EVALUATION OF SUBCHRONIC CHLORPYRIFOS POISONING ON HEMATOLOGICAL AND SERUM BIOCHEMICAL CHANGES IN MICE AND PROTECTIVE EFFECT OF VITAMIN C

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ABSTRACT — Chlorpyrifos (CPF) is one of the most widely used organophosphorous insecticides in agriculture with its attendant adverse health outcomes. This study aimed at evaluating the effect of sub-chronic oral CPF administration on hematological and serum biochemical indices, and the possible ameliorating effect of vitamin C on the indices in mice. Thirty mice divided into 3 groups of 10 mice each were used for this study. Mice in group I (control) were dosed with vegetable oil, while those in group II were given CPF (21.3 mg/kg ~ 1/5th LD₅₀) only. Mice in group III were pretreated with vitamin C (100 mg/kg) prior to dosing with CPF 30 min later (Vitamin C + CPF-treated group). This regime was given to each group of mice three times a week for a period of ten weeks. During the study period, mice were examined for signs of toxicity, and weight of each mouse was measured every week. At the end of the study period, blood samples were collected from the mice and analyzed for packed cell volume (PCV), total red blood cell (RBC), white blood cell (WBC) and total protein (TP). Serum obtained from the blood was analyzed for Na⁺, K⁺ and Cl⁻, urea, creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP). The results showed that mice in the vitamin C + CPF-treated group exhibited milder signs of toxicity and significant increase in weight gain (p<0.01) compared to the CPF-treated group. No significant increase in weight in the CPF-treated group was observed compared to the control. There was a significant increase in PCV, RBC, Hb, TP and creatinine, but a significant decrease was obtained in WBC, ALT and AST in the CPF-treated group compared to the control. All the parameters with the exception of WBC, ALT and AST (which increased significantly), were significantly decreased in the vitamin C + CPF-treated group compared to CPF-treated group. ALP was significantly elevated in the CPF-treated group compared to both the control and vitamin C + CPF-treated group. No significant changes in urea and the measured electrolytes in all three groups, except a significant decrease in the concentration of Na⁺ was observed in the CPF-treated group compared to the control. The study demonstrated that pretreatment of CPF-administered mice with vitamin C significantly altered some important hematological and serum biochemical parameters, revealing the protective action of the vitamin against some organ damage induced by CPF.

KEY WORDS: Chlorpyrifos, Vitamin C, Ameliorative effect, Hematology, Biochemical profiles, Mice

INTRODUCTION

Organophosphate (OP) compounds which have gained popularity worldwide over the persistent organochlorines are the most widely used insecticides worldwide. Despite the fact that human exposure to OP insecticides constitute a major public health hazard (Fenske et al., 2002), these compounds still account for up to 50% of all insecticides used worldwide (Casida and Quistad, 2004). Exposure to organophosphate compounds occurs predominantly on the farms (Schenker et al., 1992; Sullivan and Blose, 1992)

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where the insecticides are used for pest control. For example, in the USA in the 1990s some 2.5 million to 5 million agricultural workers were exposed to OP insecticides (Das et al., 2001). Perhaps the more terrifying is the use of OP in chemical warfare in terrorism, with homicidal intent targeted to affect a large population (Goozner et al., 2002). Generally, acute cholinergic symptoms observed following exposure to large doses of OP is associated with inhibition of cholinesterase. Low, moderate or high-dose chronic exposure to organophosphates results in pesticide-related illnesses in man (Jaga and Dharmani, 2003). Although cholinesterase inhibition is the main mechanism implicated in organophosphate toxicity, recent evidence has shown that other mechanisms may be implicated (Slotkin et al., 2006). One of the mechanisms implicated in both acute and chronic OP poisoning is oxidative stress. Several studies have demonstrated the role of oxidative stress in OP-induced poisoning in rats (Datta et al., 1992; Poovala et al., 1999; Gultekin et al., 2000; Gupta, 2001; Verma, 2001) and humans (Malkovics, 1995; Banerjee et al., 1999; Altuntas et al., 2002; Ranjbar et al., 2002; Abdollahi et al., 2004; Vidyasagar et al., 2004). Oxidative stress has been implicated in OP-induced seizures (Gupta, 2001) and also contributes to the development and severity of intermediate syndrome in acute OP poisoning (Dandapam et al., 2003). It also plays some significant role in developmental neurotoxicity of OP because the developing brain has a very low reserve of antioxidants (Bagchi et al., 1995, 1996; Crumpton et al., 2000).

Organophosphate insecticides have been shown to stimulate both enzymatic and non-enzymatic antioxidants (Gultekin et al., 2000, 2001; Jarvik et al., 2001; Altuntas et al., 2002). In addition, many antioxidants elicit protective effects on OP-induced acute poisoning in animal models (Gultekin et al., 2001; Altuntas et al., 2002). Antioxidant vitamins C and E have been shown to reduce lipid peroxidation caused by toxic substances (Appenroth et al., 1997; Gultekin et al., 2000). Although OP insecticides are genotoxic, Hoda and Sinha (1991) demonstrated that their cytogenic effect is greatly reduced by vitamin C administration.

Chlorpyrifos (CPF) (O,O-diethyl-O-(3,5,6-trichloro-2-pyridinyl) phosphorothionate) is an organophosphate pesticide widely used in both agricultural and pest control (Steenland et al., 2000). It was first marketed in the USA in 1965, and since then its use has increased rapidly, in part due to the banning of chlordane for termite application in 1988 (Steenland et al., 2000). It is one of the most widely used OP insecticides in the USA, with an annual usage of 8-10 million pounds in the agricultural sector in 1999 (Donaldson et al., 2002). Approximately 800 registered pesticide products on the market contain CPF (Smegal, 2002). CPF is also the most widely studied organophosphate compound (Slotkin et al., 2006). Despite the fact that some of its residential uses in the USA were prohibited in 2000 (US EPA, 2000), CPF is still being used in many homes. Although many studies have evaluated the role of oxidative stress and ameliorative effect of antioxidant vitamins in acute organophosphate-induced oxidative damage, there is paucity of information on the ameliorative effect of antioxidant vitamins on hematological and serum biochemical parameters induced by prolonged sublethal administration of CPF. Therefore, the aim of this study was to evaluate the protective effect of antioxidant vitamin C on clinical, hematological and some serum biochemical profiles in mice dosed subchronically with a sublethal dose of CPF.

### MATERIALS AND METHODS

**Chemicals, Animals and Treatment**

20% CPF emulsifiable concentrate (Termicot™ Sabero Organics, Gujarat Ltd., India) was reconstituted to 1% solution in vegetable oil. 100 mg/tablet of ascorbic acid (MedVit C® Dolmed Laboratory Nigeria Ltd.) was dissolved in distilled water (20 mg/ml).

Thirty healthy Swiss albino mice of both sexes weighing between 16-25 g were housed in the laboratory animal room of the Department of Veterinary Physiology and Pharmacology, Ahmadu Bello University, Zaria under standard conditions. They were fed on standard mice pellets and water was provided ad-libitum. The mice were divided at random into three groups of 10 mice each. Mice in group I were dosed with vegetable oil (control group), while those in group II were dosed with CPF per os only at a dose of 21.3 mg/kg (1/5th LD₅₀) (CPF-treated group). Mice in group III were pretreated with vitamin C orally at a dose of 100 mg/kg 30 min before administration of chlorpyrifos (21.3 mg/kg). This treatment protocol was repeated three times a week (Mondays, Wednesdays and Fridays) for a period of 10 weeks. The experiment was performed as stipulated by the guidelines on animal research of the Animal Research Ethic Committee of the Ahmadu Bello University, Zaria and in accordance with the Helsinki declaration. During the experimental period, the animals were examined for any observable signs and abnormalities, and even death. The animals...
were weighed weekly during the test period at 09.00h before feeding. At the end of the period, mice in each group were sacrificed by severing the jugular vein and blood was collected for hematological analysis. Hematological parameters evaluated included packed cell volume (PCV), hemoglobin (Hb) concentration, total erythrocyte (RBC), absolute and differential leukocyte (WBC) counts and total protein (TP). The PCV value was carried out using the method of Rodak (1995), while Hb concentration was measured using the method of Van Kampen and Zilstra (1961). RBC and WBC were evaluated according to the method of Rodak (1995). TP was determined using the method of Reitman and Frankel (1957), while ALP was determined according to the method of King and Armstrong (1934). Serum creatinine was measured using the method of Miller and Miller (1951), while urea was determined using the modified method of Natelson (1965) using diacetyl-monoxime-thiosemicarbazide procedure. In addition, the serums Na$^+$, K$^+$ and Cl$^-$ were measured by flame photometry, while Cl$^-$ was analysed using the method of Schales and Schales (1941).

Statistical analysis

Values obtained were expressed as Mean ± SEM. The mean values of the data obtained from CPF-treated group were compared to those of the control and vitamin C + CPF-treated groups, respectively, using the Student t-test. In addition, the mean value of the weight of the mice in each group at the commencement of the study (week 1) was compared with the mean weight at the termination of the study (week 10) using the Student t test. Values of p<0.01 were considered significant.

RESULTS

Mice in the control group treated with only vegetable oil did not show any apparent sign of toxicity or death. However CPF-treated mice showed varying degrees of clinical signs some few minutes after dosing. The signs included huddling, depression, conjunctivitis, mild tremor, piloerection, diarrhea and dyspnea, and death occurred in two of the mice at 7th and 9th weeks of dosing, respectively. The signs observed were related to the cholinergic crisis, a consistent sign in acute organophosphate poisoning. Except for the huddling, no other significant clinical manifestations were observed in the vitamin C + CPF-treated mice except death observed in two of the mice at about 5th and 9th weeks of dosing, respectively.

Mice in the control group showed a consistent progressive increase in weight gain over the ten-week period. There was a significant increase (p<0.01) in weight gains at the termination of the study compared to those obtained at the commencement. Mice from the CPF-treated group showed a less progressive increase in weight gain over the ten-week period, and there was no significant increase in weight gain at the termination compared to those at the commencement of the study. However, mice in the vitamin C + CPF-treated group showed a progressive increase in weight gain over the study period, and there was a significant increase in weight gain at termination compared to the commencement of the study (Figs 1 and 2).

The study has also revealed a significant increase in the value of PCV, RBC and Hb in the CPF-treated group compared to the control and vitamin C + CPF-treated groups, respectively. There was also a significant increase in the value of TP in the CPF-treated group compared to the control and vitamin C + CPF-treated groups respectively. There was a significant reduction in WBC value (4.65 ± 0.15) in the CPF-treated group compared to value obtained in the control group (7.4 ± 0.15). The leukopenia observed in the CPF-treated group was due to neutropenia. However, the value of WBC was significantly elevated in the vitamin C + CPF-treated group (8.05 ± 0.01) compared to the CPF-treated group. The effect on hematological parameters is shown in Fig. 3.

There was no significant change in the concentration of K$^+$ and Cl$^-$ between the CPF-treated group and the control, and vitamin C + CPF-treated group, respectively. Significant reduction in the concentration of Na$^+$ was observed in the CPF-treated group compared to the control. However, there was no significant change in the concentration of Na$^+$ between the vitamin C + CPF-treated and CPF-treated groups (Fig. 4).

The concentration of urea was not significantly different in the three groups, even though it is higher in the vitamin C + CPF-treated group than either the control or CPF-treated groups. The concentration of creatinine was significantly elevated in the CPF-treated group compared to the control. However, there was a significant decrease in the concentration of creatinine in the vitamin C + CPF-treated group compared to the CPF-treated group (Fig. 5). There was also a signifi-
cant reduction in the activity level of AST and ALT in the CPF-treated group compared to the control. However, the activity level of AST and ALT in the vitamin C + CPF-treated group was significantly higher than in the CPF-treated group. Conversely, there was a significant increase in the activity level of ALP in the CPF-treated group than the control and the vitamin C + CPF-treated groups (Fig. 6).

DISCUSSION

The clinical signs observed in the CPF-treated groups were consistent with cholinergic symptoms associated with cholinesterase inhibition. The signs observed in this group were more severe than in those pretreated with vitamin C. The reduction in the severity of signs of toxicity in mice pretreated with vitamin C might reveal that oxidative stress played some role in the toxicity induced by CPF.

The observation in the present study that prolonged exposure to CPF did not cause significant change was in consonant with those obtained by earlier studies (Mollelo et al., 1980; Corley et al., 1989). However, a dose-dependent effect of CPF on weight gain was demonstrated in a 13-week study in rats by Barna-Lloyd et al. (1990). Yoshida et al. (1985) in a 28-day feeding study demonstrated a dose-dependent decrease in weight gain in mice, while Barna-Lloyd et al. (1991) showed a small but significant decrease in weight gain in a two-year oral feeding study in rats. In the present study, CPF was shown to cause an insignificant increase in weight gain. However, pretreatment with vitamin C caused a significant and progressive elevation in weight gain, stressing the importance of oxidative damage in the mechanism of the poisoning. Besides, it has been demonstrated that chlorpyrifos-oxon inhibits the enzyme cholesterol ester hydrolase, with consequent elimination of normal reaction to stress (Civen et al., 1977). Therefore, weight loss observed in the CPF-treated group may be a result of the combination of oxidative stress, cholinergic stress and adrenal-mediated stress caused by the inhibition of cholesterol ester hydrolase.

The study also revealed significant elevation in the value of RBC, PCV and Hb and TP in the CPF-treated group. This apparently was connected with diarrhea, and hence the hemoconcentration observed in this group of mice, which was not observed in the vitamin C + CPF-treated group. In addition, leukopenia due to neutropenia was observed in CPF-treated mice.

Fig. 1. Effect of chlorpyrifos and/or vitamin C on pattern of weight gain.
Vitamin C protects against hematological and biochemical changes in chlorpyrifos poisoning.

Fig. 2. Comparism of the effect of chlorpyrifos and/or vitamin C on weight gain at the commencement and termination of the study.

\( ^{a} p<0.01 \) (weight at commencement compared to termination)

\( ^{b} p>0.01 \) (weight at commencement compared to termination)

\( ^{c} p<0.01 \) (weight at commencement compared to termination)

Fig. 3. Effect of chlorpyrifos and/or vitamin C on hematological parameters.

\( ^{a} p<0.01 \) (compared to CPF-treated group)

\( ^{b} p<0.01 \) (compared to CPF-treated group)
Fig. 4. Effect of chlorpyrifos and/or vitamin C on serum electrolytes.

\(^a\) p>0.01 (compared to CPF-treated group)

\(^b\) p>0.01 (compared to CPF-treated group)

Fig. 5. Effect of chlorpyrifos and/or vitamin C on serum urea and creatinine.

\(^a\) p>0.01 (compared to CPF-treated group)

\(^b\) p>0.01 (compared to CPF-treated group)

\(^c\) p<0.01 (compared to CPF-treated group)

\(^d\) p<0.01 (compared to CPF-treated group)
Activated neutrophils may be said to play a very essential role in free-radical-mediated injury through the extracellular release of the superoxide free radical (McCord et al., 1994), which is cytotoxic to the host cell including neutrophil itself, thereby resulting in its decrease. In addition, immune system abnormalities associated with depression of T lymphocytes and increased expression of CD26 associated with autoimmunity have also been described in people chronically exposed to CPF (Thrasher et al., 1993). Many pesticides have been shown to be toxic to the immune system either through the induction of cytotoxicity via apoptosis and necrosis (Corcoran et al., 1994; Rabideau, 2001). Excessive free radical generation has been implicated as an initiator of apoptosis (McConkey et al., 1991; Corcoran et al., 1994). Reactive oxygen species and free radicals have been associated with a number of events associated with immune cell regulation or apoptosis (Rabideau, 2001). Leukopenia was not observed in the vitamin C + CPF-treated group, which on the contrary even showed a significant elevation in the WBC level over those in the CPF-treated group. It was possible that vitamin C inhibited damage done to the leukocytes by the free radicals induced by CPF. Indeed, antioxidants have been shown to inhibit apoptosis (Stoain et al., 1996; Knight, 2000).

The study has also demonstrated that prolonged exposure to CPF caused significant alteration in the level of Na⁺ but not Cl⁻ and K⁺. Prolonged CPF administration was shown by this study to cause hyponatremia probably due to diarrhea observed in the CPF-treated group. However, pretreatment with vitamin C did not alter this effect on serum electrolyte level. In addition, prolonged exposure to CPF, and even pretreatment with vitamin C, did not significantly alter the urea level. However, the significant elevation in the level of serum creatinine observed in the CPF-treated group may be attributed to pathological changes in the kidneys probably associated with renal failure. Pretreatment with antioxidant vitamin C brought the level of creatinine to almost a normal level as observed in the control group. The ameliorative effect of vitamin C on creatinine level may be due to its protective role against CPF-induced renal damage, suggesting that oxidative process played a very significant role in this damage.

![Image of Figure 6](image)

**Fig. 6.** Effect of chlorpyrifos and/or vitamin C on some serum enzymes.

- a p< 0.01 (compared to CPF-treated group)
- b p< 0.01 (compared to CPF-treated group)
Prolonged exposure of mice to CPF was also shown by this study to cause a very significant decrease in the activity of ALT and AST. These findings agreed with those of Bernard Lloyd et al. (1990) in a 13-week oral toxicity study of CPF in rats. The reason for the low level of ALT and AST is not known and besides, the toxicological significance of this remains obscure. However, pretreatment with vitamin C was shown by this study to significantly improve the activity of both ALT and AST. The significant elevation in the activity of ALP indicated damage to any or all of the organs producing these enzymes such as the liver, kidneys, bones, muscle and intestinal mucosa. The activity of ALP was significantly lower in the group pretreated with vitamin C, indicating its ameliorating effect. Therefore, the result showed that pretreatment with vitamin C has an ameliorating effect on these clinical chemistry parameters, suggesting it has a protective effect on the damage induced by CPF on organs producing these enzymes, especially the liver.

Previous studies have shown that chlorpyrifos-ethyl causes an increase in lipid peroxidation and a decrease in the activity of glutathione peroxidase, superoxide dismutase and catalase (Gultekin et al., 2000, 2001), but these effects were reversed by pretreatment with vitamins C and E (Gultekin et al., 2001). Therefore, the result of the present study strongly agreed with the suggestion that oxidative stress may play a critical role in the organ and tissue damage caused by prolonged CPF exposure, as shown by the ameliorating effect of vitamin C.

In conclusion, this study has demonstrated that the antioxidant vitamin C has an ameliorative effect on clinical, hematological and serum biochemical parameters altered by prolonged administration of CPF. This implied that oxidative stress plays a significant role in the damage induced by this organophosphorous compound. In addition to its antioxidant activity, vitamin C is known to perform other actions that enhance its protective effect in OP-induced toxicity. For example, vitamin C has been shown to increase the activity of paraoxonase (Jarvik et al., 2002), an enzyme known to aid in the detoxification of organophosphate compounds. Vitamin C can also regenerate other small molecule antioxidants such as α-tocopherol, glutathione, urate and β-carotene from their respective radical species (Frei et al., 1990; Halliwell, 1996). This helps to enhance its antioxidant ability. Therefore, it is suggested that farmers, pesticide applicators, workers in the pesticide industry and other pesticide users who come in regular contact with CPF may benefit from pretreatment with vitamin C. It is recommended that this same protocol be repeated in order to elucidate the direct effect of CPF on tissues and organs since some of the serum enzymes are not tissue-specific, but only suggestive of damage to certain tissues and organs.

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