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Alleviating effects of melatonin on oxidative changes in the testes and pituitary glands evoked by subacute chlorpyrifos administration in Wistar rats

Angela J Umosen¹, Suleiman F Ambali^{1,2*}, Joseph O Ayo², Bisala Mohammed³, Chidiebere Uchendu¹¹Department of Veterinary Pharmacology, Ahmadu Bello University, Zaria, Nigeria²Department of Veterinary Physiology, Ahmadu Bello University, Zaria, Nigeria³Department of Veterinary Pathology, Ahmadu Bello University, Zaria, Nigeria⁴Department of Veterinary Physiology and Pharmacology, University of Ilorin, Ilorin, Nigeria

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ABSTRACT

Objective: To evaluate the alleviating effects of melatonin on oxidative changes in the testes and pituitary gland induced by subacute chlorpyrifos (CPF) exposure in rats. **Methods:** Forty adult male Wistar rats divided into 4 groups of 10 animals were used for the study. Group I received soya oil (2 mL/kg) while group II was administered with melatonin (0.5 mg/kg). Group III was administered CPF only (8.5 mg/kg ~ 1/10th of the LD₅₀) while group IV was pretreated with melatonin (0.5 mg/kg) and then exposed to CPF (8.5 mg/kg), 10 min later. The regimens were administered by gavage once daily for a period of 28 d. At the end of the exposure period, the rats were sacrificed and the testicular tissues and pituitary glands were evaluated for the malonaldehyde (MDA) concentration and activities of superoxide dismutase (SOD) and catalase (CAT). **Results:** CPF increased MDA concentrations and reduced the activities of SOD and CAT in the testes and pituitary gland. Melatonin pretreatment reduced the testicular and pituitary MDA concentrations and improves the SOD and CAT activities. **Conclusions:** the study showed that subacute CPF-induced oxidative stress in the testes and pituitary glands were alleviated by melatonin due to its antioxidant property.

1. Introduction

The global increase in infertility, especially in Sub-Saharan Africa with the highest prevalence is becoming a source of concern[1]. In conjunction with this are reports that indicate a significant decrease in the quality of human semen[2–4]. It is estimated that male-related disorders are probably present in up to 40% to 50% of childless couples, alone or in combination with female factors[5,6]. Environmental chemical contaminants, including pesticides such as organophosphates (OP) insecticides have been largely implicated in this reproductive deficit[7]. There is increasing evidence to suggest an association between environmental exposure to OPs and adverse reproductive outcomes[8].

Chlorpyrifos (CPF; 0,0 – diethyl 0–3,5,6–trichloro–2–pyridyl phosphothionate) is a broad spectrum OP insecticide used in the control of a wide variety of insects, including fleas, wasps, bees, cockroaches and agricultural pests[9]. Association between low sperm concentration and serum concentrations of 3,5,6–trichloropyridinol, a metabolite of CPF has been reported in humans[10]. Similarly, studies using animal models have demonstrated reduced sperm concentrations and alterations in sex hormone following repeated CPF exposure[11,12]. There are several possible mechanisms for the anti-gonadal actions of OPs: they may exert a direct inhibitory action on the testis; they may affect the pituitary, causing changes in gonadotrophin concentrations and thus subsequent spermatogenic impairment; or they may change the concentration of neurotransmitters[13]. Like other OP insecticides, the principal mechanism of CPF toxicity is due to inhibition of acetylcholinesterase (AChE) activity, resulting in the accumulation of the neurotransmitter, acetylcholine (ACh), at the cholinergic receptors in the peripheral and central

*Corresponding author: Department of Veterinary Physiology and Pharmacology
University of Ilorin, Ilorin, Nigeria.

Tel: +2348037015411

E-mail: fambali2001@yahoo.com; atunluse@gmail.com

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nervous systems[9]. However, toxicity has been reported at doses that do not inhibit AChE[14,15]. Therefore, other mechanisms including the induction of oxidative stress have been implicated in CPF-induced toxicity[16–23]. During oxidative stress, the body's antioxidant mechanisms are overwhelmed by the oxidants, resulting in cellular damage. In this type of situation, therefore, supplementation with antioxidant molecules have been shown to mitigate oxidative stress and the consequent cellular damage[17–21].

Melatonin (N-acetyl-5-methoxytryptamine), the principal secretory product of the pineal gland, produced during the dark phase of the circadian cycle, is a highly conserved antioxidant molecule[24,25]. Melatonin along with its metabolites has been shown to scavenge reactive oxygen species (ROS) or reactive nitrogen species (RNS)[26]. This cascade reaction makes melatonin a highly effective antioxidant, even at low concentrations, in protecting cells from oxidative stress[27]. Melatonin diminishes the destruction of DNA, proteins and lipids that occur as a result of their reactions with ROS and RNS[28] as it easily crosses the cell membranes and the blood–brain barrier[29]. Since antigonadal action of OPs may involve the pituitary gland and testes couple with the established role of oxidative stress in the etiopathogenesis of OP-evoked toxicity, the present study was therefore aimed at evaluating the alleviating effect of melatonin on subacute CPF-induced oxidative stress in both organs in Wistar rats.

2. Materials and methods

2.1. Chemicals and preparation

Commercial grade CPF (20% EC; Termicot® Sabero Organics, Gujarat, India) was reconstituted to 1% solution in soya oil (Grand oil® Grand Cereal, Jos, Nigeria). Melatonin tablet (5 mg, NATROL, Inc. Chatsworth, USA) was prepared by dissolving in 10 ml of distilled water to make 0.5 mg/mL.

2.2. Animals and treatments

Forty young adult male Wistar rats weighing (126 ± 3) g were obtained from the Animal House of the Department of Veterinary Physiology and Pharmacology, Ahmadu Bello University, Zaria, Nigeria. They were housed in cages and fed on standard rat pellets and water was provided ad libitum. The rats were preconditioned for two weeks in the laboratory prior to the commencement of the study. They were weighed and divided at random into four groups, consisting of 10 rats per group. Group I (S/oil) was administered soya oil (2 mL/kg) while group II (Mel) was dosed with melatonin (0.5 mg/kg; Gultekin *et al*[30]). Group III (CPF) was administered CPF only (8.5 mg/kg ~ 1/10th of the LD₅₀; Ambali[31]), while group IV (Mel+CPF) was pretreated with melatonin (0.5 mg/kg), and then administered CPF (8.5 mg/kg) 10 min later. The regimens were administered by gavage once daily for a period of 28

days.

At the end of the treatment period, the rats were killed by jugular venesection after light chloroform anesthesia. The testes and pituitary glands were evaluated for malonaldehyde (MDA) and protein concentrations, and activities of superoxide dismutase (SOD) and catalase (CAT). The study was conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals[32].

2.3. Pituitary gland and testicular tissue preparations

The pituitary glands and the testicular tissue from each animal were rapidly removed and known weight of each of the organs was homogenized in a known volume of ice cold 20 mM phosphate buffer to obtain a 10% homogenate. This was then centrifuged at $3\,000 \times g$ for 10 min to obtain the supernatant. The supernatant was then used to assess the concentration of proteins and MDA, and activities of superoxide dismutase and catalase in both tissues.

2.4. Evaluation of the effect of treatments on pituitary gland and testicular protein concentrations

The protein concentration from supernatants prepared from the pituitary gland and testicular tissues were evaluated using the method of Lowry[33].

2.5. Evaluation of the effect of treatments on testicular tissue and pituitary gland lipoperoxidation

The level of thiobarbituric acid reactive substance, malonaldehyde (MDA) as an index of lipid peroxidation was evaluated in the testicular tissue and pituitary gland samples using the double heating method of Draper and Hadley[34]. The MDA concentrations in the testes and pituitary glands were calculated by the absorbance coefficient of MDA–TBA complex $1.56 \times 10^5/\text{cm}$ and expressed in nmol/mg of protein.

2.6. Evaluation of antioxidant enzymes activities in testicular and pituitary glands

Catalase and superoxide dismutase activities in the pituitary glands and testicular tissues were evaluated using commercial kits (Northwest Diagnostics of Virginia, U.S.A.) and then expressed as IU/mg of protein.

2.7. Statistical analysis

Data obtained were expressed as mean $\pm$ SEM and subjected to statistical analysis using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc multiple comparison test using Graphpad prism 4.0, San Diego, California, USA (www.graphpad.com). Values of $P < 0.05$ were considered significant.

3. Results

3.1. Effect of treatments on testicular and pituitary gland malonaldehyde concentrations

There was a significant increase ($P<0.01$) in the concentration of testicular MDA in the CPF group when compared to S/oil, Mel or Mel+CPF group. There was no significant change ($P>0.05$) in the MDA concentration in the

Figure 1. Effect of subacute soya oil (S/oil), chlorpyrifos (CPF) and/or melatonin (Mel) exposure on testicular malonaldehyde concentration in Wistar rats ($n=10$). ^{abc} $P<0.01$ vs S/oil, Mel and Mel+CPF groups, respectively.

Figure 2. Effect of soya oil (S/oil), chlorpyrifos (CPF) and/or melatonin (Mel) on pituitary gland's malonaldehyde concentration in Wistar rats ($n=10$). ^{abc} $P<0.01$ vs S/oil, Mel and Mel+CPF groups, respectively.

Figure 3. Effect of subacute soya oil (S/oil), chlorpyrifos (CPF) and/or melatonin (Mel) exposure on testicular superoxide dismutase activity in Wistar rats ($n=10$).

Mel+CPF group when compared to either S/oil or Mel group (Figure 1).

The pituitary MDA concentration in the CPF group was significantly ($P<0.01$) higher in the CPF group when compared to S/oil, Mel or Mel+CPF group. There was no significant change ($P>0.05$) in the MDA concentration in the Mel+CPF group when compared to either the S/oil or Mel group (Figure 2).

Figure 4. Effect of soya oil (S/oil), chlorpyrifos (CPF) and/or melatonin (Mel) on pituitary gland's superoxide dismutase activity in Wistar rats ($n=10$).

Figure 5. Effect of soya oil (S/oil), chlorpyrifos (CPF) and/or melatonin (Mel) on testicular catalase activity in Wistar rats ($n=10$). ^a $P<0.05$ vs Mel group.

Figure 6. Effect of soya oil (S/oil), chlorpyrifos (CPF) and/or melatonin (Mel) on pituitary gland's catalase activity in Wistar rats ($n=10$).

3.2. Effect of treatments on testicular and pituitary gland superoxide dismutase activities

The effect of subacute exposure to soya oil, CPF and/or melatonin on testicular SOD level is shown in Figure 3. There was no significant change ($P>0.05$) in activity level of SOD between the groups. However, CPF group had the lowest mean testicular SOD activity decreasing by 11% compared to that recorded in the S/oil group. The Mel+CPF group showed a comparatively higher (6%) testicular SOD activity compared to that of CPF or S/oil group but was comparatively lower (18%) than that of the Mel group.

There was no significant change ($P>0.05$) in pituitary gland activity level of superoxide dismutase between groups. However, CPF group had the lowest SOD activity decreasing by 36% compared to the S/oil group. The Mel+CPF group showed a comparatively higher (24%) mean pituitary gland SOD activity compared to the CPF group but was comparatively lower than those recorded in S/oil (32%) or Mel (32%) group (Figure 4).

3.3. Effect of treatments on testicular and pituitary gland catalase activities

The effect of treatments on testicular CAT activity is shown in Figure 5. There was a significant decrease ($P<0.05$) in testicular activity of CAT in the CPF group when compared to the Mel group, but no significant change ($P>0.05$) when compared to that of S/oil or Mel+CPF group. The CPF group had the lowest mean CAT activity decreasing by 27% compared to the S/oil group. The Mel+CPF group showed a comparatively higher testicular CAT activity (26%) compared to the CPF group but it was comparatively lower than those in S/oil (2%) or Mel (11%) group.

There was no significant change ($P>0.05$) in pituitary gland's CAT activity between the groups. However, the CPF group had the lowest mean pituitary gland's CAT activity decreasing by 7% compared to that of the S/oil group. The Mel+CPF group showed a comparatively higher pituitary gland's CAT activity (4%) compared to the CPF group but it was comparatively lower than that of the S/oil group (24%) and the same with that of the Mel (4%) group (Figure 6).

4. Discussion

The study had also shown the ability of CPF to increase the concentration of MDA in both the pituitary gland and the testes. This confirms the increase in MDA concentration following CPF exposure in previous studies[18–23,35]. MDA is a lipoperoxidation by-product resulting from interaction of oxygen radicals with polyunsaturated fatty acids residues in membrane phospholipids and has been shown to damage proteins and DNA[36]. The high MDA concentrations of the pituitary and the testicular tissues in the CPF group are indications of the level of lipoperoxidative changes,

reflecting alteration in structural and perhaps functional status of these organs. Lipid peroxidation involves the formation and propagation of lipid radicals, the uptake of oxygen and rearrangement of the double and unsaturated lipids, resulting in a variety of degraded products that eventually cause destruction of membrane lipids[37]. However, pretreatment with melatonin has been shown by the present study to decrease the testicular and pituitary MDA concentrations, indicating its anti-lipoperoxidative effect.

Decrease in the pituitary and testicular SOD activities were recorded in the CPF group. SOD, which catalyzes the dismutation of O_2^- to H_2O_2 is the first line antioxidant defense in the body. The decreased activity in the CPF group may be due to decreased synthesis, increased degradation or even outright inactivation of the enzyme. SOD is considered the most important antioxidant enzyme in the spermatozoa where it has a key physiological role in sperm motility[38]. Therefore, the low SOD activity in the testes recorded in the CPF group may also indirectly reflect its activity status in the spermatozoa. The resulting impairment of sperm motility may have contributed to the decrease in its fertilizing capacity reported in CPF exposure. The increase in both pituitary and testicular SOD activities in the group pretreated with melatonin is a demonstration of the ability of the antioxidant agent to protect the SOD from the ravaging effect of CPF-induced ROS through boosting of the antioxidant reserves in both the testes and the pituitary glands.

Similarly, the study also showed a comparative decrease in CAT activities in both the pituitary glands and the testes in the CPF group. CAT is known to neutralize H_2O_2 to H_2O and O_2 . The decline in the CAT activity observed in CPF group may be due to the reduced conversion of O_2^- to H_2O_2 by SOD, which then leads to the accumulation of O_2^- . This accumulation of O_2^- has been shown to inhibit the activity of CAT[39] as O_2^- converts ferrous state of CAT to ferryl state, which is an inactive form of the enzyme[40]. Several studies have shown that CPF reduced the activities of both SOD and CAT[41]. The ability of melatonin to improve CAT activity is a demonstration of the ability of this antioxidant molecule to boost the endogenous antioxidant reserve and protect CAT from the ROS induced by CPF.

These lipoperoxidative changes couple with low antioxidant status represented by low level of activity of SOD and CAT in the CPF group is an indication of oxidative stress. Oxidative stress may therefore be partly responsible for the alteration in the synthesis of some sex hormones and other substances essential for sustaining reproductive activity evoked by CPF and other OPs[11,12], culminating in low sperm count. Therefore, oxidative stress may have been partly involved in the male reproductive toxicity reported in CPF poisoning[11,12].

Melatonin has been found to protect cells, tissues and organs against oxidative damage induced by a variety of free radical generating agents and processes[42]. Melatonin

acts as a direct free radical scavenger^[43] and as an indirect antioxidant via its stimulatory actions on antioxidative enzymes^[44]. The radical-scavenging potency of melatonin is much greater than that of the most important endogenous radical scavenger, glutathione, and the classical hydroxyl radical scavenger, mannitol^[45]. Melatonin has also been reported to alter the activities of enzymes that improve the total antioxidative defense capacity of the organism^[46]. Its hydrophilic and lipophilic nature also aids in its free radical scavenging and antioxidant actions. Its lipophilic nature enhances the easiness at which it crosses the morphophysiological barriers, such as the blood–brain barrier, and then effectively enters the cells and subcellular compartments^[47,48]. It distributes in all cell compartments, being especially high in the nucleus and mitochondria^[49,50]. Furthermore, several metabolites such as 6-hydroxymelatonin and N-acetyl-N-formyl-5-methoxykynurenamine that are formed when melatonin neutralizes damaging reactants are themselves scavengers suggesting that there is a cascade of reactions that greatly increase the efficacy of melatonin in stymieing oxidative mutilation. Other processes suggested to contribute to melatonin's ability to reduce oxidative stress include stimulation of glutathione synthesis (an important antioxidant which is at high concentrations within cells), reducing electron leakage from the mitochondrial electron transport chain (which would reduce free radical generation), limiting cytokine production inflammatory processes (actions that would also lower toxic reactant generation), and synergistic effects with other classical antioxidants such as vitamins C and E^[52]. All these may have aided the improvement in the antioxidant status recorded in the group pretreated with melatonin.

In conclusion, the present study has shown that the induction of oxidative stress in the testes and pituitary glands evoked by CPF may have been partly responsible for the male reproductive toxicity reported in previous studies. Melatonin was able to mitigate the oxidative changes evoked by subacute CPF exposure partly due to its antioxidant property and therefore may be useful in protecting the pituitary gland and testicular tissue from toxicity induced by the OP insecticide.

Conflict of interest statement

We declare that we have no conflict of interest.

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