Oxidative stress involvement in chronic chloropyrifos-induced hepatocellular injury: Alleviating effect of vitamin C

Keywords: Chlorpyrifos, Hepatocellular injury, Oxidative stress alleviation, Vitamin C.

Introduction

Organophosphate (OP) insecticides are one of the most widely used pesticides for agricultural and non-agricultural purposes accounting for about 50% of the global insecticide use (Casida and Quistad, 2004). The ubiquitous nature of their use has led to increasing propensity of their adverse effect on target and non-target species. Chlorpyrifos (CPF) [O,O-diethyl O-(3,5,6-trichloro-2-pyridinol) phosphorothionate] is a chlorinated OP insecticide that is widely used in agricultural, public health and domestic purposes, despite restrictions placed by United States Environmental Protection Agency on some of the latter applications. Like other OP insecticides, the mechanism of systemic toxicity of CPF resides in its ability to inhibit acetylcholinesterase (AChE) leading to cholinergic syndrome. Since toxicity occurs at doses that do not inhibit AChE or long after its restoration, other mechanisms including the induction of oxidative stress have been widely implicated. The present study was aimed at evaluating the mitigating effect of vitamin C on CPF-induced hepatocellular injury in Wistar rats.

Methods: Twenty adult male Wistar rats were divided into 4 groups of five animals in each group. The four groups were exposed by gavage to soya oil (2 ml/kg), vitamin C (100 mg/kg), CPF (10.6 mg/kg~1/8LD50) and vitamin C (100 mg/kg) + CPF (10.6 mg/kg; 30 min later), respectively for 17 weeks. The sera obtained from blood samples collected from the animals were analysed for the levels of total proteins, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyl transferase (GGT) while globulin concentration and albumin/globulin ratio were calculated. The liver homogenate was evaluated for the levels of malonaldehyde (MDA), superoxide dismutase (SOD) and catalase (CAT), and histological changes.

Results: The study showed that CPF altered the levels of the serum hepatic enzymes, hepatic MDA SOD and CAT, in addition to inducing hepatocellular degeneration. All these parameters were alleviated by pretreatment with vitamin C.

Significance: CPF-induced hepatocellular injury which was partly due to oxidative changes was mitigated by vitamin C partly due to its antioxidative activity.

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vitamin C on hepatocellular injury caused by chronic CPF exposure in Wistar rats.

Materials and methods

Chemical acquisition and preparations

Commercial grade chlorpyrifos (CPF) 20% EC, marketed as Termicor® (Sabero Organics, Gujarat limited, India), was prepared by reconstituting in soya oil (Grand Cereals and Oil Mills Ltd., Jos, Nigeria) to make 10% stock solution. A tablet (100 mg) of ascorbic acid (Med Vit. C®, Dol-Med Laboratories Limited, Lagos, Nigeria) was dissolved in 1 mL of distilled water to obtain 100 mg/mL suspension, daily prior to administration. All other reagents used in this study were of analytical grade and were obtained from Sigma Inc. (USA).

Experimental animals

Twenty adult male Wistar rats weighing between 95 and 110 g used for this study were obtained from the Laboratory Animal House of the Department of Veterinary Pharmacology and Toxicology, Ahmadu Bello University, Zaria, Nigeria. The animals were allowed to acclimatize for two weeks in the laboratory prior to the commencement of the experiment. They were fed on standard rat chow and water was provided ad libitum.

Animal treatments schedule

The rats were weighed and then assigned at random into 4 groups of 5 rats in each group. Group I (S/oil) served as the control group and were given only soya oil (2 mL/kg b.w.) while group II (VC) was given vitamin C (100 mg/kg b.w.) and supplemented with soya oil (2 mL/kg b.w.). Group III (CPF) was administered with CPF only (10.6 mg/kg b.w. ~1/8 LD50 [Ambali, 2009]) while group IV (VC+CPF) was pre-treated with vitamin C (100 mg/kg b.w.), and then dosed with CPF (10.6 mg/kg b.w.), 30 minutes later. The different regimens were administered once daily by gavage for a period of 17 weeks. The study was carried out according to the specification of the Ahmadu Bello University Animal Research Committee and in compliance with the National Research Council on Guide for Care and Use of laboratory Animals (2011). At the end of the dosing period, the rats were sacrificed by severing the jugular vein after light ether anaesthesia. Blood samples were collected into heparinized sample bottles, left incubated on the shelf for 30 min, and thereafter centrifuged at 800 x g for 10 minutes.

Evaluation of treatments on serum proteins and hepatic enzymes

The sera obtained were used to analyse for the concentrations of total proteins, albumin, and activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and gamma glutamyl transferase (GGT) using autoanalyser (Bayer Express Plus, Germany). The globulin concentration was calculated by subtracting albumin concentration from the total protein concentration. The albumin/globulin ratio was subsequently calculated.

Evaluation of treatments on hepatic lipoperoxidation

The level of thiobarbituric acid (TBA) reactive substance, malonaldehyde (MDA) as an index of lipid peroxidation was evaluated on the liver sample using the method of Draper and Hadley (1990) as modified (Yavuz et al., 2004). The principle of the method was based on spectrophotometric measurement of the colour developed during reaction of TBA with MDA. The MDA concentration in each sample was calculated by the absorbance coefficient of MDA-TBA complex 1.56 x 10^5/cm/M and expressed as nmol/mg of tissue protein. The protein concentration in the liver homogenates was evaluated using the Lowry method (Lowry et al., 1951).

Evaluation of treatments on hepatic superoxide dismutase activity

Superoxide dismutase (SOD) activity was evaluated using NWLSS™ superoxide dismutase activity assay kit (Northwest Life Science Specialities, Vancouver, WA 98662) as stated by the manufacturer, expressed as nmol/mg tissue protein.

Evaluation of treatments on hepatic catalase activity

Catalase (CAT) activity was evaluated using NWLSS™ catalase activity assay kit (Northwest Life Science Specialities, LLC, Vancouver, WA 98662) as stated by the manufacturer and expressed as nmol/mg tissue protein.

Evaluation of treatments on hepatic acetylcholinesterase activity

Acetylcholinesterase activity was evaluated using the method of Ellman et al. (1961) with acetythiocholine iodide as a substrate. Briefly, the liver sample of each animal was weighed and homogenized in a cold (0–4 °C) 20 mM phosphate buffer saline (PBS) incubated with 0.01M 5,5-dithio-bis(2-nitrobenzoic acid) in 0.1 M PBS, pH 7.0. Incubations were allowed to proceed at room temperature for 10 minutes. Then, acetylthiocholine iodide (0.075 M in 0.1 M PBS, pH 8.0) was added to each tube, and absorbance at 412 nm was measured continuously for 30 minutes using a UV spectrophotometer (T80® UV/VIS spectrometer®, PG Instruments Ltd, Leicestershire, LE 175BE, United Kingdom). The acetylcholinesterase activity expressed as IU/g tissue was calculated based on the rate of colour change per minute.

Evaluation of the effect of treatments on hepatic histological changes

The liver samples of the rats were obtained and prepared for histological processing using the method described by Luna (1960). Briefly, the liver samples were fixed in 10% formalin, embedded in paraffin and cut to 5 mm. They were then stained with haematoxylin-eosin, examined under light microscope (x400) and lesions observed were recorded.

Statistical Analysis

All data were expressed as mean ± SEM and then subjected to one-way analysis of variance followed by Tukey’s multiple comparison test. Values of p < 0.05 were considered significant.
Results

Effect of treatments on serum proteins

There was a significant decrease in the total protein concentration in the CPF group when compared to that of S/oil (p < 0.05), VC (p < 0.01) or VC + CPF (p < 0.01) group. There was no significant (p > 0.05) change in the total protein concentration in the VC + CPF group relative to S/oil or VC group (Figure 1).

There was a significant (p < 0.05) decrease in albumin concentration in the CPF group relative to that of S/oil or VC group. Although not significant (p > 0.05), the albumin concentration in the VC + CPF group was relatively higher (19%) compared to CPF group. There was no significant (p > 0.05) change in the albumin concentration in the VC + CPF group relative to that of S/oil or VC group (Figure 1).

There was no significant (p > 0.05) change in the globulin concentration in the CPF group relative to S/oil, VC or VC + CPF group. However, the mean globulin concentration in the VC + CPF group was relatively higher compared to that recorded in the S/oil (18.2%), VC (10%) or CPF (11%) group (Figure 1).

The albumin/globulin ratio was significantly (p < 0.05) lower in the CPF group when compared to that recorded in the S/oil group. Although not significant (p > 0.05), the mean albumin/globulin ratio relatively decreased by 50% in the CPF group compared to that obtained in the VC group. There was no mean difference in the albumin/globulin ratio in the CPF group relative to that of VC + CPF group (Figure 2).

Effect of treatments on serum hepatic enzymes activities

There was a significant increase (p < 0.05) in AST activity in the CPF group compared to that of S/oil, VC or VC + CPF group. There was no significant (p > 0.05) change in the AST activity in VC + CPF group when compared to that in the S/oil or VC group (Figure 3).

The ALT activity in the CPF group was significantly higher in the CPF group when compared to that in the S/oil (p < 0.05), VC (p < 0.01) or VC + CPF (p < 0.01) group. There was no significant (p > 0.05) change in ALT activity in the VC + CPF group relative to that recorded in the S/oil or VC group (Figure 3).

The ALP activity in the CPF group was significantly (p < 0.05) higher relative to the S/oil or VC group. Although there was no significant (p > 0.05) change in ALP activity in the CPF group compare to that recorded in the VC + CPF group, the ALP level obtained in the former group was comparatively higher (11%) than the latter. There was no significant (p > 0.05) change in ALP activity in the VC + CPF group relative to S/oil and VC groups, respectively (Figure 3).

The GGT activity was significantly higher in the CPF group compared to that in the S/oil (p < 0.05), VC (p < 0.01) and VC + CPF (p < 0.01) groups, respectively. The GGT activity in the VC+CPF group was not significantly (p > 0.05) different from that recorded in the S/oil or VC groups (Figure 3).

Figure 1. Effect of soya oil (S/oil), vitamin C (VC) and/or chlorpyrifos (CPF) on serum proteins concentration in Wistar rats.

\(^{a} p < 0.05\) vs S/oil group, \(^{b} p < 0.01\) vs VC and VC + CPF groups, respectively; \(^{d} p < 0.05\) vs VC group

Figure 2. Effect of soya oil (S/oil), vitamin C (VC) and/or chlorpyrifos (CPF) on albumin/globulin in Wistar rats.

\(^{c} p < 0.05\) vs S/oil group.

Figure 3. Effect of soya oil (S/oil), vitamin C (VC) and/or chlorpyrifos (CPF) on liver enzymes activities in Wistar rats.

\(^{a} p < 0.05\) vs S/oil, VC and VC + CPF groups, respectively; \(^{b} p < 0.01\) vs VC and VC + CPF groups, respectively.
Effect of treatments on hepatic malonaldehyde concentration and activities of superoxide dismutase and catalase

There was a significant increase in the hepatic MDA concentration in the CPF group relative to that in the S/oil (p < 0.05), VC (p < 0.01) and VC + CPF (p < 0.05) groups, respectively. There was no significant (p > 0.05) change in the hepatic MDA concentration in the CPF group relative to that of the S/oil or VC group (Figure 4).

Figure 4. Effect of soya oil (S/oil), vitamin C (VC) and/or chlorpyrifos (CPF) on malonaldehyde concentrations in Wistar rats.
*abc* p < 0.01 vs S/oil, VC and VC + CPF groups, respectively.

There was a significant decrease in the hepatic SOD activity in the CPF group when compared to that recorded in the S/oil (p < 0.05), VC (p < 0.01) and VC + CPF (p < 0.05) groups, respectively. There was no significant (p > 0.05) change in the hepatic SOD activity in the VC + CPF group relative to that recorded in the S/oil (p > 0.05) or VC (p > 0.05) group (Figure 5).

Figure 5. Effect of soya oil (S/oil), vitamin C (VC) and/or chlorpyrifos (CPF) on superoxide dismutase activity in Wistar rats.
*abc* p < 0.01 vs S/oil, VC and VC + CPF groups, respectively; *c* p < 0.01 vs VC group.

The hepatic catalase activity in the CPF group was significantly lower relative to that recorded in the S/oil (p < 0.05), VC (p < 0.01) or VC+CPF (p < 0.05) group. There was no significant (p > 0.05) change in the VC + CPF group relative to that obtained in the S/oil (p < 0.05) or VC (p > 0.05) group (Figure 6).

Figure 6. Effect of soya oil (S/oil), vitamin C (VC) and/or chlorpyrifos (CPF) on liver enzymes activities in Wistar rats.
*abc* p < 0.05 vs S/oil and VC + CPF groups, respectively; *c* p < 0.01 vs VC group.

Effect of treatments on hepatic histological changes

The effect of treatments on hepatic histology is shown in Figure 7. There was widespread hepatocellular degeneration and sinusoidal dilatation in the CPF group. Sinusoidal dilatation was also recorded in the VC + CPF group. No apparent histological abnormality was recorded in the S/oil and VC groups.

Discussion

The increased hepatic MDA concentration and decreased SOD and catalase activities recorded in the CPF group is a further demonstration of the ability of CPF to cause oxidative stress. Oxidative stress, which results when there is imbalance in the level of prooxidants versus antioxidants in favour of the former, is known to cause cellular damage. The ability of CPF to cause hepatocellular oxidative changes has been demonstrated in many studies (Goel et al., 2005; Aly et al., 2010; Ambali et al., 2010, 2011a, b). The increased hepatic MDA concentration and decreased SOD and catalase activities recorded in the CPF group in this study is a further demonstration of the ability of CPF to cause oxidative stress. Malonaldehyde is one of the products of membrane lipid peroxidation that has been shown to cause membrane damage, impaired membrane function, structural integrity, reduced membrane fluidity and inactivation of membrane-bound enzymes (Gutteridge and Halliwell, 2000; Shittu et al., 2012). It has been used as a marker of oxidative stress.

Superoxide dismutase, a first line antioxidant enzyme dismutates the superoxide (O$_2^-$) to hydrogen peroxide (H$_2$O$_2$) and molecular oxygen. The H$_2$O$_2$ is subsequently hydrolysed into water (H$_2$O) and O$_2$ by CAT, an enzyme whose activity was reduced in the CPF group in the present study. The decrease in CAT activity
may be linked to CPF-induced decrease in SOD activity, which eventually reduced the conversion of $O_2^-$ to $H_2O_2$, the substrate for CAT. The increased prooxidant (MDA) concentration and the decreased activities of antioxidant enzymes (SOD and CAT) cause hepatocellular oxidative stress. Oxidative stress resulting from increased production of reactive oxygen species (ROS) in OP poisoning arises from the metabolism of the insecticide by cytochrome P450s (Łukaszewicz-Hussain, 2010). The cytochrome P450s are monooxygenases that catalyse oxidation by addition of one atom of molecular oxygen into the substrate (OP) by an electron transport pathway (Jakoby and Ziegler, 1990; White 1991; Chambers et al., 2001). Moreover, the ability of CPF and some other OPs to inhibit mitochondrial adenosine triphosphate (ATP) production through the uncoupling of oxidative phosphorylation could also lead to the generation of ROS (Ishii et al., 2004). Vitamin C was shown in the present study to decrease MDA concentration and increased SOD and CAT activities due to its antioxidant effect. The mechanism of free-radical scavenging ability of vitamin C may be due to its ability to abstract hydrogen from ascorbate, to become monodehydroascorbate, which soon gains another electron to become dehydroascorbate (Wilson, 2002). The ROS are reduced to water, while the oxidized forms of ascorbate are relatively stable, unreactive and does not cause cellular damage (Krishnamoorthy et al., 2007). Vitamin C has been previously shown to mitigate oxidative parameters in the liver of CPF-intoxicated mice (Aly et al., 2010).

The significantly lower serum protein and albumin concentrations in the CPF group recorded in the present study have also been reported in previous studies (Peeples et al., 2005; El-Banna et al., 2009; Bayomy et al., 2016). The significantly lower albumin/globulin ratio in the CPF group showed that the lower total proteins concentration in this group was due largely to a lower albumin concentration. The low protein and albumin concentrations may be partly due to impairment of liver synthesis consequent to hepatocellular degeneration. The ability of albumin to bind and, subsequently hydrolyse CPF-oxon has been demonstrated in previous studies (Ortigoza-Ferado et al., 1984; Sultatos et al., 1984). Indeed, Peeples et al. (2005) have proposed albumin as a biomarker of OP poisoning. Furthermore, lipid hydroperoxides, the lipid peroxidation products of polyunsaturated fatty acids (PUFA) which have been shown to cause protein degradation and denaturation (Chiba and Iwata, 2002) may have contributed to the low serum protein and albumin obtained in the CPF group in the present study.

Pre-treatment of the rats with vitamin C alleviated the deficit in total serum protein and albumin caused by CPF, partly through mitigation of hepatic degenerative changes. In addition, the
ability of vitamin C to increase the activity of paraoxonase (PON), especially PON I (Jarvik et al., 2002), which aids in the detoxification of the OP compounds (Shih et al., 1998) may have sparing effect on albumin since they compete for binding with the CPF and subsequent detoxification. The alleviation of oxidative changes in the liver may have reduced the production of lipid hydroxides, thereby limiting the degradation and denaturation of proteins.

The relative increase in globulin concentration in the CPF group relative to that of the control corroborates earlier observation by Subbotina and Bellonozhko (1968) following exposure to an OP compound, sevin. This may be due to induction of immunological abnormalities, since many pesticides have been shown to be toxic to the immune system through the induction of cytotoxicity (Corcoran et al., 1994; Rabideau, 2001). Indeed, some of the chronic neurotoxic effects of OP insecticides may be due to their ability to induce the formation of antibodies to nervous tissue. Antibodies to myelin basic protein, neurofilament triplet protein, and glial fibrillary acidic proteins have all been exhibited after OP exposure (McConnell et al., 1999). In addition, antibodies to smooth muscle, parietal cells, brush borders, thyroid follicles as well as antinuclear antibodies have been demonstrated in OP poisoning (Thrasier et al., 1993). All these antibodies may be partly responsible for the apparent increase in globulin concentration observed in the present study. Pre-treatment with vitamin C further increased the CPF-induced hypergobulinemia. This may be due to the ability of the vitamin C to stimulate the immune system by enhancing T-cell proliferation which may have ultimately assisted the B-cells to synthesize immunoglobulin (Campbell et al., 1999; Naidu, 2003). Vitamin C has been shown to protect the immune system and improve natural killer cell activities, lymphocyte proliferation, chemotaxis, and delayed-type hypersensitivity (Wintergerst et al., 2006).

The central role of the liver in the detoxification of xenobiotics makes it highly susceptible to injury. Therefore, the extent of hepatic injury and consequent dysfunction are indirectly assessed via the measurement of the activities of serum enzymes (including ALT, AST and ALP). The significantly higher AST activity observed in rats chronically exposed to CPF compared to that of the control revealed its ability to cause liver damage. Although AST is less specific for hepatic injury, since its high level may also signal damages to the cardiac and skeletal muscles, red blood cells, kidney and brain tissue, it has been suggested that increased AST activity in the serum is a sensitive marker of hepatic lesion, even if the damage is of a subclinical nature (Kauppinen, 1984; Meyer and Harvey, 1998).

Similarly, chronic exposure to CPF only resulted in significant elevation of the activity of ALT, a more sensitive marker of liver damage (Ballantyne, 1988; Haschek et al., 2010). This shows that chronic exposure to CPF caused damage to the hepatocytes. Alanine aminotransferase and AST are enzymes produced by the hepatocytes that are involved in the metabolism of amino acids and synthesis of proteins. In dying or damaged cells, these enzymes leak into the blood stream. Therefore, the observed elevation of these serum enzymes could be attributed to their release from the cytoplasm into the blood circulation after hepatic injury. The release of these enzymes could further lead to hepatic necrosis and inflammatory reactions. The elevation in ALT activity level observed in the present study in rats chronically exposed to CPF agrees with the findings from previous studies in laboratory animals (Yoshida et al., 1985; Tanvir et al., 2015, 2016). Similarly, results obtained from a study involving pesticide sprayers have shown high liver enzyme activities in the serum of these subjects (Patil et al., 2003). This result however contradicted the previous findings in rats (Szabo et al., 1988; Barna-Lloyd et al., 1990) and mice (Ambali et al., 2007, 2011a). These studies reported low AST and ALT activities following repeated CPF exposure, although the implication of low activities of these enzymes is not clear. High ALT activity observed in the rats exposed to CPF only in the present study may be due to increased hepatic oxidative and degenerative changes. Indeed, CPF has been shown to cause reduction in the hepatic tissue contents of antioxidant vitamins C (Raina et al., 2015) A and E (Spodniewska and Barski, 2016) due to the pro-oxidative properties of the pesticide. Furthermore, CPF, due to its lipophilicity, could accumulate in the liver to an appreciable extent causing direct cytotoxicity (Spodniewska and Barski, 2016).

The present study has shown that pre-treatment with vitamin C caused a relative decrease in the AST and ALT activities. This demonstrates a relative protective effect of antioxidant vitamin on CPF-induced liver damage. Histopathological findings in vitamin C-pre-treated group also revealed an improvement in hepatic histoarchitecture demonstrating hepatic protection. Therefore, it may be inferred that oxidative stress plays a significant role in CPF-induced liver and/or muscle damage, and that pre-treatment with antioxidant vitamin C ameliorated the damage.

The increased ALP activity in rats chronically exposed to CPF has been reported in previous studies (Goel et al., 2005; Ambali et al., 2010; 2011a, b; Tanvir et al., 2015). Increased ALP activity is not only seen in biliary tract obstruction and hepatopathological changes, it is also associated with bone, placental, renal or intestinal damage. However, when juxtaposed with the increased GGT activity in the CPF group, it is most probable that the increased ALP activity was due to hepatobiliary injury. Gamma glutamyl transferase produced solely by the liver, is involved in transportation of amino acids and peptides into the cells. It is a good indicator of hepatobiliary disease (Stojtević et al., 2008) as it is not affected in bone pathology (Haschek et al., 2010).

The restoration of AST, ALT, ALP and GGT activities in the group pre-treated with vitamin C demonstrated the protective effect of the antioxidant vitamin on hepatocellular injury provoked by CPF. The restoration of hepatic enzyme activities may have been due to reduction of CPF-provoked oxidative and cytotoxic changes in the hepatocytes because of the antioxidant and free radical scavenging properties of vitamin C.

The widespread hepatocellular degeneration and dilated sinusoids recorded in the CPF group, which may have been associated with oxidative injury and direct cytotoxicity evoked by the insecticide agrees with that recorded by previous studies (Tripathi and Srivastav 2010; Al-Shap et al., 2015; Tanvir et al., 2015; Ezzi et al., 2016). Accumulation of ROS has long been shown to play a vital role in mediating apoptosis among differ-
ent cell types (Pierce et al., 1991) via mitochondrial and non-mitochondrial dependent pathways (Sinha et al., 2013). Pretreatment with vitamin C was shown to mitigate hepatic histotoxicity induced by CPF apparently due to its antioxidant properties. Vitamin C may have neutralized the ROS that causes lipoperoxidation of the plasma and mitochondrial membranes, thereby stabilizing them, preventing the release of proapoptotic mediators.

Conclusion

In conclusion, the present study has shown that chronic CPF exposure causes hepatocellular injury partly due to oxidative damage as revealed by biochemical and histological parameters. However, vitamin C alleviated the hepatocellular injury partly due to its antioxidant property. Therefore, individuals that are predisposed to exposure to CPF and other OP insecticides as a result of their occupation or the environment they live in may benefit from the hepatoprotective effect of vitamin C.

Conflict of interest

The authors declare that they have no conflict of interest.

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