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Full Length Research paper

The attenuating effect of zinc on *Propoxur*-induced oxidative stress, impaired spermatogenesis and deranged steroidogenesis in wistar rat

¹Oyewopo, A.O., ²Saalu, L.C., ³Osinubi, A.A., ⁴Imosemi, I.O., ¹Omotoso G.O., ¹Adefolaju G.A.

Department of Anatomy, University of Horin, Horin, Nigeria.

²Department of Anatomy, Lagos State University College of Medicine, Ikeja, Nigeria. ³Department of Anatomy, College of Medicine, University of Lagos, Lagos, Nigeria.

⁴Department of Anatomy, University of Ibadan, Ibadan, Nigeria.

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Propoxur (PPX), a carbamate pesticide is known to cause reproductive toxicity partly through induction of cellular oxidative stress. In the present study, the ability of Zinc (Zn), a known antioxidant to ameliorate testicular oxidative stress was investigated in adult male Wistar rats exposed to PPX (10 mg/kg body weight per day) orally for 30 days. The results from this study showed that there was a significant reduction (p < 0.05) in the growth rate and relative weights of testes of PPX-exposed rats compared to the control. PPX-exposed rats also displayed a significant (p < 0.005) decreases in plasma testosterone levels, epididymal sperm count and spermatozoa motility when compared to the control values. Furthermore, there was a statistically significant increase in malondialdehyde (MDA) and a significant (p < 0.005) decrease in the activities of Glutathione Peroxidase (GPx) in the testes of PPX-exposed animals. Zn supplementation in the *PPX*-exposed rats restored the activities of testicular GPx and the testicular content of MDA to the levels of the control group. Co-treatment with Zn also normalized the deranged sperm parameters and hormonal profiles. We conclude that Zn administration minimized the evidences of testicular oxidative damage and reversed the impairment of spermatogenesis and steroidogenesis induced by *PPX* in the rat.

Key words: Propoxur, oxidative stress, rat, testosterone, zinc.

INTRODUCTION

Propoxur (PPX) is an active agent of insecticide called baygon, which was introduced in 1959 (Cincinnati, 1986). PPX (2-isopropoxy-phenyl-N-methylcarbamate), a nonsystemic carbamate insecticide replaced Dichloro Diphenyl Trichloro ethane (DDT) in the control of black flies and mosquitoes (Hallenbeck and Barns, 1986), Presently it is used against mosquitoes in outdoor areas, against flies in agricultural settings, against fleas and ticks on pets, as an acaricide, on lawns and turf for ants, on flowering plants, and in private dwellings and public buildings. It is also used as a molluscicide. It is effective against cockroaches, aphids and leafhoppers (EXTOXNET, 1996). Because widespread use of PPX in public health programmes and households, health hazards resulting from this situation are presently inevitable (Sanvidhan et al., 2006). It is in this regards

that PPX has generated considerable interest.

Like all N- methyl carbamates and organophosphorous insecticides, propoxur is believed to act by inhibiting acetylcholinesterase activity (Baron, 1991; Timbrell, 2000). This results in accumulation of acetylcholine in the synapse, which in turn can stimulate cholinergic receptors producing both nicotinic and muscarinic effects in the organism, such as muscle contractions and secretion in many glands (Taylor, 1990). The biochemical mechanism by which PPX mediate cytotoxicity is however currently unclear. It has however been postulated that its action may be connected with increased generation of free radical and reactive oxygen species (ROS), together with alteration of enzymatic antioxidative systems such as glutathione redox system (Sanvidhan et al., 2006). The ultimate effect of all these pathways is increased oxidative stress and cellular toxicity.

Zinc (Zn) is a trace element essential for living organisms. More than 300 enzymes require Zn for their

^{*}Corresponding author E-mail: chiasaalu@hotmail.com; Tel: 234-8033200876

activity. It also plays an important role in the DNA protein replication, transcription, and synthesis, influencing cell division and differentiation (Anderson and Desnick, 1979). It has been noted that Zn has a relationship with many enzymes in the body and can prevent cell damage through activation of the antioxidant system (Angel et al., 1986a; Angel et al., 1986b; Arroyo et al., 1987). Zinc is an essential component of the oxidant defense system and functions at many levels (Powell, 2000; Ozturk et al., 2003; Ozdemir and Inanc, 2005). One study has shown that Zn deficiency in the diet paves the way for cell damage in the rat testis (Shaheen and El-Fattah, 1995). Furthermore, Zn deficiency increases lipid peroxidation in various rat tissues, whereas the Zn supplementation corrects the impairment (Saxena et al., 1989). Even though the biochemical process by this beneficial role of Zn is still under investigation, its acute effects are generally thought to involve two mechanisms: protection of protein sulfhydryls or reduction in the formation of OH from H₂O₂ through the antagonism of redox-active transition metals, such as iron and copper (Bray and Bettger, 1990). However, virtually all of the beneficial effects of long-term administration of zinc can be linked to the induction of some other substance that serves as the ultimate antioxidant. In this regard, the most studied effectors are the metallothioneins.

The metallothioneins are a group of low-molecular-weight (6000–7000 kDa) metal- binding proteins containing 60–68 amino acid residues, of which 25–30% are cysteine. They contain no aromatic amino acids or disulfide bonds and can bind 5–7 g zinc (mol/protein) (Bernhard et al. 1987; Kagi and Kojima 1987; Kagi and Hunziker 1989). The metallothioneins have been shown to have antioxidant effects under a variety of conditions, including radiation exposure (Matsubara 1987), toxicity from anticancer drugs such as doxorubicin (Satoh et al. 1988, Yin et al. 1998) and others (Lazo and Pitt 1995; Lazo et al. 1998), ethanol toxicity (Harris, 1990) and oxidatively mediated mutagenesis (Rossman and Goncharova, 1998).

Many reports exist in the literature evaluating the ability of several antioxidants to protect various organs from PPX-induced toxicity. None however utilized Zn, an important element in the oxidative defense system.

The present study was designed to investigate the capability of Zn to reverse PPX-induced effects on the testicular oxidative status, spermatogenesis, and steroidogenesis in the rat.

MATERIALS AND METHODS

Chemicals

Technical grade (99.4% purity) propoxur was obtained from M/s Bayer AG, Monheim, Gernany. Zinc chloride was obtained from Merck, Darmstadt, Germany and olive oil was from Roberts Laboratories Limited, Belton, England.

Animals and interventions

Experimental procedures involving the animals and their care were conducted in conformity with International, National and institutional guidelines for the care of laboratory animals in Biomedical Research and Use of Laboratory Animals in Biomedical Research as promulgated by the Canadian Council of Animal Care (CCAC, 1985). Further the animal experimental models used conformed to the guiding principles for research involving animals as recommended by the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals (American Physiological Society, 2002).

The rats were procured from a breeding stock maintained in the Animal House of Lagos State University College of Medicine (LASUCOM). The animals were housed in well ventilated wirewooden cages in the Animal Facility of the Department of Anatomy, LASUCOM, Ikeja, Lagos

An approval was sought and obtained from the Departmental adhoc Ethical Committee. The rats were maintained under standard natural photoperiodic condition of twelve hours of light alternating with twelve hours of darkness (i.e. L: D; 12:12) with room temperature of between 25°C to 26°C and humidity of 65 ± 5 %. They were allowed unrestricted access to water and rat chow (Feedwell Livestock Feeds Ltd, Ikorodu, Lagos, Nigeria). They were allowed to acclimatize for 28 days before the commencement of the experiments. The weights of the animals were estimated at procurement, during acclimatization, at commencement of the experiments and twice within a week throughout the duration of the experiment, using an electronic analytical and precision balance (BA210S, d= 0.0001 g) (Satorius GA, Goettingen, Germany).

Forty male adult (11 to 13 weeks old) Wistar rats weighing 185-210 g were used for this research work. The rats were randomly divided into four groups of ten rats each such that the average weight difference between and within groups did not exceed ± 20% of the average weight of the sample population. Group A received daily olive oil 0.5 ml/kg body wt, (orally). Group B animals were given intraperitoneal (ip) normal saline 0.5 ml/kg body wt/day. Porpoxur was dissolved in olive oil of pharmacological quality and administered orally to group C animals at a dose of 10 mg/kg/day using syringe and 20 gauge Ryle's tube in a dose volume of 10 ml/kg. Volumes were adjusted weekly for body weight.

Zinc chloride was ground in mortar-pestle, suspended in normal saline and given to group D at a dose of 20 mg/kg body wt/day, (ip). Group E animals received daily dose of both porpoxur (10 mg/kg body wt/orally) and Zinc (20 mg/kg body wt; ip). The rats were treated with vehicle or test dose chemicals for 30 days.

Animal sacrifice and sample collection

The rats were at the time of sacrifice first weighed and then anaesthetized by placing them in a closed jar containing cotton wool sucked with chloroform anaesthesia. Blood sample was collected from the heart of each rat immediately after sacrifice with the aid of a 21G needle mounted on a 5 mL syringe (Hindustan Syringes and Medical Devices Ltd., Faridabad, India). The blood samples were collected into tubes containing 2% sodium oxalate and centrifuged at 3000 rpm for 10 minutes using a table top centrifuge (P/C 03) and the serum extracted.

The abdominal cavity was opened up through a midline abdominal incision to expose the reproductive organs. Then the testes were excised and trimmed of all fat. The testes weights of each animal were evaluated .The testes were weighed with an electronic analytical and precision balance (BA 210S, d=0.0001-Sartoriusen GA, Goettingen, Germany). The testes volumes were measured by water displacement method. The two testes of each rat were measured and the average value obtained for each of the two parameters was regarded as one observation. Serum and the

testes of each animal were stored at – 25°C for biochemical assays.

Sperm characteristics

The testes from each rat were carefully exposed and removed. They were trimmed free of the epididymides and adjoining tissues.

Epididymal sperm concentration: Spermatozoa in the right epididymis were counted by a modified method of Yokoi and Mayi, (2003). Briefly, the epididymis was minced with anatomic scizzors in 5mL physiologic saline, placed in a rocker for for 10 minutes, and allowed to incubate at room temperature for 2 minutes. After incubation, the supernatant fluid was diluted 1:100 with solution containing 5 g sodium bicarbonate and 1 mL formalin (35%). Total sperm number was determined by using the new improved Neuber's counting chamber (haemocytometer). Approximately 10 µL of the diluted sperm suspension was transferred to each counting chamber of the haemocytometer and was allowed to stand for 5 minutes. This chamber was then placed under a binocular light microscope using an adjustable light source. The ruled part of the chamber was then focused and the number of spermatozoa counted in five 16-celled squares. The sperm concentration was the calculated multiplied by 5 and expressed as [X] x 10⁶ /ml, where [X] is the number of spermatozoa in a 16-celled square.

Sperm progressive motility: This was evaluated by an earlier method by Sonmez et al, (2005). The fluid obtained from the left cauda epididymis with a pipette was diluted to 0.5 mL with Tris buffer solution. A slide was placed on light microscope with heater table, an aliquot of this solution was on the slide, and percentage motility was evaluated visually at a magnification of x 400. Motility estimates were performed from three different fields in each sample. The mean of the three estimations was used as the final motility score. Samples for motility evaluation were stored at $35^0 c$.

Sperm morphology: The sperm cells were evaluated with the aid of light microscope at x 400 magnification. Caudal sperm were taken from the original dilution for motility and diluted 1:20 with 10% neutral buffered formalin (Sigma-Aldrich, Oakville, ON, Canada). Five hundred sperm from the sample were scored for morphological abnormalities (Atessahin *et al.*, 2006). Briefly, in wet preparations using phase-contrast optics, spermatozoa were categorized. In this study a spermatozoon was considered abnormal morphologically if it had one or more of the following features: rudimentary tail, round head and detached head and was expressed as a percentage of morphologically normal sperm.

Testosterone assay

Radioimmunoassay (RIA) for serum and testicular testosterone was carried out with a testosterone 125I RIA Kit (ICN, Biochemical, Immunotech, Marseille, France) according to the manufacturer's protocol. Radioactivity was determined by gamma scintillation counting. All samples were run in duplicate in a single assay to avoid interassay variation.

Assay of glutathione peroxidase (GPx) activity

Glutathione peroxidase activity was measured by the method described by Rotruck et al. (1973). The reaction mixture contained 2.0 ml of 0.4M Tris- HCl buffer, pH 7.0, 0.01 ml of 10mM sodium azide, 0.2 ml of enzyme. 0.2 ml of 10mM glutathione and 0.5 ml of 0.2mM. H₂O₂. The contents were incubated at 37 $^{\circ}$ C for 10 minutes followed by the termination of the reaction by the addition of 0.4 ml 10% (v/v) TCA, centrifuged at 5000 rpm for 5 minutes. The absorbance of the product was read at 430nm and expressed as nmol/mg protein.

Estimation of lipid peroxidation (Malondialdehyde)

Lipid peroxidation in the testicular tissue was estimated calorimetrically by thiobarbituric acid reactive substances TBARS method of Buege and Aust (1978). A principle component of TBARS being malondialdehyde (MDA), a product of lipid peroxidation. In brief, 0.1 ml of tissue homogenate (Tris-Hcl buffer, pH 7.5) was treated with 2 ml of (1:1:1 ratio) TBA-TCA-HCl reagent (thiobarbituric acid 0.37%, 0.25 N HCl and 15% TCA) and placed in water bath for 15 min, cooled. The absorbance of clear supernatant was measured against reference blank at 535nm. Concentration was calculated using the molar absorptivity of malondialdehyde which is $1.56 \times 10^5 \, \mathrm{M}^{-1} \, \mathrm{cm}^{-1}$ and expressed as nmol/mg protein.

Statistical analysis

All data were expressed as mean \pm SD of number of experiments (n = 10). The level of homogenecity among the groups was tested using Analysis of Variance (ANOVA) as done by Snedecor and Cochran 1980. Where heterogenecity occurred, the groups were separated using Duncan Multiple Range Test (DMRT). A value of p < 0.05 was considered to indicate a significant difference between groups (Duncan, 1957).

RESULTS

Body Weight Changes

Table 1 shows that rats in controls (olive oil only and normal saline only treated) and Zn only groups significantly (p < 0.05) increased in weight by 20% of their initial mean live weight. Both PPX- administered groups lost weights when compared with their initial weights. However the weight loss by the PPX-administered alone rats was higher than the losses by the group received Zn along with the PPX treatment.

Weights and Volume of testes mean

The testicular weights, testis weight/body weight ratio and volumes of the PPX -alone rats were the least, being significantly lower (p < 0.05) compared to the mean testicular weights, testis weight/body weight ratio and volumes of the PPX rats that in addition had Zn, which in turn were also lower but not significantly (p > 0.05) lower than those of the controls and Zn-alone rats (Table 1).

Testicular oxidative stress

Activities of testicular enzyme- glutathione peroxidase (GPx): The GPx activities following Zn administration approximated that of the animals that were given either olive oil or normal saline alone. PPX, however, markedly (p < 0.005) decreased the enzyme activity compared to control values. Administration of Zn along with PPX significantly (p < 0.05) increased the GPx activity in testicular tissue compared to animals treated with PPX

Table 1. The changes in gross anatomical parameters of Wistar rats.

Treatment groups	Initial Body Weight (g)	Final Body Weight (g)	Body Weight Diff.(g)	Testis Weight (g)	Testis Volume (mL)	Testis Wt/ Body Wt ratio
Α	191.7± 4.5	191.6 ± 4.3	0.1	1.55 ± 0.8	1.55 ± 0.6	0.007
В	190.3±3.6	190.4± 3.6	0.1	1.56± 0.5	1.58± 0.7	0.006
С	198.4 ± 5.7	175.6 ± 3.6	22.8**	$0.78 \pm 0.6**$	$0.77 \pm 0.4**$	0.004**
D	189.2 ± 7.8	1893 ± 4.5	0.1	1.50 ± 0.4	1.58 ± 0.3	0.006
E	199.6 ± 4.5	186.5 ± 4.4	13.1*	$1.33 \pm 0.7^*$	$1.35 \pm 0.5^*$	0.005*

^{*, **}represent significant decreases at p < 0.05 and p < 0.005 respectively when compared to control values. Values are means \pm S.E.M. n = 10 in each group.

Group A: Olive oil only (0.5 mL/kg body wt//day/oral)

Group B: Normal saline only (0.5 mL/kg body wt//day/ip)

Group C: Porpoxur dissolved in olive oil (10 mg/kg body wt /day/oral)

Group D: Zinc mixed with normal saline (20 mg/kg body wt/day/ ip)

Group E: Porpoxur (10 mg/kg body wt /day/oral) and Zinc (20 mg/kg body wt/day/ ip)

Table 2. Testicular Antioxidative Enzyme (GPx), testicular MDA and serum TT

Treatment Groups	GPx (nmol/mg protein)	MDA (nmol/mg protein)	TT (ng/ml)
Α	0.80 ± 0.05	0.72 ± 0.05	1.64 ± 0.03
В	0.79 ± 0.15	0.69 ±0.04	1.63 ± 0.05
С	0.44 ± 0.16**	2.23 ±0.4**	0.85 ± 0.03 *
D	0.81 ± 0.07	0.74 ± 0.03	1.62 ± 0.03
E	0.60 ±0.15*	1.23 ±0.06*	1.53 ± 0.05
_ A	0.80 ± 0.05	0.72 ± 0.05	1.64 ± 0.03

 $^{^*}$, ** represent significant differences at p<0.05 and p<0.005 respectively compared to controls. Values are means \pm S.E.M. n = 10 in each group.

Group A: Olive oil only (0.5 mL/kg body wt/ /day/oral)

Group B: Normal saline only (0.5 mL/kg body wt//day/ip)

Group C: Porpoxur dissolved in olive oil (10 mg/kg body wt /day/oral)

Group D: Zinc mixed with normal saline (20 mg/kg body wt/day/ ip)

Group E: Porpoxur (10 mg/kg body wt /day/oral) and Zinc (20 mg/kg body wt/day/ ip)

alone. As shown in Table 2, Zn – co treatment with PPX did not result in significant changes in GPx activity compared with the control.

Testicular content of malondialdehyde (MDA): Following treatment with Zn, the testicular MDA level was not significantly different from the control groups. PPX significantly elevated the testicular MDA by about 3-folds compared to the control values (Table 2).

Co-administration with Zn exhibited a marked (p < 0.005) reduction in the testicular MDA level compared to PPX alone treated rats. However, the lipid peroxide level was still significantly higher (p < 0.05) than control values (Table 2).

Serum testosterone (TT)

The serum TT levels of the two control groups and those of the group that was given only Zn approximated each other. There was however a statistically significant (p <

0.005) reduction in the serum TT levels of the animal that received PPX without an accompanying Zn therapy. As shown in table 2, the animals that were given both PPX and Zn showed demonstrated only a non-significant (p > 0.05) decrease in their serum TT.

Sperm parameters

Sperm count and sperm motility: The Zn- alone Wistar rats did not demonstrate any significant (p > 0.05) difference in their sperm count and motility when compared to the control values. The animals that were given PPX without co-treatment with Zn showed a significant (p < 0.005) reduction in both their sperm count and motility as compared to the control groups and those groups that had both Zn and PPX challenge (Table 3).

Sperm progressivity and sperm morphology: The results of the sperm progressivity and morphology were similar to that of the sperm count and motility stated

Table 3. Sperm parameters

Treatment	Sperm count (x10 ⁶ /ml)	Sperm motility %	Sperm progressivity	Sperm morphology	
Groups				% Normal	% Abnormal
Α	137.5 ± 6.7	99.0 ± 1.0	a1	91.5 ± 1.3	8.4 ± 1.4
В	136.4 ± 5.5	98.3 ± 1.7	a1	92.5 ± 1.2	7.5 ± 1.3
С	$30.3 \pm 2.8**$	25.3 ± 1.9**	b1*	28.7 ± 1.4**	69.3 ± 1.1**
D	136.3 ± 1.4	93.4 ± 1.2	a1	93.5 ± 1.1	6.5 ± 1.4
Е	135.5 ± 2.4	95.5 ± 2.5	a1	90.2 ± 3.5	9.8 ± 1.2

^{*, **} represent significant differences at P<0.05 and P< 0.005 respectively when compared to the control values..

Values are means \pm S.E.M. n = 10 in each group.

Group A: Olive oil only (0.5 mL/kg body wt//day/oral)

Group B: Normal saline only (0.5 mL/kg body wt//day/ip)

Group C: Porpoxur dissolved in olive oil (10 mg/kg body wt /day/oral)

Group D: Zinc mixed with normal saline (20 mg/kg body wt/day/ ip)

Group E: Porpoxur (10 mg/kg body wt /day/oral) and Zinc (20 mg/kg body wt/day/ ip)

earlier. The Zn- alone group similarly showed normal sperm progressivity and morphology. The animals that were given PPX without co-treatment with Zn showed a significant (p < 0.05 and p < 0.005) reduction in both their sperm progressivity and normal sperm morphology rates, respectively as compared to the control groups and the group that had a Zn co-pretreatment (Table 3). Similarly, Zn co-treatment abated the derangement in sperm progressivity and percentage abnormal sperm morphology.

DISCUSSION

The dramatic increase in using carbamate compounds such as porpoxur (PPX) as substitutes for chlorinated hydrocarbon insecticides has resulted in a new dimension of occupational hazard in the agricultural industry (Baron, 1991). Furthermore, because of its widespread use in public health, it has enjoyed a considerable attention on account of its potential health hazards. In deed there are reports detailing the cytotoxic effects of PPX on the rat testis (Sikka and Nigun, 2005; Ngoula et al., 2007). The present work was therefore designed to investigate the potential testiculoprotective effect of Zn as an antioxidant on PPX-induced testicular toxicity.

The biochemical mechanism by which PPX mediate cytotoxicity is currently unclear. It has however been postulated that its action may be connected with increased generation of free radical and reactive oxygen species (ROS), together with alteration of enzymatic antioxidative systems such as glutathione redox system. The ultimate effect of all these pathways is increased oxidative stress and cellular toxicity (Timbrell, 2000).

The results of the present study indicate that administration of PPX in a dose of 10 mg/kg body weight (orally) decreased the absolute testicular weights, testicular weight/body weight ratio and testicular volumes of rats. This could be attributed to severe parenchyma atrophy in the seminiferous tubules following PPX challenge. In deed similar findings were reported by previous investigators (Sikka and Nigun, 2005; Ngoula et al., 2007). In addition testicular atrophy and degenerative changes of the seminiferous tubules have been reported in experimental animals with various carbamate insecticides (Ezeasor, 1990; Chapin et al., 1994).

Evaluation of lipid peroxidation activities of antioxidant enzymes such as GPx in biological tissue have been always used as markers for tissue injury and oxidative stress (Priestman, 2008; Saalu et al., 2009; Saalu et al., 2010). The result from this study demonstrate that testicular and oxidative damage induced by PPX administration are also manifested by a significant increase in the activities of GPx and the testicular content of MDA. More remarkable was the findings that the increased testicular MDA as well as the concomitant increase in activities of GPX were recovered by Zn cotreatment.

This increased oxidative stress induced by PPX could damage the sperm membranes, proteins and DNA (Ngoulaa et al., 2007). This may explain the reduced sperm concentration, sperm motility and sperm progressivity, with accompanying increase in abnormal sperm rates as seen in PPX alone group of rats. Again the capacity of Zn to abate these effects was adequately demonstrated with a normalization of these sperm parameters following Zn supplementation.

The findings of our studies also showed a significant decrease in serum testosterone in animals that were

In this study a spermatozoon was considered abnormal morphologically if it had one or more of the following features: rudimentary tail, round head and detached head.

a₁ = rapid linear progressive motility, b₁ = show sluggish linear or non-linear motility.

given PPX without Zn. This could be ascribed to a direct toxic effect of the insecticide on testicular function, to an alteration in the internal endocrine environment or a direct effect on the central nervous system (Afifi *et al.*, 1991).

Co-treatment with Zinc prevented cytotoxity of the testes exposed to PPX. This suggests PPX interference with Zinc-related metabolic functions. The competitive mechanism of interaction is a plausible mechanism of protection of Zinc in relation to PPX toxicity. Radio-labeled Zinc has been reported to be incorporated into elongated spermatids and to display competitive interaction with heavy metals for incorporation (Saxena et al., 1989). Another possible mechanism of protection for Zinc is metallothionein induction. Supplemental dietary Zinc prevented PPX-induced testicular Zinc depletion and injury. Our results showed that the GPx, activity is distinctly lower in the testes of PPX-exposed rats.

The interaction between PPX and essential trace elements could be one of the reasons for decreased antioxidant enzymes in the rat testis. This effect was related to induction of oxidative stress. In the present study, Zn supplementation effectively reversed PPX induced toxicity in the antioxidant system of the rat testis. Zinc is an essential trace mineral that acts as an antioxidant by neutralizing free radical generation (Powell, 2000). Souza et al. (2004) suggested that Zn protection against the cytotoxicity of PPX may be related to the maintenance of normal redox balance inside the cell. Zn could exert a direct antioxidant action by occupying the iron or copper binding sites of lipids and proteins (Bray and Bettger, 1990), Zinc-deficient male rats have higher levels of Lipid peroxidation and protein oxidation, which lead to reduced testicular growth and oxidative stress in PPX -exposed rats.

In conclusion, propoxur induced testicular oxidative stress, caused impairments of sperm parameters mediated deranged steroidogenesis, all of which were largely abated by Zinc co-administration.

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