Orginal article

The Effect of Pesticide Chlorpyrifos (Dursban^{R}) on Sperm Parameters and Testicular Tissue in Mice

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Abstract
Environmental pollution by the pesticide chlorpyrifos has been documented as the most commonly detected pesticide in food and water posing a potential hazard to human health. Therefore, the present study was conducted to determine the effect of pesticide chlorpyrifos on sperm parameters and testicular tissue in mice. Sperm count, sperm motility and sperm morphology parameters are very important semen characteristics and are strong predictors of male fertility. The reproductive toxicity of the pesticide chloropyrifos was studied in adult male mice via intraperitoneal administration of chlorpyrifos (20 mg/kg) on alternate days for a period of 15 days. The results of this study showed a decrease in all sperm parameters, especially sperm motility and histological lesions of testicular tissue. The histological lesions emphasize the positive correlation between cytogenetic damage and abnormal sperm parameters. These results support the hypothesis that exposure to pesticides may be associated with decreased semen quality and hence infertility.

Key words: Chlorpyrifos, Pesticide, Sperm analysis, Testes, Mice

Introduction

Several studies have reported an apparent decline in semen quality (Carleson *et al.* 1992; Auger *et al.*, 1995; Schisterman *et al.* 2014) and an increase in male infertility (de Kretser, 1997; Schisterman *et al.* 2014) over the past 50 years. The reasons for these detrimental male reproductive changes appear to be environmental rather than genetic due to the short time period over which they have occurred. Therefore, scientific research has mainly focused on environmental chemical contaminants. These include: synthetic chemicals (e.g. trichloroethylene (TCE), polychlorinated biphenols (PCBs), dioxin), heavy metals (e.g. cadmium, lead, arsenic, nickel), plastics (e.g. bisphenol A (BPA), phthalates), and pesticides (e.g. vinclozolin, Diazinon (DZN), dichlorodiphenyltrichloroethane (DDT), methoxychlor) (Anway *et al.* 2005; Olea and Fernandez, 2007; Stouder and Paoloni-Giacobino, 2010; Dwivedi and Flora, 2011; Manikkam et al., 2012; Skinner *et al.* 2013; Al-Griw *et al.* 2015; Adamkovicova *et al.* 2016). In wildlife chemical pollution has frequently been associated with adverse male reproductive effects (e.g. damage to the testes and spermatozoa) (Colborn *et al.* 1993; Guillette and Gunderson, 2001; Multigner *et al.* 2008). Recently, Martenies and Perry (2013) suggested that exposure to pesticides environmentally or occupationally may be associated with decreased sperm count in men. Thus, the evaluation of reproductive toxicity of common pesticides is of great importance to public and environmental health.

The pesticide chlorpyrifos (O,O-diethyl O-3,5,6-trichloro-2-pyridyl Phosphorothioate) is the most widely used organophosphate insecticide **(**Betancourt *et al.*, 2006**).** Chlorpyrifos account for up to 50% of all insecticide application worldwide (Casida and Quistad, 2004). Chlorpyrifos is a broad spectrum organophosphate insecticide used in the control of agriculture pests and household insects such as fire ants, cockroaches, and fleas (Eaton *et al.* 2008). Commercially, it is available in different trade names such as Dursban, Lorsban, Agromil, Dhanwan, Suscon Green, Empire, Equity, Dorson, and Omexan (Watts, 2013; John and Shaike, 2015). Varying concentrations of chlorpyrifos pollution in the environment have been reported (George *et al.* 2014; Lari *et al.* 2014; Marasinghe *et al.* 2014; Gulati *et al.* 2015), posing a potential hazard to human health. It has been reported that chlorpyrifos is linked to brain deformities (Rauh *et al.*, 2012), neural tube defects (Abdelmalek *et al.* 2016) and impaired fetal growth (Whyatt *et al.* 2004) in human fetuses. However, few studies have evaluated the impact of chlorpyrifos pesticide exposure on male reproductive health (Joshi *et al.* 2007; Mandal and Das, 2011; Sai *et al.* 2014). Therefore, the aim of this study was to evaluate the reproductive toxicity of the insecticide chloropyrifos in male mice. Sperm parameters (sperm count, sperm motility and sperm morphology) and histology of testicular tissue were investigated.

Materials and Methods

Study area

The Pesticide chlorpyrifos (O,O-diethylO-3,5,6-trichloropyridin-2-ethyl phosphorothioate) under the trade name Dursban 4 EC (emulsifiable concentrate) manufactured by Dow Agrosciences was obtained from a local pesticide store, supplied by Libyan Agrochemicals & Agriculture Supplies Specialized Co.. It was emulsified in water immediately before use and

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administrated to animals intraperitoneal (i/p) at a dose of 20 mg/Kg.

Animals and Treatments

Animals used in this study were mature male Swiss albino mice (*Mus musculus*) 6 to 8 weeks old, weighing 25 to 28 gm. They were breed in the animal house of the Zoology Department, Faculty of Science, University of Tripoli. The mice were housed in plastic cages containing wooden flakes under husbandry, and maintained at room temperature 22 ± 3°C under natural light/dark photoperiod. The mice were fed with a standard mouse diet and drinking water *ad libitum*. They were randomly divided into two groups: treated and control of five males each. The treated mice were administered intraperitoneal (i/p) injections of chloropyrifos 20 mg/kg body weight every alternate day for two weeks and the control mice did not receive any treatment. The mice were observed daily for survival and clinical signs of toxicity. At the end of the treatment period, all mice were sacrificed by cervical dislocation and sperms were isolated from vas deferens and sperm analysis was evaluated.

Sperm count

Sperm count was determined by Neubauer hemocytometer counting chamber following the method of Wang *et al*. (1995). Sperm samples were collected from vas deferens, the count was repeated at least three times to minimize error and mean value was taken for calculation. Each vas deferens was gently squeezed and thoroughly stripped in a clean small glass Petri dish containing 1 mL of physiological normal saline (0.9% NaCl). The sperm suspension was incubated for five minutes at 37 °C to allow sperm separation, after incubation sperm suspension was thoroughly mixed with a fine pipette, 100 uL of the diluted sperm suspension was placed on counting chamber. The number of motile and immotile sperms was counted under a light microscope at 400x magnification. The calculated results were expressed as percent motility or immotility (e.g. the number of motile sperm/total number of sperm x 100). For total sperm count, the number of motile and immotile sperms was added and multiplied by dilution factor and expressed as $x10^6$/mL.

Sperm Morphology

Sperm morphology examination was done by making sperm smears from the sperm suspension. One drop of sperm suspension was placed on a clean microscopic slide and a sperm smear was made, allowed to air dry, and then stained with 1% eosin for ten minutes. These smears were observed at 400X magnification using a standard light microscope and the number of normal and abnormal sperms was determined. The calculated results were finally expressed as percent: the number of abnormal sperm/total number of sperm x 100. At least 500 sperms were counted from each animal to determine sperm morphology and abnormalities. The criteria for abnormal sperm morphology include the following: the shape of sperm head, the presence or absence of hook, and tail shape.

Histological examination

Testes were fixed in 10% buffered formalin, passed through ascending series of ethanol and then through xylene and embedded in paraffin wax. Tissues were sectioned at thickness of 7 uM and stained with hematoxylin and eosin (H and E) and microscopically analyzed and photographed.

Statistical Analysis

The data was analyzed by one way analysis of variance (ANOVA) using MSTATE-C version 4 software. The statistical significance of difference between the control and treated groups for sperm parameters: sperm count, sperm morphology, and sperm motility was determined with Duncan's test; a probability value of $P \leq 0.05$ was considered statistically significant.

Results

The pesticide chlorpyrifos had toxic effect on all sperm parameters: sperm count, sperm motility, and sperm morphology analyzed in this study, as well as histopathological changes in testicular tissue.

The effect of chlorpyrifos on sperm parameters

There was a decrease in sperm count of treated mice ($22.4x10^6$) in comparison with control group ($25.4x10^6$); however the probability value (P = 0.400) indicate that this decrease was not significant (Table 1). The greatest toxic effect of chlorpyrifos was observed on sperm motility. There was very significant (P=0.000) decline in the mean percentage of sperm motility of treated mice (4.7%) verses control (94.2%) group (Table 1).

Table1. Sperm parameters in control and chlorpyrifos treated mice.

Sperm parameter	Control group	Chlorpyrifos group
Sperm count	$25.4x10^6$/mL	$22.4x10^6$/mL
Percent of motile sperms	94.2%	4.7%*
Percent of abnormal sperm morphology	4.8%	14.2%**
Percent of normal sperm morphology	95.2%	85.8%

***** P=0.000**, **** P<0.022

There was a significant increase (P<0.022) in the mean percentage of morphologically abnormal sperm shapes in treated mice (14.2%) with respect to control (4.8%) group (Table 1). The morphological different forms of sperm shape abnormalities in this study include banana head, coiled tail, thin tail, and ring tail (Figure 1).

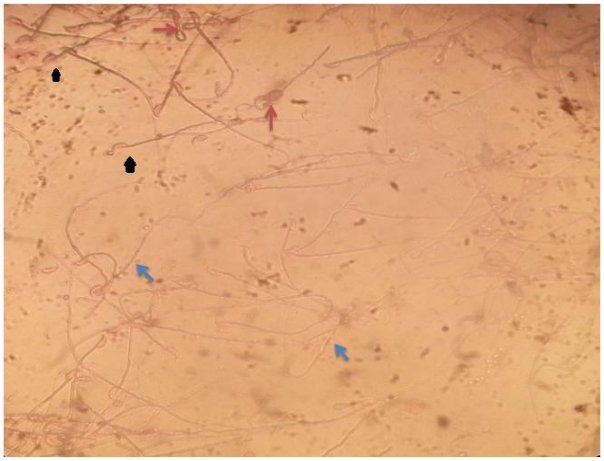

Figure 1. Photomicrograph of abnormal sperm morphology in chlorpyrifos treated group. Coiled tail (red arrows), thin tail (blue arrows), and banana head (black arrows). 1.0% eosin stain, 400X.

The effect of chlorpyrifos on testicular tissue

Light microscopic examination of testicular tissue in the control group shows normal histological structure, normal pattern of seminiferous tubules with orderly arranged spermatogenic cells and high spermatozoa concentration in the lumen, and little interstitial space (Figure 2). Meanwhile, the testes of chlorpyrifos treated group revealed marked damage in histoarchitecture and organization of seminiferous tubules (Figure 3) compared to control group (Figure 2). The histological lesions of testicular tissue include: distortion of seminiferous tubules, reduction in lumen width of seminiferous tubules, reduction in spermatozoa numbers in the lumen of the seminiferous tubules, derangement and sloughing of normal germinal epithelial cells (spermatogonia) lining seminiferous tubules, increased interstitial space, and vasodilatation of interstitial blood vessels (Figure 3).

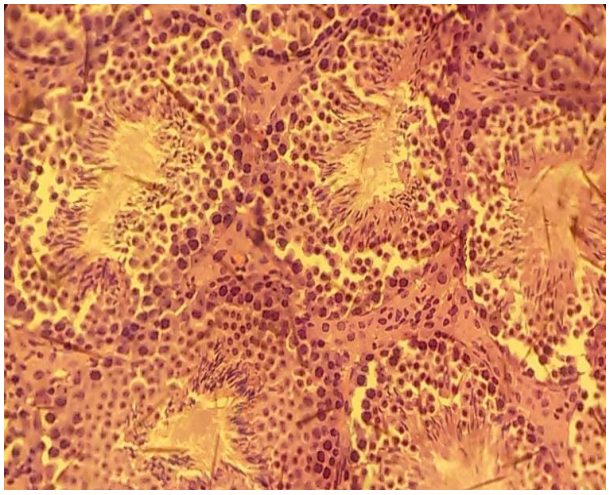

Figure 2. Normal histological structure of the testes in the control group showing normal pattern of seminiferous tubules with many spermatozoa in lumen, orderly arranged spermatogenic cells, and little interstitial space. H and E stain, 400X.

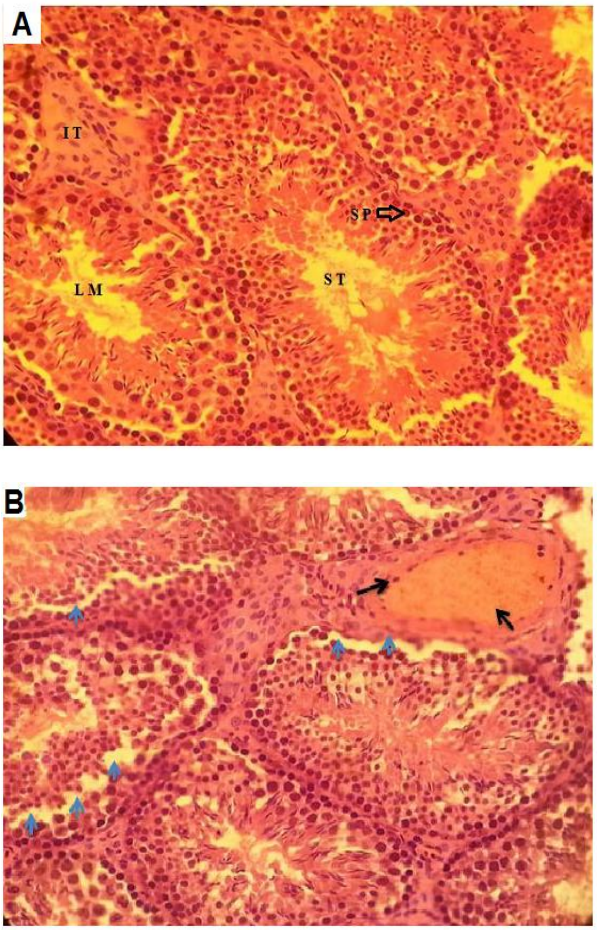

Figure 3. Histological section of testes of male mice treated with chlorpyrifos showing disorganized seminiferous tubules (ST), narrow lumen (LM), and few spermatozoa (A); sloughing of spermatogonia (sp) blue arrows, increased interstitial space and dilation of blood vessels (black arrows (B). H and E stain, 400X.

Discussion

In the present study, reproductive and histopathological studies were performed on the testes of adult male mice after i/p administration of pesticide chlorpyrifos at a concentration of 20 mg/Kg (which represent 1/3 of oral LD_{50}) over a period of two weeks. The results indicate decreased sperm count, significant decline in sperm motility and normal sperm morphology as well as testicular tissue damage of the mice exposed to chlorpyrifos. These findings are similar to those reported from experimental animal (Joshi *et al.* 2007; Zidan, 2009; Farag *et al.* 2010; El-Kashoury and Tag El-Din, 2010; Alaa-Eldin *et al.* 2017) and clinical human studies (Padungtod *et al.*, 2000; Meeker *et al.* 2004; Perry *et al*. 2011; Marteniesa and Perry, 2013).

Joshi *et al.* (2007) studied the toxic effects of chlorpyrifos at dose levels of 7.5, 12.5 and 17.5 mg/kg /d administered orally to male rats for 30 days on testicular histology, biochemistry, sperm dynamics and testosterone levels; their results indicated that Chlorpyrifos induces severe testicular damage, decreased testosterone levels, and reduction in sperm count (Joshi *et al.* 2007). Similarly, adult male mice were treated by oral gavage with chloropyrifos at doses

of 5, 15, and 25 mg/kg/d for 4 weeks resulted in decrease of the percent of morphologically normal spermatozoa in 15 and 25 mg/kg/d dose groups; however, sperm motility and count were decreased in all treated groups compared to the control (Farag *et al.* 2010). Exposure to three trade names of formulated chlorpyrifos from different Egyptian manufactures (chlorozan, pestpan, and pyriban) administrated orally to male rats at dose of 23.43, 21.40 and 17.43 mg/kg b.w. with 5 doses per week for 28 days resulted in decreased sperm motility and impairment of spermatogenesis (El-Kashoury and Tag El-Din, 2010). In addition, administration of chlorpyrifos to rats at a dose of 6.75 mg/kg b.w./daily by oral gavage for 12 weeks resulted in reduced testicular weight, decreased sperm count, motility and viability, significantly increased percent of morphologically abnormal spermatozoa, and significant increments in sperm DNA fragmentation index (DFI) with respect to control group (Alaa-Eldin *et al.* 2017).

In this study, the percentage of morphologically normal sperm was low due to large number of sperms with abnormal tail morphology, especially coiled tail. These results are consistent with Joshi *et al.* (2007), Zidan (2009), Farag *et al.* (2010), El-Kashoury and Tag El-Din (2010), and Alaa-Eldin *et al.* (2017). Abnormal tail morphology significantly reduces the fertilization capabilities of sperm because of spermatozoa movement dysfunction (Selvaraju *et al.* 2011).

Epidemiological human data reveal a potential association between exposure to chlorpyrifos and decreased semen quality. A survey on Chinese pesticide factory workers showed that exposure to organophosphate insecticides was associated with decreased sperm count and motility (Padungtod *et al.* 2000). Association between low sperm count and serum concentrations of 3,5,6-trichloropyridinol (a metabolite of chlorpyrifos and chlorpyrifosmethyl) has been reported in men (Meeker *et al.* 2004; Marteniesa and Perry, 2013). Measurable levels of urinary 3,5,6-trichloro-2-pyridinol was reported in more than 90% of males in the United States (CDC, 2003).

The changes in sperm parameters may be attributed to impairment of spermatogonia proliferation and maturation, which might be due to oxidative stress or to low levels of testosterone hormone (Saradha and Mathur, 2006). Testicular tissues are vulnerable to oxidative injury (Mendez-Alvarez *et al.* 2002) and defective sperm function is associated with an increase in lipid peroxidation derived free radicals and impaired antioxidant defense (Attia *et al.* 2012). Chlorpyrifos toxicity involves formation of reactive oxygen species (ROS) (El-Kashoury and Tag El-Din, 2010; Mandal and Das, 2011), inhibition of the activities of marker enzymes: alkaline and acid phosphates (ALP and ACP) and lactate dehydrogenase (LDH) (El-Kashoury and Tag El-Din, 2010), and significant decrease in activities of antioxidant enzymes including catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) which counteract the toxicity of ROS (Attia *et al.* 2012). ROS impede sperm motility by reducing mitochondrial membrane potential and decreasing ATP availability (Wang *et al.* 2003; Tremellen, 2008).

In this study, light microscopic examination of testes revealed that chlorpyrifos induced numerous histopathological changes in testicular tissues. Several previous studies confirm this result (Joshi *et al.* 2007; Akhtar *et al.* 2009; Zidan, 2009; Kalender *et al.* 2012; Sai *et al.* 2014; Alaa-Eldin *et al.* 2017); who found mild to severe histopathological changes in the testis of rodents (rats and mice) at various dose levels of chlorpyrifos . The histopathological changes included: degeneration and atrophy of seminiferous tubules with large interstitial space, decreasing number of spermatogenic cells in seminiferous tubules, sloughing of germinal epithelial into the lumen of seminiferous tubules and loss of sperms, edema, congested blood vessels, and exudate in interstitial tissue of the testis. The histological lesions induced by chlorpyrifos support the findings in this study and indicate that the exposure to chlorpyrifos caused a severe disturbance of spermatogenesis, with a marked decline in sperm quality; it also emphasize the positive correlation between cytogenetic damage and abnormal sperm parameters.

Conclusion

Sperm analysis is one of the end points in reproductive toxicology studies. Sperm count, sperm motility and sperm morphology parameters are very important semen characteristics and are strong predictors of male fertility (Perreault and Cancel, 2001; Buraimuh *et al.* 2012). Small changes in sperm counts are known to have adverse effects on human fertility. Reduced motility and tail abnormalities are distinctive features observed in idiopathic asthenozoospermia and teratozoospermia in humans; these disorders are major causes of male infertility (Maruyama *et al.* 2016). Humans are exposed to environmental chemicals from numerous sources. Recently, the focus has been on adverse reproductive outcomes associated with widespread and permanent contamination by pesticides (Gulati *et al.* 2015).

Environmental pollution by the pesticide chlorpyrifos has been documented as the most commonly detected pesticide in food and water posing a potential hazard to human health (Marasinghe *et al.* 2014; John and Shiake, 2015). Therefore, the present study was designed to determine the effects of pesticide chlorpyrifos on sperm parameters and testicular tissue in mice. The current study demonstrated adverse effects of chloropyrifos exposure on sperm parameters at sub-lethal dose 20 mg/kg. The results presented in this study support the hypothesis that exposures to pesticides may be associated with decreased semen quality and hence infertility. Therefore, it is recommended to restrict the use of chlorpyrifos and to focus on alternative safe methods (e.g. biological control) to overcome the pollution and toxicity problems associated with chlorpyrifos usages.

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