Effect of Melatonin on Chlorpyrifos-Induced Alterations in Reproductive Hormones and Semen Characteristics in Wistar Rats

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ABSTRACT

The study was conducted to determine the effect of subacute chlorpyrifos exposure on some hormonal and semen characteristics and, the ameliorative effects of melatonin. Twenty four adult male Wistar rats divided into 4 groups of 6 animals each were used for the study. Rats in group I (S/oil) received soya oil (2 ml/kg) while those in group II (Mel) were given melatonin (0.5 mg/kg). Rats in group III (CPF) were exposed to CPF only (8.5 mg/kg ~ $1/10^{th}$ of the LD_{50}) while rats in group IV (Mel + CPF) were pretreated with melatonin (0.5 mg/kg) and then exposed to CPF (8.5 mg/kg), 10 minutes later. The regimens were administered by gavage once daily for a period of 28 days. At the end of the study period, the rats were sacrificed, and sera obtained from the blood samples were analyzed for testosterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH). Testicular weight, testicular acetylcholinesterase (AChE) activity, epididymal sperm count and sperm motility were also evaluated. The results showed that pretreatment with melatonin ameliorated the CPF induced alterations in epididymal sperm count, sperm motility, testicular weight and levels of testosterone, FSH and LH. In conclusion, subacute CPF-induced alterations in sex hormones and semen characteristics were ameliorated by melatonin probably due to its antioxidant property.

Keywords: Chlorpyrifos, Melatonin, Follicle stimulating hormone, Luteinizing hormone, Testosterone, Semen characteristics.

INTRODUCTION

Pesticides usage has increased rapidly in the last fifty years due to intensification of farming in order to obtain higher yields. The over-dependence on chemicals not only resulted in a high cost of

production, but also irreparable damage to the environment and long term health problems in humans and animals[1]. One of such chemicals is the organophosphate (OP) compound. There is increasing evidence to suggest an association between environmental exposure to certain agricultural pesticides and adverse reproductive outcomes in men and women exposed to OPs[2].

Chlorpyrifos (CPF; 0, 0 – diethyl 0-3, 5, 6-trichloro-2-pyridyl thiophosphonate) is a broad spectrum OP pesticide used for agricultural and public health purposes[3]. Its principal mechanism of toxicity is the inhibition of acetylcholinesterase (AChE); however, the induction of oxidative stress has also been incriminated in its toxicity[4-6]. Occupational studies have shown significant associations for maternal as well as paternal exposure to chemicals like chlorpyrifos and adverse reproductive outcomes[7].

Animal studies have shown that antioxidants protect tissue from CPF-induced toxicity[4,8]. Recently, great attention has been given to antioxidants by a reason of their medical use. This is because antioxidants perform an important role in the maintenance of the integrity of the living organisms and the association of many human diseases with oxidative stress[9].

Melatonin (N-acetyl-5-methoxytryp-tamine), the principal secretory product of the pineal gland, produced during the dark phase of the circadian cycle, is a highly conserved antioxidant molecule. Melatonin along with its metabolites has been shown to scavenge ROS or RNS[10]. This cascade reaction makes melatonin highly effective, even at low concentrations, in protecting cells from oxidative stress[11]. Melatonin is also known to easily cross cell membranes and the blood-brain barrier[12]. Although the effects of CPF on reproductive hormones and sperm quality have been evaluated, there is paucity of information on the protective role of antioxidant, melatonin on CPF-induced toxicity. The present study was undertaken to evaluate the effect of subacute CPF exposure on some reproductive hormones and semen characteristics, and the ameliorative effect of melatonin.

MATERIALS AND METHODS

Chemicals, Animals and Treatments

Commercial CPF (20% E.C) (Termicot® Sabero Organics, Gujarat, India) were reconstituted to 1% solution in soya oil, while melatonin tablet (5 mg, NATROL, Inc. Chatsworth, USA) was dissolved in 10 ml of distilled water (0.5 mg/ml).

Twenty four young adult male Wistar rats, 7-8 weeks of age and weighing between 101-132 g obtained from the animal house of the Department of Veterinary Pharmacology and Toxicology, Ahmadu Bello University, Zaria were used for the study. They were housed in cages in the Laboratory of the Department and given access to standard rat chow and water was provided *ad libitum.*

Experimental groupings

The rats were weighed and divided into four groups, consisting of 6 rats per group. Rats in Group I (S/oil) was dosed with soya oil, 2 ml/kg. Rats in group II (Mel) were dosed with melatonin dissolved in water (0.5 mg/kg)[13], while those in group III (CPF) were given CPF only (8.5 mg/kg or $1/10^{th}$ of the LD_{50} previously determined). Rats in group IV (Mel + CPF) were pretreated with melatonin (0.5 mg/kg), and CPF (8.5 mg/kg) was administered 10 min later. The regimens were administered by gavage once daily for a period of 28 days. At the end of the study period, the rats were sacrificed by jugular venesection after light ether anaesthesia and blood samples were collected from each rat into test tubes. Thereafter, the blood was allowed to clot

and then centrifuged at 800 × g for 10 min to obtain the sera used for analysis of testosterone, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). The testicles were harvested, weighed and used to determine AChE levels. The study was carried out according to the specification of the Ahmadu Bello University Animal Research Committee and Guide to the Care and Use of Laboratory Animals[14].

Determination of Concentration of Follicle-Stimulating Hormone, Luteinizing Hormone and Testosterone

The concentrations of serum FSH, LH and testosterone were assayed using their enzyme immunoassay (EIA) kits (Syntron Bioresearch Inc., Carlsbad, U.S.A.).

Evaluation of the Effect of Treatments on Testicular Acetylcholinesterase Activity

Acetylcholinesterase activity in the testes was determined by Ellman[15], using acetylthiocholine iodide as a substrate. Briefly, the testes of each animal were weighed using the mettle weighing balance (Mettler® P161, Mettler instrument AG, CH806, Geifensee, Zurich, Switzerland) and then homogenized in cold (0–4 °C) 20 mM phosphate-buffered saline, incubated with 0.01M5,5-dithio-bis(2-nitrobenzoic acid) in 0.1 M phosphate-buffered saline, pH 7.0. Incubations were allowed to proceed at room temperature for 10 min. Then, acetylthiocholine iodide (0.075 M in 0.1 phosphate-buffered saline, pH 8.0) was added to each tube, and absorbance at 412 nm was measured continuously for 30 min. Each testis in every group were prepared in triplicate and acetylcholinesterase activity calculated based on the rate of colour change per minute, using the extinction coefficient of 1.36×10^4 expressed as nmoles/min/mg protein.

Determination of Some Sperm Characteristics and Epididymal Sperm Count

The testes were removed, and semen carefully collected from the caudal epididymis onto a glass slide. Percentage of mass motile spermatozoa was assessed immediately under light microscope at x 100[16]. The epididymal sperm count was also evaluated using the right caudal epididymis[17]. Counting was done using an improved Neubaeur counting chamber under a light microscope at the magnification of x 400.

Statistical analysis

Values obtained were expressed as mean ± SEM and subjected to statistical analysis using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc multiple comparison test. Graphpad prism version 4.0 (San Diego, California, USA) was used for the analyses. Values of $P < 0.05$ were considered significant.

RESULTS

There was no significant change ($P > 0.05$) in testicular AChE activity between the groups. However, CPF group had the lowest activity, decreasing by 12% compared to the S/oil group. The Mel + CPF group showed a comparatively higher testicular AChE activity compared to the CPF (34%), Mel (19%) and S/oil (26%) groups (Figure 1).

There was no significant change ($P > 0.05$) in testicular weight between the groups. However, CPF group had the lowest testicular weight, decreasing by 22% compared to the S/oil group. The Mel + CPF group showed a comparatively higher testicular weight (22%), compared to the CPF group, with no significant difference when compared with the S/oil and Mel group (Figure 2).

There was no significant change (P > 0.05) in FSH concentration between the groups. However, Melatonin group had the highest concentration, increasing by 20% compared to the S/oil. However, the Mel + CPF group showed a comparatively lower concentration (11%) compared to the Melatonin group (20%) but it was comparatively higher than that in the CPF (5%) group (Figure 3).

There was also no significant change (P > 0.05) in luteinizing hormone concentration between the groups. However, CPF group had the lowest concentration, decreasing by 11% compared to the S/oil group. The Mel + CPF group showed a comparatively higher serum LH concentration (17%) compared to the CPF group and even when compared to those in S/oil and Mel (13%) group (Figure 4).

There was no significant change (P > 0.05) in testosterone concentration between the groups. However, CPF group had the lowest concentration, decreasing by 16% compared to the S/oil group. The Mel + CPF group showed a comparatively higher serum testosterone concentration (6%) compared to the CPF group, but it was comparatively lower than those in the S/oil and Mel (15%) group (Figure 5).

There was a significant decrease in sperm concentration in the CPF group when compared to either S/oil (P < 0.01), Mel (P < 0.05) or Mel + CPF group (P < 0.05). The CPF group had the lowest sperm concentration, decreasing by 81% compared to the S/oil group. The Mel + CPF group showed a comparatively higher sperm concentration (31%) compared to the CPF group, but it was comparatively lower than that in the S/oil group and at *par* with the Mel group (31%) (Figure 6).

There was also a significant decrease in sperm motility in the CPF group when compared to either S/oil (P < 0.001), Mel (P < 0.01) or Mel + CPF (P < 0.01) group.

However, CPF group had the lowest sperm motility, decreasing by 60% compared to the S/oil group. The Mel + CPF group showed a comparatively higher sperm motility (51%) compared to the CPF group, but it was comparatively lower than those in the S/oil (23%) and Mel (3%) group (Figure 7).

DISCUSSION

The study revealed a comparatively lower AChE activity in the testis of rats in the CPF group, indicating a higher level of AChE inhibition in the group. This agrees with the findings of Shittu *et al*.[18]. The alteration in the AChE activity may be partly responsible for the depreciating level of spermatogenesis recorded in the CPF group. Pretreatment with melatonin improved the AChE activity in the testes. Indeed, some antioxidants such as vitamin C and E have been shown to increase AChE activity following its inhibition by OPs[19]. The restoration of AChE activity by melatonin may, therefore, be partly responsible for the improvement in the sperm concentration recorded in the Mel + CPF group.

The comparatively lower testicular weight recorded in the CPF group demonstrates the adverse effect of CPF on testicular weight changes. Significant decrease in testicular weight following CPF intoxication was recorded by Joshi *et al*.[7] and Afaf *et al*.[20]. Similarly, Colborn *et al*.[21] showed that impairment in the release of LH by OPs was responsible for the gonadal changes. Furthermore, spermatogenic arrest and inhibition of steroid biosynthesis in Leydig cells may have contributed to the reduced testicular weight [22]. In the present study, pretreatment with melatonin was shown to ameliorate the decrease in weight induced by CPF, probably due to antioxidant and tissue protecting properties of melatonin.

The low FSH concentration in the CPF treated rats obtained in the present study is consistent with those recorded by Zidan *et al*.[23] and Shittu *et al*.[18]. This decrease in FSH concentration may be due to the effect of the OP insecticide on the pituitary gland and hypothalamus, as increased lipoperoxidation of the pituitary gland was reported in our previous report[5]. The effect of this is the impairment in GnRH synthesis and low FSH output by the hypothalamus and pituitary gland, respectively.

The present study showed a decrease in serum LH concentration in the CPF group compared to the other groups. Significant decrease in LH concentration has been recorded following CPF-methyl and CPF exposure, in Wistar rats by Zidan *et al*.[23] and Shittu *et al*.[24], respectively. The oxidative damage to the hypothalamus, the center responsible for the secretion of gonadotrophin-releasing hormone (GnRH) which stimulates LH and FSH release by the pituitary gland may partly play a significant role in the LH deficits in the CPF group. OP insecticides such as CPF, fenthion, fenithrithion and dimethoate have been shown to act like androgen receptor antagonists or suppressors of gene expression related to gonadotrophin synthesis (LH and FSH) or steroido-genesis[25]. Recio *et al*.[26] showed that LH and FSH are mostly affected by OP-induced hypothalamic-pituitary disruption. The rise in LH concentration in the melatonin pretreated rats may be due to the antioxidant effect of the agent, protecting the pituitary gland and the hypothalamus from CPF-induced oxidative changes.

The comparatively low testosterone concentration observed in the CPF group compared to the control has been reported in previous studies[7,23,27]. Similarly, Meeker *et al*.[28] showed a direct relationship between concentrations of testosterone and that of 3, 5′- trichloropyridinol, the metabolite of CPF in humans. This decrease in serum testosterone concentration may be due to low serum LH concentration in the CPF-treated rats. This is because circulating LH is responsible for maintaining normal testosterone concentration by stimulating Leydig cells to release testosterone[29]. Krause[30] reported that the decreased testosterone might be due to direct damage to Leydig cells or to a lowered stimulation of these cells by LH. It has also been shown that OPs cause decline in serum steroid hormone levels by increasing steroid catabolism and elimination or directly inhibiting steroid hormone production[31]. The cumulative effect of the responses was a decrease in the concentrations of steroidal hormones, LH, FSH and testosterone. Thus, the improvement in testosterone concentration observed in the group pretreated with melatonin may be due to a relative increase in serum LH and the antioxidant effect of melatonin.

The sperm count is one of the most important factors that affect fertility[32]. The present study recorded a significant low caudal epididymal sperm count in the CPF group compared to the values obtained in other groups. This finding agrees with those of other workers following intoxication with CPF[24,27,33]. This may be due to low FSH, LH and, subsequently, testosterone concentra-tions in the CPF group. Testosterone is required for differentiation of sex organs and production of sperms[34]. Maintaining serum LH concentration is critical for initiating and supporting spermatogenesis and even fertility[35], hence the lowered serum LH in the CPF group may be involved in the impairment of spermatogenesis. In fact, circulating FSH has long been considered a valuable marker of Sertoli cell function and spermatogenesis[36]. Sertoli cells, the major epithelial components of the seminiferous epithelium, are essential for the control of

spermatogenesis, because they supply nutrients which ensure germ cell proliferation and differentiation[37]. Many Sertoli cell functions are, however, regulated by FSH. In the testis, spermatogenesis takes place in the tubular compartment, and steroidogenesis in the interstitial compartment. Despite being anatomically separated, both compartments are closely connected with each other in a paracrine manner. The integrity of both compartments is necessary for quantitative and qualitative normal sperm production[38]. Therefore, lower sperm count in the CPF group may be due to suppression of gonadotrophin[39], and the direct and indirect effects (due to oxidative damage) on the testicular elements such as seminiferous tubules, especially the Sertoli and Leydig cells. In addition, CPF-induced oxidative damage directly to the spermatozoa may alter its genomic integrity, even with rupture of DNA strands[40]. Melatonin was able to improve the CPF-induced sperm count deficit, perhaps due to its antioxidant effect. Melatonin has been shown to have effects on the testicular metabolism in adult rats, suggesting that it plays some role in the modulation of the testicular function[41].

The result also showed a decrease in the sperm motility in the group exposed to CPF only. This finding agrees with those observed in mice[7] and in rats[27,33] following CPF intoxication. Sperm motility is an important functional sperm characteristic which is of value in predicting sperm fertilizing capacity, and it has been used extensively as a marker of seminal vesicle function[42]. Any perturbation on sperm motility would seriously affect the fertilizing ability[43]. Marked inhibition of sperm motility recorded in the CPF group may also be due to low level of ATP content[44]. Sperm motility may be affected by altering enzymatic activities of oxidative phospholytic process. Oxidative phospholytic process is required for adenosine triphosphate (ATP) production, a source of energy for the forward movement of spermatozoa. Full ATP pool is crucial for normal spermatozoa movement; and a slight deprivation of ATP leads to reduction in motility, which may cause infertility[45]. The observed decrease in sperm motility could also be attributed in part to the concomitant abnormality of the sperm. Pretreatment with melatonin restored the CPF-evoked decrease in sperm motility. This may be due partly to the ability of the antioxidant to protect the sperm from oxidative damage and provide a relatively good environment in the epididymis for sperm maturation. In conclusion, CPF induced alterations in reproductive hormones and some sperm characteristics in subacute exposure Wistar rats, and supplementation with melatonin was able to minimize the changes induced by CPF.

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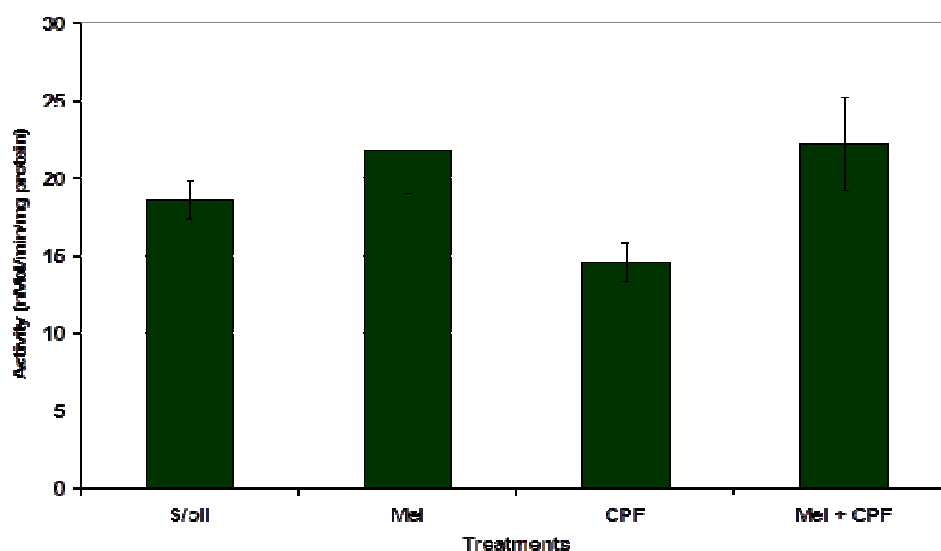
Figure 1. Effect of soya oil, chlorpyrifos and/or melatonin on testicular acetylcholinesterase activity in Wistar rats (n= 6)
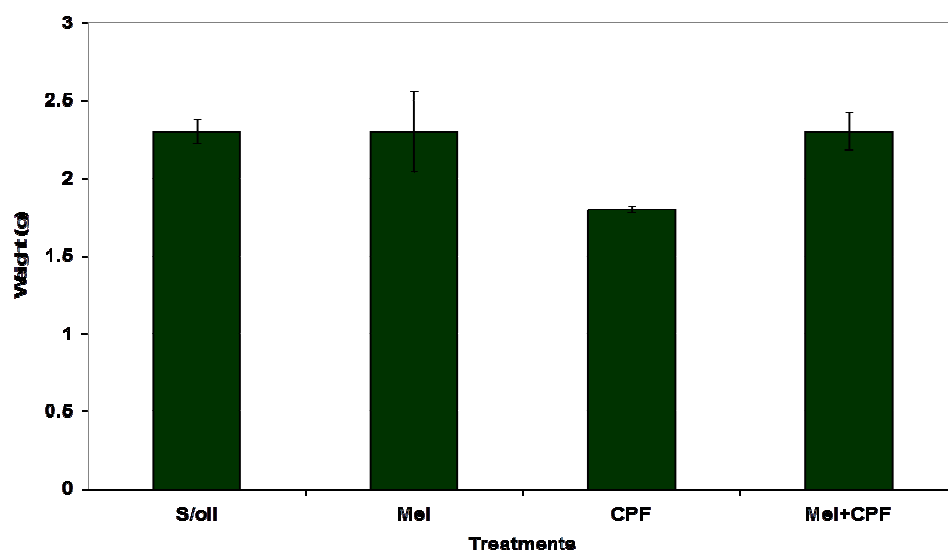
Figure 2. Effect on testicular weight in Wistar rats dosed with soya oil, CPF and/or melatonin (n= 6)
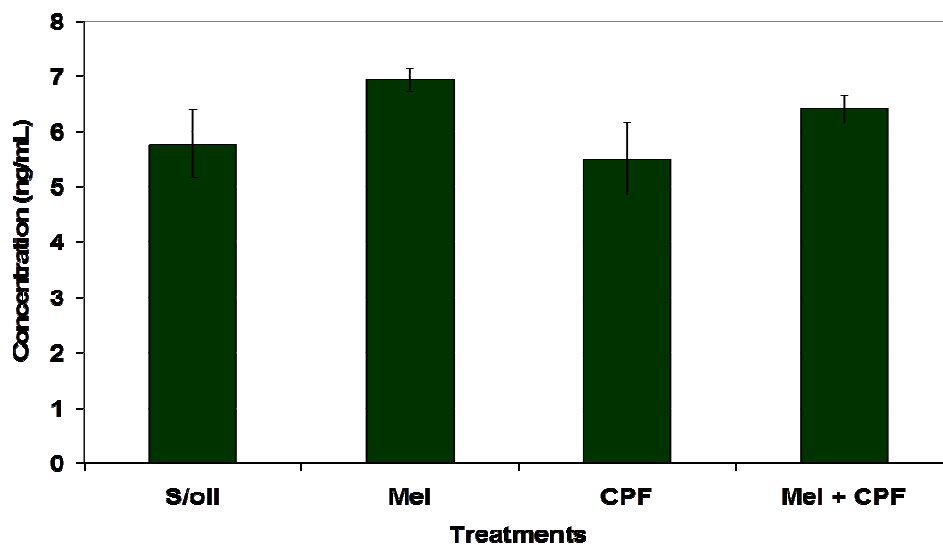

Figure 3. Effect of soya oil, chlorpyrifos and/or melatonin on follicle stimulating hormone concentration in Wistar rats (n= 6)
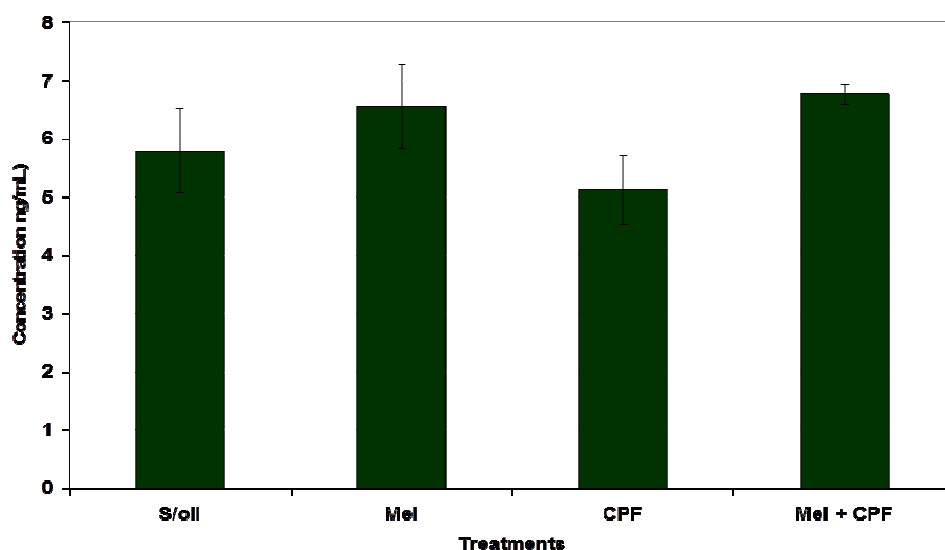
Figure 4. Effect of soya oil, Chlorpyrifos and/or melatonin on luteinizing hormone concentration in Wistar rats (n= 6)
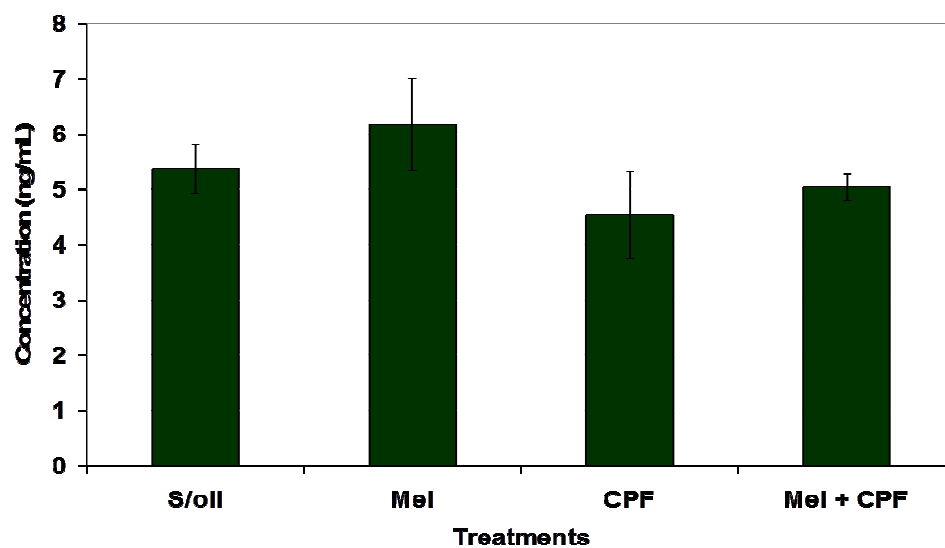

Figure 5. Effect of soya oil, chlorpyrifos and/or melatonin on testosterone concentration in Wistar rats (n= 6)
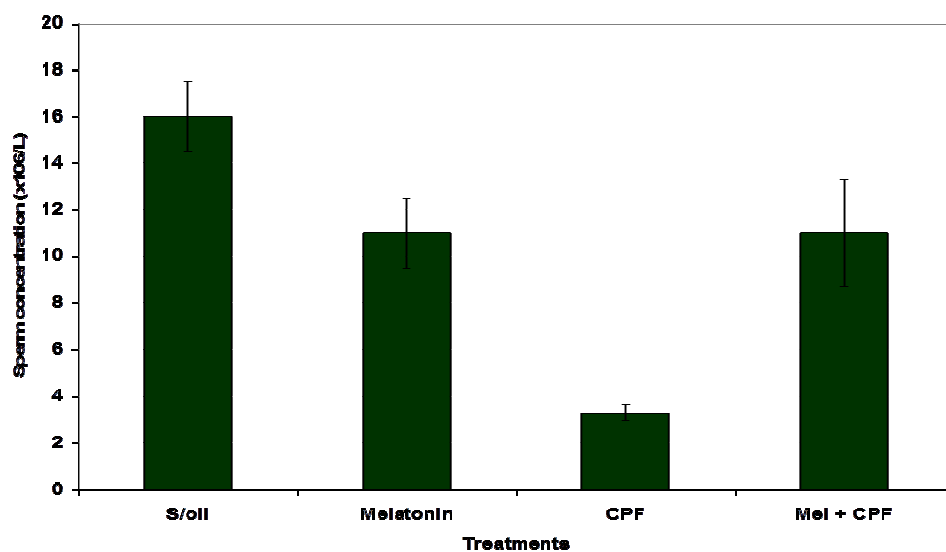
Figure 6. Effect on sperm concentration in Wistar rats dosed with soya oil, CPF and/or melatonin (n= 6). [a]Value in CPF group significantly lower compared to S/oil (P < 0.01), Mel (P < 0.05) and Mel + CPF (P < 0.05) groups, respectively
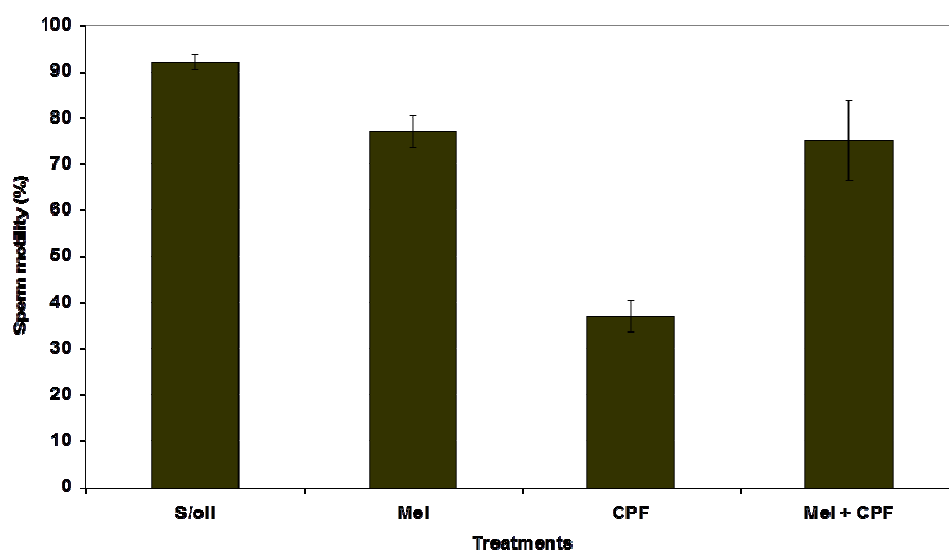

Figure 7. Effect on sperm motility in Wistar rats dosed with soya oil, CPF and/or melatonin (n= 6). [a]Value in CPF group significantly lower compared to S/oil (P < 0.001), Mel (P < 0.01) and Mel + CPF (P < 0.01) groups, respectively