Chlorpyrifos-Induced Clinical, Hematological and Biochemical Changes in Swiss Albino Mice- Mitigating effect by co-administration of vitamins C and E

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Short title: Vitamins C and E mitigate chlorpyrifos-induced pathological changes

Abstract

Background. Induction of Oxidative stress is one of the molecular mechanisms in chlorpyrifos toxicity. *Objective*. To evaluate the effect of prolonged CPF exposure on clinical, hematological and biochemical parameters in mice and the possible ameliorative effect of coadministration of vitamins C and E. *Methods*. 40 mice divided into 4 groups of 10 animals in each group served as subjects for this study. Groups I and II were administered corn oil (2 ml/kg) and combination of vitamins C (100 mg/kg) and E (75 mg/kg), respectively. Group III were exposed to CPF only (21.6 mg/kg ~ 1/5th of the previously determined LD_{50} of 108 mg/kg), while group IV were pretreated with combination of vitamins C (100 mg/kg) and E (75 mg/kg) and then administered CPF (21.6 mg/kg) 30 min later. The regimens were administered orally once daily for a period of 10 weeks. The mice were examined for signs of toxicity and weekly body weight changes. Blood and serum samples obtained from sacrificed animals at the end of the study were evaluated for some hematological and biochemical parameters, respectively. *Results*.Vitamins pretreatment ameliorated cholinergic toxic signs and changes in body weight, PCV, Hb, RBC and WBC count induced by CPF. CPF-evoked alteration in Na^+, K^+, Cl^-, TP, urea, creatinine, ALP and MDA levels were ameliorated by pretreatment with the vitamins. ALT and AST activities lowered by CPF was further reduced by vitamins pretreatment. *Conclusion*. Vitamins C and E protected mice from subchronic CPF-induced alteration in clinical, hematological and serum biochemical parameters. [Life Science Journal 2010;7(3):37-44]. (ISSN: 1097-8135).

Keywords: Chlorpyrifos; hematology; serum biochemistry, lipid peroxidation; vitamins C and E.

Abbreviations
CPF= Chlorpyrifos
OP= organophosphate
MDA= Malonaldehyde
PCV= Packed cell volume
Hb- Hemoglobin
RBC= Red blood cells
WBC= White blood cells
Na^+= Sodium
K^+= Pottasium
Cl^-= Chloride
TP= Total proteins
AST= Aspartate aminotransferase
ALT= Alanime aminotransferase
ALP= Alkaline phosphatase

Introduction

Chlorpyrifos (CPF) (*O,O*-diethyl 0-[3,5,6-trichloro-2-pyridinol phosphorothionate) is a broad-spectrum OP insecticide that is widely used in agriculture and domestic pest control [1]. Toxicity associated with this insecticide led to the restriction of some of its domestic uses by United State Environmental Protection Agency in 2000. Despite its restriction, CPF still remains one of the most widely used insecticides. According to Steenland et al. [2], CPF is applied about 20 million times per year in US to houses and lawns, and 82% of adults have detectable levels of the 3,5,6-trichloropyridinol, the metabolite of CPF in their urine. Like the other OPs, CPF toxicity has been largely associated with irreversible inhibition of acetylcholinesterase (AChE) resulting in accumulation of acetylcholine in the cholinergic receptors[3]. However, other putative mechanisms have been implicated in molecular mechanisms of CPF toxicity. Among these, the induction of oxidative stress has received tremendous attention[4-8].

The mammalian cells reduced the adverse effect of lipid peroxidation via the utilization of both enzymatic and non-enzymatic antioxidants, which scavenge for free radicals in the system. Oxidative stress results when the endogenous antioxidants have been overwhelmed by the rate and extent of free radical generation. Therefore, during oxidative stress, an increase in the exogenous supply of antioxidants improves the capacity of the tissue to cope with high antioxidant demands. Several studies have suggested high effectiveness following administration of two antioxidants in combating oxidative stress in the body[9-10]. It has been shown that the combination of vitamins C and E reduced lipid peroxidation induced by CPF[4-6]. We have earlier demonstrated the ameliorative effect of vitamin C on some of clinical, hematological and biochemical changes induced by repeated CPF administration in mice[7]. Therefore, the aim of this study was to evaluate the effect of CPF on clinical, hematological and serum biochemical changes in mice, and the possible ameliorative effect of the combination of vitamins C and E.

Materials and Methods

Chemicals

Commercial grade CPF (Termicot®, Sabero organics, Gujarat Limited, India), Vitamin C tablet (Medvit C®, Dol-Med Laboratory, Nigeria) and vitamin E (α-tocopherol, Paterson Zochonis, Nigeria) were used for this study. Both

the CPF and vitamin E were reconstituted appropriately in corn oil immediately prior to use.

Animals and Treatments

Forty Swiss albino mice of both sexes weighing between 17 and 21g served as subjects for this study. The mice were fed on standard mice pellets and water was provided *ad libitum.* They were randomly divided into four groups. Group I (control) received corn oil only (2 ml/kg) while group II (VC+VE group) were co-administered vitamins C (100 mg/kg) and E (75 mg/kg). Group III (CPF group) received CPF only (21.6 mg/kg~ equivalent of 1/5th LD_{50} of 108 mg/kg determined in the preliminary study). Group IV (VC+VE+CPF group) were pretreated with coadministred vitamins C (100 mg/kg) and E (75 mg/kg) followed by exposure to CPF (21.3 mg/kg), 30 minutes later[7,11]. These regimens were administered *per os* three times every week days (Mondays, Wednesdays and Fridays) for a period of ten weeks. During the test period, the animals were observed for any abnormal clinical signs and death, and body weight changes evaluated on weekly basis. The experiment was performed according to the guidelines on animal research of the Animal Research Ethic Committee of the Ahmadu Bello University, Zaria.

Evaluation of hematological parameters

At the end of the test period, the mice were sacrificed by decapitation after light ether anesthesia, and blood samples (2 ml) collected into heparinised sample bottles were examined for packed cell volume (PCV), hemoglobin (Hb) concentration, total red blood cells (RBC) and absolute and differential white blood cell (WBC) counts using the method described by Dacie and Lewis[12].

Evaluation of serum biochemical parameters

Another set of blood samples collected into test tubes were allowed to clot and then centrifuged at 1000 x g for 10 minutes to obtain the serum. The serum was evaluated for the levels of TP, electrolytes (Na^+, K^+ and Cl^-), urea, creatinine, AST, ALT and ALP. AST and ALT were determined using the method of Reitman and Frankel[13], while ALP was evaluated according to the method of King and Armstrong[14]. Serum creatinine was measured as described by Miller and Miller[15], urea was determined using the modified method of Natelson [16], using diacetyl-monoxime-thiosemicarbazide procedure. In addition, the serum Na^+ and K^+ were measured by flame photometry, while Cl^- was analysed using the method of Schales and Schales [17].

Evaluation of serum malonaldehyde concentration

Serum malonaldehyde (MDA) concentration as an index of lipoperoxidative changes was evaluated using the method of Draper and Hadley[18] as modified[19]. For this purpose, 1.25 ml of 100 g/L trichloroacetic acid solution was added to 0.25 ml serum in each centrifuge tube and placed in a boiling water bath for 15 min. After cooling in tap water, the mixture was centrifuged at 1000 x g for 10 min, and 1 ml of the supernatant was added to 0.5 ml of 6.7 g/L TBA solution 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, 645, Japan) at 532 nm. The concentration of MDA was calculated by the absorbance coefficient of MDA-TBA complex 1.56×10^5 /cm, and expressed in μmol/ml.

Statistical analysis

Values obtained were expressed as Mean ± SEM and then subjected to one way analysis of variance followed by Tukey's multiple comparison test. The mean body weight of the mice in each group at the commencement of the study (week I) was compared with that obtained at the termination of the study (week X) using the Student's *t*-test. The statistical analysis was done using graphpad prism version 4.0 (www.graphpad.com). Values of $P<0.05$ were considered significant.

Results

Effects of treatments on clinical signs

The control and VC+VE groups did not show any apparent sign of toxicity. Toxic signs observed in the CPF group included huddling, depression, conjunctivitis, mild tremor, piloerection, soft fecal bolus (mild diarrhea) and dyspnea. Death occurred in two of the mice at 7th and 9th weeks of dosing, respectively. VC+VE+CPF group showed milder toxic signs compared to those in the CPF group, and these included huddling, depression, rough hair-coat and tremor.

Effect of treatments on body weight changes

The effect of the treatments on body weight changes is shown in Figures 1 and 2. A consistently progressive increase in body weight was recorded in mice in the control, VC+VE and VC+VE+CPF groups. A significant increase ($P<0.01$) in body weight gains was recorded at termination compared to at commencement of the study in the control, VC+VE with percentage weight increase of 32% and 42%, respectively. The CPF group showed a less progressive increase in their dynamics of body weight gain over the ten week period, and there was no significant change in their body weight at termination (21.4 ± 2.2g) compared to the value obtained at the commencement (20.8 ± 3.5g) of the study, with a percentage weight increase of 3%. On the other hand, VC+VE+CPF group demonstrated a progressive elevation of body weight gain over the study period, and there was a significant increase ($P < 0.01$) in body weight at termination (24.5 ± 2.9g) compared to that obtained at the commencement (19.5 ± 3.5g) of the study with a percentage body weight increase of 20%.

Effect of treatments on hematological parameters

The effect of the various treatments on PCV, Hb and RBC concentrations is shown in Figures 3, 4 and 5, respectively. A significant increase in PCV ($P < 0.05$), Hb concentration ($P < 0.01$) and RBC counts ($P < 0.05$) was recorded in the CPF group compared to the control. The PCV, Hb and RBC concentrations in VC+VE+CPF group were not significantly different ($P> 0.05$) from those obtained in the control and VC+VE groups. There was a significant decrease in PCV ($P < 0.01$), Hb ($P < 0.05$) and RBC ($P < 0.01$) in the VC+VE+CPF group compared to the CPF group. The WBC in the CPF group was significantly lower ($P < 0.01$) than those obtained in the control, VC+VE and VC+VE+CPF groups, respectively. Differential leukocyte count showed that neutropenia was the cause of leukopenia observed in the CPF group. On the other hand, the WBC concentration in the VC+VE+CPF group was not significantly different ($P> 0.05$) from those obtained the control and VC+VE groups, respectively. Similarly, there was a significant elevation ($P < 0.01$) in WBC in the

VC+VE+CPF group compared to the CPF group (Figure 6).

Effect of treatments on serum biochemical parameters

A significant increase ($P < 0.01$) in the concentration of Na^+ was obtained in the control compared to the VC+VE and VC+VE+CPF groups, respectively. The Na^+ concentration in the CPF group was significantly higher compared to VC+VE and VC+VE+CPF groups, respectively. K^+ concentration in the control group was not significantly different from those obtained in the CPF and VC+VE+CPF groups. However, the K^+ concentration in the CPF group was significantly higher ($P < 0.01$) compared to VC+VE+CPF groups. There was no significant change in the Cl^- concentration in the CPF group compared to the control and VC+VE groups, respectively. However, a significant increase ($P < 0.05$) in the Cl^- concentration was obtained in the CPF group compared to the VC+VE+CPF group (Figure 7).

The TP concentration was significantly higher ($P < 0.05$) in the CPF group compared to the control and VC+VE groups, respectively. No significant change ($P > 0.05$) in the TP concentrations was obtained in the VC+VE+CPF group compared to the control (Figure 8).

The urea concentration in the VC+VE+CPF group was not significantly different ($P > 0.05$) from those obtained in the control and VC+VE groups. However, there was a significant increase ($P < 0.01$) in the urea concentration in the CPF group compared to the VC+VE+CPF group. The creatinine level in the CPF group was significantly increased ($P < 0.01$) compared to the control and VC +VE groups. Similarly, a significant rise ($P < 0.01$) in creatinine concentration was obtained in the VC+VE+CPF group compared to the control and VC+VE groups, respectively (Figure 9).

The effect of treatments on serum enzyme's activity is shown in Figure 10. There was a significant reduction ($P < 0.01$) in the activity of ALT in the CPF group compared to the control. The ALT activity was also significantly lowered ($P < 0.01$) in the VC+VE+CPF group compared to the control and CPF groups, respectively. The level of AST in the control group was significantly higher ($P < 0.01$) compared to the CPF and VC+VE+CPF groups, respectively. The activity of ALP in the CPF group was significantly elevated compared to the control ($P < 0.05$), VC+VE ($P < 0.01$) and VC+VE+CPF ($P < 0.01$) groups, respectively.

Effect of treatments on serum malonaldehyde concentration

The effect of treatments on serum thiobarbituric reactive acid substance, MDA is shown in Figure 11.The serum MDA concentration was significantly increased ($P < 0.01$) in the CPF group compared to the control, VC+VE and VC+VE+CPF groups, respectively. No significant change ($P > 0.05$) in the MDA concentration was recorded in the control group compared to the VC+VE and VC+VE+CPF groups, respectively.

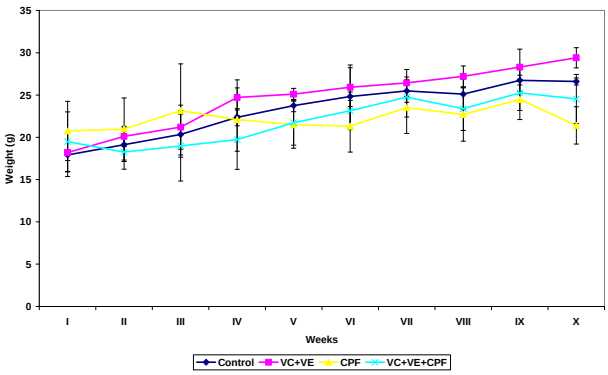

Figure 1: Effects of chlorpyrifos and the combination of vitamins C and E on dynamics of body weight throughout the period of study

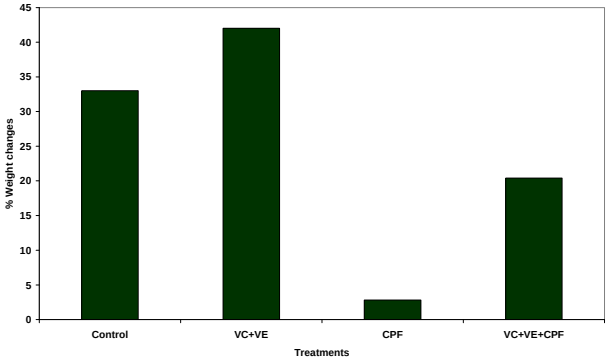

Figure 2: Percentage weight changes of mice administered chlorpyrifos (CPF) and vitamins C (VC) and E (VE)

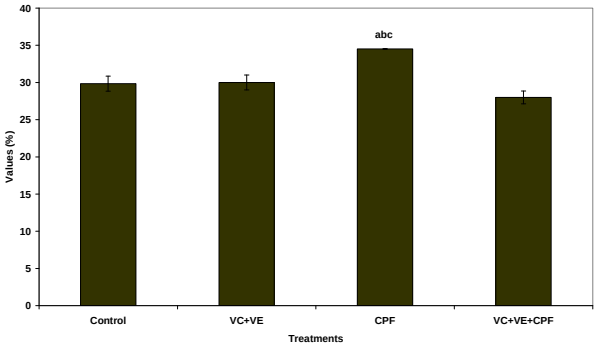

Figure 3: Effects of chlorpyrifos (CPF) and coadministration of vitamins C (VC) and E (VE) on packed cell volume in mice. [a] $p < 0.05$ versus control; [b] $p < 0.05$ versus vitamin C+vitamin E group; [c] $p < 0.05$ versus vitamin C+vitamin E+CPF group

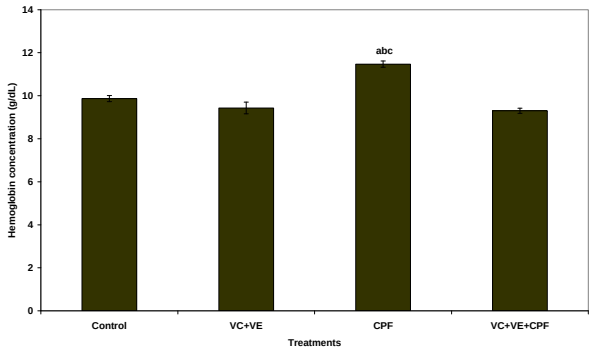

Figure 4: Effects of chlorpyrifos (CPF) and coadministration of vitamins C (VC) and E (VE) on hemoglobin concentration in mice. [a] $p < 0.05$ versus control; [b] $p < 0.01$ versus vitamin C+vitamin E group; [c] $p < 0.01$ versus vitamin C+vitamin E+chlorpyrifos group.

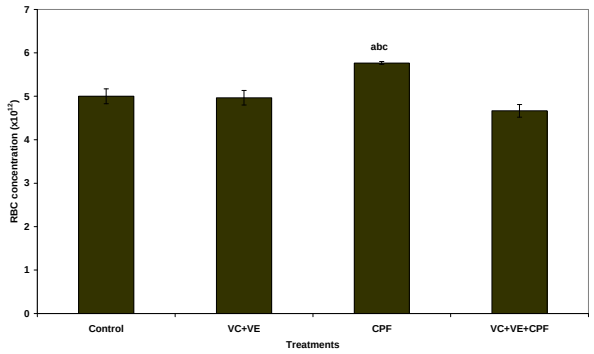

Figure 5: Effects of chlorpyrifos (CPF) and coadministration of vitamins C (VC) and E (VE) on red blood cell count in mice. [a] $p < 0.05$ versus control; [b] $p < 0.05$ versus vitamin C+vitamin E group; [c] $p < 0.05$ versus vitamin C+vitamin E+chlorpyrifos group.

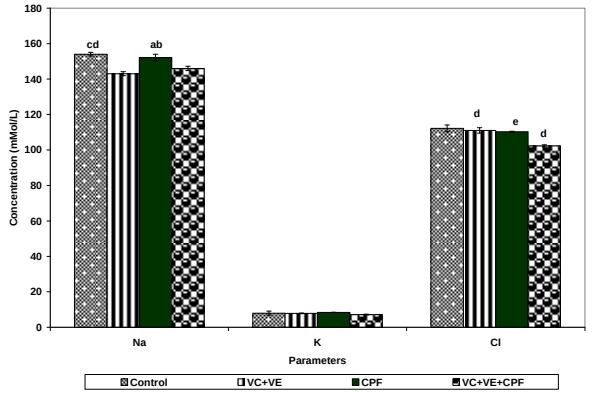

Figure 7: Effects of chlorpyrifos (CPF) and coadministration of vitamins C (VC) and E (VE) on serum electrolytes. [a] $p < 0.05$ versus vitamin C + vitamin E group; [b] $p < 0.01$ versus vitamin C+vitamin E+chlorpyrifos group; [c] $p < 0.01$ versus vitamin C+vitamin E group; [d] $p < 0.01$ versus vitamin C+vitamin E+ chlorpyrifos group; [e] $p < 0.01$ versus vitamin C+vitamin E+chlorpyrifos group; [f] $p < 0.01$ versus vitamin C+vitamin E+ chlorpyrifos group.

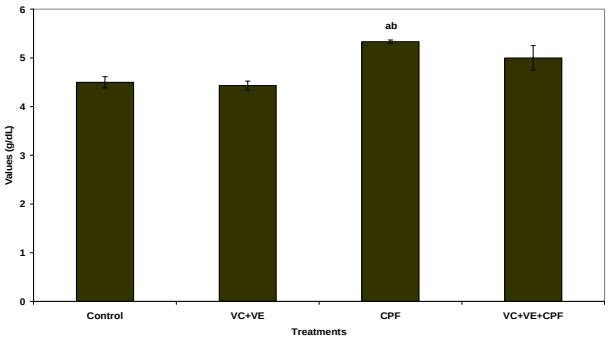

Figure 8: Effects of chlorpyrifos (CPF) and coadministration of vitamins C (VC) and E (VE) on total protein concentration. [a] $p < 0.01$ versus control; [b] $p < 0.01$ versus vitamin C+vitamin E group.

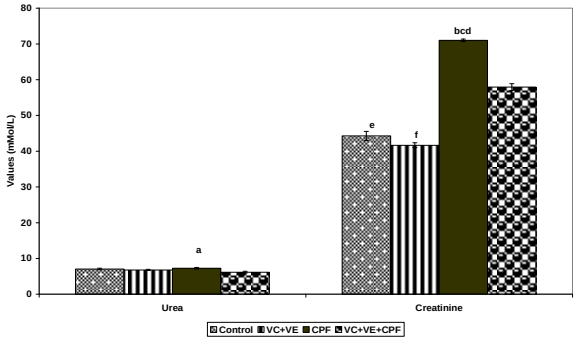

Figure 9: Effects of chlorpyrifos (CPF) and coadministration of vitamins C (VC) and E (VE) on serum urea and creatinine concentration. [a] $p < 0.05$ versus vitamin C+ vitamin E + chlorpyrifos group; [b] $p < 0.01$ versus control; [c] $p < 0.01$ versus vitamin C + vitamin E group; [d] $p < 0.01$ vs vitamin C + vitamin E + chlorpyrifos group; [e] $p < 0.01$ versus vitamin C + vitamin E group; [f] $p < 0.01$ versus vitamin C + vitamin E + chlorpyrifos group.

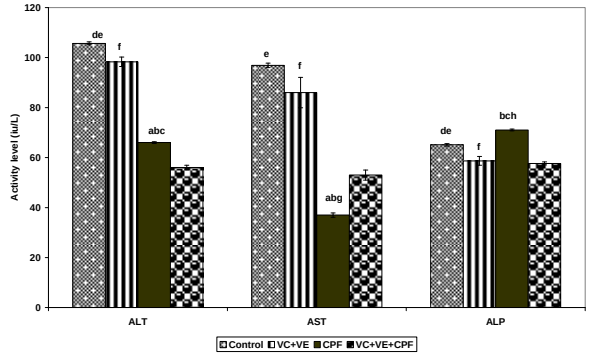

Figure 10: Effects of chlorpyrifos (CPF) and coadministration of vitamins C (VC) and E (VE) on serum enzymes concentration. [a] $p < 0.01$ versus control; [b] $p < 0.01$ versus vitamin C + vitamin E ; [c] $p < 0.01$ versus vitamin C + vitamin E + chlorpyrifos group; [d] $p < 0.01$ versus vitamin C + vitamin E group; [e] $p < 0.01$ versus vitamin C + vitamin E + chlorpyrifos group; [f] $p < 0.01$ versus vitamin C + vitamin E + chlorpyrifos group; [g] $p < 0.05$ versus vitamin C + vitamin E + chlorpyrifos group; [h] $p < 0.05$ versus control.

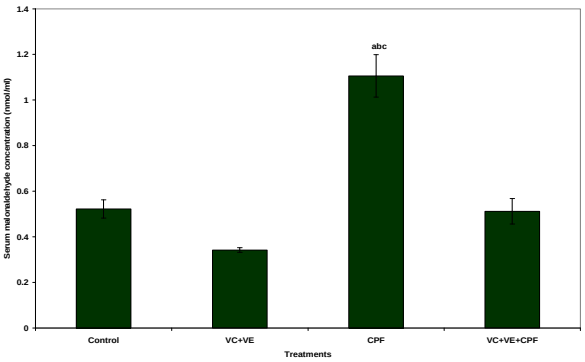

Figure 11: **Effects of chlorpyrifos (CPF) and coadministration of vitamins C (VC) and E (VE) on serum malonaldehyde concentration.** [a] $p < 0.01$ versus control; [b] $p < 0.01$ versus vitamin C + vitamin E ; [c] $p < 0.01$ versus vitamin C + vitamin E + chlorpyrifos group.

Discussion

The study also showed that prolonged CPF administration did not cause significant change in body weight of mice. A mere 3% increase in weight gain over a period of 10 weeks demonstrated the adversity caused by prolonged CPF exposure. This finding was in accord with the results obtained in earlier studies[8, 21-23]. However, this result contravened those recorded in other studies[24,25], which showed a dose-dependent decrease in body weight of mice treated with CPF. In the present study, the low weight gain observed in the CPF group compared to what was observed in the other groups despite normal feed consumption may have resulted from alteration in food utilization, perhaps through interaction with enzymes and hormones essential for normal metabolism. Chronic CPF exposure has been shown to cause vacuolization of *zona fasciculata*[25,26], therefore, altering the elaboration of cortisol, which plays essential role in metabolism. Similarly, CPF exposure has been associated with hypothyroidism[8,27], resulting in alteration of metabolic rate. Furthermore, CPF-oxon, the active metabolite of CPF has been shown to inhibit cholesteryl-ester hydrolase, which is an enzyme essential in the promotion of normal reaction of the body to stress[28]. The combination of these effects may have caused mild body weight gains observed in the CPF group. On the contrary, pretreatment with combination of vitamins C and E resulted in a consistent increase in weight gain throughout the study period, and the weight recorded at termination was significantly higher than what was obtained at the commencement of the study. Although the 20% increase in weight gain obtained in the vitamin pretreated group was not as high compared to 33% recorded in the control, it is much higher than those observed in the CPF group. This showed that oxidative stress is an essential mechanism involved in CPF-induced adversity on body weight.

The clinical signs observed in the CPF groups were consistent with cholinergic symptoms observed in OP poisoning. These symptoms resulted from the AChE inhibition caused by CPF and its active metabolites, CPF-oxon, leading to accumulation of acetylcholine in the cholinergic receptors [3]. The mitigation of clinical signs and mortality in mice pretreated with vitamins C and E demonstrated the protective effect of the vitamins on CPF-induced toxic signs and death. This shows the role of oxidative stress in toxic signs evoked by CPF. Apart from its direct effect on free radical, vitamins C and E have been shown to partially restore the activity of AChE[20], which may have contributed to the mild toxic signs observed in the vitamins pretreated groups.

The significant elevation of PCV, Hb concentration and RBC counts in mice administered CPF only may be due to the mild diarrhea and the resultant hemoconcentration. However, vitamins C + E pretreatment significantly suppressed the adverse hematological effect by CPF. The leukopenia observed in the CPF group showed its immunotoxic potentials. The neutropenia in the CPF group may be related to the essential role played by neutrophil in free-radical mediated injury by inducing extracellular release of superoxide and other free radicals[29]. This also leads to neutrophil destruction resulting in their decrease in the peripheral circulation. Pretreatment with combination of vitamins C and E significantly improved the concentration of leukocytes in the circulation, indicating that oxidative stress plays an essential role in the leukopenia induced by prolonged CPF administration. Vitamin E is an essential intracellular antioxidant in the cytomembranes responsible for the maintenance of cellular integrity[30]. Therefore, the membrane stabilization by vitamin E may have played a significant role in the improvement of the cellular integrity of the neutrophils, preventing the release of the cell damaging free radicals. Similarly, vitamin C may have assisted in this role by scavenging for free radical in the extracellular medium, and regeneration of active vitamin E.

Exposure to prolonged CPF exposure did not significantly alter the Na^+ concentrations compared to the control, despite the mild diarrhea provoked by the insecticide. However, pretreatment with the vitamins lowered the Na^+ concentration significantly compared to the CPF group. Prolonged CPF administration did not significantly alter the serum level of K^+. Similar to what was observed with Na^+, pretreatment with vitamins did significantly reduce the K^+ concentration compared to the CPF group. CPF exposure did not also significantly alter the serum Cl^- concentration compared to the control. On the contrary, pretreatment with the vitamins significantly lowered the Cl^- concentration compared to the CPF group. The reason for decrease in Na^+, K^+ and Cl^- concentrations in the two groups administered vitamins C and E compared to the CPF group is unknown. The increased TP concentration in the CPF group may have been due to hemoconcentration, resulting from the mild diarrhea. Pretreatment with the vitamins did ameliorate the high TP concentration resulting from CPF exposure.

The increased urea concentration in the CPF group showed that the insecticide caused pathological changes in the liver. The reduced urea concentration in group pretreated with vitamins C and E was an indication of their protective effect in CPF-induced lipoperoxidative damage to the liver. Similarly, the high creatinine concentration evoked by prolonged CPF exposure was ameliorated by combination of vitamins C and E. This showed that the antioxidant vitamins protected the kidneys from the lipoperoxidative changes provoked by CPF. Pretreatment with a combination of vitamins C and E did significantly

reduce the creatinine level compared to those observed in the CPF group. This showed that the vitamins protected the kidney from damages provoked by CPF, probably due to their free radical scavenging ability.

The low ALT and AST activity in mice exposed to prolonged CPF agreed with the previous findings[8,25]. Currently, the toxicological significance of low ALT and AST activities is not known. However, pretreatment with the vitamins resulted in a significant improvement in the level of AST but not ALT, which was further lowered. The high ALP activity in mice exposed to prolonged CPF indicated pathological changes in the organs such as the liver, skeletal muscles and bones producing this enzyme. The significant reduction in ALP activity in group pretreated with vitamins C and E demonstrated their protective effect on CPF-induced tissue damage, probably as a result of their antioxidant effect. Studies have shown that CPF causes damage to the liver[6,8, 31,32]. It has been demonstrated that pesticide mixture including CPF induced 8-OH-2-deoxyguanosine in the liver of rat, indicating free radical DNA damage[33]. CPF has been shown to impair antioxidant enzyme activities either directly or through the induction of free radicals [4,34], resulting in oxidative stress. Therefore, the ameliorative effect of vitamins C and E on serum enzymes activities reaffirmed the role of oxidative stress in CPF-induced organ damage and the protective effect of antioxidant vitamins.

The increased serum MDA concentration observed in CPF group indicated that the insecticide evoked lipoperoxidative damage to the tissue through free radical induction. This findings agreed with results obtained in the previous studies[8,33, 35-37].Tissue lipid peroxidation is a degradative phenomenon as a consequence of free radical chain production and propagation which affects mainly polyunsaturated fatty acids[38]. The significantly low MDA concentration in vitamins pretreated group showed their ability to quench CPF-induced tissue lipoperoxidative damage. This may have been responsible for amelioration of the CPF-provoked clinical, hematological and biochemical deficits. Vitamins C and E have been shown to act synergistically as antioxidants[39-40]. Vitamin E acts in the lipid component of the membrane to prevent lipid peroxidation, whereas vitamin C is hydrophilic and an important antioxidant in the biological fluid[41]. Vitamin C also has a sparing effect on vitamin E by facilitating the regeneration of α- tocopherol[42-43]. Furthermore, vitamins C and E have been shown to restore the decreased activities of the antioxidant enzymes, superoxide dismutase and catalase, caused by CPF-ethyl[4] thereby boosting the body's antioxidant reserve. Apart from its antioxidant effect, other non-antioxidant related effect of the vitamins may have been involved in the tissue protective effect observed in the present study. Vitamins C and E have been shown to increase the activity of paraoxonase[44], which is involved in the detoxification of OP compounds. Furthermore, vitamin C is known to serve as cofactors in many essential enzymes involved in metabolism[45,46].

In conclusion, the present study has shown that oxidative stress plays an essential role in CPF-mediated injury and the combination of vitamins C and E ameliorated the injury through its free radical scavenging effect. Therefore, the administration of both vitamins C and E may be of value to farmers and other workers who are frequently exposed to CPF in reducing tissue injury mediated by this OP compound.

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