Medical and Health Sciences European
Med. H. Sci. E. J.
Volume: 7; Issue: 05,
September-October, 2023
ISSN: 2469 – 6774
Impact Factor: 4.42

Advance Scholars Publication
Published by International Institute of Advance Scholars Development
https://aspjournals.org/Journals/index.php/mhsej

CHRONIC EXPOSURE OF PLANT BASED INSECTICIDE PYRETHRIN AFFECTS THE REPRODUCTIVE ORGANS OF MALE WISTAR RATS

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Keywords:
Pyrethrin Insecticides, Endocrine disruptors, male reproductive functions, Sperm

Abstract: *Insecticides derived from plants, such as pyrethrin, have gained popularity as a safer alternative to synthetic insecticides. Pyrethrin Plant based insecticides are now commonly used in agriculture, households to control pests and thought to be safe. Chronic exposure to pyrethrin insecticides may have adverse effects on the environment and human health. However, its effects especially on male reproductive system especially during chronic exposure has not been studied to any reasonable extent. In this study, we investigated the effects of chronic exposure to plant-derived insecticide pyrethrin on sperm and the male reproductive system of adult Wistar rats. Fifteen adult male Wistar rats used were divided into 3 groups. Group 1 was the control while groups 2 and 3 served as test groups. Group 1 received 2 puffs of distilled water daily, while groups 2 and 3 were exposed to 2 puffs (Low dose) and 3 puffs twice daily (morning and night) for six weeks. Serum samples obtained from the rats were assayed for reproductive hormones (Testosterone, Estradiol, Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH)) using Enzyme-Linked Immunosorbent Assay (ELISA). Testes, epididymis, seminal vesicles and the prostate glands were removed for histology, while sperm motility and concentration were determined using sperm from the caudal epididymis and vas deference. Sperm movement kinetics of the rats was determined using Computer Assisted Sperm Analysis (CASA) and histology was determined using*

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Haematoxylin and Eosin (H&E) staining.
There was no significant difference in the relative reproductive organs weight (testis, seminal vesicle, epididymis) among the three groups but there was a significant change ($p<0.05$) in the prostate glands in group 2 and 3 compared to the control. There were no significant differences in Estrogen, Testosterone, FSH and LH values in the test groups of rats when compared with the control but there was an observable increase. There was no significant difference in the motility and kinetics of the sperm. There was a significant increase in sperm concentration as well as a significant reduction in the numbers of immotile sperm of groups 2 and 3 respectively compared to the control group. Interestingly, tissue damage was observed in the testis, epididymis, seminal vesicles and prostate gland was seen using H&E staining.
Chronic exposure to pyrethrin insecticides did not significantly affect sperm but induced tissue destruction of the male reproductive organs. This may lead to decreased secretions in the testes, epidydimis, seminal vesicles and prostrate glands that may affect male reproductive functions.

INTRODUCTION

Pyrethrins (pyrethrum) are a mixture of natural chemical compounds found in the extract of *chrysanthemum* flowers [*Tanacetum* (= *Chrysanthemum* = *Pyrethrum*) *cinerariaefolium*]. Pyrethrum extract has six different compounds with insecticidal activity. These active compounds in the chrysanthemum flower extract are named pyrethrins (Todd *et al.* 2003). Moreover, pyrethrins are registered as pesticides and found worldwide (Bond *et al.*, 2014). Pyrethrins also have the advantage over other synthetic insecticides of being rapidly broken down upon exposure to light and air, are metabolized quickly, and can be used in the production of organic farm products. They are generally considered to be non-polluting (Sun *et al.*, 2020). Pyrethrins are used on a variety of agricultural crops and for structural and public health pest control. Worldwide, about 200,000 kilograms of Pyrethrins are used each year (Crossby, 1995). Despite being considered as safe, Pyrethrins are a common cause of insecticide poisonings (Saxena *et al.*, 1974). Pyrethrins can also affect physiological processes that are not related to the nervous system. Researchers at the Osaka City Institute of Public Health and Environmental Sciences (Japan) showed that pyrethrins inhibit mitochondria; the cellular bodies that convert food to usable energy in rat livers (Yamano *et al.*, 1993; Adams, 1994). Karel et al., (1975) also found out that animals fed on large doses of pyrethrins may experience liver damage. Rats fed pyrethrin at high levels for two years showed no significant effect on survival, but slight, damage to the livers was observed. Injection of pyrethrins caused gerbil blood sugar levels to rise between 30 and 70 percent. Blood sugar

peaked an hour after treatment, but the increase persisted for several days (Karel *et al*., 1975). Pyrethrins have been observed to be associated with increased cancer risks among farmers and have also caused cancer in laboratory test (Brown *et al*., 1990). National Cancer Institute reported increased risk factors for leukemia among farmers exposed to pyrethrins used for pest control on livestock (Ebubanks, 1997; WHO, 2000).

However, there have been no studies to investigate the effects of chronic exposure of Pyrethrin insecticides on the male reproductive organs. We report the effects of chronic exposure to plant-derived insecticide pyrethrin on sperm and the male reproductive system of adult Wistar rats.

MATERIALS AND METHOD

Animals

Fifteen adult male Wistar rats were used. The Wistar rats were kept in cages in the LASUCOM Animal House and maintained at room temperature with approximately 12 hours dark and 12 hours light cycle. The rats were feed standard rat chow and water ad labitum during this study. LASUCOM Animal Research Ethics Committee approved the study with approval number (Ref. No: AREC/2022/03).

Experimental design

The rats were divided into three groups (1, 2 and 3) with 10 rats in the first two groups and 7 in the last group. Group 1 (7 Male Wistar Rat) was the control while groups 2 (10 Male Wistar Rat) and 3(10 Male Wistar Rat) were the test groups. Two of the test groups were exposed to spray of insecticides in sprayed puffs from the aerosolized insecticide (Kill a dream). The insecticide contains 0.4% of perythrin (1, 2, 3). Group 1 (the Control group) received distilled water daily, while Groups 2 and 3 where groups were exposed to spray of insecticides in sprayed puffs. Group 2 were sprayed 2puffs (Low dose) and Group 3 were sprayed 3 puffs twice daily (morning and night) for six weeks. After six weeks, groups 1-3 were sacrificed. The rats were administered intraperitoneally with 0.2ml/100g (de Carvalho *et al*., 2011) of Ketamine and were monitored for indications of anaesthesia such as sleepiness and loss of consciousness before they were sacrificed when reflexes were lost. Blood sample of each rat was collected by retroorbital method using heparinised tube and/or cardiac puncture using 5mls syringe. The samples were placed in plain bottles and allowed to clot, followed by centrifuging at 2500 rpm for 20 minutes using a desktop centrifuge (Surgifriend centrifuge, Model SMBO-2, England). This process separates the sera from the blood cells. The sera were aliquoted into an Eppendorf tube and stored at -20◦. The frozen blood samples were used for hormonal assay. The testes, epididymis, seminal vesicles and prostates were carefully dissected free of fat and weighed using a digital weighing balance. Some samples of all the tissues removed were fixed and preserved in 10% buffered formalin.

Hormonal Assay

The serum from Groups 1-3 were tested for Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH), Oestradiol, and testosterone. The procedure had previously been described (Ajonuma et al. 2017a, Ajonuma et al 2017b). Briefly, hormonal assay enzyme linked immunosorbent assay (ELISA) kits used were purchased from Monobind Inc., CA, USA. The assays were done according to the instructions of the manufacturer. Assay kits were brought to room temperature. The

following steps were taken: The needed number of micro strips were placed in the frame, with 12 wells for the calibrator samples and 12 wells for each serum sample .50µl of Calibrator sample were placed into the wells using pipette, 50µl of serum were also placed in the required wells and 50µl of hormonal Conjugate Reagent was dispensed into the wells and then incubated for 60 minutes at 37 degrees Celsius. The strips were rinsed five times with a pre-diluted washing solution after they had been incubated (containing surfactant in buffered saline) and a 20x dilution of the washing solution concentrate with distilled water was used to make the washing solution.50µl of 3,3,5,5'-Tetramethylbenzidine (TMB) substrate A solution was added into each of the wells followed by the addition 50µl of TMB Substrate B solution into all the well this was thoroughly mixed and incubated for 20 minutes at 26 degrees Celsius.100µl of stop solution was added into each of the wells using a pipette. Optical density (OD) was measured at 450nm/620nm on a STAT Fax 4700 ELISA micro plate reader.

Heamatoxylin and Eosin (H&E) Tissue Staining

This was done on testes, epididymis, prostate, and seminal vesicles samples that had previously been fixed in 10% formalin and described (Ajonuma *et al*., 2005) with some changes. The fixed tissue was placed in a paraffin wax mould, and the wax was blasted onto the surface until a thin film of wax solidified. The tissue-containing mould was placed in a container of cold water. It stayed submerged in the wax until it hardened. At 5 p.m., the paraffin block was trimmed and placed on ice for 1 hour before being set in place on the microtome and sections were cut. In a heated bath at 40°C, the thin part is floated with 20% alcohol.

The thin portion was selected and dried at 75°C on a hot plate. The piece was immersed in water for 10 minutes before being stained with Hematoxylin. The stained slice was washed in water before being differentiated in a 1% acid-alcohol solution. This was rinsed for 1 minute in water before being counter stained with 1% Eosin for 1 minute.

The excess stain was washed away, and the piece was dehydrated in steps of 70%, 90%, and 100% alcohol for 15 seconds each. The section was then cleaned in Xylene and mounted in dihydroxy phthalate xylol (DPX). A light microscope (Olympus, China) was used to examine the stained section, and photographs of the section were acquired using the attached camera.

Epididymal Sperm Analysis

The cauda epididymis was minced and incubated for 5 minutes at 37°C in 2ml of normal saline. A clear, debris-free solution was obtained, which was utilized to assess sperm concentration, motility, vitality, movement characteristics, and sperm DNA status).

Sperm Motility and Movement Kinetics

This was carried out as previously described (Ajonuma et al., 2002) and in this study using the JH-6004 Computer Assisted Sperm Analysis (CASA) machine (Jiangsu Jiahua Electronic Instrument Co., Ltd. China). The stage warmer of the CASA was set at 37°C and the software launched on the computer before commencement of analysis. 10µL of the sperm incubated in sperm washing medium at 37°C was then loaded on improved Neubauer Hemocytometer, covered with a cover slip and

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placed on the microscope warming stage. The microscope was adjusted appropriately to get a clearer image as seen in the microscope and also appropriately captured by the Camera attached to the microscope as displayed on the computer screen. The X40 magnification was used and this magnification was also selected in the multiples of the CASA machine software. At least a minimum of 100 sperm at a minimum of 5 views were evaluated. The impurities were automatically excluded by the CASA software. The results were printed out by using the printer attached to the machine.

The following parameters were accessed by the CASA machine average path velocity (VAP, μm/s), curvilinear velocity (VCL μm/s), and Straight line velocity (VSL μm/s). Amplitude of lateral head (ALH, μm), Beat Cross Frequency (BCF, Hz) in Hertz, straightness (STR: VAP/VCL×100%) in percentage (%), Wobble (WOB) in percentage (%) and Mean Move Angle Degree (MAD) in degrees (°), as well as sperm motility (progressive motility, local motility, and immotile spermatozoa) measured in percentage (%).

Sperm Concentration

The sperm concentration was carried out according to WHO (2010). 950μl of sperm diluent fluid were added to 50μl of sperm epididymal sperm in an Eppendorf tube. 10ul of this mixture was loaded into improved Neubauer Hemocytometer. The sperm was counted in the 5 small spaces within the large center square using Light microscope (magnification x 40) and the total sperm counted is expressed as sperm/ml-sperm counted x1,000,000/ml.

Statistical Analysis

Results were presented as mean SEM. The mean across groups were analysed using Analysis of Variance (ANOVA) and Turkey post hoc test. Statistical significance was accepted at $p \leq 0.05$. Statistical analyses were carried out using Graph Pad Prisms, version 8.0, Graph Pad Inc, USA.

RESULTS

The Effect of Pyrethrin on the Reproductive Organ of the Wistar rat

Relative Testicular Weight

In table 1, group 2 (P value=0.177) and group 3 (P value=0.601) showed no significant difference when compared to the Control (group 1).

Relative Epididymis Weight

In table 1, group 2 (P value=0.801) and group 3 (P value=0.521) showed no significant difference when compared to the Control (group 1).

Relative Seminal Vessicle Weight

In table 1, group 2 (P value=0.545) and group 3 (P value=0.698) showed no significant difference when compared to the Control (group 1).

Relative Prostate Gland Weight

In table 1, group 2 (P value=0.035) is significantly increased ($P<0.05$) when compared to the control, while group 3 (P= 0.787) showed no significant difference when compared to control, there was also a reduction in group 3 (P=0.011) when compared group 2

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Table1: Effect of Pyrethrin on the reproductive organs of the male Wistar rats

Groups	RTW (g)	REW (g)	RSVW (g)	RPW (g)
Group 1 (control) n=5	1.29±0.09	0.55±0.03	0.61±0.05	0.14±0.01
Group 2 (low dose) n=5	1.10±0.05	0.52±0.01	0.70±0.02	0.19±0.02*
Group 3 (high dose) n=5	1.19±0.07	0.49±0.04	0.53±0.09	0.12±0.01*

RTW= Relative Testis Weight, REW= Relative Epididymis weight, RSVW= Relative Seminal Vesicle Weight, RPW= Relative Prostrate Weight and n= number of animals.

The effect chronic exposure of pyrethrin on Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH), Testosterone, Estradiol and Testosterone estradiol ratio

Pyrethrin caused no significant difference in the serum levels of Follicle Stimulating Hormone (FSH) among the test groups of the Wistar rats but there was an elevation of the FSH levels seen in group 3 that was not significant (Fig 1a). There was no significance difference in the serum levels of the Luteinizing Hormone (LH) of the test groups when compared to the control group but a spike was observed in the serum levels of LH among group 3 (Fig 1b). There was no significance difference in the serum level of the testosterone of the test group when compared to the control group (Fig 1c). There was no significance difference in the serum levels of estradiol of the test groups when compared to the control group. However, a pattern of reduction in the test groups 2 and was observed (Fig 1d). Pyrethrin caused no significant difference on the Testosterone / estradiol ratio (Fig 1e).

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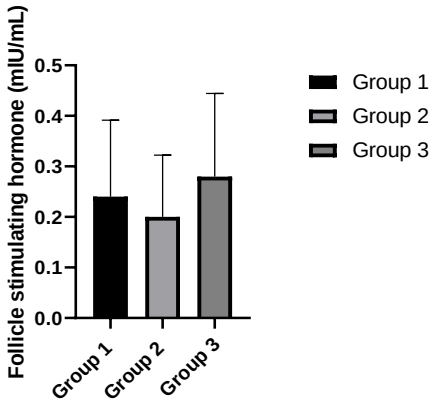

Fig 1a: Effect of chronic exposure of pyrethrin on serum Follicle stimulating hormone of male rats P= 0.6989, Group 1= control, Group 2= low dose, Group 3=high dose (n=5)

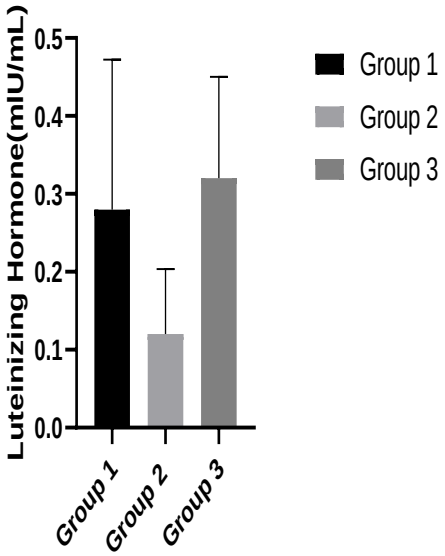

Fig. 1b: Effect of chronic exposure of pyrethrin on serum LH of male Wistar rat P= 0.1037, Group 1= control, Group 2= low dose, Group 3=high dose (n=5)

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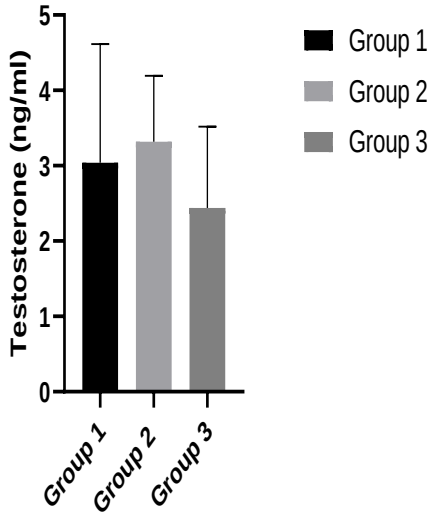

Fig. 1c: Effect of chronic exposure of pyrethrin on serum Testosterone on male Wistar rat P=0.5218, Group 1= control, Group 2= low dose, Group 3=high dose Cn=5)

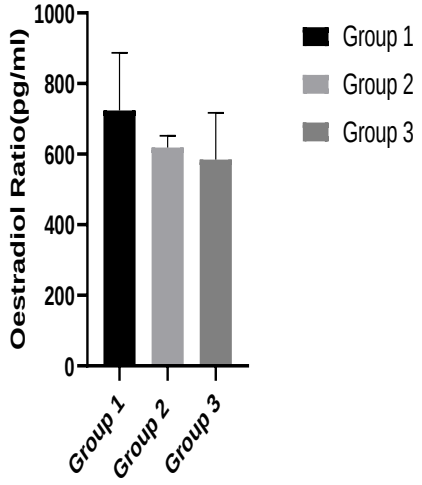

Fig. 1d:Effect of chronic exposure of pyrethrin on serum oestradiol on male Wistar rat p=0.2149, Group 1= control, Group 2= low dose, Group 3=high dose (n=5)

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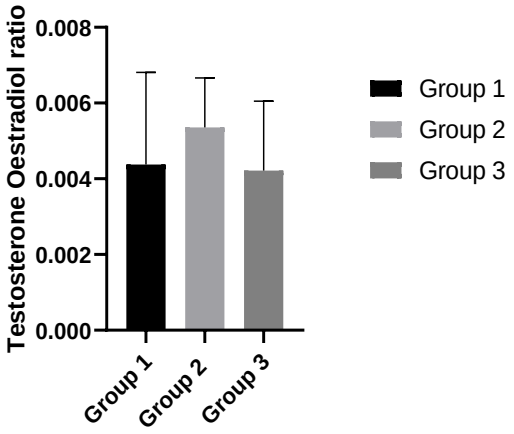

Fig. 1e: Effect of chronic exposure of pyrethrin on serum on testosterone oestradio ratio of male Wistar rat P=0.606, Group 1= control, Group 2= low dose, Group 3=high dose (n=5)

The Effect of Chronic Exposure of Pyrethrin on Computer Assisted Sperm Analysis (CASA) Parameters of Male Rats

Effect of Chronic Exposure of Pyrethrin on Percent of motile Sperm and immotile sperm

Pyrethrin caused no significant difference on the percent motile of sperm on the test group compared to the control group (Fig. 2a). Pyrethrin caused no significant difference on the percent of immotile sperm on the test group compared to the control group. However, a pattern of reduction was observed among the test groups 2 and 3 (Fig. 2b).

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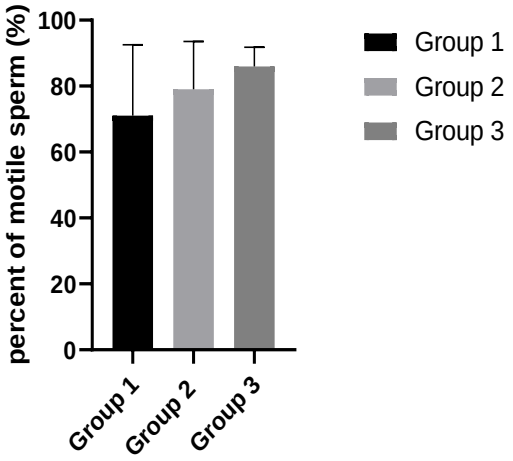

Fig. 2a: Effect of chronic exposure of pyrethrin on the percent of motile sperm on male Wistar rat p=0.3384, Group 1= control, Group 2= low dose, Group 3=high dose (n=5)

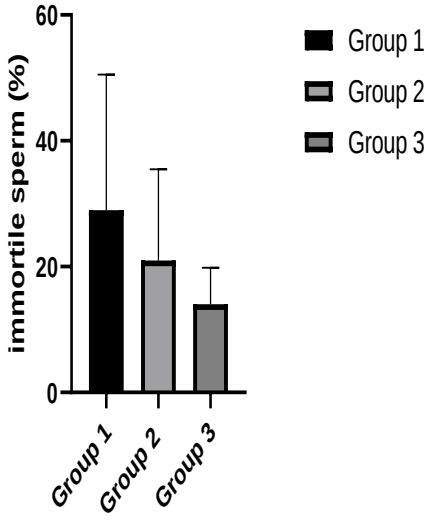

Fig. 2b: Effect of chronic exposure of pyrethrin on the percent of immotile sperm on male Wistar rat p=0.3384, Group 1= control, Group 2= low dose, Group 3=high dose (n=5)

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The effect of chronic exposure of pyrethrin on the sperm concentration of ale Wistar rat

Pyrethrin causes significant increase (p> 0.05) on the sperm concentration on the test group 2 compared to the test group 3 and control (Fig 3).

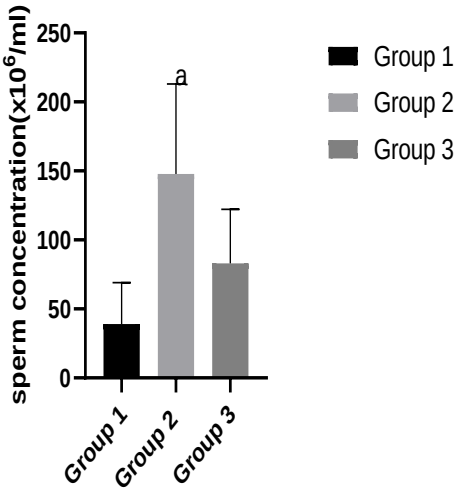

Fig 3: The Effect of chronic exposure of pyrethrin on sperm concentration on male Wistar rat [a]p= 0.011, Group 1= control, Group 2= low dose, Group 3=high dose (n=5)

The Effect of Chronic Exposure of Pyrethrin on Computer Assisted Sperm Analysis Parameters (Sperm Movement Kinetics) of Male Wistar Rats

Pyrethrin caused no significant difference on the average path velocity (Fig 4a), curvilinear velocity (Fig 4b), straight line velocity (Fig 4c), amplitude of lateral head (Fig 4d), beat cross frequency (Fig 4e), Linearity (Fig 4f), on straightness (Fig 4g), mean angle displacement (Fig 4h) of the test group of the male Wistar rats compared to the control. However there was an increased pattern observed in the test group 2 and 3 for all parameters.

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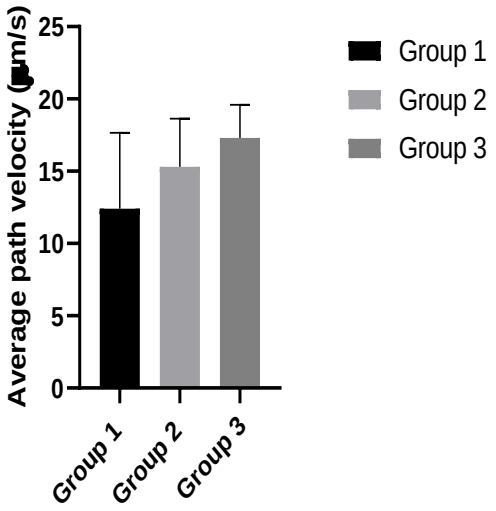

Fig 4a: Effect of chronic exposure of pyrethrin on the average path velocity of sperm in male rat p=0.1674, Group 1= control, Group 2= low dose, Group 3= high dose (n=5)

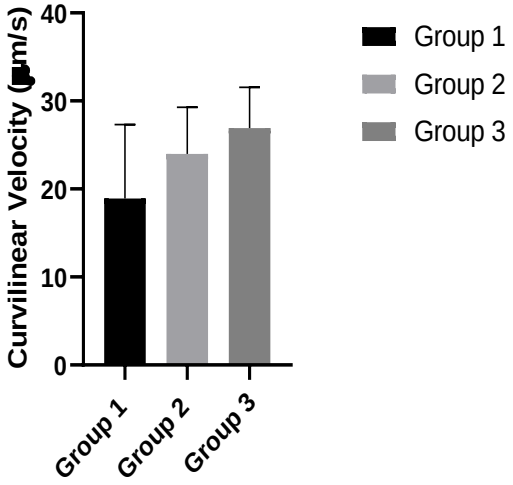

Fig 4b: Effect of chronic exposure of pyrethrin on the curvilinear velocity of sperm in male rat p=0.1713,n=5, Group 1= control, Group 2= low dose, Group 3= high dose (n=5)

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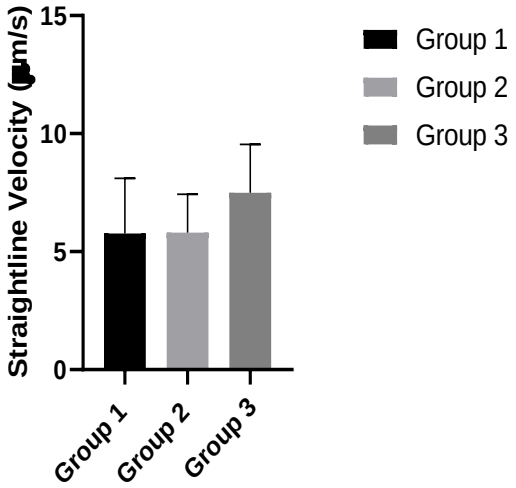

Fig 4c: Effect of chronic exposure of pyrethrin on the straightline velocity of sperm on male Wistar rat p=0.3432, Group 1= control, Group 2= low dose, Group 3=high dose (n=5)

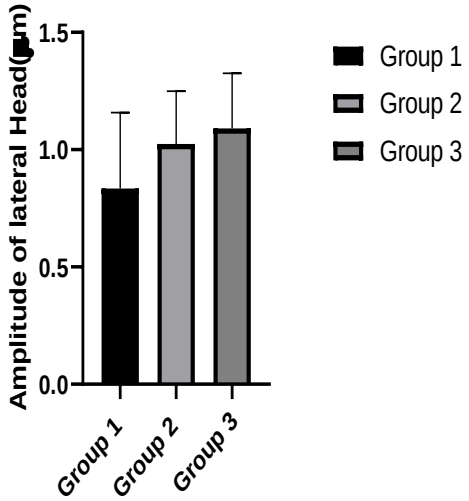

Fig 4d: Effect of chronic exposure of pyrethrin on the Amplitude of lateral head of sperm in male Wistar rat p= 0.3192, Group 1= control, Group 2= low dose, Group 3= high dose (n=5)

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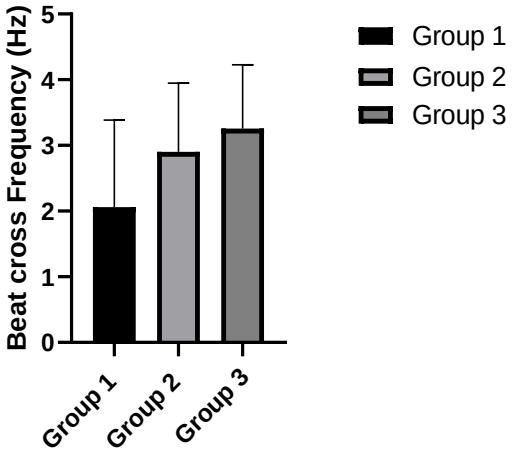

Fig 4e: Effect of chronic exposure of pyrethrin on the beat cross frequency of sperm on male Wistar rat p=0.2644, Group 1= control, Group 2= low dose, Group 3= high dose (n=5)

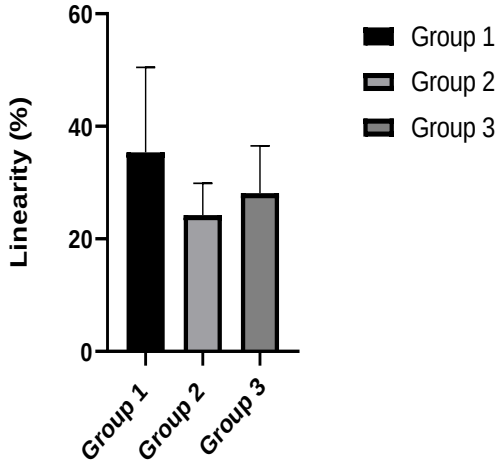

Fig 4f: Effect of chronic exposure of pyrethrin on the linearity of sperm in male rat p= 0.2731, Group 1= control, Group 2= low dose, Group 3= high dose (n=5)

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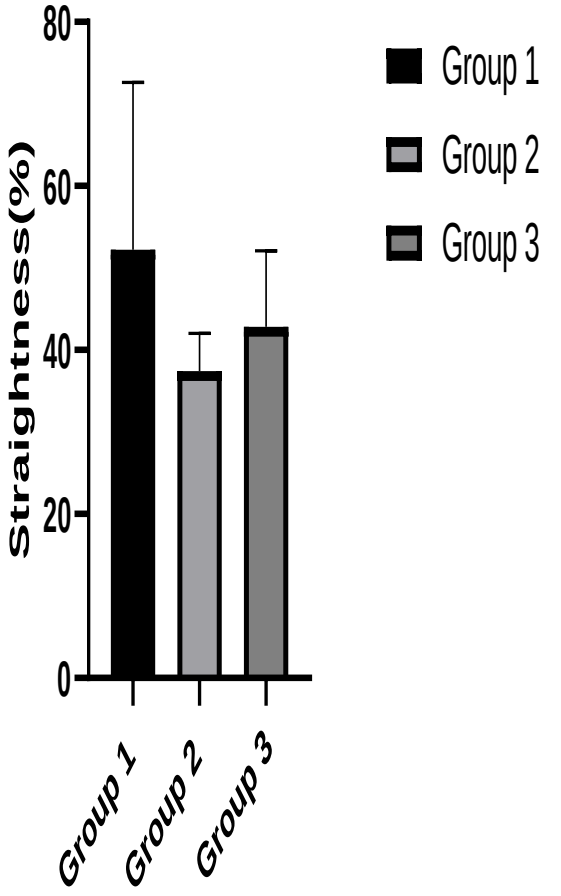

Fig 4g :Effect of chronic exposure of pyrethrin on the straightness of sperm in male rat p= 0.2390, Group 1= control, Group 2= low dose, Group 3= high dose (n=5)

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Volume: 7; Issue: 05,
September-October, 2023
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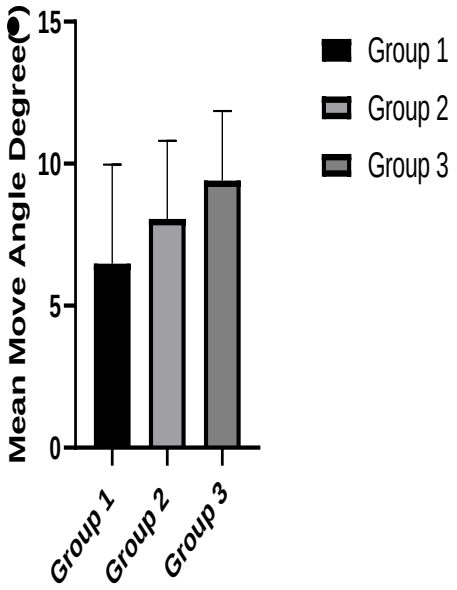

Fig 4h :Effect of chronic exposure of pyrethrin on the mean move angle degree of sperm in male rat p= 0.3208, Group 1= control, Group 2= low dose, Group 3= high dose (n=5)

Effect of Chronic Exposure of Pyrethrin on the H and E -Stained Section of the Testis of male Wistar rats

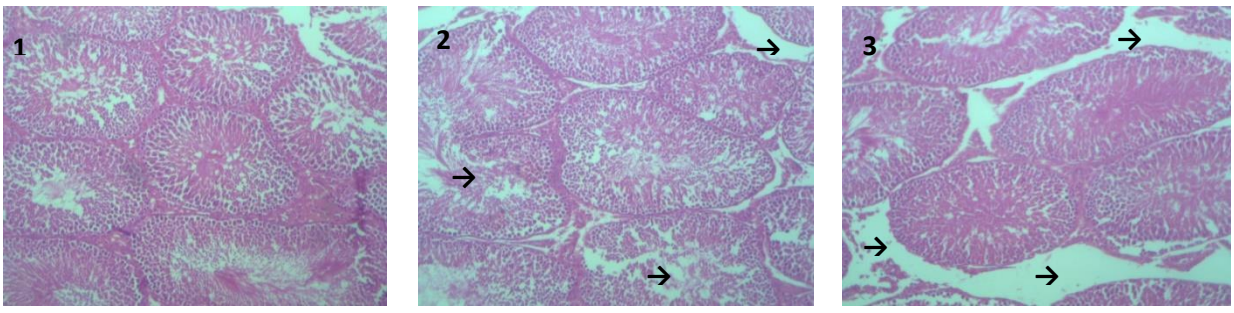

Fig. 5. Photomicrograph of the testis of group 1, 2 and 3. Group 1 which is the control, the seminiferous tubules were normal within testis and the interstitial tissue between them, the sertoli cells are normal in the seminiferous. Group 2 are rats that were fed on low dose, the seminiferous

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tubules seem distorted within the testis, with a little space in the between the interstitial tissue as indicated with the arrows. Group 3, the arrows indicates a basal around the seminiferous tubules, but the interstitial tissues were normal as well as the sertoli cells. Magnification ×10

Effect of Chronic Exposure of Pyrethrin on the H and E -Stained Section of the Epididymis of male Wistar rats

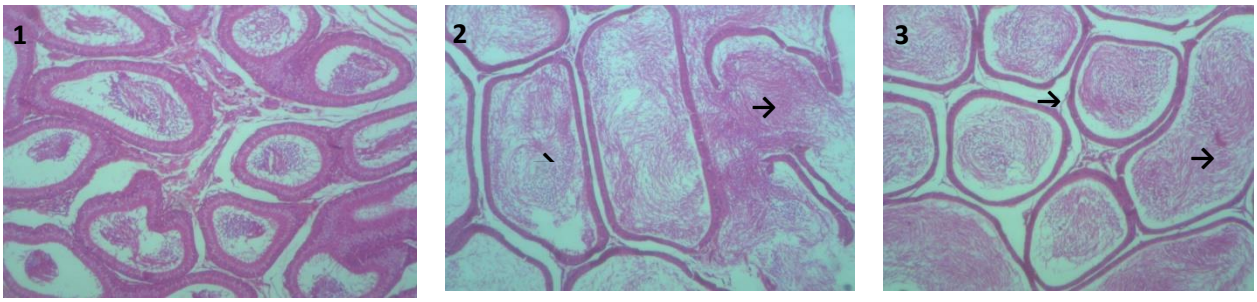

Fig. 6. Photomicrograph of the Epididymis of group 1, 2 and 3. Group 1 which is the control, observable normal functioning and stocked with sperm, the epididymis had a lumen. The lining of the epididymis was healthy. For group 2, the epididymis has architectural distortion and it was distended increase septal spaces due to edema, then group 3 the epididymis has architectural distortion but slightly distended. Magnification ×10

Effect of Chronic Exposure of Pyrethrin on the H and E -Stained Section of the Seminal vesicles of male Wistar rats.

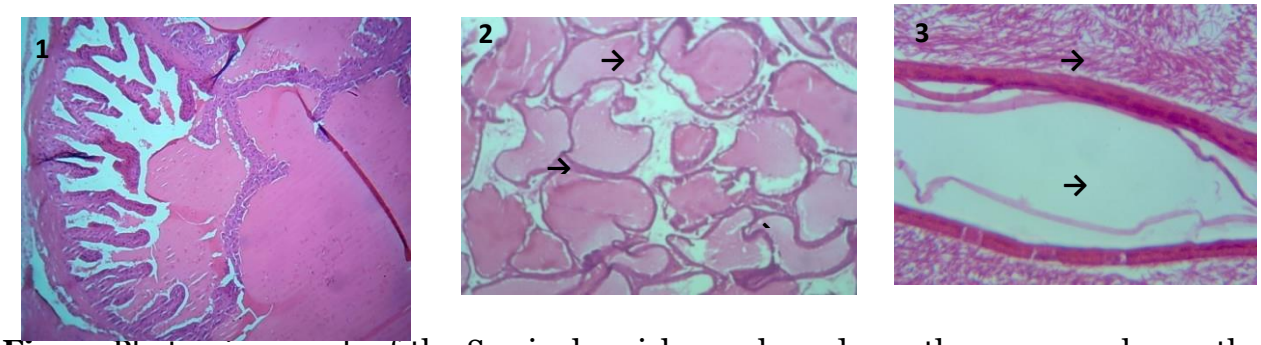

Fig. 7. Photomicrograph of the Seminal vesicle of group 1, 2 and 3. Group 1 (control) the interstitial lining of the seminal vesicles was normal. For group 2 that were administered of low dose, the arrows shows that there was excess proliferation of cell in the seminal vesicle. For group 3 that were place on high dose, the stroma is densely fibrotic (the muscle

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is reacting to it), there is increase widen of the septal spaces. Magnification ×10

The Effect of Chronic Exposure of Pyrethrin on the H and E -Stained Section of Prostate gland male Wistar rat

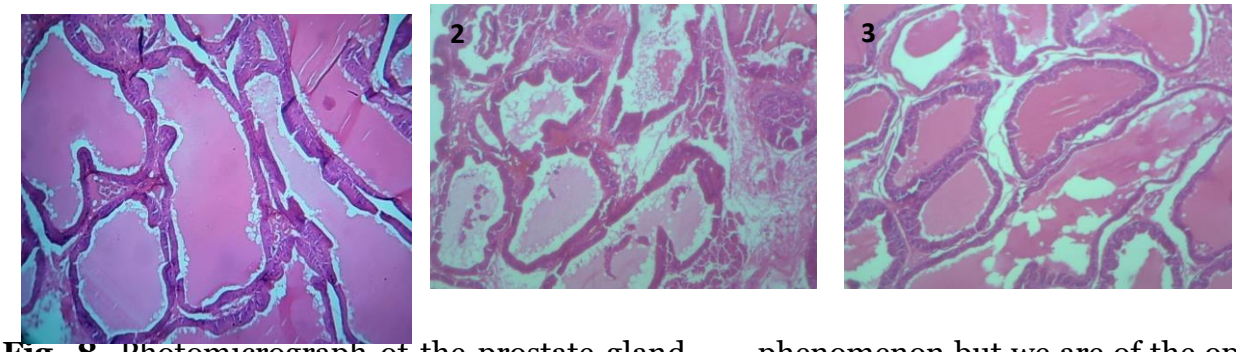

Fig. 8. Photomicrograph of the prostate gland of group 1, 2 and 3. For group 1 on control dose, the prostate gland's structural features and prostatic secretions were both normal. For group 2 (low dose) they had normal grandular cells but less prostatic secretions. Group 3 (high dose) presented expanded grandular cells due to edema but there were a lot of prostatic secretions. Magnification ×10

DISCUSSION

This study to the best of our knowledge is the first to determine the effects of chronic exposure of pyrethrin based insecticides on the reproductive organs of the male Wistar rats. We observed that chronic exposure of pyrethrin based insecticides to male Wistar rats did not significantly affect the relative weights of testis, seminal vesicles and epididymis. However there was a significant increase on the relative prostate gland weights of the low dose (Group 2) and a very significant reduction on high dose (Group 3) of Wistar rats when compared to the low dose (Table 1). Presently, it is not very clear what may have been the cause of this phenomenon but we are of the opinion that this will definitely affect the prostatic secretions.

There were no significant differences in gonadotropins (FSH and LH) levels in groups 2 and 3 when compared to control. There was a no significant decrease in the high dose group of testosterone levels as well as in the estrogen low and high doses This could indicate that longer chronic exposure to pyrethrin could lead to decrease in both testosterone and estrogen levels affecting reproductive processes (Fig. 1a – e).

Sperm motility was normal in this study. Sperm concentration was slightly elevated and the numbers of immotile sperm were decreased in both low and high doses (fig.2 and 3). Sperm movement kinetics especially those that are involved in sperm capacitation and acrosome reaction, hyperactivation and fertilization were not in any way seen to be severely affected (fig. 4 a - h). However, it is not known presently if this might change over time of increased exposure.

Epithelial tissue destruction and distortion were seen in the somniferous tubules of the

testes of groups 2 and 3 (fig. 5). Similarly, tissue destruction and distortion were seen in the epidydimal epithelial cells. Less quantity of sperm was also seen in the epidydimal lumen of the male Wistar rats of groups 2 and 3 (fig. 6) while they were normal in the controlled group. For seminal vesicles, the controls were normal while those of low dose showed excess proliferation of cells. High dose group showed densely fibrotic stroma and increased widen septal spaces (fig. 7). Prostate glands structural features and secretions were both normal in the control group. Low dose group had distorted epithelial but normal glandular cells but with less prostatic secretions. While high dose group in addition, had expanded glandular cells due to edema (fig. 7).

In summary, chronic exposure of male Wistar rats to pyrethrin insecticides did not significantly affect sperm but induced tissue destruction of the male reproductive organs. This may lead to decreased secretions in the testes, epidydimis, seminal vesicles and prostrate glands that may affect male reproductive functions.

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