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Original Research

Evaluation of chronic chlorpyrifos-induced reproductive toxicity in male wistar rat: protective effects of vitamin C

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Key Words
Acetylcholinesterase; Chlorpyrifos; Reproductive hormones; Sperm count; Testicular histology; Vitamin C

Abstract
The aim of the present study was to evaluate the effect of vitamin C on reproductive toxicity, induced by chronic chlorpyrifos (CPF) exposure in male Wistar rats. Twenty adult male Wistar rats were divided into 4 groups of 5 animals in each group. Group I received soya oil (2 ml/kg); group II was given vitamin C only (100 mg/kg); group III was administered CPF only (10.6 mg/kg; ~1/8th LD_{50}), while group IV was pretreated with vitamin C and then exposed to CPF, 30 min later. The regimens were administered by gavage once daily for 15 weeks. At the end of the treatment period, the animals were sacrificed by jugular venesection after light chloroform anesthesia, and sera obtained from the blood samples were analyzed for follicle-stimulating hormone (FSH), luteinizing hormone (LH) and testosterone concentrations. Pituitary gland and the testicular tissues of each rat were quickly dissected, removed and assayed for the levels of glycogen and acetylcholinesterase (AChE) activity. The right caudal epididymis was evaluated for spermatozoa concentrations. The results showed that decrease in concentrations of spermatozoa, luteinizing and follicle-stimulating hormones, testosterone, testicular glycogen, and inhibition of pituitary gland and testicular AChE activities caused by CPF were ameliorated by vitamin C.

INTRODUCTION

Increasing evidence has shown a global decline in the sperm count of men [1]. This decline, which may be a cause of an increasing deficit in human fertility rates [2], has been linked to chemical contaminants including pesticides [3]. Observations suggestive of a causal relationship between pesticide exposure and reproductive dysfunctions have been reported [4]. Organophosphates (OPs) are one of the most widely used insecticides, accounting for about 70% of the global insecticidal use [5].

Chlorpyrifos (CPF; *O,O*-Dimethyl-O-(3,5,6-trichlor-2-pyridinyl)thiophosphat) is a broad spectrum OP insecticide that is widely used in agriculture, horticulture, and in domestic and urban pest control [6]. CPF, like other OP insecticides, has been shown to alter reproductive function by altering the activity of the pituitary-thyroid and pituitary-adrenal axes [7] giving credence to its endocrine disrupting effect. Inverse relationship between CPF exposure and sperm count has been established in humans [8] and laboratory animals [9]. Although the main mechanism of chlorpyrifos toxicity is inhibition of acetylcholinesterase (AChE) activity [10], toxicity does occur at doses that did not inhibit AChE [11], prompting search for other mechanisms. The induction of oxidative stress is one of the non-cholinergic mechanisms implicated in CPF toxicity [12].

Oxidative stress, which results from an imbalance of prooxidant/antioxidant ratio in favor of the former, is known to cause tissue damage [13]. Under normal circumstances, the body is endowed with an array of enzymatic and non-enzymatic antioxidant to combat the menace posed by oxidative stress. However, in the

face of increased oxidative challenge as reported in CPF poisoning [14], supplementation with exogenous source of antioxidant molecules becomes pertinent. Vitamin C is a non-enzymatic antioxidant molecule that has been used in mitigating CPF-induced toxicity in the liver [15], hematopoietic system [16], retina [17], pituitary glands and testes [12]. The aim of the present study was therefore to evaluate the mitigating effect of vitamin C on reproductive toxicity induced by chronic CPF exposure in male Wistar rats.

MATERIALS AND METHODS

Experimental design

Twenty adult male Wistar rats (*Rattus norvegicus*), weighing between 116-151 g used for the study were obtained from the Animal House of the Department of Veterinary Physiology and Pharmacology, Ahmadu Bello University, Zaria, Nigeria. They were housed in plastic cages and fed with standard rats' chow, while tap water was provided *ad libitum*. They were acclimatized for 2 weeks in the laboratory prior to the commencement of the study. The procedures involved in the handling of the Laboratory Animals follow the Guiding principle for the Care and Use of Laboratory Animals in research [18] and declaration of Helsinki.

Chemical preparations

Commercial grade CPF (20% EC) marketed as Termicot® (Sabero Organics, Gujarat, India) was used for the study. It was reconstituted in soya oil (Grand Cereals and Oil Mills Ltd, Jos, Nigeria), while vitamin C tablet (100 mg) (Med Vit C®; Dol-Med Laboratories Limited, Lagos, Nigeria) was dissolved in distilled water to obtain 100 mg/ml suspension prior to daily administration.

Animal treatment schedule

The rats were divided at random into four groups containing five rats per group. Group I (S/oil) serve as control and received soya oil (2 ml/kg), while group II (VC) was administered with vitamin C only (100 mg/kg [19]). Group III (CPF) received CPF only (10.6 mg/kg; ~1/8th of the LD_{50}), while group IV (VC+CPF) was pretreated with vit. C (100 mg/kg) and then administered CPF (10.6 mg/kg), 30 min later. The regimens were administered orally by gavage once daily for a period of 15 weeks. During this period, the rats were observed for signs of toxicity and death.

At the end of the treatment period, the animals were sacrificed by jugular venesection after light chloroform anesthesia, and sera obtained from the blood samples were analyzed for follicle-stimulating hormone (FSH), luteinizing hormone (LH) and testosterone concentrations. Pituitary gland and the testicular tissues of each rat were quickly dissected, removed and assayed for the levels of glycogen and AChE activity. The right caudal epididymis was evaluated for spermatozoa concentrations.

Effect of treatments on pituitary gland and testicular acetylcholinesterase activity

Acetylcholinesterase activity was evaluated using the method of Ellman *et al* [19]. Briefly, the pituitary gland and testicle of each rat were individually homogenized in cold (0-4°C) 20 mM phosphate buffer saline (PBS), incubated with 0.01 M 5,5-dithio-bis(2-nitrobenzoic acid) in 0.1 M PBS, pH 7. Incubations were allowed to proceed at room temperature for 10 min. Then acetylthiocholine iodide (0.075 M in 0.1 M PBS, pH 8) was added to each tube, and absorbance at 412 nm was measured continuously for 30 min using UV spectrophotometer (T80+ UV/VIS Spectrometer, PF Instruments, Wibtoft, England). The assay of each organ was done in triplicate. AChE activity was calculated based on the rate of color change per min using the extinction coefficient of 1.36×10^4 and was expressed as nanomoles/min/mg protein. The protein content of the tissue was determined using the method of Lowry *et al* [20].

Determination of serum follicle-stimulating hormone, luteinizing hormone and testosterone concentrations

The FSH and LH and testosterone concentrations were evaluated using commercial kits (Microwell FSH and LH enzyme-linked immunosorbent, and testosterone enzyme-linked immunosorbent assay kits, Syntron Bioresearch, Carlsbad, CA, USA.)

Evaluation of epididymal sperm count

The epididymal sperm count was evaluated using the method of Yarube *et al* [21]. Briefly, the right caudal epididymis of each rat was carefully removed, washed with phosphate buffer, dried with blotting paper and immediately homogenized in 1 ml of 0.5% formol saline. 1 ml of the aliquot was diluted (1:200) using erythrocyte diluting pipette. Counting was done using new improved Neubauer counting chamber (Marienfeld, Germany) and the cells were counted using a light microscope (Olympus, Tokyo, Japan) at the magnification of x400.

Determination of testicular glycogen concentration

The gravitational method of Good *et al* [22] was used for the evaluation of testicular glycogen concentration. The results were expressed in grams of glycogen per 100 g of the testicular tissue.

Effects of treatment on testicular histopathology

Histopathological slides of the testicular tissues were prepared using the method described by Luna [23]. The tissue samples were fixed in Bouin's solution for 48 h, embedded in paraffin, passed through graded

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concentration of alcohol and cut at 5 µm using microtome. The sections were then stained with hematoxylin-eosin dye, which was mounted in a neutral deparaffined xylene medium for light microscope at magnification of x200 and lesions observed were recorded.

Statistical analysis

Data obtained were expressed as mean ± SEM and then subjected to one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test using Graph Pad version 4.0. Values of $P < 0.05$ were considered significant.

RESULTS

Clinical signs

There was no death recorded in any of the groups. Rats in S/oil, VC and VC+CPF groups did not show any apparent sign of toxicity. However, toxic signs such as lacrimation, congested ocular mucous membranes and mild tremor were observed in the CPF group.

Pituitary gland and testicular acetylcholinesterase activities

The effects of treatments on pituitary gland and testicular AChE activities are as shown in Figs.1 and 2, respectively. The pituitary and testicular AChE activities were significantly reduced ($P < 0.01$) in the CPF group when compared to that obtained in the S/oil, VC+CPF group. There was no significant change ($P > 0.05$) in the pituitary gland and testicular AChE activities in the VC + CPF group compared to that of S/oil or VC groups.

Follicle-stimulating hormone concentration

There was no significant change ($P > 0.05$) in the FSH concentration between the groups. However, the mean FSH concentration in the CPF group was relatively lower compared to that recorded in S/oil (6%), VC (16.4%) or VC+CPF (5%) group (Table 1i).

Luteinizing hormone concentration

The LH concentration in the CPF group was significantly lower, compared to the S/oil ($P < 0.05$), VC ($P < 0.01$) or VC+CPF ($P < 0.01$) group. There was no significant change ($P > 0.05$) in the LH concentration in the VC+CPF group relative to that of S/oil or VC group (Table 1ii).

Testosterone concentration

The effect of treatments on serum testosterone concentration is shown in Table 1iii. There was a significant decrease in the serum testosterone 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 change ($P > 0.05$) in the testosterone concentration in the VC+CPF group compared to that of S/oil or VC group.

Epididymal sperm count

The epididymal sperm count in the CPF group was significantly lower ($P < 0.01$) compared to that recorded in S/oil and VC group. Although the difference was not significant ($P > 0.05$), the sperm count of VC+CPF group was 46% higher than the concentration recorded in the CPF group. The epididymal sperm count in the S/oil group was significantly ($P < 0.05$) higher that of the VC+CPF group (Table 1iv).

Testicular glycogen concentration

There was a significant decrease ($P < 0.01$) in the glycogen concentration in the CPF group, compared to that recorded in the S/oil, VC or VC+CPF group (Table 1v).

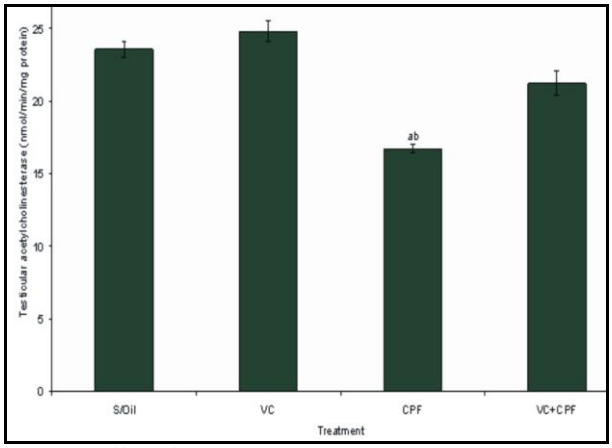

Figure 1. Effect of chronic exposure to soya oil (S/oil), vitamin C (VC) and/ or chlorpyrifos (CPF) on testicular AChE activity in adult male Wistar rats; [ab]$P < 0.05$ versus S/oil and VC groups.

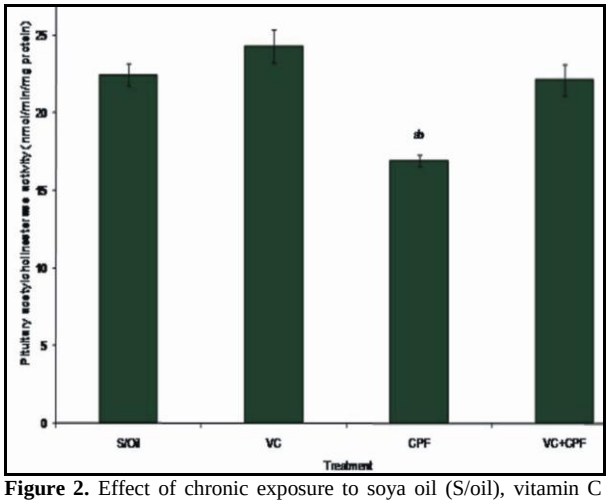

Figure 2. Effect of chronic exposure to soya oil (S/oil), vitamin C (VC) and/or chlorpyrifos (CPF) on pituitary AChE activity in adult male Wistar rats; [a]$P < 0.05$ vs VC+CPF, [b]$P < 0.01$ vs S/oil and VC groups.

Table 1. Values o FSH, LH, testosterone, epididymal sperm count and glycogen concentrations of rats in the study groups

	Parameters	Groups			
		S/oil	VC	CPF	VC + CPF
i	**FSH concentration (ng/ml)**	8.97 ± 0.68	10.13 ± 0.58	8.47 ± 1.2	8.9 ± 0.56
ii	**LH concentration (ng/ml)**	5.1 ± 0.42*****	7.7 ± 1.3******	4.1 ± 0.32	5.3 ± 0.38
iii	**Testosterone (ng/ml)**	5.9 ± 0.42*****	6.2 ± 0.34******	3.6 ± 0.15	9.5 ± 1.1******
iv	**Epididymal sperm count (x10^6/ml)**	43 ± 4******	60 ± 7.2******	7 ± 1.5	13 ± 1.8*****
v	**Glycogen concentration (g/100 g tissue)**	42 ± 1.2******	42 ± 2.1******	26 ± 1.5	38 ± 2.6******

Values are means ± SEM; *****P < 0.05 *vs* CPF or VC+CPF; ******P < 0.01 *vs* CPF or VC+CPF.

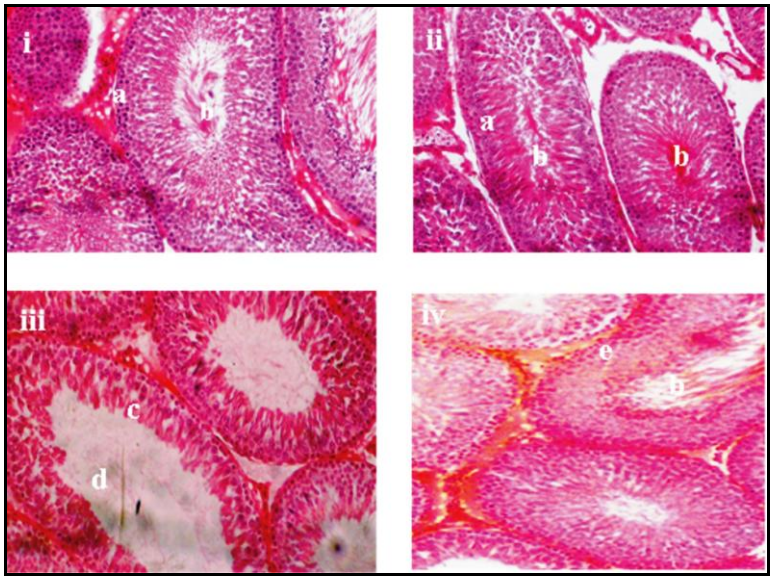

Figure 3.
Photomicrograph of sections of testes of rats dosed with S/oil (i),
vitamin C (ii),
chlorpyrifos (iii),
and Vitamin C + Chlorpyrifos (iv);
showing intact seminiferous tubules (a),
spermatozoa in the lumen of seminiferous tubules (b),
degenerative seminiferous tubules (c),
seminiferous tubules devoid of spermatozoa (d),
and relatively intact seminiferous tubules (e);
(H&E x 200).

Testicular histopathology

Fig.3 shows the effect of treatment on testicular histology. There was no apparent lesion in the testes of S/oil and VC groups. The testes of CPF group showed degeneration of the spermatogenic cells and seminiferous tubule with the lumen of the latter devoid of spermatozoa. There was a relative improvement in the testicular histoarchitecture in the VC+CPF with a relatively intact spermatogenic cells and seminiferous tubules, and an apparent increase in the number of spermatozoa in the lumen of seminiferous tubules compared to that of the CPF group.

DISCUSSION

The lacrimation and tremor recorded in rats exposed to CPF only are part of the cholinergic signs arising from inhibition of AChE leading to accumulation of ACh in the cholinergic receptors [24]. However, rats pretreated with vitamin C did not manifest any apparent toxic sign, which agrees with observations of previous workers [15]. The result indicates that vitamin C ameliorated the CPF-induced toxicosis, apparently due to its ability to restore AChE activity, which is reported in the present and those conducted previously [25]. Furthermore, the ability of vitamin C to increase the activity of paraoxonase-1 (PON1) [26], an esterase enzyme that aids in the detoxification of OPs [27] may have aided in the mitigation of the toxicosis.

The reduction in AChE activity in the pituitary glands and testes of rats exposed to CPF only in the present study is in consonance with findings of previous works in humans [28] and animals [29]. OPs, including CPF are known to bind to the serine portion of the AChE [29], a membrane-bound enzyme [30] causing its inhibition. In addition, the ability of CPF to induce testicular oxidative stress [12] may have altered the integrity of the testicular membrane, hence that of the membrane-bound enzymes, including AChE [31]. Tsakiris *et al* [32] established a relationship between hydroxyl radical and AChE inhibition. Ambali *et al* [33] have also shown the ability of vitamin C to restore

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AChE activity inhibited by CPF. Pretreatment with vitamin C restored the deficits in pituitary gland and testicular AChE activites. This may be partly due to the antioxidant activity of vitamin C, thereby scavenging for reactive oxygen species induced by CPF, hence preserving the membrane bound enzyme.

The reproductive functions in various species may be assessed from the alterations in plasma/or serum sex hormonal levels. Therefore, quantitative and qualitative estimations of both tissue and serum sex hormonal levels are a good assessor of reproductive integrity in both animals and man [34]. Significant decreases in the levels of serum sex hormones are known to be associated with suppressed reproductive functions. Exposure to several chemical agents has been reported to cause reproductive dysfunctions in different subjects [21].

The relatively lower FSH concentration recorded in the CPF group in the present study was in agreement with established findings of other workers [35]. The apparently lower FSH concentration may have been partly responsible for the low sperm count in the CPF group since FSH is actively involved in spermatogenesis [36].

The lower LH concentration in the CPF group agreed with the result obtained by previous workers [37]. Interference of CPF with LH and FSH concentrations may be due to its ability to suppress the gene involved in gonadotrophin synthesis or interfere with steroidogenesis [38]. The prolonged brain AChE inhibition by CPF, resulting in interference with neuro transmission may have suppressed the brain synthesis and/or release of gonadotropins (LH and FSH), apparently due to inhibition of their releasing hormone [39] from the hypothalamus. Therefore, the ability of OPs to disrupt the hypothalamo-pituitary endocrine function may have been responsible for the alterations in the concentrations of the gonadotropins [40]. The low LH concentration can have a far-reaching effect on the Leydig cells, involved in testosterone production in males [41]. Pretreatment with vitamin C apparently restored the FSH and LH concentrations. This shows that oxidative stress is partly involved in the low gonadotropin concentrations in the CPF group, perhaps due to protection of the hypothalamus and the pituitary glands from CPF-induced oxidative damage [12]. The ability of vitamin C to protect brain neurons and glial cells from CPF-induced lesions which has been demonstrated in our laboratory [42], may have improved the activity at the hypothalamo-pituitary axis, thereby aiding the gonadotropins synthesis and release. Furthermore, the ability of vitamin C to restore pituitary AChE activities may have improved neuronal activity, resulting in increased synthesis and/or release of the FSH and LH.

The significant reduction in the testosterone concentration in the CPF group agreed with the result obtained by Joshi *et al* [9] and Kang *et al* [37]. Similarly, Meeker *et al* [8] demonstrated an inverse association between 3,5,6-trichloro-2-pyridinol, a metabolite of CPF, and sperm count in humans. The low serum testosterone concentration may be linked to the inhibitory effect of OP insecticides on the secretion of pituitary gonadotropins (FSH and LH), which are involved in testosterone biosynthesis [9]. Reduced testosterone concentration may also occur due to direct damage to the Leydig cells [35]. Indeed, oxidative and degenerative changes in the testes of rats chronically exposed to CPF have been previously demonstrated in our laboratory [12]. Juxtaposed with this is the testicular degeneration evoked by CPF in the present study. Thus, the low testosterone concentration in the CPF group may be partly due to oxidative damage to the pituitary gland and the testicular tissues. The improvement in testosterone concentration in group pretreated with vitamin C showed the ameliorating effect of the antioxidant vitamin. The effect may be related to the enhancement of gonadotropin secretion and release due to reduced peroxidative damage to the pituitary gland and improvement in testicular tissue integrity, which were observed in the present study. Furthermore, the improved AChE activity in the pituitary gland of VC+CPF group apparently increases neuronal activity, thereby enhancing hormonal secretion.

The reduction in epididymal sperm count in the CPF group agreed with the result obtained by previous workers [9]. The lowered sperm reserves in the CPF group may be partly due to induction of oxidative stress in the testes [12], thereby providing unconducive environment for spermatogenesis in the seminiferous tubules. Besides, the spermatozoa on their own are highly susceptible to oxidative damage by excessive ROS due to the high concentration of polyunsaturated fatty acids within their plasma membrane [43]. Low concentrations of scavenging enzymes and glutathione [12, 44], and high production of free radicals, resulting from mitochondrial respiration and deficient DNA repair mechanisms [45] may have contributed to the deficit in sperm count in the CPF group. Although the spermatozoa require short exposure to low amounts of free radicals for spermatic capacitation and hyper-activation [44], the ROS must however be continuously inactivated to levels that will not be injurious to the spermatozoa. The spermatozoa possess an array of cellular antioxidant defense mechanisms such as SOD, catalase and ascorbic acid [46], but an increased oxidative challenge, as recorded during the CPF exposure in our previous studies [12] must have overwhelmed the antioxidant defense system, leading to oxidative damage in the testes. This may have

apparently led to acceleration of germ cell apoptosis, resulting in a decline in sperm count, and altered gonadal integrity and function [47]. The reduced sperm count in the CPF group may have also resulted from reduced testosterone concentration due to the low LH output and oxidative damage to the Leydig cells, since the hormone aids epididymal sperm maturation [43]. Similarly, the apparently low FSH concentration in the CPF group may have contributed to the low sperm count in this group since this hormone is important in spermatogenesis [36]. The degenerative changes in the seminiferous tubules and their lumen being devoid of spermatozoa confirmed the low sperm count in the CPF group in the present study. Pretreatment with antioxidant vitamin C has been shown in the present study to cause a relative increase in sperm count. This may be partly due to the antioxidant properties of the vitamin, which may have aided in maintaining the integrity of testes, especially the seminiferous tubules, Sertoli cells and the epididymis, thereby providing favourable environment for spermatogenesis and sperm maturation. Similarly, the improved LH, FSH and testosterone concentrations and restoration of the testicular AChE activity may have aided the improvement in the sperm count in the VC+CPF group.

The significant decrease in testicular glycogen concentration in CPF group agreed with result obtained from previous works [9]. Reduced glycogen level may be due to alterations in glucose metabolism induced by CPF [9, 48]. OPs have been shown to cause hyperglycemia [49], which may eventually result in reduced tissue glycogen concentration. The liver, which is the most important organ in glucose homeostasis, is an important target for CPF toxicity [48]. In addition, the attempt by the body to overcome the toxic stress induced by CPF has been shown to result in expenditure of energy, hence stimulation of glycogenolysis and gluconeogenesis [50]. The decrease in testicular glycogen content in the CPF group may also be due to its utilization in the detoxification of OPs or their metabolites through the process of glucuronidation, involving the interaction of toxic metabolites and glucose phosphate [51]. Although this requires further investigation, the consequence of low glycogen in similar processes has been shown to cause reduction in spermatozoa concentration [52]. Pretreatment with vitamin C has been shown to apparently restore the glycogen level. Ambali *et al* [49] have shown that vitamin C restored the deficit in pancreatic glycogen level evoked by co-administration of CPF and lead (Pb) in rats. The improvement in glycogen concentration may partly be due to the ability of the vitamin to protect the liver from CPF-induced toxic stress. Therefore, the body was spared of the enormous energy-utilizing process required to respond to toxic assaults. This, apparently, preserved the tissue glycogen from glycogenolysis, and thus restored the glycogen level.

In conclusion, vitamin C has been shown in this study to mitigate the reproductive toxicity evoked by chronic exposure to CPF in rats. Therefore, this vitamin may be used to mitigate male reproductive toxicity induced by CPF and by extension other OP insecticides. This may be partly due to its antioxidant and anticholinesterase restoration properties.

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CONFLICTS OF INTEREST

None were declared

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