cell. It plays a vital role in the detoxification process by destroying free radicals formed in the cells. So, deficiency of GR causes greater lipid peroxidation leading to cell damage [38]. Glutathione peroxidase (GPx) is a major defense system against oxidative damage to essential intracellular compounds (e.g., proteins and poly-unsaturated fatty acids); particularly by effective reduction of hydroperoxide to water. Also, Glutathione-s-transferase (GST) is involved in the detoxification process due to its ability to conjugate GSH with lipid peroxidation products [39].

It is well documented that the antioxidant enzymes, such as SOD, GST and CAT act as free radical scavengers by limiting the effects of ROS on the tissues, and thus they protect the cell from injury [40]. These enzymes work together in order to eliminate ROS and any deviation in the physiological concentrations. The conversion of superoxide radical to H_2O_2 is catalyzed by SOD, while CAT converts H_2O_2 to water. Therefore, these enzymes have capability

to alleviate the hazards of ROS [35].

In agreement with the results shown in Figures 1-6, many studies reported elevation of lipid peroxidation (LPO) and CAT and decline of SOD, as well as the glutathione-enzyme group following exposure to OP pesticides, such as chlorpyrifos [15-17], and diazinon [18-21]. Also, it was reported that treatment of rats with CPF has resulted in increasing oxidative stress in the body. This was evidenced by high elevation of thiobarbituric acid reactive substances (TBARS), accompanied with a decrease in the levels of antioxidative stress enzymes (e.g., SOD, CAT and GPx) in liver, kidney and spleen [41]. The elevated activity of catalase in insecticide-treated rats in this study may be due to the adaptive response to the generated free radicals indicating the failure of the total antioxidant defense mechanism to protect the tissues from damage caused by free radicals [35].

Cytochrome P_{450} (CYP_{450}) enzyme system plays an important role in the bio-activation of OP insecticides through catalysing oxidation of one atom molecular oxygen into a substrate (organophosphate) by an electron transport pathway [42]. In this reaction ROS are generated. In the current study, results revealed that the tested insecticides, CPF, DIZ and their combination induced significant decrease in the level of (CYP_{450}) (Fig. 7). These results are in agreement with those reported by Yamano and Morita [43]. At high doses of diazinon, a noticeable decline in CYP_{450} level and decrease in activity of drug-metabolizing enzymes were indicatives for destruction of CYP_{450}. This may also refer to the inhibition of heme synthesis [44]. Many pesticides belonging to OC and OP compounds were reported to inhibit the activity and alter the expression of various CYP_{450} isoforms. These changes may increase sensitivity of the cells against reactive endogenous metabolites or other xenobiotics. Therefore, it has been suggested that the inhibition of cytochrome activity by OP compounds may contribute to the development of Parkinson's disease due to rendering the neurons more sensitive to toxic metabolites of neurotransmitters [45].

Testosterone (T) is the main steroid sex-hormone in male rats. It is secreted by leydig cells of the testes under the control of complex neuroendocrine interactions [46]. The present findings revealed pronounced decrease of (T) level following exposure of male rats to CPF, DIZ and their mixture (Figure 8).

Several pesticides were previously reported the decline of this hormone in rats treated with OC pesticides [46,47]. Also, the OP insecticides may decline the steroid hormone levels by increasing steroid catabolism and elimination or inhibition of steroid hormone production. Specifically, diazinon was reported to reduce testosterone level because it induced reduction in spermatogenesis and fertility in animals and has the capacity to disrupt reproductive function in animals [48].

Thyroid hormones (e.g., T4) might be able to regulate the activities of (SOD), (CAT) and (GPx) enzymes in lymphoid organs and skeletal muscles [49]. The role of thyroid hormones in metabolic pathways and antioxidant enzyme activities are well known in many species such as rats [50] and camel [51]. The results of the present study indicated that the tested insecticides caused decline of thyroxine (T4) level in the treated rats (Figure 9). These findings are supported by the results of several investigators who addressed the thyroid inhibitory nature of OP insecticides [52], and CPF+Pd mixture administered to Wistar rats [53].

The observed decrease in the level of T_4 in the insecticide-treated groups of rats may refer to some damage in the thyroid gland due to oxidative stress induction and functional impairment of the pituitary-thyroid axis by insecticides such as CPF [54].

The study of the role of some essential metals to modulate the effects of toxicants is an area of increasing interest. In this respect, several studies have shown that zinc possesses antioxidant properties and thus can protect the cell from oxidative damage induced by certain xenobiotics. Apart from its direct antioxidant effect by occupying iron and copper binding sites on lipids, proteins and DNA, zinc also plays a structural role in maintaining the integrity of Cu-Zn-SOD as a cofactor, and in glutathione regulation which is vital to cellular antioxidant defense [22]. Moreover, zinc plays an essential role in cellular glutathione regulation which is a vital process to cellular antioxidant defense [55]. Therefore, many studies have tested the ameliorative effect of some vitamins and natural products in CPF-induced oxidative stress; however, there are few studies on the ameliorative effect of zinc against oxidative stress induced by CPF in rat [16,17]. To the best of our knowledge, there are no similar studies on DIZ and/or CPF+DIZ mixture with respect to co-administration with zinc. Thus, the present study evaluated the ameliorative effect of zinc against oxidative stress, as well as hormonal disturbance induced by CPF, DIZ and their mixture (CPF+DIZ).

According to Mansour and Payrastre [15], it was possible to assess the effect of the tested pesticides in the present study on the measured biochemical and hormonal parameters in a "quantitative manner" by calculating the percentage of change in pesticides-treated groups relative to control untreated groups. By other words, such changes indicate how much deviation than normal values due to pesticide treatments.

On the other hand, we estimated the "Amelioration Index; AI" by comparing the results of a given biochemical parameter in pesticides + Zn groups with the results of control groups, to assess the ameliorative effect of Zn. As AI approaching "1", as the amelioration reaching high degree of normalization to the control value. In this context, (Table 1) presents the net results of biochemical parameters that enabled us to estimate percent of changes due to exposure to the tested pesticides and the ameliorative efficiency of Zn when co-administered with these pesticides.

Treatment	Biochemical Parameters								
	SOD	MDA	CAT	GPX	GR	GST	CYP450	T	T4
Control (a)	**104.87**	**1.35**	**0.48**	**0.87**	**85.37**	**1.07**	**0.136**	**4.86**	**3.89**
Chlorpyrifos									
CPF (b)	**82.19**	**2.29**	**0.67**	**0.74**	**66.20**	**0.79**	**0.036**	**1.90**	**2.32**
CPF+Zn (c)	**99.31**	**1.61**	**0.57**	**0.87**	**78.31**	**0. 95**	**0.112**	**3.16**	**3.16**
% of Change*	**-21.6**	**69.6**	**39.6**	**-14.9**	**-22.5**	**-26.2**	**-73.5**	**-60.9**	**-40.4**
Ameliorative Index**	**0.95**	**1.2**	**1.2**	**1.0**	**0.92**	**0.89**	**0.82**	**0.65**	**0.81**
Diazinon									
DIZ (b)	**84.15**	**2.29**	**0.62**	**0.75**	**61.48**	**0.79**	**0.039**	**1.94**	**2.45**
DIZ+Zn (c)	**97.41**	**1.61**	**0.54**	**0.88**	**72.28**	**0.92**	**0.122**	**3.07**	**3.15**
% of Change*	**-19.8**	**69.6**	**29.2**	**-13.8**	**-28.0**	**-26.2**	**-71.3**	**-60.1**	**-37.0**
Ameliorative Index**	**0.93**	**1.2**	**1.1**	**1.0**	**0.86**	**0.86**	**0.90**	**0.63**	**0.81**
Chlorpyrifos + Diazinon									
CPF+DIZ (b)	**74.23**	**2.83**	**0.99**	**0.65**	**56.17**	**0.71**	**0.029**	**1.13**	**1.96**
CPF+DIZ+Zn (c)	**91.37**	**1.96**	**0.67**	**0.78**	**67.51**	**0.85**	**0.085**	**2.83**	**2.91**
% of Change*	**-29.2**	**109.6**	**106.2**	**-25.3**	**-34.2**	**-33.6**	**-78.7**	**-76.8**	**-49.6**
Ameliorative Index**	**0.87**	**1.4**	**1.4**	**0.9**	**0.79**	**0.79**	**0.63**	**0.58**	**0.75**

Table 1. Percent of change in some biochemical parameters, related to oxidative stress and hormonal disturbance, in male rats induced by chlorpyrifos (CPF), diazinon (DIZ) and the mixture (CPF+DIZ), and the ameliorative effect of zinc supplementation.

- Data refer to Figs. 1- 9 and each value is a mean of 8 values.

* % of change in the biochemical parameter in question (effect of pesticide) = [(b – a) / a] x 100;

**Amelioration Index (AI) (effect of zinc co-administration) = c / a

- Biochemical Parameters abbreviations:

SOD: Superoxide Dismutase; MDA: Malondialdehyde; CAT: Catalase; GPX: Glutathione Peroxidase; GR: Glutathione Reductase; GST: Glutathione-S- Transferase; CYP 450: Cytochrome P450; T: Testosterone; T4: Thyroxine.

In the CPF treatments, the following percent of changes and amelioration indices (AI) due to Zn co-administration were, SOD: (-21.6%; AI = 0.95); MDA: (69.6%; AI = 1.2); CAT: (39.6%; AI = 1.2); GPx: (-14.9%; AI = 1.0); GR: (-22.5%; AI = 0.92); GST: (-26.2; AI = 0.89); CYP450: (-73.5%; AI = 0.82); T: (-60.9%; AI = 0.65); and T4: (-40.4%; AI = 0.81). In the treatments of DIZ alone, the highest change was accounted to MDA (69.7%), CYP 450 (-71.3%) and T (-60.1%). Co-administration of Zn resulted in amelioration indices of 1.2, 0.90 and 0.63, respectively.

Treatments of the mixture (CPF+DIZ) caused changes in the levels of the measured biochemical parameters. The changes were very higher than those occurred in the single treatments. For example, the changes reached 109.6% for MDA, 106.2% for CAT, -78.7% for CYP 450 and -76.8% for T. High AIs (e.g., 1.4) were obtained for MDA and CAT, while low AIs were obtained for T (0.58) and CYP450 (0.63). Such extremely deviated AI values than 1.0 may refer to interaction between the mixture component (CPF+ DIZ+Zn). For instance, a high value such as 1.4 could be seen as an indicative of "synergism", while a low value such as 0.6 as an indicative of "antagonism". However, in light of the available data (Tables 1), it's difficult to conclude the nature of such interaction (e.g., synergism or antagonism), and yet remains a suggestion acquires more investigation.

Conclusion

The results of the present study revealed that the acute oral toxicity of CPF to male rats was higher than that of DIZ, by a factor of magnitude approaching five folds. Deviation (i.e. % of change) of the values of the measured biochemical parameters in different insecticide treatments compared with control (normal) values (Table 1) gave an indication to the toxicity of the tested doses of CPF, DIZ and CPF+DIZ.

Generally, both CPF and DIZ individually induced similar or slightly different alterations, while the mixture (CPF+DIZ) induced alterations higher than those recorded for each of the individual insecticides.

However, with few exceptions, the amelioration indices (AIs) were closely around 1.0 which indicates high ameliorative effect of zinc supplementation with the pesticides. Extremely AIs (Table 1) lower than 1.0 (e.g., 0.58 and 0.63; for testosterone and cytochrome P450, respectively) or higher than 1.0 (e.g., 1.4; for MDA and CAT), were mainly attributed to some CPF+DIZ+Zn treatments; results may refer to interaction between the mixture components and the efficiency of zinc to alleviate oxidative stress in favor of the above mentioned biochemical parameters. Also, the study has shown, for the first time, the ability of zinc to ameliorate the oxidative stress induced by diazinon and its combination with chlorpyrifos. Zinc may therefore be useful as a powerful antioxidant agent against toxic damage induced by CPF, DIZ and their combination, especially in individuals who are occupationally exposed daily to low doses of such pesticides.

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Conflict of Interest

The authors declare that there was no conflict of interest.

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