Acetyl-L-carnitine attenuates haemotoxicity induced by subacute chlorpyrifos exposure in Wistar rats

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ABSTRACT

The present study was aimed at evaluating the effect of subacute CPF exposure on haematological changes and the ameliorating effect of acetyl-L-carnitine (ALC) in Wistar rats. Twenty-eight adult male Wistar rats divided into 4 groups of 7 animals each were used for the study. Groups I and II were given soya oil (2 ml kg⁻¹) and ALC (300 mg kg⁻¹), respectively. Group III received CPF only (8.5 mg kg⁻¹ ~ 1/10th of the LD₅₀) while group IV was pretreated with ALC (300 mg kg⁻¹), and then dosed with CPF (8.5 mg kg⁻¹), 30 min later. The regimens were administered by gavage once daily for 28 days. The study showed that haematological parameters such as total red blood cell, packed cell volume, haemoglobin concentration, mean cell volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, platelets, absolute total and differential white blood cell count, neutrophil-lymphocyte ratio and erythrocyte osmotic fragility altered by CPF were ameliorated by pretreatment with ALC.

Key words: Chlorpyrifos, haematology, acetyl-L-carnitine, amelioration, oxidative stress.

INTRODUCTION

Chlorpyrifos, a broad spectrum chlorinated organophosphate (OP) insecticide with relatively low acute toxicity is extensively used globally in agriculture and residential pest control [1, 2]. The main mechanism of toxicity is its ability to irreversibly inhibit acetylcholinesterase (AChE) in the central and peripheral nervous system [3], leading to accumulation of acetylcholine (ACh) at cholinergic receptor with consequent neurotoxicity [4]. However, recent evidence has shown that other mechanisms independent of AChE inhibition has been implicated in its toxicity [5, 6]. One of the mechanisms implicated in both acute and chronic CPF poisoning that has received considerable attention is oxidative stress [7, 8, 9].
Acetyl-L-carnitine (ALC) is a vital co-factor for the mitochondrial oxidation of fatty acids that results in ATP production in peripheral tissues [10-12]. It has an antioxidant activity and may be involved in ameliorating cellular dysfunction via an inhibition of lipid hydroperoxidation [10, 13]. Some studies have suggested neuroprotective role of ALC [12, 14], as it provides protection from lesions induced by neurotoxic agents [15, 16]. It is effective in various pathologic conditions characterized by increased oxidative stress, and also ameliorates oxidative injury of organs in animal models via its free radical scavenging and antioxidant properties [17].

Pesticides use in both developing and developed nations have increased dramatically in recent times due to compelling need to feed the ever-increasing human and animal population and to improve public health. But the hazards posed by low-level exposure are insidious and often damaging to the system. This has however been accompanied by adverse effects in non target species such as humans and animals [18]. The long-term effect of acute and chronic CPF intoxication has centred mainly on neurological consequences. The effect of acute OP exposure is obvious, although few studies have evaluated the effect of prolonged low-level exposure to CPF on haematological parameters [8, 19-24], as alterations in haematological parameters are used as indicators of the health status of the individual. The present study therefore, evaluates the effect of subacute CPF exposure on haematological parameters, the ameliorative role of ALC in CPF-induced haematoxicity.

MATERIALS AND METHODS

Experimental Animals
Twenty eight male Wistar rats (10-12 weeks old) weighing 110-150g used for this study were obtained from the Animal House of the Department of Veterinary Physiology and Pharmacology, Ahmadu Bello University, Zaria, Nigeria. They were fed on standard rat pellets and water was provided ad libitum.

Chemicals
Commercial grade CPF, Termicot® (Sabero Organics, Gujarat Limited, India) used for the study was reconstituted in soya oil (10%) prior to daily administration. ALC capsules (500 mg/capsule), L-carnipure® (Ideasphere Inc. America Fork UT84003 U.S.A) was reconstituted also in soya oil prior to daily administration.

Treatment Protocol
The rats were weighed and then divided into 4 groups with each group having 7 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 ALC) were administered ALC only (300 mg kg⁻¹) while those in group III (labeled CPF) were dosed with CPF only at 8.5 mg kg⁻¹. Group IV (labeled ALC + CPF) were pretreated with ALC (300 mg kg⁻¹) and then dosed with CPF (8.5 mg kg⁻¹), 30 minutes later. The different regimens were administered daily by oral gavage for a period of 4 weeks. At the end of the treatment period, the rats were sacrificed by severing the jugular vein after light chloroform anaesthesia. The study was carried out according to the specification of the Ahmadu Bello University Animal Research Committee.
Erythrocyte Osmotic Fragility

*In vitro* erythrocyte osmotic fragility was evaluated in all the rats in each group using the method described by [25] as modified by [26], using different amounts of sodium chloride (pH 7.4) from 0.0, 0.1, 0.3, 0.5, 0.7 and 0.9 g/L of distilled water. Briefly, freshly obtained whole blood from each rat was pipetted into the test tubes containing varying concentration of NaCl and then followed by careful, gentle mixing and incubation for 30 minutes at room temperature, 26-28°C. The samples were then centrifuged at 600 g for 10 minutes using a centrifuge model IEC HN-SII (Damon IJEC Division, UK). The supernatant was transferred into a glass cuvette and the absorbance of the supernatant measured colorimetrically with Spectronic 20 (Bausch and Lomb, USA) at a wave length of 540 nm. The percent haemolysis for each sample was then calculated thus:

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\text{Percent haemolysis} = \frac{\text{Optical density of test solution}}{\text{Optical density of standard solution}} \times 100
\]

Haematological Evaluation

Two millilitre of blood collected into heparinized sample bottles were analyzed for haematological parameters such as Packed Cell Volume (PCV), Haemoglobin (Hb), total Red Blood Cell (RBC), Mean Cell Volume (MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC), total and differential White Blood Cell (WBC) count and total platelets count using an automatic haematological assay analyzer, Advia 60® haematology system (Bayer Diagnostic Europe Ltd., Ireland). Blood smears were also stained with Giemsa for differential WBC count [27], while the neutrophil-lymphocyte ratio was calculated.

Statistical Analysis

Data obtained as Mean ± SEM were subjected to one-way analysis of variance (ANOVA) followed by Tukey’s test using GraphPad Prism Version 4.0. Values of P < 0.05 were considered significant. Percentages were also used to express differences between groups where P > 0.05.

RESULTS

Effect of Treatments on Packed Cell Volume

There was no significant difference (P > 0.05) in PCV between the groups (Fig. 1). However, the CPF group had the lowest PCV as it slightly decreased by 14.7% compared to the S/oil group. There was a 7.5% increase in PCV in ALC + CPF group over the CPF group.

Effect of Treatments on Haemoglobin Concentration

No significant difference (P > 0.05) in Hb concentration was recorded between the groups. The lowest mean Hb value was however, recorded in the CPF group as it slightly decreased by 8% compared to the S/oil group (Fig. 2). However, the pretreatment group, ALC + CPF slightly increased by 5% when compared to the CPF group.

Effect of Treatments on Total Red Blood Cell Concentration

There was no significant difference (P > 0.05) in RBC concentration between the groups. The lowest mean RBC count was observed in the CPF group which decreased by 5% compared to the
S/oil group. However, there was 8% increase in RBC concentration in the ALC + CPF group, when compared to the CPF group (Fig. 3).

**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). The MCHC value in the CPF group slightly rose by 6% compared to the S/oil group. However, the MCHC in the ALC+CPF group increased by 2%, relative to the CPF group. On the other hand, the MCH value in the CPF group decreased by 3% compared to the S/oil group, while there was a 2% decrease in the ALC+CPF group, relative to the CPF group. Furthermore, there was a 10% decrease in the CPF group, relative to the S/oil group, while there was a marginal decrease (0.7%) in ALC+CPF group compared to the CPF group.

**Effect of Treatments on Platelets Count**
No significant change (P > 0.05) in the platelet count was observed between the treatment groups. However, the platelet count slightly decreased in the CPF (13%) compared to the S/oil group. There was a marginal increase in the ALC + CPF (1.2%) group when compared to the CPF group (Fig. 5).

**Effect of Treatments on White Blood Cells**
There was no significant change (P > 0.05) in total white blood cell count between the groups (Fig. 6). The WBC slightly increased in the CPF group by 8% compared to the S/oil group. However, there was a 10% decrease in WBC in the ALC + CPF group, when compared to the CPF group.

**Effect of treatments on differential leucocyte count**
The effect of treatment on differential leucocyte count is shown in Fig. 7. There was no significant difference in the neutrophil and lymphocyte count between the groups. However, the lymphocyte count in the CPF group was the highest compared to either the S/oil, ALC or ALC+CPF groups, while the lymphocyte count in the ALC+CPF decreased by 17%, relative, to the CPF group.

**Effect of Treatments on Neutrophil/Lymphocyte ratio**
There was no significant difference (P > 0.05) in the neutrophil/lymphocyte ratio between the groups (Fig. 8). The neutrophil/lymphocyte ratio was lowest in the CPF group compared to the other groups as it decreased by 3% compared to the S/oil group. However, there was a 53% increase in neutrophil/lymphocyte ratio in the ALC + CPF group, relative to the CPF group.

**Effect of Treatments on In vitro Erythrocyte Osmotic Fragility**
Generally, irrespective of the treatment groups, percent haemolysis decreased with increasing NaCl concentration. Haemolysis was complete (100 %) in the standard solvent (distilled water). There was no significant difference (P > 0.05) in the degree of erythrocyte osmotic fragility among the rats in the various groups at 0.1, 0.3, 0.5, 0.7% and 0.9% of NaCl concentration. However, at 0.5% of the NaCl concentration, the CPF group showed the highest mean percentage of erythrocyte osmotic fragility, while the ALC + CPF group showed a comparative decrease (48%) in percentage of erythrocyte osmotic fragility, relative to CPF group. At 0.7% of NaCl concentration, the CPF group also showed the highest mean erythrocyte fragility, while the
ALC + CPF group showed a 25% decrease in erythrocyte fragility when compared to the CPF group (Fig. 9). Furthermore, at 0.9% of the NaCl concentration, there was a 16% decrease in erythrocyte osmotic fragility in the ALC + CPF group compared to the CPF group, while the CPF group showed the highest mean percentage of erythrocyte osmotic fragility.

**DISCUSSION**

The toxic effect of OPs such as CPF results in adverse effect on different body systems including the haematopoietic system [28]. The lower values of RBC, PCV and Hb observed in the CPF group in the present study agreed with those obtained in earlier studies [21, 24, 29, 30]. This indicates that repeated exposure to CPF causes anaemia. Various reasons have been ascribed to the anaemia associated with CPF and other Ops exposure. This may however, be related to disruption of erythropoiesis or an increase in RBC destruction [31, 32]. The destruction of red blood cells is postulated to occur by either membrane oxidation or haemoglobin denaturation [33]. It may also be due to the ability of OP compound to decrease tissue iron concentration [24], interference with Hb biosynthesis and shortening of RBC life span [34] or even increase in erythrocyte fragility [19, 20, 22]. Earlier workers have demonstrated the role of oxidative stress in the pathophysiology of anaemia [19-22, 35, 36]. The RBC is vulnerable to lipoperoxidative changes because of its direct association with molecular oxygen, high content of metal ions catalyzing oxidative reaction and availability of high amount of PUFA, which are susceptible to lipid peroxidation [37].

The MCH and MCHC did not alter significantly within the groups, thus indicating that CPF did not alter haemoglobin amount and concentration. The slight decrease in MCV seen and the apparently normal MCH and MCHC may indicate normocytic normochromic anaemia.

The study also showed an increase in the percentage erythrocyte fragility in the CPF group compared to the control group. This agreed with the findings of [19, 20, 22]. This shows the ability of subacute exposure to CPF to compromise the integrity of the RBC membrane apparently from increased oxidative damage to the erythrocyte membrane [38]. The oxidative modification of the erythrocyte membrane has been shown to increase the fragility of RBC [19-21, 23, 39]. Since CPF is lipophilic, it may enhance lipoperoxidation by directly interacting with the cellular plasma membrane [40]. The increase in the *in vitro* erythrocyte osmotic fragility in rats exposed to CPF only confirmed the observation that this can be used as indirect method of evaluating lipid peroxidation in animals [41]. Process of lipid peroxidation decreases hydrophobic characteristic of bilayer membrane of erythrocyte altering affinity and interaction of protein and lipids, thereby impairing the functioning and homeostasis of erythrocyte membranes [42]. Therefore, the anaemia observed in this study may be as a result of a combination of factors.

The improvement in the RBC, PCV and Hb in the rats pretreated with VC and/or ALC suggest their ameliorative effect on the CPF-induced anaemia. This underscores the role of oxidative stress in CPF-induced anaemia. The reason for the improvement in PCV, RBC and Hb concentrations in the ALC pretreated group may be due to the ability of ALC to improve RBC survival may be through enhanced erythrocyte membrane stability and rise in red blood cell osmotic resistance, and also by increasing the action of erythropoietin on bone marrow [43-45].
Fig 1: Effect of soya oil, chlorpyrifos and/or acetyl-L-carnitine on packed cell volume in Wistar rats.

Fig 2: Effect of soya oil, chlorpyrifos and/or acetyl-L-carnitine on haemoglobin concentration in Wistar rats.

Carnitine is known to have beneficial effect on sickle-cell [46] and chronic renal failure anaemia [47]. Similarly, carnitine has been shown to reduce deformability of red blood cell, increase haematocrit, haemoglobin levels, erythrocyte count and survival time [44, 48]. The improvement in erythrocyte osmotic fragility seen in the ALC pretreated group may be due to its ability to aid the maintenance of RBC membrane stability [43] and also decrease osmotic fragility [49]. ALC
exhibited either a primary antioxidant effect by inhibiting free radical propagation or more likely functioned as a secondary antioxidant by repairing oxidized polyunsaturated fatty acid esterified in membrane phospholipids [50].

![Graph](image1.png)

**Fig 3:** Effect of soya oil, chlorpyrifos and/or acetyl-L-carnitine on red blood cell concentration in Wistar rats.

![Graph](image2.png)

**Fig 4:** Effect of soya oil, chlorpyrifos and/or acetyl-L-carnitine on red blood cell indices in Wistar rats.

The study revealed an apparent increase in the WBC concentration in the group exposed to CPF only. The reason for the apparent leukocytosis is not known. This finding agreed with those of [30] in rats and in cat fish [29]. However, this finding contradicted the result of previous studies which showed that repeated CPF exposure causes leukopaenia [8, 21, 24]. The leucocytosis recorded in the CPF group is due to lymphocytosis. This was further demonstrated by the

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decrease in the neutrophil/lymphocyte ratio in the CPF group. The implication of the apparent leucocytosis is not known, especially as it relates to the immune system and deserves further studies. However, Joshi et al. [51] attributed leucocytosis following exposure of fish to lindane and malathion to increase in antibody production. Okechukwu et al. [29] also suggested that the leucocytosis may be due to hypersensitivity reaction to CPF leading to antibody production. Pretreatment with ALC moderated the CPF-induced leukocytosis. The reason for the apparent restoration of CPF-evoked leucocytosis by ALC is not known for certain but may be related to its modulatory role on the immune system [52].

![Graph](image)

**Fig 5:** Effect of soya oil, chlorpyrifos and/or acetyl-L-carnitine on platelet count in Wistar rats.

The present study also recorded a relative decrease in platelet count of rats exposed to CPF only. This however, disagrees with that reported by other workers [21, 53]. However, the thrombocytopaenia may be related to CPF-induced oxidative damage to the platelet membranes. The platelet membranes, apart from being thinner than those of the erythrocytes, it is also less resistant to oxidative attack [54]. Therefore, the vulnerability of thrombocyte membrane to oxidative damage may have been partly responsible for the apparent thrombocytopaenia in the present study. It is expected that increased oxidative stress may lead to increase in platelet lysis. Ohyashiki et al. [55] showed that lipid peroxidation increases in the platelet exposed to free oxygen radicals. Paradoxically, relative thrombocytopaenia was also recorded in the ALC group. This may be due to the overwhelming presence of the antioxidants beyond the existing oxidative demand of the cell, thereby resulting in prooxidant effect. Improvement in platelet count was recorded in the group pretreated with ALC indicating the role of oxidative stress in the CPF-induced thrombocytopaenia. This may be due to the antioxidant properties of ALC, thereby improving the integrity of the thrombocyte membrane.
Fig 6: Effect of soya oil, chlorpyrifos and/or acetyl-L-carnitine on total white blood cell count in Wistar rats.

Fig 7: Effect of soya oil, chlorpyrifos and/or acetyl-L-carnitine on differential leucocyte count in Wistar rats.
Fig 8: Effect of soya oil, chlorpyrifos and/or acetyl-L-carnitine on neutrophil/lymphocyte ratio in Wistar rats.

Fig 9: Effect of soya oil, chlorpyrifos and/or acetyl-L-carnitine on erythrocyte osmotic fragility in Wistar Rats.
In conclusion, the present study has shown that pretreatment with ALC attenuates CPF-induced alteration in haematological parameters partly due to its antioxidant property. Therefore, individuals who are constantly exposed to low-dose CPF and perhaps other OP insecticides may be protected from OP-induced haemotoxicity by pretreatment with ALC.

REFERENCES
