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Effects of Dimethoate on Ovarian Follicles, Fertility Parameters and the Protective Role of Vitamin C in Female Albino Wistar Rat (Rattusnorvegicus)

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Abstract

Background: Dimethoate (DM) (O, O-dimethyl-5-methyl carbomyolphosphorodithioate) is an organophosphate insecticide with a contact and systemic action. It is widely used against a broad range of insect pest and disease vectors such as mosquito and houseflies. The extensive use of dimethoate may pose a serious health hazards to animals and humans due to its persistence in soil and crops. In humans, the main risk groups to higher-dose dimethoate exposure include dimethoate producers, pesticide workers, farm owners and those that use it at home against mosquito and houseflies.Although dimethoate is moderately toxic (the reported acute oral LD50 value for rats is 310mg/kg/day, and for humans is anticipated to be about 30mg/kg) [1], and studies have shown that its toxicity may negatively affect many organs and systems such as the liver, kidney, respiratory, nervous, reproductive and immune systems [2, 3]. Some proposed mechanisms of organophosphates toxicity include the inhibition of acetylcholinesterase in the nervous system [4] and through electrophilic attack on the cellular constituents of tissues/organs, with simultaneous generation of reactive oxygen species [5]. Organophosphate pesticides may cause reproductive toxicity through several mechanisms, which may include direct damage to the structure of the cells, interference with biochemical processes necessary for normal cell function and biotransformation resulting in toxic metabolites [6]. The complexities and chains of physiological conditions that surround the toxicity of organophosphates can elicit and maintain or exacerbate several pathological conditions [7]. Hence the need arises for researchers to initiate and sustain researches of their effects on several organs and systems of the body.

Objective: The aim of this study was to investigate the effects of dimethoate on ovarian follicles, fertility parameters and the protective role of vitamin c in female albino wister rat (Rattusnorvegicus).

INTRODUCTION

Experimental Chemicals

Dimethoate (40% EC) used in this study was manufactured by Jiangsu Tenglong Biological and Medical Co. Ltd., Jiangsu province, China and was procured from the Solutor Agro & Allied Chemicals Company, Shop A2, Mile 1 market, Port Harcourt. The vitamin C was purchased from a local chemist store at Choba, Port Harcourt, while all other chemicals were obtained from the local distributors of scientific materials in Rivers State, Nigeria.

Experimental Design

Sixty four (64) female rats were acclimatized for two weeks, weighed and later divided into eight groups of sixteen rats each and treated as follows: Groups two, three and four were treated with 8.86, 12.4 and 20.68mg/kg/day of dimethoate, and were later designated as low dose (LD), medium dose (MD) and high dose (HD) respectively. Group five was treated with the low dose (LD) of dimethoate + 200mg/kg of vitamin C, group six was treated with the medium dose (MD) of dimethoate + 200mg/kg of vitamin C, group seven was treated with the high dose (HD) of dimethoate + 200mg/kg of vitamin C, group eight was treated with only the 200mg/kg of vitamin C, while group one was administered with 1ml of

distilled water per rat (to serve as the control). All dimethoate (DM) and vitamin C (VC) doses were dissolved in distilled water and given to the rats via oral gavage for forty two (42) consecutive days. At the end of the treatment (43rd day), some animals were scarificed and dissected, thenovarian tissues were taken for follicular and luteal count whilethe remaining treated female rats were cohabited with fertile untreated males and used for the assessment of their fertility status.

Histological Examination of the Ovary

Ovaries of the control, dimethoate and vitamin C treated rats (4 rats form each group) were carefully removed and fixed in 10% buffered formalin for 48 hours in properly labeled test tubes. The tissues were grossed and the best portions were put into labeled tissue cassettes and transferred to 95% alcohol for 2 hours for dehydration. Tissues were passed through xylene II for 2 hours to remove the alcohol and later embedded with paraffin wax and cut into sections of 2 to 3 µm thickness, using the microtome. Thereafter, sections were placed on slides, allowed to drain, arranged on a slide rack and gently warmed for 10 minutes, using the hot air oven.

The slides were then stained with haematoxylin for 10 minutes, rinsed in running tap water for 5 minutes and counter stained with eosin for 3 minutes. Slides were again dehydrated in absolute alcohol and then cleared in xylene and examined for follicular and luteal count, using the light microscope. Microphotographs of the slides were taken, using the Moticam1000 camera at 100x magnification.

Follicular and Luteal Count

The stained ovary slides were examined for follicular and luteal count. Parameters that were counted in the ovary include primary follicles, secondary follicles, graafian follicles (tertiary follicles), atretic follicles and corpus lutea. Primary follicles were identified based on the presence of primary oocyte surrounded by a layer of granulosa cells. Secondary follicles were counted based on the presence of many layers of granulosa cells surrounding the oocyte, the appearance of a follicular antrum (a gap containing a fluid known as liquor folliculi) within the granulosa layer, and the appearance of the zonapellucida. Graafian follicles (the follicular stage before ovulation) were counted based on the presence of a large follicular antrum that makes up most of the follicle [8].

Atretic follicles are follicles undergoing atresia by the apoptosis of granulosa cells which are replaced by fibrous materials. The oocyte also degenerates and the basement separating the oocyte from the granulosa cells thickens to become the glassy membrane. A follicle was considered to be undergoing atresia when a minimum of 5% pyknotic (apoptic) granulosa cells were found in a single section or the oocyte showed signs of degeneration. Care was taken not to repeat the counting of the same follicle more than once. Corpus lutea were counted based on the presence of a high number of hypertrophied (shrunken) granulosa cells with rich blood supply in the corpus luteum or an increase in fatty substance with rich blood supply giving the appearance of a gland [9].

Fertility Parameters

At the end of the treatment course, the remaining female rats (dams) of the control and treated groups (8 rats per group) were cohabited with proven fertile untreated males (4:1) for 10 days. Female rats were examined for the presence of sperm or vaginal smear (plug) at the vaginal orifice. Those with vaginal smear were considered to have successfully mated or copulated. They were caged separately and observed till delivery (21-24 days). Pups were removed and examined for liter size and mating success while the liter weights were taken and recorded, using a digital weighing balance.

Furthermore, fertility index (number of pregnant animals/number of females mated X 100), gestation index (number of pups born alive/number of total pups born X 100), and parturition index (number of females delivered/number of pregnant animals X 100) were also calculated and recorded.

Determination of the Protective Role of Vitamin C

The ameliorative properties of vitamin C (VC) on dimethoate toxicity of the female rats was determined by comparing the analyzed ovarian parameters in the vitamin C treated groups (groups five, six, seven & eight) with those of the dimethoate treated groups (groups two, three and four) and the control (group one). Fertility parameters across the eight groups were also examined to find out whether the vitamin C administration was able to restore the dimethoate induced level of the analyzed parameters to normal levels.

Statistical Analysis

Data in this study were expressed as mean±SEM. Data were analysed for significant difference between the control and treated groups at a significant level of $p<0.05$. Data analysis tools include the Statistical Package for Social Science (SPSS) version 22, One Way Analysis of Variance (ANOVA) and microphotographs.

RESULTS AND DISCUSSION

Result of the Effect on Follicular and Luteal Count

Effect on corpus lutea

There was significant decrease (P< 0.05) in the mean number of corpus lutea in the dimethoate treated groups when compared with the control while their respective dimethoate + vitamin C treated groups showed an insignificant increase when compared with those that received only the dimethoate. There was no significant difference between the control (CT) and group eight (VC) with the highest mean numbers of 4.67 and 4.33 respectively (Table 1). There was also no significant difference between groups three (MD) and five (LD+VC), and between groups two (LD), four (HD), six (MD+VC) and seven (HD+VC) (Plates 3, 5 a & b).

Effect on atretic follicle

The mean number of atretic follicles was significantly increased (P< 0.05) both in the dimethoate treated groups and in the groups that received the dimethoate + vitamin C as compared to the control group. However, the lowest mean number of 4.00 was reported in group eight (VC) which was significantly different from the control group which had a mean number of 5.00 (Table 1). There was no significant difference between groups three (MD) and five (LD+VC), and between groups six (MD+VC) and seven (HD + VC) (Plates 2, 4, 5b).

Effect on graafian follicle

There was significant decrease (P< 0.05) in the mean number of graafian follicles in both the groups that received only dimethoate and those that received dimethoate + vitamin C as compared to the control (Table 1). A dose dependent decrease was reported between the groups that received the dimethoate, with no recorded mean number for group four (0.00) whereas no significant difference was reported between the groups that received the dimethoate + vitamin C. There was significant difference between the control (CT) and group eight (VC) with recorded mean numbers of 2.00 and 1.33 respectively (Plates 1, 4).

Effect on primary follicle

The mean number of primary follicles was significantly decreased (P< 0.05) in the dimethoate treated groups when compared to the control. The decrease in number of primary follicles was in a dose related fashion, with the lowest mean number of 2.33 reported in group four (HD) (Table 1). However, a non-dose related increase was recorded in the groups that received dimethoate + vitamin C when compared with those that received only the dimethoate. There was no significant difference between the control (CT) and group eight (VC) with reported high mean numbers of 6.67 and 6.00 respectively (plates 1, 3, 5 a & b).

Effect on secondary follicle

A significant decrease (P< 0.05) in the mean number of secondary follicles was reported in both the dimethoate and dimethoate + vitamin C treated groups as compared to the control. There was no significant difference between the dimethoate treated groups and the dimethoate + vitamin C treated groups, whereas there was significant difference between the control and group eight (VC) with reported mean members of 2.33 and 1.33 respectively (Table 1). There was also no significant difference between groups two (LD), five (LD+VC) and eight (VC), and between groups four (HD) and six (MD+VC) which recorded the lowest mean number of 0.67 (Plates 5 a & b).

Table 1: Effects of dimethoate and vitamin C on follicular and luteal count

Treatment groups	Ovarian parameters				
	CL	AF	GF	PF	SF
GRP 1 (CT)	4.67 ± 0.67^{b}	5.00 ± 1.53^{a}	2.00 ± 1.15^{b}	6.67 ± 1.20^{bc}	2.33 ± 0.88^{c}
GRP 2 (LD)	3.33 ± 0.33^{ab}	5.00 ± 1.53^{a}	1.00 ± 0.58^{bc}	3.67 ± 0.33^{abc}	1.33 ± 0.33^{a}
GRP 3 (MD)	2.67 ± 0.33^{a}	5.33 ± 0.67^{bc}	0.33 ± 0.33^{ab}	3.00 ± 0.58^{ab}	1.00 ± 0.58^{a}
GRP 4 (HD)	3.00 ± 0.58^{ab}	5.67 ± 0.33^{c}	0.00 ± 0.00^{a}	2.33 ± 0.33^{a}	0.67 ± 0.33^{bc}
GRP 5 (LD+VC)	2.67 ± 0.33^{a}	5.33 ± 1.20^{bc}	$o.33\pm 0.33^{ab}$	5.33 ± 1.45^{c}	1.33 ± 0.33^{a}
GRP 6 (MD+VC)	3.33 ± 0.33^{ab}	6.33 ± 1.45^{ab}	0.33 ± 0.33^{ab}	4.00 ± 1.00^{abc}	0.67 ± 0.33^{bc}
GRP 7 (HD+VC)	3.33 ± 0.33^{ab}	6.67 ± 0.88^{ab}	0.33 ± 0.33^{ab}	4.67 ± 1.20^{abc}	1.00 ± 0.58^{a}
GRP 8 (VC)	4.33 ± 0.88^{b}	4.00 ± 0.58^{b}	1.33 ± 0.67^{bc}	6.00 ± 1.15^{bc}	1.33 ± 0.67^{a}

CL = Corpus lutea, AF = Atretic follicle, GF = Graafian follicle, PF = Primary follicle, SF = Secondary follicle.

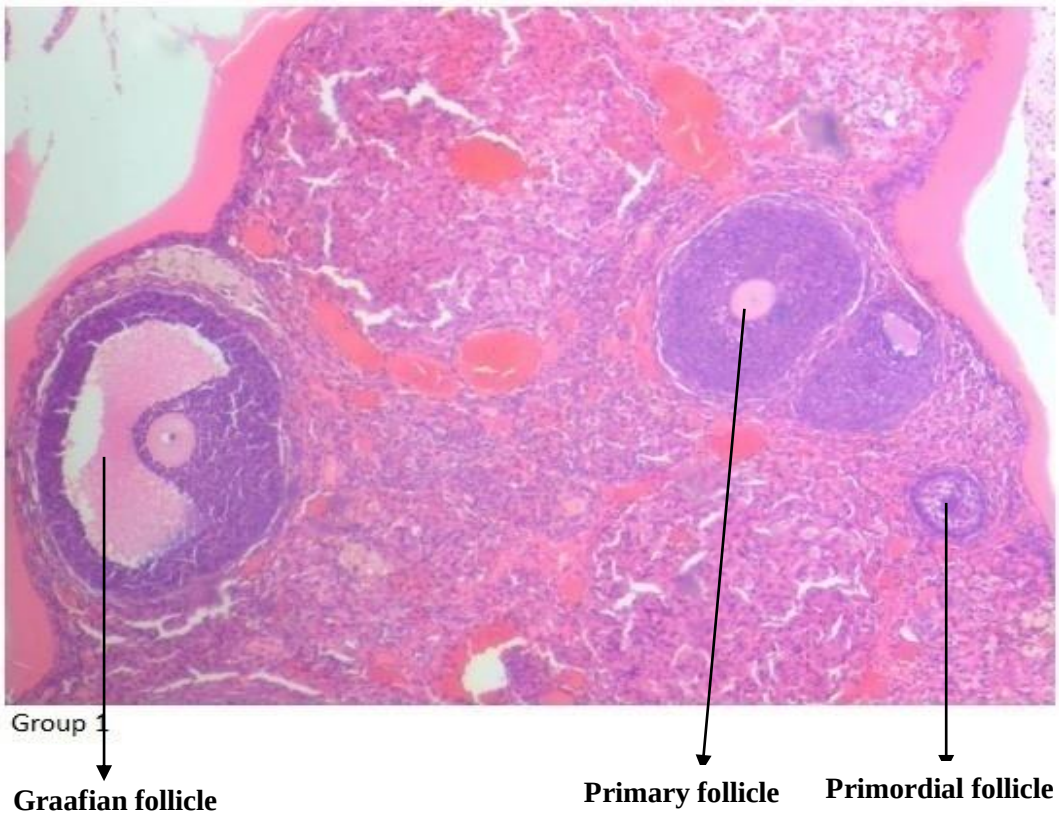

Plate 1: Ovary section showing graafian follicle, primary follicle and primordial follicle

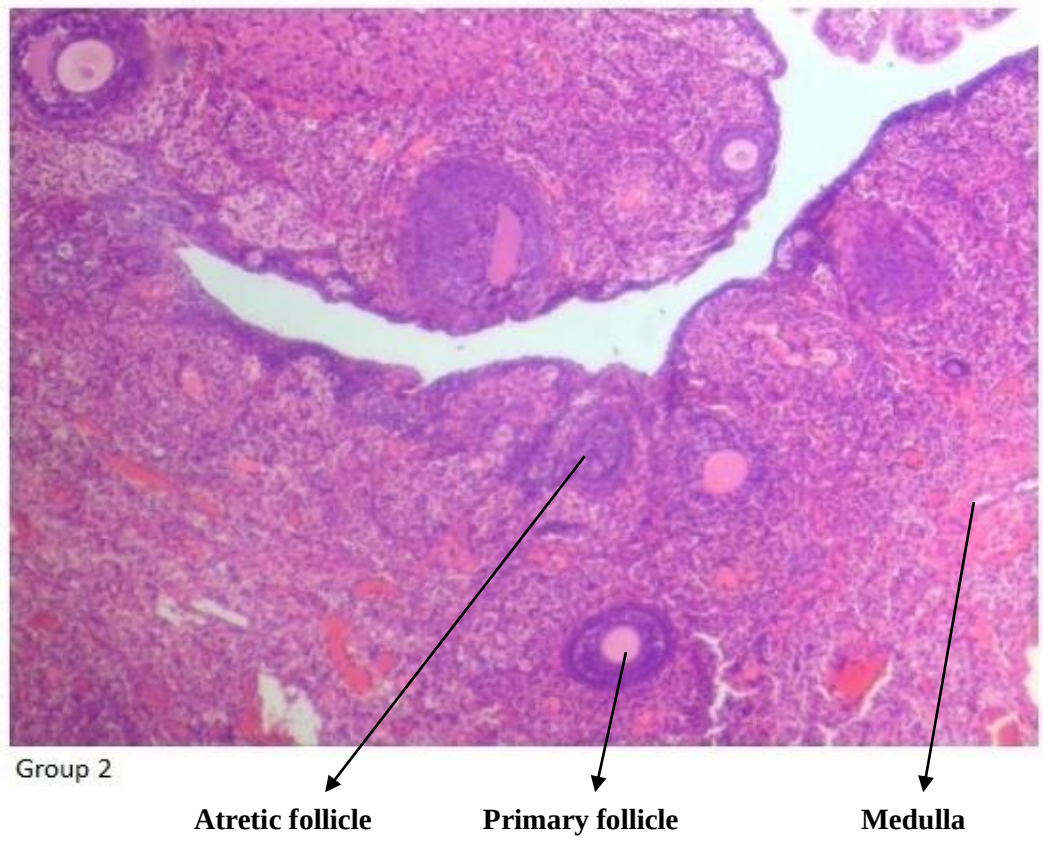

Plate 2: Ovary section showing atretic follicle, primary follicle and medulla

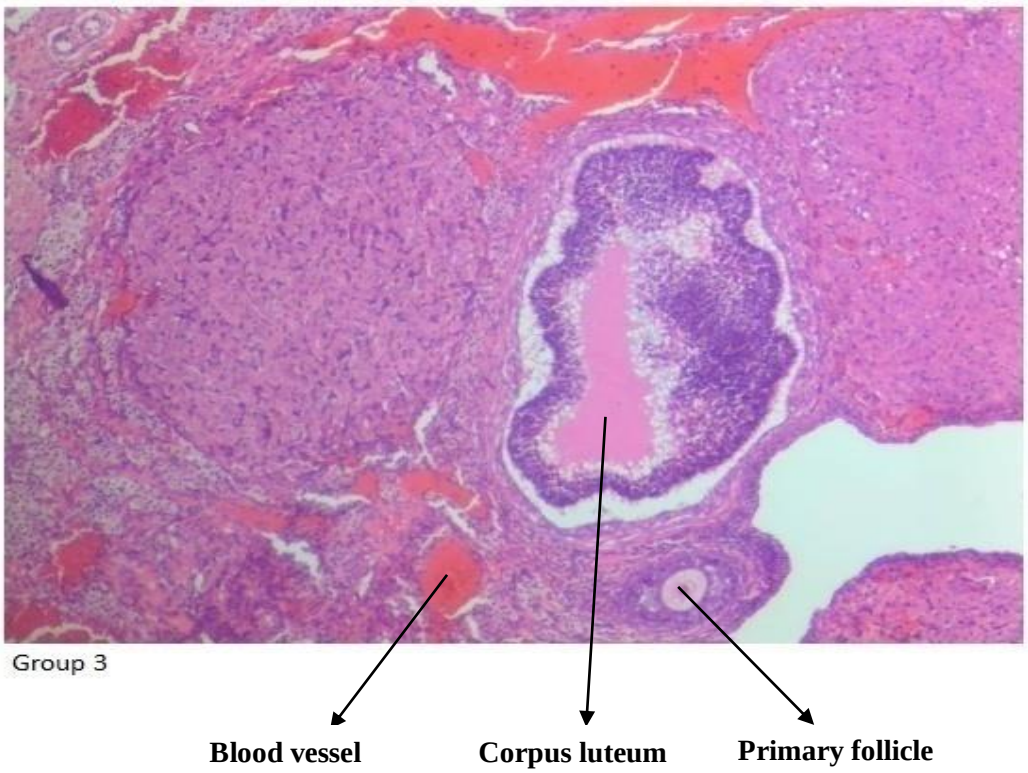

Plate 3: Ovary section showing blood vessel, corpus luteum and primary follicle

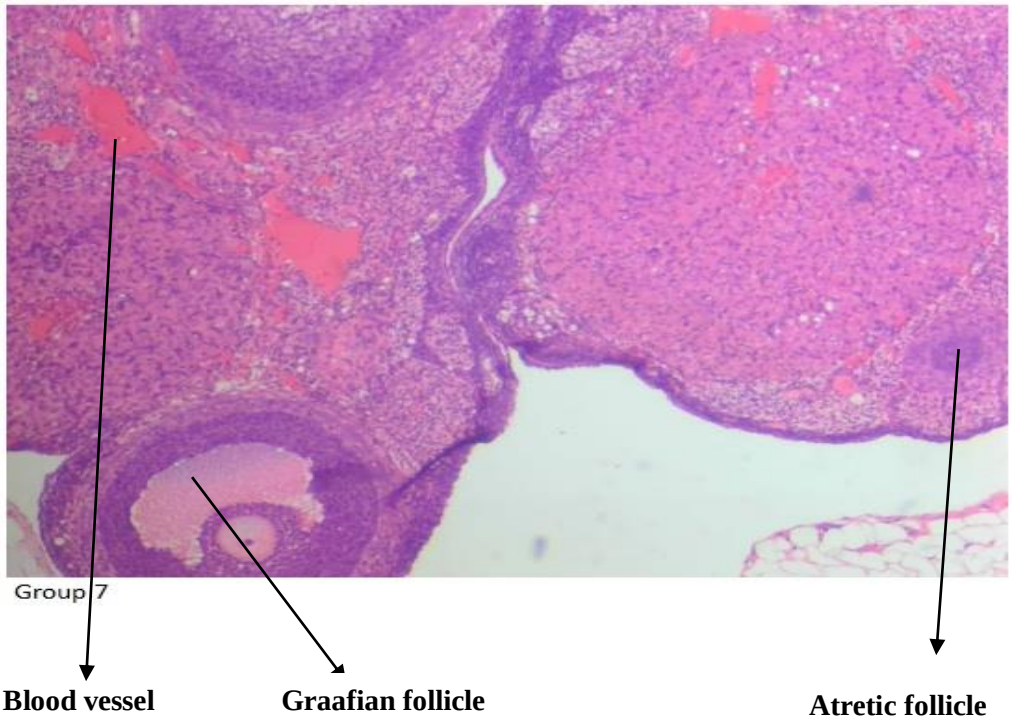

Plate 4: Ovary section showing blood vessel, graafian follicle and atretic follicle

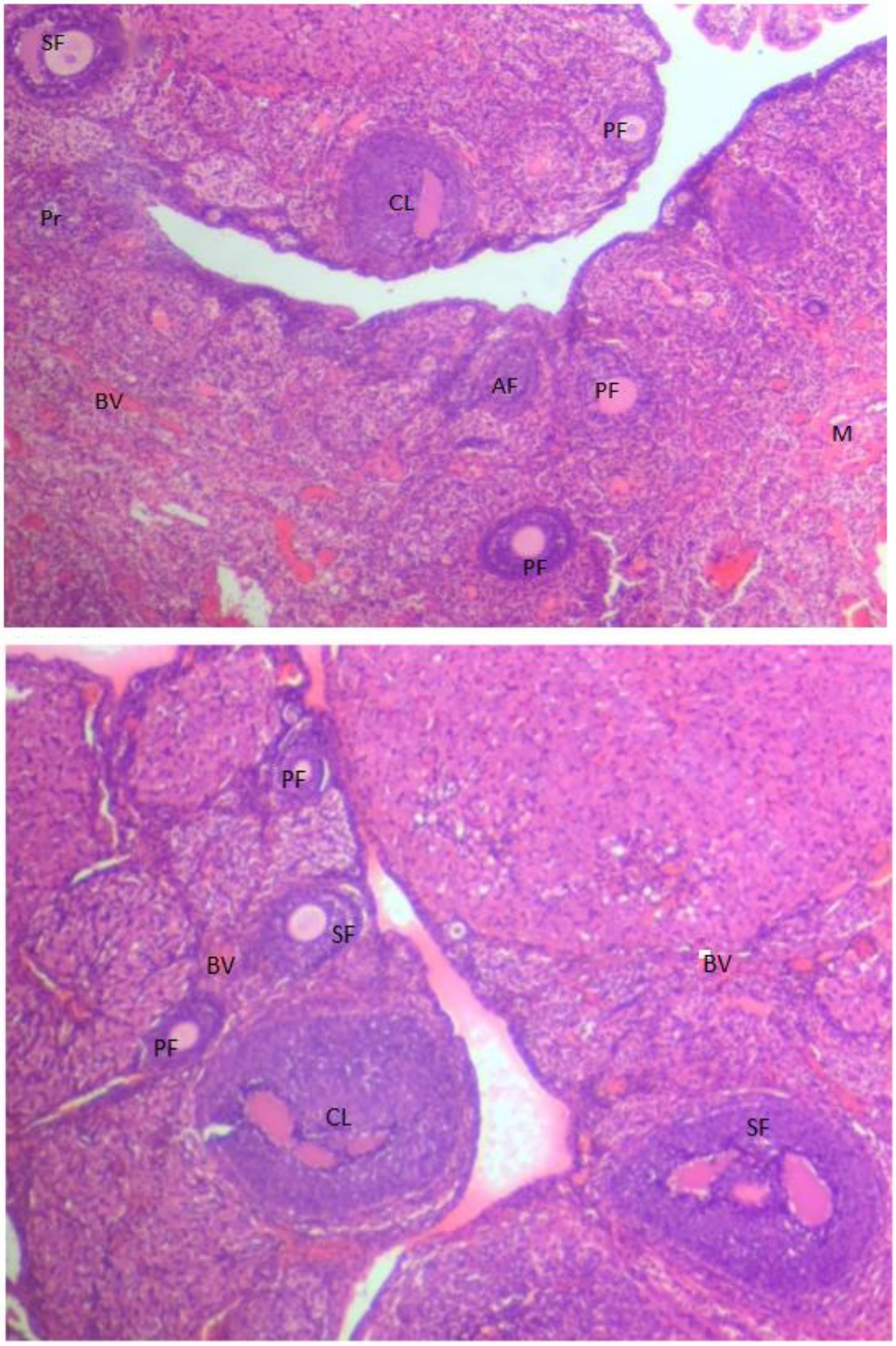

Plate 5a & b: Ovary sections showing labeled ovarian parameters.

Key: CL=Corpus luteum, SF=Secondary follicle, PF=Primary follicle, AF=Atretic follicle, Pr=primordial follicle, M=Medule, BV=Blood vessel.

There was a significant decrease ($P< 0.05$) in the mean number of corpus lutea, graafian, primary and secondary follicles with a significant increase in the number of atretic follicles in the dimethoate treated groups as compared to the control. These changes (increase/decrease) were more drastic with increase in dose level, which indicate a dose dependent

adverse action of the dimethoate in female rats. The observations in the present study that dimethoate caused a decrease in number of healthy follicles with an increase in atretic follicles are in agreement with reports on the effects of other pesticides in rats [10]. Estrogen is essential for normal folliculogenesis and is crucial for the survival of ovarian follicles [11]. Hence, the reduction in serum estrogen level resulting from dimethoate in the present study might be due to the decrease in the number of corpus lutea, graafian, primary and secondary follicles and the concomitant increase in atretic follicles. The study also reported a slight increase in the mean number of corpus lutea, graafian, primary and secondary follicles whereas the atretic follicles showed significant increase in both the dimethoate and the dimethoate + vitamin C treated groups.

The increase in atretic follicles in both the dimehoate and dimethoate + vitamin C treated groups could be attributed to the toxic effect of dimethoate on folliculogenesis while the slight increase in number of corpus lutea, graafian, primary and secondary follicles in the dimethoate + vitamin C treated groups could be due to the protective role of vitamin C to the damaging effect of the dimethoate on folliculogenesis.

Result of the Effect on Fertility Parameters

Effect on litter size, litter weight and mating success

There was significant decrease ($P< 0.05$) in mean litter size of the dimethoate treated groups when compared to the control while their respective dimethoate + vitamin C treated groups showed an insignificant increase as compared with the dimethoate treated groups, and with no significant difference between the control (CT) and group eight (VC) which had the highest recorded mean litter size of 5.20 (Table 2).

There was significant decrease ($P< 0.05$) in the mean litter weight of the dimethoate treated groups as compared to the control whereas an insignificant increase was reported in the dimethoate + vitamin C treated groups when compared with their corresponding dimethoate treated groups. There were no significant difference between groups six (MD+VC), seven (HD+VC) and eight (VC) with the highest mean litter weight reported in the control (5.03 g) (Table 2).

Analysis on mating success showed a percentage decrease ($P< 0.05$) in the dimethoate treated groups compared to the control whereas there was a percentage increase in the dimethoate + vitamin C treated groups when compared with their respective dimethoate treated groups. There was no percentage difference between the control (CT) and group eight (VC) with a 63% mating success, and between groups five (LD+VC), six (MD+VC) and seven (HD+VC) which had a 38% mating success (Table 2, Figure 1).

Table 2: Effects of dimethoate and vitamin C on litter size, litter weight and mating success

Treatment groups	Fertility parameters		
	Litter size	Litter weight (g)	% Mating success
GRP 1 (CT)	5.20 ± 0.21^{a}	5.03 ± 0.04^{d}	$^{5}/_{8}(63\%)$
GRP 2 (LD)	4.67 ± 0.13^{b}	4.75 ± 0.09^{bc}	$^{3}/_{8}(38\%)$
GRP 3 (MD)	5.00 ± 0.05^{bc}	4.45 ± 0.07^{b}	$^{2}/_{8}(25\%)$
GRP 4 (HD)	3.00 ± 0.00^{c}	4.27 ± 0.03^{a}	$^{1}/_{8}(13\%)$
GRP 5 (LD+VC)	5.33 ± 0.28^{cb}	4.66 ± 0.06^{b}	$^{3}/_{8}(38\%)$
GRP 6 (MD+VC)	5.00 ± 0.10^{bc}	4.88 ± 0.04^{cd}	$^{3}/_{8}(38\%)$
GRP 7 (HD+VC)	4.33 ± 0.04^{b}	4.93 ± 0.03^{cd}	$^{3}/_{8}(38\%)$
GRP 8 (VC)	5.20 ± 0.22^{a}	4.96 ± 0.04^{cd}	$^{5}/_{8}(63\%)$

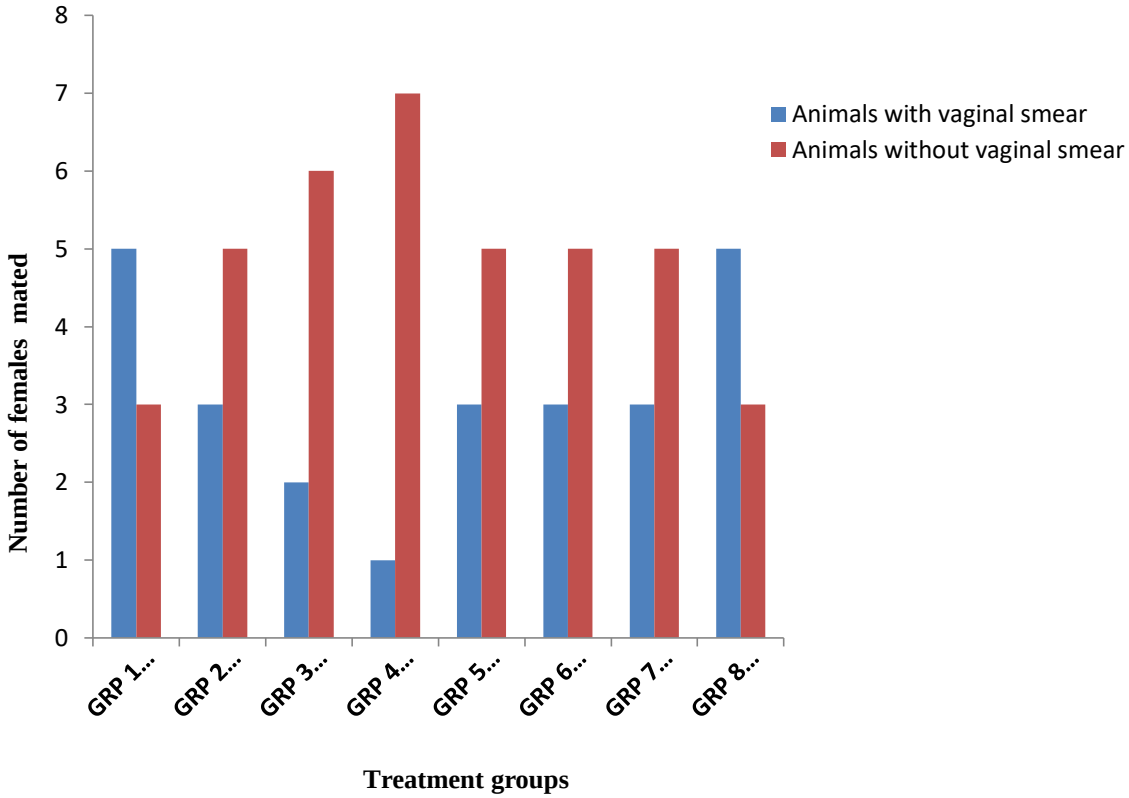

Figure 1: Summary of the effect of dimethoate and vitamin C on mating success

Percentage effect on fertility, gestation and parturition indices

These was dose dependent percentage decrease for the groups that received the dimethoate only when compared to the control whereas a percentage increase was reported in the groups that received the dimethoate + vitamin C when compared with the dimethoate treated groups. There was 63% fertility for the control and group eight (VC) while a 39% fertility was reported for all the dimethoate + vitamin C treated groups, with the least percentage fertility (13%) recorded in group four (HD) (Table 3).

Gestation index showed a percentage decrease for all the dimethoate treated groups when compared with the control whereas an insignificant percentage increase was recorded for the dimethoate + vitamin C treated groups as compared to their respective dimethoate treated groups. There was 100% gestation index for the control and group eight (VC) while the lowest percent (67%) was reported in group four (HD). There was 100% parturition index for all the eight groups, as this was confirmed by the number of females delivered divided by the number of pregnant animals (Table 3).

Table 3: Percentage effect of dimethoate and vitamin C on fertility, gestation and parturition indices

Treatment groups	Fertility parameters		
	Fertility index	Gestation index	Parturition index
GRP 1 (CT)	$^{5}/_{8}$(63%)	$^{26}/_{26}$(100%)	$^{5}/_{5}$(100%)
GRP 2 (LD)	$^{3}/_{8}$(38%)	$^{13}/_{14}$(93%)	$^{3}/_{3}$(100%)
GRP 3 (MD)	$^{2}/_{8}$(25%)	$^{8}/_{10}$(80%)	$^{2}/_{2}$(100%)
GRP 4 (HD)	$^{1}/_{8}$(13%)	$^{2}/_{3}$(67%)	$^{1}/_{1}$(100%)
GRP 5 (LD+VC)	$^{3}/_{8}$(38%)	$^{15}/_{16}$(94%)	$^{3}/_{3}$(100%)
GRP 6 (MD+VC)	$^{3}/_{8}$(38%)	$^{13}/_{15}$(87%)	$^{3}/_{3}$(100%)

GRP 7 (HD+VC)	$^{3}/_{8}$(38%)	$^{9}/_{13}$(69%)	$^{3}/_{3}$(100%)
GRP 8 (VC)	$^{5}/_{8}$(63%)	$^{26}/_{26}$(100%)	$^{5}/_{5}$(100%)

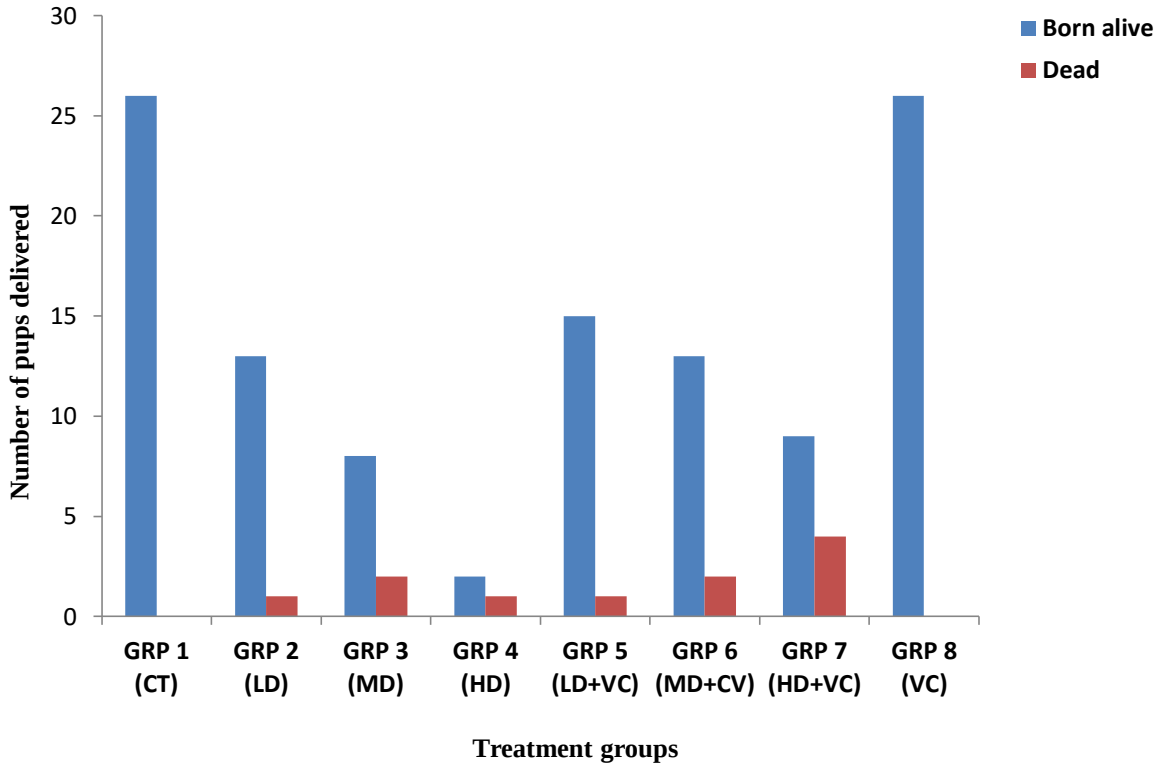

Figure 2: Summary of live and dead pups delivered after the dimethoate and vitamin C treatment

The present study showed a dose dependent decrease in litter size, litter weight and percentage mating success, fertility index and gestation index in the dimethoate groups compared to the controls. These reductions may be due to the toxic effect of dimethoate on reproductive organs, reproductive hormones and the ovarian oxidative damage that were earlier observed in this study. This suggests that for the accomplishment of reproduction and fertility normal functioning of accessory organs is essential. This result is consistent with that of [12] who reported an increased foetalresorption and reduced number of live feotuses in animals treated with dimethoate. The present result is also in agreement with that of [13] who showed that methoxychlor exposure to parental generation, both parental and F1 generations exhibited prolonged estrous cycle, and decline in fertility index, number of implantation sites and litter size. However, there was 100% parturition index for all the treatment groups including the control in this study. This was confirmed by the number of females delivered divided by the number of pregnant animals, and this indicates that the dimethoate exposure had no influence on parturition.

The present study also recorded an increase in litter size, litter weight and percentage mating success, fertility index and gestation index for all the groups that received dimethoate+ vitamin C when compared with the dimethoate treated groups. The increase in values of fertility parameters in the dimethoate + vitamin C treated groups could be due to the protective effect of vitamin C to the toxic effect of dimethoate on the examined fertility parameters.

CONCLUSION

This study revealed that the subchronic exposure of wister rats to dimethoate insecticide at dose levels of 8.86, 12,4 and 20.68mg/kg/day induced reproductive toxicity in female rats manifested by decreases in litter size, litter weight, mating success, fertility index, gestation index, number of healthy follicles with a concomitant increase in atretic follicles. However, the ultimate effects were observed in the high dose (HD) group.In contrast, co-administration of vitamin Cwith the insecticide antagonized the ovarian follicles and fertility toxicity. Based on the above findings, it is plausible that vitamin C may provide a cushion for prolonged therapeutic options against insecticide-induced ovarian follicles and fertilitytoxicity without harmful side effects.

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COMPETING INTERESTS
The authors have declared that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

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