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DICHLORVOS CURTAILS SEXUAL BEHAVIOR AND FERTILITY IN MALE SPRAGUE-DAWLEY RATS

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ABSTRACT
Dopamine (DA) activity in the medial preoptic area (MPOA) contributes to the control of sexual behavior in adult male rat. The presence of testosterone is permissive for DA release in the MPOA, both during basal conditions and in response to a female. Testosterone is the major driver of male reproductive development and function. This study was carried out to evaluate the effect of dichlorvos DDVP on testosterone and dopamine in relation to sexual behavior and fertility in male Sprague-Dawley rats. Experimental rat (n=18) Weighing 245.17±10.18 were divided into three groups. Two groups containing seven rats each were given water contaminated with 0.01% and 0.05% DDVP, respectively per day as drinking water and the control group containing four rats was given water without DDVP. The rats were fed with 30 days. Female animals were introduced to the male animals twice per week to observe any sexual behavioural changes. Blood chemistry parameters and hematological profile were determined using auto-analyzer, semen quality by the neuberhaematocytometer, histopathology of the brain and testis, assay of dopamine in the serum and MPOA by HPLC and assay of testosterone using chemiluminescent immunoassay. There was no significant difference ($p>0.05$) in the levels of creatinine, urea, alkaline phosphatase, aspartate aminotransferase activities, red blood cell count, hemoglobin, haematocrit level of rat administered DDVP when compared with control. Histopathology of the brain and testis revealed no abnormalities. However, there was a significant increase ($p<0.05$) in the level of testosterone and dopamine across the groups orally administered DDVP. Sperm count and sperm motility decreased significantly in group treated with 0.05% DDVP with an increase in sperm abnormality. Therefore, data of this study revealed that DDVP may increase the sexual behavior of rats by interfering with the synthesis of testosterone in the leydig cells which increased DA in the MPOA, but can impair fertility due to poor semen quality that may have resulted from the action of DDVP on sertolli cells.

INTRODUCTION
The U.S Environmental Protection Agency (EPA) defines a pesticide as "any substance or mixture of substances intended for preventing, destroying, repelling, or lessening the damage of any pest," which may include plants, weeds, animals, insects, and fungus (Elhalwagy *et al.*,, 2008). Organophosphate insecticides represent one group of pesticides that is widely used and has been shown to have toxic effects in human and animals (Eskenazi *et al*., 1993). Dichlorvos (2,2-dichlorovinyl dimethyl phosphate) is one of the classes of insecticides referred to as organophosphate compound used to control households and stored product insects and also to control ectoparasites in domestic animals (Bennett, 2001).

The World Health Organization has classified dichlorvos as a highly hazardous and toxic compound (WHO, 1992).The exposure of individual to dichlorvos could be either through direct or indirect route. Direct exposure occurs in farmers and individuals who personally apply pesticides for agricultural, occupational or residential purposes. Indirect exposure occurs through drinking water, air and food and this represent routes of long term low level exposures (Alavanja *et al.,* 2004).The increasing knowledge of the reproductive toxicity of environmental chemicals has raised public concern as to whether the current use of pesticides could adversely affect human (Chattergee *et al.,* 1998). There is an increased concern that exposure to pesticides may adversely affect the reproductive system of humans and other non-target organisms. It has also been reported that exposure to agricultural pesticides may affect male fertility (Taylor *et al*., 2010).

Dopamine (DA) activity in the medial preoptic area (MPOA) contributes to the control of sexual behavior in adult male rat (Juan and Elaine, 2005). The neurotransmitter dopamine is important for male sexual behaviour and it is one neurotransmitter system that may be upregulated by hormones (Juan and Elaine, 2005). Dopamine facilitates male sexual behaviour in numerous species and is release before or during copulation in three

integral neuronal systems with distinct sexual role: in the nigrostaital system, dopamine enhances readiness for sexual response; in the mesolimbic system, it promote sexual appetitive behaviour; finally, in the medial preoptic area), a region at the rostral end of the hypothalamus, which is important for endocrine activity and essential for the expression of male sexual behaviour, it increases sexual motivation, genital reflexes and copulation (Hull *et al*., 1999).

The presence of testosterone is necessary for the precopulatory DA release and for copulation itself (Hull *et al*., 1995). Dopamine is not thought to directly elicit behaviour, but it is thought to allow hormonally primed output pathways to have easier access to sexually relevant stimuli (Hull *et al.*, 1999).Testosterone upregulate NOS activity in the MPOA, which produces more NO, which in turn promotes DA release in both basal and sexual situations; the increased MPOA Dopamine release, in turn, promotes sexual motivation, genital reflexes, and copulation (Elaine *et al.*, 1995).

Several works have been done to unveil the relationship between dopamine release in the medial preoptic area and testosterone level before or after copulation. According to Elaine *et al* (1995), the presence of testosterone is permissive for DA release in the MPOA, both during basal conditions and in response to a female. Hence, this study was carried out to evaluate the effect of dichlorvos on testosterone and dopamine in relation to sexual behaviour and fertility in male Sprague-Dawley rats.

MATERIALS AND METHODS

Material

Sniper®: The bottle (100ml) of 1000 EC Sniper® insecticide used was purchased from Mushin local market, Lagos State, Nigeria. It contained 1000g/L 2,3-Dichlorovinyl dimethyl phosphate (DDVP) as its active ingredient. It is manufactured by BeihajHepij pesticide Co. limited, China.

Experimental Animal: Eighteen Sprague-Dawley rats weighing between 243g and 262g were used for the study. The rats were purchased from the Animal Laboratory Centre, College of Medicine, University of Lagos, Lagos State, Nigeria, and were kept in the metallic cages at room temperature. The rats were acclimatized for two weeks under a 12h light/dark cycle and allowed free access to rat chow *for 30days.* Animal care and handling were carried out according to standard protocol and institutional ethical guidelines for animal experimentation practice.

Experimental Design: Eighteen Sprague-Dawley rats were placed in three groups and treated with dichlorvos through drinking water for 30 days according to the method of Ethelbert *et al.* (2015).

Group (1) contained four rats and they were given water without dichlorvos.

Group (2) contained seven rats and administered 0.01% DDVP/day.

Group (3) contained seven rats and administered 0.05% DDVP/day.

The rats were observed at least once daily for behavioural changes; signs of intoxication, mortality, morbidity, food and water consumption were monitored daily. Receptive female rat was introduced to the male rats twice in a week after the first week of DDVPexposure to observe the effect on their sexual behaviour.

Sample Collection: At the end of the experimental period, blood samples were collected from the fasted rats by ocular bleeding using plain capillary tubes into clean and labelled test-tubes (plain tubes and EDTA tubes). The blood samples in the plain tubes was allowed to clot at room temperature and centrifuged at 2000 rpm for 10min to obtain the serum which was transferred into a clean, dry bottle and stored at 4^{o}C until further analysis, for the determination of testosterone, blood chemistry and Plasma dopamine level.

The rats were sacrificed by cervical dislocation and the tissues of interest which were excised, namely testes, epididymis and brain. The samples for The brain and the testis samples histopathology were 4^{0}C and water collected in well labelled universal bottles and fixed with 10% formaldehyde immediately. The brain sample for dopamine assay was stored in 10% phosphate buffer solution and stored at 4^{o}C. The epididymis was prepared for fertility evaluation.

METHODS

Biochemical analysis: Biochemical parameters analyzed include: alkaline phosphatase, creatinine, urea, aspartate amino transferase, alanine amino transferase and total cholesterol levels were carried out using COBAS C111 autochemistry analyzer.

Evaluation of Fertility: Spermatozoa were obtained by mincing the cauda epididymis in a petri dish containing a known volume of saline.

Sperm Motility: The motility of the sperm was evaluated directly after mincing by placing a drop of the diluted semen on a glass slide and covered immediately with a cover slip. The glass slide was viewed under the microscope with X40 objective lens. Non-motile sperm numbers were first determined and followed by counting of total sperm. Sperm motility was expressed as percent of motile sperm of the total sperm counted, according to Linder *et al*. (1986).

Sperm Count: The spermatozoa concentration was carried out by diluting a portion of the semen with Sodium barbiturate (1:20), then mixed together, after that a drop of the mixture was delivered into the Neubaure haemocytometer in each side of the counting chamber.The haemocytometer was viewed under X10 objective lens of microscope and sperm were counted in the three large squares, and expressed as sperm concentration in millions were recorded. The average of the three squares (field) was recorded and the total sperm count was computed.

Sperm Morphology: A drop of Eosin stain was added to the sperm suspension and kept for 5 min at 37°C. After that, a drop of sperm suspension was placed on a clean slide and spread gently to make a thin film. The film was air dried and then observed under a microscope for changes in sperm morphology, according to the method of El-Kashoury *et al.,*(2010). The criteria chosen for head abnormality were; no hook, excessive hook, amorphous, pin and short head, white for the tail the abnormalities recorded were; coiled flagellum and bent flagellum tips, the results are expressed as the percentage overall of abnormal form.

Hispathological Examination: The brain and testes Tissue were fixed in 10% formaldehyde solution and used for histopathological examination. The MPOA of brain was cut out, a section of the testis was cut out also and these tissues were placed inside a well labelled tissue embedding cassette. The tissues were processed using an automatic tissue processor for 17h. The tissues were washed by dehydration in increasing gradients of ethanol and finally cleared in toluene. The tissues were then embedded in molten paraffin wax. Sections with a thickness of 5μm were cut and stained with hematoxylin and eosin. The slides were dehydrated by dipping in ascending grades of alcohol, cleared in xylene and mounted with D.P.X (Dibutylpthalate, polystyrene, xylene). The slides were read under X400 magnification, to process the organ photomicrographs.

HPLC Assay of Dopamine: Dopamine concentration in the serum as well as in the MPOA area were determined by HPLC. The brain was dissected to isolate the MPOA using the anatomy rat brain atlas and weighed, after which it was homogenized using Teflon grass homogenizer with 3ml of 10% phosphate buffer solution (PBS). The homogenates were centrifuged at 4000rpm for 5 min and the supernatant was separated from the pellet into a clean well- labelled plain tube. Both the serum and supernatant were concentrated to 1ml in a 50^{o}C water bath, then 0.6ml of serum and supernatant were deproteinised by adding 1.2ml of acetonitrile, centrifuged at 4000rpm for 5 min, separated the supernatant and allowed to concentrate to 0.6ml. The clear supernatant was injected directly into a station auto-analyser HPLC system to determine dopamine. Supernatant fluid was isocratically eluted through Ultrasphere RP analytical column (SMT OD5-100/15 RP C18 150*4.6mm) with 5µm particle size. The mobile phase contained ACN: 25mMKH_2PO_4 (40:60)%. The flow rate was 0.5mL/min.

Chemiluminescent Immunoassay of Testosterone: Testosterone assay was carried out according to the method of Sarkar *et al.,* (2000), with a slight modification by chemiluminescent immunoassay using Cobas e411 analyzer. Serum testosterone was assayed using a competitive chemiluminescent immunoassay kit. The testosterone in the sample competes with acridium ester-labelled testosteronefor binding to polyclonal rabbit and testosterone antibody in the solid phase. The polyclonal rabbit anti-testosterone antibody is bound to monoclonal mouse anti-rabbit antibody, which is coupled to paramagnetic particles. The assay uses a testosterone-releasing agent to release bound testosterone from the endogenous binding proteins.

STATISTICAL ANALYSIS

Statistical significance was established using one-way analysis of variance (ANOVA) and the data were reported as mean ± standard deviation. Significant differences were established at $p \leq 0.05$.

RESULTS

Observation of the Sexual Behavior of the Sprague-Dawley rats for 30 days

The responsiveness and the rate at which the Sprague-Dawley male rats chased and mounted the receptive female rats do not diminish throughout the duration of DDVP administration.

The total cholesterol level increased from 69.59±4.82 mg/dL in the control to 102.90±9.28 mg/dL in the rats exposed to 0.01% dichlorvos, then reduced to 65.58 ±7.51mg/dl in the rats exposed to 0.05% dichlorvos. There was no Significant ($p<0.05$) difference in levels of urea, creatinine, alkaline phosphatase and aspartate aminotransferase activities when compared to the control. ALT activity reduced significantly across the groups exposed to dichlorvos when compared to control as shown in table 1.

Table 1: Total cholesterol level, liver and Kidney function of rats results are presented orally administered dichlorvos for 30days.

	Control N= 4	0.01% DDVP N= 4	0.05% DDVP N= 4
ALT (U/L)	71.20 ± 10.70	53.45 ± 4.05 *	47.75 ± 3.25 *
AST (U/L)	212.00 ± 42.00	195.55 ± 50.55	185.45 ± 16.15
ALP (U/L)	266 ± 0.70	344.30 ± 98.90	253.50 ± 42.00
TCHOL(mg/dL)	69.59 ± 4.82	102.90 ± 9.28 *	65.58 ± 7.51 #
CRE (mmol/L)	87.90 ± 12.70	89.00 ± 3.10	73.10 ± 3.90 #
UREA(mmol/L)	5.70 ± 9.96	7.45 ± 0.85	6.80 ± 0.70

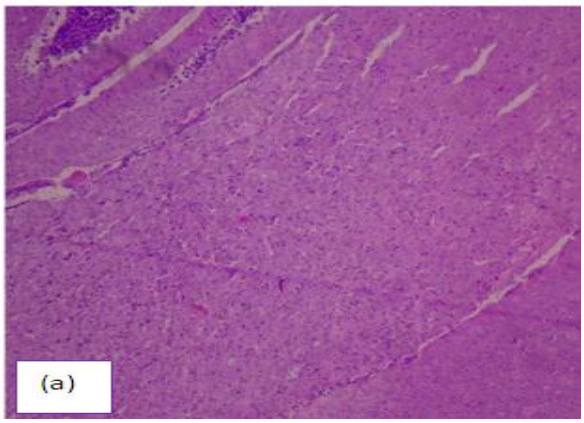
* = p<0.05 significant when compared to control, results are presented as mean ± S.D
# = p<0.05 significant when compared within group exposed to DDVP

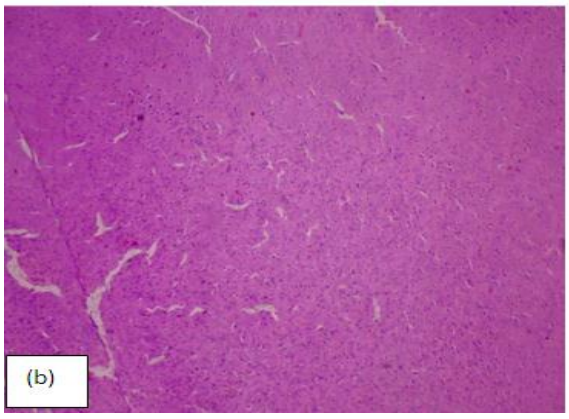
Effects of dichlorvos pesticide on sperm count, sperm motility and sperm abnormalities of the male rats are presented in Table (2) below. The results showed a significant reduction in sperm motility of rats exposed to 0.05% dichlorvos when compared to control. The sperm motility and sperm counts reduced with increase in pesticide concentration with lowest sperm motility of 66.50±1.58 and sperm count of 96.88±2.35 $X10^6$ recorded in the rats exposed to 0.05% pesticide. There was also a significant (p<0.01) increase in sperm abnormalities in rats exposed to 0.05% dichlorvos when compared to control.

Table 2: Semen Quality of Sprague-Dawley rats orally administered dichlorvos for 30 days.

	Sperm motility (%) N= 4	Sperm morphology (%) N= 4	Sperm count (*10^6) N= 4
Control	83.00 ± 0.63	1.00 ± 0.02	98.88 ± 2.45
0.01% DDVP	94.50 ± 0.95 *	1.00 ± 0.63	105.75 ± 2.43 *
0.05% DDVP	66.50 ± 1.58 * #	2.5 ± 0.32 * #	96.88 ± 2.35

* = p<0.05 significant when compared to control, Results are presented as mean ± S.D
# = p<0.05 significant when compared within group exposed to DDVP





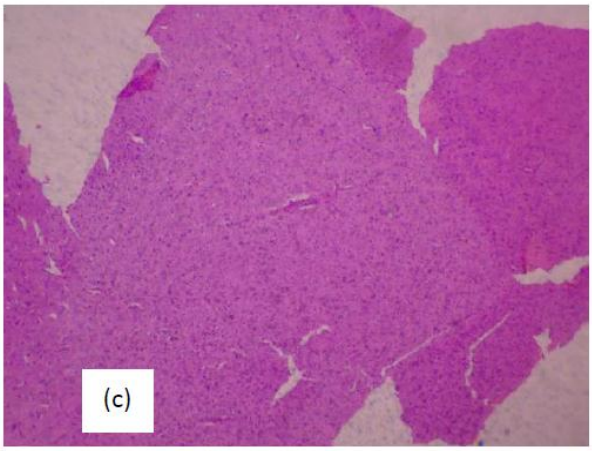
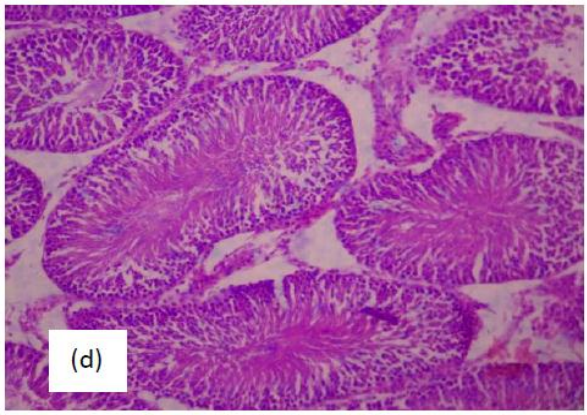
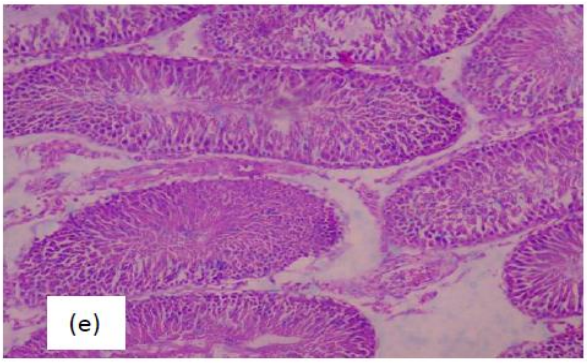


Figure 1: Photomicrographs of haematoxylin stained and Eosin fixed sectionsof adult male Sprague–Dawley rat brain orally administered dichlorvos for 30 days. (a) Control rat showing brain architecture. (b) Rats exposed to 0.01dichlorvos showed no lesion. (c) Brain section of rat exposed to 0.05% dichlorvos showed no abnormality. Original magnification X400.





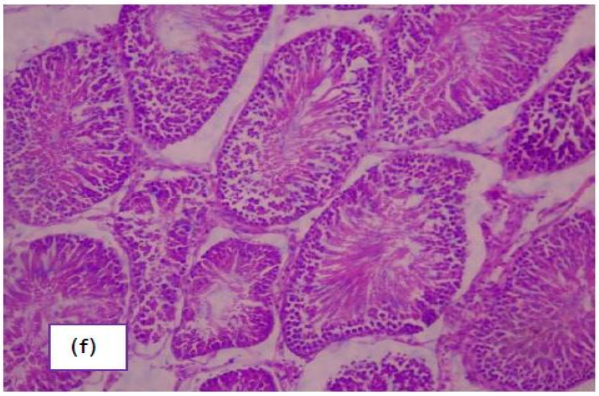


Figure 4.3: Sections of Photomicrographs of haematoxylin stained and Eosin fixed sections of adult male Sprague–Dawley rat testis orally administered dichlorvos for 30 days. (d) Control rat showing normal testis architecture. (e) rats exposed to 0.01 dichlorvos showed no abnormality (f) Testis section of rat exposed to 0.05% dichlorvos showed no abnormality. Original magnification X400.

There was a significant increase in the level of dopamine released in the MPOA, in rat exposed to 0.05% dichlorvos and there is also a significant increase in the concentration of dopamine found in circulation in rats exposed to dichlorvos (Table 3) when compared to the control at $p< 0.05$.

Table 3: Brain and Serum dopamine levels of rats orally administered dichlorvos for 30days.

	Brain Dopamine in MPOA (µg/g)	Serum Dopamine (µg/ml)
Control	90.10 ± 7.22	11.04 ± 0.38
0.01% DDVP	97.57 ± 4.07	15.23 ± 0.57 *
0.05% DDVP	112.30 ± 6.00 * #	15.75 ± 0.76 *

* = p<0.05 significant when compared to control. Results are presented as mean ± S.D
# = p<0.05 significant when compared within group exposed to DDVP

Table 4 shows significant increase in the level of testosterone in the rats exposed to dichlorvos compared to the control. The highest testosterone (12.1nmol/L) was recorded in the group exposed to 0.05% dichlorvos.

Table. 4: Testosterone levels of rats orally administered dichlorvos for 30days.

	Testosterone (nmol/L) N= 4
Control	9.9 ± 0.15
0.01% DDVP	10.8 ± 0.16 *
0.05% DDVP	12.1 ± 0.27 * #

* = p<0.05 significant when compared to control
# = p<0.05 significant when compared within group exposed to DDVP

DISCUSSION

Organophosphates are among the most widely used synthetic insecticides (Pedigo, 1991). The widespread use of organophosphates has stimulated research into the possibility of affecting the reproductive activities of humans and animals exposed to them (Suresh *et al.*, 2007). Dichlorvos, a typical organophosphate insecticide, has wide applications in both agriculture and domestic use.

The liver is an important internal organ, because it detoxifies the body of many toxins and synthesizes a wide range of proteins. Liver function tests (LFTs) are groups of blood assays that provide information about the state of the liver. Hepatotoxicity involves only mild symptoms initially; therefore, early detection is vital (Olaniyi *et al*., 2003). ALT and AST are known markers for liver damage (Loganathan *et al*., 2005). Dose dependent alanine aminotransferase reduction in rats administered dichlorvos in this study was suspected to be due impairment in liver functions as an organ that detoxify toxins, but this could not be ascertain as there was no significant difference in the activity of aspartate amino transferase and alkaline phosphatase in rats treated with dichlorvos when compared to control to indicate any hepatocellular damage.

Serum urea and creatinine levels were also determined in the present study; however, there was no significant change in the levels of urea and creatinine in all treated groups relative to the control. Many pesticides can cause some toxic and adverse effects on kidney tissues (Elhalwagy *et al*., 2008) Urea and creatinine levels are kidney function parameters. Pesticides have been shown to cause various histopathological changes in the kidney tissue of experimental animals (Elhalwagy *et al.*, 2008). However, in the present study, at the dose and duration of study, there was no evidence of kidney damage. The effect of dichlorvos on serum cholesterol was not consistent. The cholesterol level increased with pesticide exposure but later reduced. This corroborated with the report of Ethelbert *et al*. (2015).

The histological examination of the brain and testis of rat exposed to dichlorvos from sniper in this present study revealed no abnormality. This is in constrast with Ethelbert *et al.*,(2015) who observed lesion in the testis of rat given 0.01% and 0.05% dichlorvos and Oluwafemi (2014) who observed lesion in brain section of rats treated with 50mg/kg b.w of dichlorvos. Increase in sperm abnormality, decrease sperm count and decrease sperm motility at the higher percentage of dichlorvos from this study revealed the impact of dichlorvos on the semen quality of the rats. This is in agreement with Faris (2008), Chaudhary and Joshi (2003). They attributed the low sperm count to impairment of testicular functions which affected the activities of the enzymes responsible for spermatogenesis. Studies by Bretveld *et al*.,(2007) showed that pesticides may directly damage spermatozoa, alter sertoli cell or leydig cell function or disrupt the endocrine function in any stage of hormonal regulation. It could also be that at the higher dose of dichlorvos, the potency of the chemical had killed some of the sperm or incapacitated others hence the low semen quality. Sanchez- Pena *et al*. (2008) found that sperm DNA is sensitive to organophosphate pesticide exposure and it seemed to play an important role in the genesis of sperm chromatin alterations. The chromatin structure of abnormal sperm reflects a variety of anomalies during spermatogenesis after exposure to chemicals (Spano *et al.*, 1996). Other organophosphate pesticides reported to reduce semen quality include malathion (Contreras,2007) and Chloropyrifos (Joshi *et al.*, 2007).

Furthermore, this study revealed a dose dependent increase in the level of testosterone with corresponding increase in the level of dopamine in the brain MPOA and serum. Sarkar *et al*. (2000) reported an increase in mean serum concentration of testosterone in organophosphate-treated rat. According to Sarkar *et al*. (2000) increases in serum testosterone concentrations may be explained by the fact that the treatment with organophosphate caused an increase in serum LH concentrations. In male rats, circulating LH is responsible for maintaining normal increased serum testosterone concentrations (Ellis and Desjardins, 1982). Secondly, organophosphate pesticides may act directly on Legdig cells to stimulate testosterone synthesis. In contrast, Ethelbert *et al*. (2015) reported a significant reduction in testosterone in rats exposed to dichlorvos, Mansour *et al*. (2008)and Uzon *et al*. (2009) reported reduction in testosterone in rats treated with malathion. Reviewed in (Hull *et al.*, 2006) provided support for the hypothesis that DA action is downstream of testosterone in the causal change of events that result in the activation of male-typical sexual behaviour and it is well documented that testosterone acts as a permissive hormone, and a basal level is needed to stimulate sexual behaviour (Meisel and Sachs, 1994).

CONCLUSION

This present study revealed that dichlorvos may interfere with the synthesis of testosterone in the leydig cells. The results showed that a dose dependent increase in testosterone may increase sexual behaviour. Increase in testosterone, from the study corresponds with an increase in dopamine in the medial preoptic area of the brain, a region where dopamine released enhances responsiveness to stimuli from an oestrous female and increases the probability, rate, and efficiency of copulation. Furthermore, at the concentration of dichlorvos used and the Duration of the study, dichlorvos does not exert any toxicity effect on the brain and testis, but could have interfered directly with the production of sperm in the sertoli cell, hence the low sperm quality.

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