Chlorpyrifos-Induced Alteration of Hematological Parameters in Wistar Rats: Ameliorative Effect of Zinc


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Abstract: Chlorpyrifos (CPF) toxicity has been shown to be partly mediated through induction of oxidative stress. In the present study, experiments were conducted with the aim of evaluating the ameliorative potentials of zinc on CPF-evoked alteration in hematological parameters in Wistar rats. Twenty adult male Wistar rats were divided at random into 4 groups of 5 animals per group. Rats in group I served as the control and were given only soya oil (2 mL kg⁻¹) while those in group II were administered zinc only (50 mg kg⁻¹). Rats in group III were dosed with CPF only (10.6 mg kg⁻¹, ~1/8th of LD₅₀). Those in group IV were co-administered zinc (50 mg kg⁻¹) and CPF (10.6 mg kg⁻¹). The different regimens were administered orally once daily for a period of 8 weeks. At the end of the study period, blood samples collected after sacrificing the animals were analyzed for hematologic parameters such as Packed Cell Volume (PCV), Hemoglobin (Hb), Red Blood Cells (RBC), Mean Cell Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), hemoglobin indices, platelets, absolute and differential White Blood Cell (WBC) counts, neutrophil-lymphocyte ratio and erythrocyte malonaldehyde (MDA) concentration. The results revealed that alterations in the values of PCV, Hb, RBC, platelets, absolute and differential WBC and MDA induced by CPF were ameliorated by co-administration with zinc. In conclusion, the study has shown that attenuations of CPF-evoked alterations in hematological parameters by zinc may be partly mediated through its antioxidant properties.

Key words: Organophosphate insecticides, chlorpyrifos, hematology, lipoperoxidation, amelioration, zinc

INTRODUCTION

Chlorpyrifos, a phosphorothioate broad spectrum moderately toxic Organophosphate (OP) insecticide remains one of the most widely used in agriculture and public health, despite the restriction of some of its domestic uses by the United States Environmental Protection Agency in 2000. The primary mechanism of toxicity is associated with its ability and especially that of its metabolite, CPF-oxon to inhibit acetylcholinesterase (AChE), an enzyme that normally terminates neurotransmission at cholinergic synapses (Eaton et al., 2008). However, other mechanisms independent of AChE inhibition have been reported since, toxicity occurs at doses that did not inhibit the enzyme (Slotkin, 2004, 2005; Slotkin et al.,

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Therefore, rather operating through a single defined mechanism, CPF toxicity involves several families of mechanisms. Among these, the induction of oxidative stress has been given considerable attention (Gultekin et al., 2001; Ambali et al., 2007; Tuzmen et al., 2008). Prolonged exposure to CPF has been shown to cause anemia (Ambali, 2009). Although, the mechanism of CPF-induced anemia has not been elucidated, earlier study from our laboratory suggests that increased erythrocyte fragility apparently due to oxidative damage to the erythrocyte membranes may be partly involved (Ambali et al., 2009).

Zinc is the second most abundant trace element in the body. It is particularly important in cellular function because it is an integral component of numerous proteins, including metalloenzymes, structural proteins and transcriptional factors (Zhou et al., 2007). The antioxidant effect of zinc have been clearly demonstrated in several studies (Korbash et al., 1989; Goel et al., 2005; Brocardo et al., 2007). Zinc has never been shown to interact directly with oxidant species but rather prefers to exert its effects in an indirect manner (Powell, 2000). Adequate zinc intake is an important determinant of the antioxidant capacity (Kouy et al., 2004; Kamp and Donangelo, 2008; Oliveira et al., 2009). The present study investigated the ameliorative effect of zinc on CPF-evoked hematological alteration in Wistar rats.

MATERIALS AND METHODS

Experimental Animals

Twenty male Wistar rats (12-14 weeks old) weighing 115-126 g used for this study were obtained from the Animal House of the Department of Veterinary Physiology and Pharmacology, Ahmadu Bello University, Zaria. They were fed on pellets made from grower’s mash, maize bran and groundnut cake in the ratio of 4:2:1 and water was provided ad libitum. The study was conducted in the Toxicology Laboratory of the Department.

Chemicals

Commercial grade CPF, Termitoc® (Sabero Organics, Gujarat Limited, India) used for this study was reconstituted in soya oil (1%) prior to daily administration. Zinc gluconate tablet (50 mg; Nature Field, USA) was obtained from a Pharmaceutical store in Zaria, Nigeria. They were reconstituted in distilled water (50 mg mL⁻¹) prior to daily administration.

Experimental Protocol

The rats were weighed and then divided at random into 4 groups with each group having 5 animals. Rats in group I served as the control (labeled S/oil) and were given only soya oil (2 mL kg⁻¹). Rats in group II (labeled Zn) were administered zinc only (50 mg kg⁻¹) while those in group III (labeled CPF) were dosed with CPF only at 10.6 mg kg⁻¹, 1/8th of LD₅₀ (Ambali, 2009). Group IV (labeled Zn+CPF) were co-administered with zinc (50 mg kg⁻¹) and CPF (10.6 mg kg⁻¹). The different regimens were administered by oral gavage once daily for a period of 8 weeks. At the end of the study period, the rats were sacrificed by severing the jugular vein after light ether anesthesia. The study was carried out according to the specifications of the Ahmadu Bello University Animal Research Committee and in accordance with Helsinki Declaration.

Hematological Evaluation

Two milliliter of blood collected into heparinized sample bottles were analyzed for hematological parameters such as Packed Cell Volume (PCV), Hemoglobin (Hb), total Red Blood Cells (RBC), Mean Cell Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean
Corpuscular Hemoglobin Concentration (MCHC), total White Blood Cell (WBC) and total platelets count using an automatic hematological assay analyzer, Advia 60® Hematology system (Bayer Diagnostics Europe Ltd., Ireland). Blood smears were also stained with Giemsa for differential WBC count (Gulye et al., 1988), while the neutrophil-lymphocyte ratio was calculated. The hemoglobin index was obtained using the formula described by Smirnov et al. (1987).

Evaluation of Erythrocyte Lipid Peroxidation

Three milliliter of heparinized blood sample obtained from each animal was centrifuged at 1000x g and the plasma discarded. Erythrocyte packets were prepared by washing erythrocytes three times in cold isotonic saline (0.9% w/v). The washed erythrocytes were used to analyze for malondialdehyde (MDA) concentrations using the double heating method of Draper and Hadley (1990) as modified by Altuntas et al. (2002). The principle of the method was based on spectrophotometric measurement of the colour produced during the reaction of thiobarbituric acid (TBA) with MDA. Briefly, 2.5 mL of 100 g L⁻¹ trichloroacetic acid solution was added to 0.5 mL of erythrocytes in a centrifuge tube and placed in a boiling water bath for 15 min. After cooling in tap water, the mixture was centrifuged at 1000x g for 10 min and 2 mL of the supernatant was added to 1 mL of 6.7 g L⁻¹ TBA in a test tube and placed in a boiling water bath for 15 min. The solution was then cooled in tap water and its absorbance measured using a UV spectrophotometer (Jenway, 6405 model, Japan) at 532 nm. The concentration of MDA was calculated by the absorbance coefficient of MDA-TBA complex, 1.56×10⁴/cm/M and expressed in nmol g⁻¹ of hemoglobin. The hemoglobin concentration was determined using the method described by Dacie and Lewis (1991).

Statistical Analysis

Values obtained as Mean±SEM were subjected to one-way Analysis of Variance (ANOVA) followed by Tukey test using GraphPad Prism version 4.0 for windows from GraphPad Software, San Diego, California, USA (www.graphpad.com). Values of p<0.05 were considered significant.

RESULTS

Effects of Treatments on Packed Cell Volume

The PCV of rats in the CPF group was significantly lower (p<0.05) compared to either the S/oil, Zn or Zn+CPF group. There was no significant change in the PCV of rats in the Zn+CPF group compared to either the S/oil or Zn group (Fig. 1).

Effect on Hemoglobin Concentration

The Hb concentration in the CPF group was significantly lower (p<0.05) compared to either the S/oil, Zn or Zn+CPF group. There was no significant difference (p>0.05) in the PCV of Zn+CPF group compared to the S/oil and Zn groups (Fig. 2).

Effect of Treatments on Total Red Blood Cell Concentration

The effect of treatments on RBC concentration is shown in Fig. 3. The RBC concentration in the CPF group was significantly lower (p<0.05) compared either the S/oil or Zn group. Anisocytosis was also observed in the CPF group compared to the other groups. The RBC concentration in the Zn+CPF group was marginally higher than those in the CPF group but it was not significant (p>0.05).
Fig. 1: Effect of soya oil, zinc and chlorpyrifos on packed cell volume. *p<0.01 versus soya oil group; †p<0.05 versus zinc group; ‡p<0.05 versus zinc+chlorpyrifos group. Values are Mean±SEM of 5 animals per group.

Fig. 2: Effect of soya oil, zinc and chlorpyrifos on hemoglobin concentration in Wistar rats. *p<0.05 versus soya oil group; †p<0.01 versus zinc group; ‡p<0.05 versus zinc+chlorpyrifos group. Values are Mean±SEM of 5 animals per group.

Fig. 3: Effect of soya oil, zinc and chlorpyrifos on red blood cell concentration in Wistar rats. *p<0.05 versus soya oil group; †p<0.05 versus zinc group. Values are Mean±SEM of 5 animals per group.

**Effect of Treatments on Red Blood Cell Indices**

The values obtained for MCV, MCH and MCHC were not significantly different (p>0.05) between the groups (Fig. 4)
Fig. 4: Effect of soya oil, zinc and chlorpyrifos on red blood cell indices in Wistar rats. Values are Mean±SEM of 5 animals per group.

Fig. 5: Effect of soya oil, zinc and chlorpyrifos on platelet count in Wistar rats. *p<0.01 versus soya oil group; †p<0.05 versus zinc group, ‡p<0.01 versus zinc+chlorpyrifos group. Values are Mean±SEM of 5 animals per group.

**Effect of Treatments on Platelet Count**

The platelet count in the CPF group was significantly elevated compared to either the S/oil (p<0.01), Zn (p<0.05) or Zn+CPF (p<0.01) group. The platelet count in the Zn+CPF group did not significantly vary (p>0.05) from those obtained in the S/oil and Zn groups (Fig. 5).

**Effect of Treatments on White Blood Cells**

The WBC count in the CPF group was significantly lower (p<0.01) compared to either the S/oil, Zn or Zn+CPF group. There were no significant variation (p>0.05) in the WBC count in S/oil group compared to either Zn or Zn+CPF group (Fig. 6).

**Effect of Treatments on Differential Leukocyte Count**

The effect of treatments on differential leukocyte count is shown in Fig. 7. There was no significant difference in the neutrophil count between the groups. On the other hand, the lymphocyte count in the CPF group was significantly lower (p<0.01) compared to either the S/oil, Zn or Zn+CPF group. No significant change (p>0.05) in the lymphocyte concentration was recorded in the Zn+CPF group compared to either the S/oil or Zn group.
Fig. 6: Effect of soya oil, zinc and chlorpyrifos on total white blood cell count in Wistar rats. *p<0.05 compared to soya oil group. **p<0.01 versus soya oil; ***p<0.05 versus zinc; 'p<0.05 versus zinc+chlorpyrifos group. Values are Mean±SEM of 5 animals per group.

Fig. 7: Effect of soya oil, zinc and chlorpyrifos on differential white blood cell count in Wistar rats. *p<0.01 versus soya oil group. **p<0.01 versus soya oil group; ***p<0.05 versus zinc group; 'p<0.01 versus zinc+chlorpyrifos group. Values are Mean±SEM of 5 animals per group.

Fig. 8: Effect of soya oil, zinc and chlorpyrifos on lymphocyte/neutrophil ratio in Wistar rats. *p<0.01 versus soya oil group. **p<0.01 versus soya oil group; ***p<0.05 versus zinc group; 'p<0.01 versus zinc+chlorpyrifos group. Values are Mean±SEM of 5 animals per group.


**Effect of Treatments on Neutrophil/Lymphocyte Ratio**

The neutrophil/lymphocyte ratio in the CPF group was significantly higher (p < 0.01) compared to either the S/oil, Zn or Zn+CPF group. The neutrophil/lymphocyte ratio of Zn+CPF group was not significantly different (p > 0.05) from those obtained in the S/oil and Zn groups (Fig. 8).

**Effect of Treatments on Erythrocyte Lipoperoxidative Changes**

There was a significant elevation (p < 0.01) in the erythrocyte MDA concentrations in the CPF group compared to either the S/oil, Zn or Zn+CPF group. The MDA concentration in the Zn+CPF was not significantly different (p > 0.05) from those obtained in the S/oil and Zn+CPF groups (Fig. 9).

**DISCUSSION**

The lower values of PCV, Hb and RBC observed in the CPF group in the present study agreed with those obtained in earlier studies (Barna-Lloyd *et al.*, 1991; Goel *et al.*, 2006; Ambali, 2009). This indicates that repeated CPF exposure causes anemia. The lack of significant change in the MCV in all the groups shows that CPF does not alter erythrocyte size. However, the anisocytosis in the CPF group agreed with the observation of Goel *et al.* (2006). Anisocytosis is a feature that is commonly found in anemic and other blood conditions. The hemoglobin index of CPF group was greater than 1 (data not shown), indicating hypochromia (Smirnov *et al.*, 1987). The reason for the anemia in the CPF group is not known. It may however be related to disruption of erythropoiesis or an increase in RBC destruction (Vural *et al.*, 1986). Goel *et al.* (2006) observed a decrease in serum iron concentration which eventually results in reduced hemoglobin production. An earlier report from our laboratory has shown that repeated CPF exposure increases erythrocyte fragility due to increased lipoperoxidative changes in the erythrocyte membranes (Ambali *et al.*, 2009). The increased MDA concentration in the erythrocytes of rats exposed to CPF only is indicative of lipoperoxidative damage, which agreed with a previous report from our laboratory (Ambali *et al.*, 2009) and those recorded by other workers (Gullekin *et al.*, 2001;
Mansour and Mossa, 2009). Lipid peroxidation is a process that is determined by the extent of peroxide-deforming free radical mechanism on the Polyunsaturated Fatty Acids (PUFA) (Tuzmen et al., 2008). The MDA is considered to be the most significant indicator of membrane lipid peroxidation resulting from the interaction between Reactive Oxygen Species (ROS) and cell membranes (Halliwell and Gutteridge, 1990, 1995). The RBC is vulnerable to liperoxidative changes because of its direct association with molecular oxygen, high content of metal ions catalyzing oxidative reactions and availability of high amount of PUFA, which are susceptible to lipid peroxidation. Inability to repair membrane damage and regenerate and poor antioxidant enzymes composition of the plasma medium in which they are bathed are some of the other factors that enhance the vulnerability of the RBC to lipid peroxidation (Marklund et al., 1982; Eblen and Tomur, 2006). By-products of lipid peroxidation causes profound alterations in the structural organization and functions of the cell membrane including decreased membrane fluidity, increased membrane permeability, inactivation of membrane-bound enzymes and loss of essential fatty acids (Ginkel and Sevanian, 1994). CPF is lipophilic and may enhance lipid peroxidation by directly interacting with the cellular plasma membrane (Hazarika et al., 2003). The overall effect of these on the RBC is the reduction in its membrane integrity and life span. Apart from the direct effect of oxidative stress, we have earlier demonstrated the ability of CPF to cause degenerative changes in the kidneys (Ambali, 2009), which may alter the production of erythropoietin, an hormone critical to functional erythropoiesis. Furthermore, CPF has been shown to cause chromosomal abnormality in the bone marrow (Anonymous, 2008). Therefore, the anemia observed following repeated CPF exposure is due to a combination of factors.

The significant improvement in the PCV, Hb and RBC concentrations and anisocytosis in rats co-administered zinc and CPF suggests the ameliorative effect of the trace element. The ameliorative effect of zinc on the indices of anemia agreed with the observation of Goel et al. (2006). The positive association between the improvement in anemia indices and amelioration of the erythrocyte liperoxidative changes demonstrated that oxidative damage probably plays an important role in CPF-induced anemia. The ameliorative effect of zinc on CPF-induced anemia may have arisen from its antioxidant properties (Powell, 2000). Cellular zinc exists in only one redox state (II); thus, it cannot undergo redox reactions that are commonly responsible for the generation of reactive oxygen species (Zhou et al., 2007). Apart from its direct antioxidant effect via occupation of iron and copper binding sites on lipids, proteins and DNA thereby preventing hydroxyl radical formation near these structures (Powell, 2000; Prasad and Kucuk, 2002), zinc also plays a structural role in the maintenance of the integrity of Cu-Zn superoxide dismutase as a cofactor (Coudray et al., 1992; Sahin and Kucuk, 2003) and in the regulation of glutathione that is vital to cellular antioxidant defense (Parrat et al., 1997). In addition, zinc protects sulphhydryl group against oxidation thereby preventing protein from oxidation, hence stabilizing the cellular thiol pools (Kraus et al., 1997; Verhaegh et al., 1998). Furthermore, zinc regulates metallothionein, a highly conserved low molecular weight cystein rich metal chelating antioxidant protein, which has been demonstrated to share a lot of features with glutathione (Quesada et al., 1996; Zhou et al., 2007). Goel et al. (2005) have shown that CPF intoxication causes reduction in metallothionein level, a feature that was ascribed to zinc deficiency. Zinc deficiency has been associated with increased erythrocyte fragility (Kraus et al., 1997; O’Dell, 2000) apparently due to the alteration in erythrocyte membrane integrity. A decrease in membrane zinc content could cause subtle changes in membrane rheology and leave vacant potential binding sites for redox-active metals such as iron and copper (Driscoll and Bettger, 1992) that can promote membrane-lipid oxidation (Verstraeten et al., 2004) and alterations in membrane lipid
composition (Driscoll and Bettger, 1992). This leads to disturbance in the physical properties of the lipid bilayers (Verstraeten et al., 2004). Although, the present study did not measure the level of zinc in the erythrocyte membrane, it is no gain saying that CPF-induced zinc deficiency as observed in a previous study (Goel et al., 2005) may have played a significant role in the pathophysiology of anemia observed in the present study. Therefore, zinc supplementation may have attenuated CPF-induced zinc deficiency and hence ameliorates the anemia. Zinc supplementation has been shown to cause positive haematological parameters in athletes (Kilic et al., 2004).

The study also recorded a significant increase in platelet count of rats exposed to CPF only. This agreed with the findings of Szabo et al. (1988). The implication of thrombocytosis in rats exposed to low-dose CPF is not known. However, thrombocytosis is associated with coagulopathic disorders characterized by widespread clotting in the tissue, resulting in embolism, tissue ischemia and tissue/organ damage. ROS has been shown to facilitate platelet activation (Krotz et al., 2004; Essex, 2009). Zinc supplementation has been shown by the present study to normalize the CPF-evoked thrombocytosis. Although, the mechanism responsible for this is not clear, it may be partly due to the antioxidant effect of zinc resulting in lowered ROS elaboration.

Repeated CPF exposure has been shown by the present study to cause lymphopenic leukopenia. This finding agreed with that obtained in our earlier studies (Ambali et al., 2007; Ambali, 2009). The lymphopenia observed in the CPF-exposed rats may be due to either decreased production and/or increased rate of removal due to rapid destruction. Although, the present study did not observe significant change in the neutrophil content of the blood, Goel et al. (2006) observed neutrophilia following CPF exposure. The high neutrophil:lymphocyte ratio in the CPF group in the present study however corroborated high neutrophil count observed in the earlier study (Goel et al., 2006) indicating that the animals exposed to this chemical are under stress. Earlier works have shown that pesticides are toxic to the cells of the immune system through the induction of necrosis and apoptosis. Immune cells are particularly sensitive to oxidative stress due to the high percentage of PUFA in their plasma membranes and the increased production of ROS (Knight, 2000). Oxidative stress provoked by CPF may have played a significant role in cellular necrosis and apoptosis. Supplementation with zinc has been shown in the present study to attenuate CPF-evoked leukopenia and increased neutrophil:lymphocyte ratio. This demonstrated that zinc supplementation improved the CPF-induced lymphopenia and stress. Zinc plays an essential role in the T-cell function. Zinc ions have been found to induce blast transformation in human lymphocytes (Rühl et al., 1971; Berger and Skinner, 1974). Zinc is therefore unique as the simplest mitogen known (Wellinghausen and Rink, 1998). Similarly, zinc is an essential cofactor for thymulin, an important thymic hormone secreted by thymic epithelial cells (Hadden, 1992). Apart from inducing markers of differentiation in immature T cells in the thymus, thymulin also has effects in the periphery where it influences mature T cells (Wellinghausen and Rink, 1998). Furthermore, the protective effect of zinc on WBC may be attributed to their antioxidant properties either as a vital component of enzymatic antioxidant Cu-Zn SOD or due to their ability to antagonize the catalytic properties of the redox-active transition metals iron and copper with respect to the promotion of hydroxyl radicals formation from hydrogen peroxide and superoxide (Powell, 2000). Zn also causes inhibition of both endogenous as well as induced lipid peroxidation thereby resulting in stabilization of biomembranes (Dhawan et al., 1992; Srivastava et al., 1993).

In conclusion, the present study has shown that zinc supplementation attenuated CPF-induced alteration in hematological parameters partly due to its antioxidant activity. Therefore, individuals who are constantly exposed to low-dose of CPF and perhaps other OP insecticides may benefit from toxicity protection offered by zinc supplementation.
REFERENCES


