Co-administration of Vitamins C and E Ameliorates Chronic Chlorpyrifos-induced Erythrocyte Osmotic Fragility in Wistar Rats

Suleiman F. Ambali, Joseph O. Ayo, Samuel A. Ojo, and King A.N. Esievo

1Department of Veterinary Physiology and Pharmacology
2Department of Veterinary Anatomy
3Department of Veterinary Pathology and Microbiology
Ahmadu Bello University, Zaria, Nigeria

Abstract: Studies were conducted to evaluate the effect of chronic low-dose chlorpyrifos (CPF) administration on erythrocyte osmotic fragility, the role of lipoperoxidative changes and the ameliorative effect of co-administration of vitamins C and E on CPF-induced osmotic fragility. To accomplish these objectives, 20 rats divided into 4 groups of 5 animals in each group served as subjects for this study. Rats in group I received soya oil only (2 ml/kg), while those in group II were co-administered vitamin C (100 mg/kg) and vitamin E (75 mg/kg) only. Rats in group III were given only CPF (10.6 mg/kg, ~ 1/8 LD₅₀), while those in group IV were co-administered vitamin C (100 mg/kg) and vitamin E (75 mg/kg) followed by CPF (10.6 mg/kg), 30 minutes later. The regimens were given by gavage once daily for 17 weeks. Blood collected from the animals at the end of the test period were analyzed for erythrocyte osmotic fragility and malonaldehyde (MDA) concentration as an index of lipid peroxidation. The study showed that repeated CPF exposure caused increased erythrocyte fragility and MDA concentration. Pretreatment with vitamins C and E ameliorated the CPF-induced increase in erythrocyte fragility and lipoperoxidative changes in Wistar rats.

Key words: Insecticides, Organophosphate, Chlorpyrifos, Chronic poisoning, Vitamin C, Vitamin E, Erythrocyte fragility, Lipid peroxidation, Oxidative stress, Rats

INTRODUCTION

Human and animal populations throughout the world are exposed on daily basis to low levels of environmental contaminants. Pesticides such as organophosphate (OP) insecticides are one of the most important environmental contaminants as they remain inevitably present as residues in food from both vegetal and animal origins (Bolognesi and Morasso, 2000).

Chlorpyrifos (CPF) is a phosphorothionate chlorinated OP insecticides that has been widely used for a variety of agricultural and public health purposes (Mansour and Mossa, 2009). Like many other OP insecticides, CPF acts by inhibiting acetylcholinesterase (AChE) leading to accumulation of acetylcholine in the synaptic cleft, resulting in muscarinic, nicotinic and central cholinergic effects (Eaton et al., 2008). However, it has been shown that CPF is capable of inducing pathological changes at doses that did not cause AChE inhibition or long after restoration of AChE activity (Pope et al., 1992; Cañasadas et al., 2005). Therefore, other additional mechanisms are involved in CPF toxicity. The induction of oxidative stress is one of the molecular mechanisms that have been implicated in CPF-induced toxicity (Gultekin et al., 2001; Ambali et al., 2007, 2010; Ambali, 2009).

Studies have shown that repeated exposure to CPF results in anemia (Goel et al., 2006; Ambali, 2009). The pathophysiological basis of anemia in CPF poisoning still remains speculative. Goel et al. (2006) attributed the anemia to decreased serum concentration of iron, an essential element in hemoglobin synthesis. However, the possibility that other mechanisms may be involved in CPF-induced anemia exists. Increased lipid peroxidation and oxidative stress have been observed in the erythrocytes of rats following repeated CPF exposure (Gultekin et al., 2001; Goel et al., 2006; Mansour and Mossa, 2009; Ambali et al., 2010). To combat the menace of lipoperoxidative changes and oxidative stress, erythrocytes are endowed with arrays of endogenous antioxidant defense systems, including vitamins C and E, which act as potent scavengers of free radicals.

Corresponding Author: Suleiman F. Ambali, Department of Veterinary Physiology and Pharmacology
Phone number: +234 8037015411
E-mail: fambali2001@yahoo.com
radicals (FRs) (Kollanjiappan et al., 2002). However, in situation of increased oxidative challenge as observed in CPF poisoning, these antioxidant machineries are overwhelmed resulting in oxidative stress. By-products of lipid peroxidation have been shown to cause profound alterations in structural organizations and functions of the cell membranes (Van Ginkel and Sevanian, 1994). It is suspected that this may be playing a role in CPF-induced anemia, since lipoperoxidative damage to the erythrocyte membrane compromises its integrity. The osmotic fragility used as an indication of oxidative hemolysis measures the sensitivity of the erythrocytes to changes in osmotic pressure and has been used to measure the integrity (Kollanjiappan et al., 2002) and metabolism (Rai et al., 2009) of the erythrocytes.

Vitamin C is a water-soluble vitamin that is found intra- and extracellularly as ascorbate (Chihuailaf et al., 2002). It is a natural antioxidant that prevents the increased production of FRs induced by oxidative damage to lipids and lipoproteins in various cellular compartments and tissues (Sies et al., 1992). It has been shown to react directly with superoxide (Nishikimi, 1975; Hemila et al., 1985), hydroxyl radicals (Bielski, 1982) and singlet oxygen (Bodannes and Chan, 1999). It is generally regarded as a primary first-line protective agent that repairs or nullifies FRs by donating a single electron, followed by a proton to yield a chemically reduced non-radical product and ascorbyl radical. The ascorbyl radical dismutates to ascorbate and dehydroascorbic acid (Carr et al., 2000; Halliwell, 2001). Vitamin E is the major lipid soluble antioxidant present in all cellular membranes where it protects the membranes against lipid peroxidation (Machlin, 1980). Therefore, from the foregoing, these two vitamins play important role in the maintenance of structural integrity of the erythrocytes protecting it from oxidative damage. Therefore, the objective of the present study is to evaluate the effect of repeated CPF exposure on erythrocyte osmotic fragility, the role of lipid peroxidation and the ameliorative effect of pretreatment with the combinations of vitamins C and E.

MATERIALS AND METHODS

Experimental Animals:
Twenty adult male Wistar rats (10-12 weeks old) weighing between 95 and 110g used for this study were obtained from the Laboratory Animal House of the Department of Veterinary Physiology and Pharmacology, Ahmadu Bello University, Zaria, Nigeria. They were allowed to acclimatize for two weeks prior to the commencement of the experiment. The rats were fed on pellets made from grower mash, maize bran and groundnut cake at a ratio of 4:2:1 and water was provided ad libitum.

Chemical Acquisition and Preparations:
Commercial grade chlorpyrifos, 20% EC, marketed as Termicot® (Sabero Organics, Gujarat limited, India), was obtained from an Agrochemical Store in Zaria, Nigeria. It was prepared by dissolving in soya oil (Grand Cereals and Oil Mills Ltd., Jos, Nigeria) to make 10% stock solution. Vitamin E (100 mg/capsule) from Pharco Pharmaceuticals, Egypt, was obtained from a reputable Pharmaceutical Store in Zaria. Prior to daily use, each capsule was aspirated into a syringe and then reconstituted with soya oil to 100% v/v.

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 at the dose of 2ml/kg. Rats in group II (labeled VCE) were co-administered with vitamins C and E only at a dose of 100 mg/kg and 75 mg/kg, respectively, while those in group III (labeled as CPF) were administered with CPF only at a dose of 10.6 mg/kg (~1/8th LD₅₀). Rats in group IV (labeled VCE+CPF) were pretreated with combination of vitamin C (100 mg/kg) and vitamin E (75 mg/kg), and then dosed with CPF at a dose of 10.6 mg/kg, 30 minutes later. The different regimens were administered orally by gavage once daily for a period of 17 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 specification of the Ahmadu Bello University Animal Research Committee and in accordance with Helsinki Declaration.

Evaluation of Erythrocyte Osmotic Fragility:
Blood collected into heparinized sample bottles were analyzed for erythrocyte osmotic fragility using the method described by Faulkner and King (1970) as modified by Oyewale (1982). Briefly, freshly obtained heparinized blood from each rat was pipetted into the test tubes containing 0.0, 0.1, 0.3, 0.5, 0.7, 0.9 g/L of NaCl (pH 7.4) and then followed by careful mixing and incubation for 30 minutes at room temperature, 26-28°C. The test tubes were then centrifuged at 2000 x g for 10 minutes using a centrifuge model IEC HN-SII.

The supernatant was transferred into a glass cuvette and the absorbance of the supernatant measured colorimetrically with Spectronic 20 (Bausch and Lomb, USA) at wavelength of 540 nm. The percent hemolysis for each sample was then calculated thus:

\[
\% \text{ haemolysis} = \frac{\text{Optical density of test solution}}{\text{Optical density of standard solution}}
\]

**Evaluation of Erythrocyte Malonaldehyde Concentration:**

The erythrocyte malonaldehyde (MDA) concentration, as a marker of lipid peroxidation was determined by the double heating method of Draper and Hadley (1990) as modified by Altuntas et al. (2001). The principle of the method was spectrophotometric measurement of the colour produced during the reaction to thiobarbituric acid (TBA) with MDA. Briefly, 2.5 ml of 100 g/L 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 1000 x g for 10 min, and 2 ml of the supernatant was added to 1 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, 6405 model, Japan) at 532 nm. The concentration of MDA was calculated by the absorbance coefficient of MDA-TBA complex, 1.56x10^5 cm^-1 M^-1, and expressed in nanomoles per gram of hemoglobin. The hemoglobin concentration was determined using the method of 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 AND DISCUSSION**

**Effect on In Vitro Erythrocyte Osmotic Fragility:**

There was complete hemolysis (100%) in the control solvent (distilled water). Generally, there was no significant change (P < 0.05) in the degree of erythrocyte fragility among the rats in the various groups at 0.7, and 0.9 g/L of NaCl. On the other hand, there were significant changes in the degree of erythrocyte fragility between groups at 0.1, 0.3 and 0.5 g/L of NaCl. At 0.1 g/L NaCl concentration, a significant decrease (P < 0.05) in erythrocyte fragility was observed in VC + VE group compared to the control, CPF and VC+VE+CPF groups, respectively. A significant increase in erythrocyte fragility was recorded in the CPF group compared to the control (P < 0.05), VC+VE+CPF, (P < 0.05) and VC+VE (P < 0.01) groups, respectively, at 0.3 g/L NaCl concentration. Similarly, at 0.5 g/L NaCl concentration, a significant increase in erythrocyte fragility was observed in the CPF group compared to the control (P < 0.05), VC+VE+CPF (P < 0.01) and VC+VE (P < 0.01) groups, respectively. (Figure 1)

**Effect on Erythrocyte Malonaldehyde Concentration:**

The effect of treatments on erythrocyte MDA concentration is shown in Figure 2. The MDA concentration as index of lipid peroxidation was significantly higher (P < 0.01) in rats in the CPF group compared to those obtained in the S/oil, VC+VE and VC+VE+CPF groups. There was no significant change in the MDA concentration in VC+VE+CPF group compared to the VC+VE and the S/oil groups, respectively.

**Discussion:**

The present study has revealed the ability of repeated CPF exposure to caused an increased erythrocyte fragility. This observation was positively correlated with increased MDA concentration in the erythrocyte membranes. This showed that increased lipoperoxidative damage may have been partly responsible for the increased erythrocyte fragility observed in group exposed to CPF only. The normal function of erythrocytes is wholly dependent on intact erythrocyte membranes. Toxic effect of many environmental chemicals and pesticides is due in large part to their effect on erythrocyte membranes (Datta et al., 1992). Erythrocytes and the erythrocyte membranes are critical target in the lipid peroxidation process due to constant exposure to high oxygen tension, high level of iron and richness in polyunsaturated fatty acids (PUFA) (Kollanjappan et al., 2002) coupled with their inability to possess nucleus and other organelles (Dordeviç et al., 2008). Process of
lipid peroxidation decreases hydrophobic characteristics of bilayer membrane of erythrocytes, altering affinity and interaction of proteins and lipids. In that way, the normal functioning of membrane and homeostasis of erythrocytes are impaired (Dargel, 1991). Lipid radicals in the membrane can modify the structure and function of the membrane, resulting in a loss of cell homeostasis (Pajovic et al., 2006). Reactive oxygen species can equally affect the proteins resulting in modification of enzymes activity and damage to the membrane transport proteins may produce disturbed cellular ionic homeostasis, leading to alterations in intracellular calcium and potassium that triggers a series of changes in the cell (Kerr et al., 1992).

Fig. 1: Effect of soya oil, vitamins C+ E, chlorpyrifos, vitamins C+ E+ chlorpyrifos on erythrocyte osmotic fragility.

Fig. 2: Effect of soya oil, vitamins C+E, chlorpyrifos and vitamins C+ E + chlorpyrifos on erythrocyte malonaldehyde concentrations.

*Comparison of chlorpyrifos group and soya oil group (P < 0.01). *Comparison of Vitamins C+ E + chlorpyrifos group and soya oil group (P > 0.05). *Comparison of vitamins C+E+ chlorpyrifos group and chlorpyrifos group (P < 0.01). *Comparison of vitamins C + E group and chlorpyrifos group (P < 0.01). *Comparison of Vitamins C +E group and soya oil group (P < 0.01). *Comparison of vitamins C + E + chlorpyrifos group and vitamins C +E group (P < 0.01). Values are means ± SEM of 5 animals per group.
Erythrocytes are endowed with high level of antioxidant enzymes and molecules, however, under the condition of enhanced lipid peroxidation as observed in rats exposed to CPF only, these protective molecules are overwhelmed resulting in impaired cellular integrity. FRs have been found to damage membrane ATPase and increased potassium efflux from the cell, a process that greatly contributes to cell lysis (Brovelli et al., 1977; Kollanjippan et al., 2002).

Co-administration of vitamins C and E has been shown by the present study to ameliorate the CPF-induced erythrocyte fragility. This may have partly resulted from the antioxidant properties of the vitamins as observed by reduced lipoperoxidative damage to the erythrocyte membranes in groups pretreated with both vitamins. Combinations of vitamin C and E have been shown to reduce erythrocyte lipid peroxidation changes induced by exposure to CPF-ethyl (Gultekin et al., 2001) and malathion (Durak et al., 2009).

Vitamin C is a water-soluble antioxidant in the biological fluids (Frei et al., 1989, 1990), which owes its biochemical and physiological actions to ability to donate electron (Padayatty et al., 2003). It has been shown to be more efficient than other plasma components, including α-tocopherols, at blocking the initiation of lipid peroxidation by trapping peroxyl radicals in the aqueous phase before it can react with lipids in plasma and cell membranes (Craven et al., 1997). Devi et al. (2007) reported that supplementation with vitamin C decreased the induction of oxidative stress in the erythrocytes.

Similarly, vitamin E is a universal participant of antioxidant defense reactions in biological membranes, since it acts at all steps of membrane oxidative damage (Evstigneeva et al., 1998). It acts as a first line defense against peroxidation of PUFA (Singh et al., 2000). A positive association between vitamin E and lipid peroxide formation has been reported (Chow, 1991). In addition, decrease in glutathione concentration observed during oxidative damage results in decreased vitamin E concentration (Kollanjippan et al., 2002). Increased erythrocyte fragility is a cardinal symptom observed in vitamin E deficient individuals (Horwitt, 1960, 1962; IOM, 2000) since the vitamin has been shown to have membrane-stabilizing effect (Niki et al., 1991; Palozza and Krinsky, 1994). Therefore, the increased MDA concentration recorded in the erythrocytes of rats exposed to CPF only may be associated with a deficient vitamin E, which may have increased the susceptibility of the erythrocytes to oxidative attack. It then implies that the decreased erythrocyte fragility in group pretreated with a combination of vitamins C and E may be due to the replacement of vitamin E that has been lost from CPF-ethyl (Gultekin et al., 2001) and malathion (Durak et al., 2009).

In conclusion, the present study has demonstrated that chronic CPF exposure results in increased erythrocyte lipid peroxidation, which may have been partly responsible for the increased erythrocyte osmotic fragility. Thus, the increased erythrocyte fragility may have played a significant role in the anemia observed following repeated CPF exposure in previous studies (Goel et al., 2006; Ambali, 2009). Pretreatment with combination of vitamins C and E has equally been shown by the present study to ameliorate the erythrocyte membrane lipoperoxidative damage, which must have been partly responsible for the significant reduction in the erythrocyte fragility.

REFERENCES


