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Original Research Article

Follicular Counts and Biochemical Evaluation in Ovary of Wistar Rats Exposed to Esbiothrin-based Mosquito Repellant

Akunna G.G[*1], Nwafor J[1], Egwu O.A[1], Akingbade A.M[2], Olasehinde O.R[3], Faeji C.O[4], Ibitoye B.O[5]
[1]Department of Anatomy, Faculty of Basic Medical Sciences, Federal University Ndufu Alike Ikwo, Ebonyi State
[2]Department of Anatomy, College of Medicine and Health Sciences, Afe Babalola University, Ado Ekiti, Ekiti State
[3]Department of Medical Biochemistry, College of Medicine and Health Sciences, Afe Babalola University, Ado Ekiti, Ekiti State
[4]Department of Medical Microbiology and Parasitology, College of Medicine and Health Sciences, Afe Babalola University, Ado Ekiti
[5]Department of Anatomy, Faculty of Basic Medical Sciences, College of Medicine, University of Ibadan, Oyo State

***Corresponding author**
Akunna G.G
Email: ggakunna@gmail.com

Abstract: In this study, the effect of Esbiothrin-based mosquito coil exposure on the ovary of Wistar rats where studied. Twenty (20) Wistar rats (8 to 10 weeks old) weighing 120-130 g were divided into four groups (A, B, C and D) of five rats each. The rats in group A served as the negative control group while the rats in B, C and D were exposed via whole body inhalation to the commercially available mosquito coil smoke for 8 hours (7am-3pm), 12 hours (7am-7pm) and 16 hours (7am-11pm) every day for 3 weeks respectively. The rats were sacrificed after 21 days using cervical dislocation. There was a significant ($p<0.05$) decrease in primary follicles, secondary follicles, graffian follicles, the level of Follicle stimulating hormone, Luteinizing hormone and Progesterone when compared to models in control group. Also, there was a significant ($p<0.05$) increase in the level of Malondiadehyde and atretic follicles when compared to animals in the control group. Taken together therefore, it was concluded that Esbiothrin-based mosquito coil is toxic to the ovary of female Wistar rat.
Keywords: Esbiothrin, Mosquito Coil, Ovary, Histo-morphometry.

INTRODUCTION

Hundreds of cases of insect-borne diseases occur every year, representing a major threat to global public health. Vector-borne diseases account for around 17% of the estimated global burden of infectious diseases. Malaria is mostly a disease of hot climate. In the community level vector control is a major approach in reducing the incidence of this disease, however for an individual, personal protection against mosquito bite remains the first line of defense.

Organophosphorus insecticides represent one of the most widely used classes of insecticides with high potential effect during human exposure in both rural and residential environments [1]. The major active ingredients of the mosquito coil are pyrethrins (Esbiothrin) which account for about 0.3–0.4% of coil mass. Humans are most likely to be exposed to Esbiothrin dermally or by inhalation during the use of mosquito coil. Epidemiologic studies have shown that long-term exposure to mosquito coil smoke can induce asthma and persistent wheeze in children [2, 3]. Toxicological effects of mosquito coil smoke on rats include focal declination of the tracheal epithelium, metaplasia of epithelial cells, and morphologic alteration of the alveolar macrophages [4, 5].

Male infertility is a vexing clinical issue and in recent years, an important relationship between exposure to mosquito repellant and organ toxicity and male infertility [6] has been established. Although the male semen may seem to be the target for investigative and restorative interventions and scrutiny, results are evident in females [7, 8] hence the incidence of female infertility in a population has important demographic since it relies on their partners who may have reproductive issues [9]. Recent reports have indicated that female fertility decline with age as a result of decreased ovary function and genomic damage. Chemotherapeutic agents, radiation, some pharmaceutical agents and a variety of household materials have been implicated in female reproductive dysfunction. They act either as direct ovarian toxins or through a steroidal pathway [9, 1]. Infertility and pesticide exposure has been correlated in several studies with many environmental xenobiotic chemicals, such as

polychlorinated biphenyls (PCBs), Dichloro diphenyl-trichloro ethane (DDT), dioxin, insecticides, herbicides and some pesticides known to have estrogenic effects [11-13]. The main purpose of this study is to investigate the effect of Esbiothrin-based mosquito coil on the ovary of female albino rat using histo morphometric, histochemical and biochemical evaluations.

MATERIALS AND METHODOLOGY

Mosquito Coil

Mosquito coils (Ali Out! green mosquito coils) were purchased from retail outlet in Abakaliki Main market located in Abakaliki, Ebonyi State, Nigeria. This brand (Ali Out! green mosquito coil) commercially purchased for the experiment contain Esbiothrin (EBT) 0.1%w/w as active ingredient and inert ingredient 99.9% w/w. Each coil that was used measured 12 cm in diameter, 72 cm length and 12 g in weight.

Animals

Twenty female Wistar rats (8 to 10 weeks old) weighing 120-130 g were obtained from the animal house of Department of Biotechnology and Plant Science Ebonyi State University (EBSU) Abakaliki. They were allowed to acclimatize for 2 weeks and were fed freely with rat pellets purchased from Okeysons livestock feed Limited, Abakaliki. Relatively constant environmental condition were maintained with proper aeration and good source of light (12h light-12h dark and 24degree C ± 3degree C). Food and water were provided ad libitum. The weighing and observations were done before and after the exposure of the animals to the coils respectively.

The weights of the animals were estimated at procurement, during acclimatization, at commencement of the experiments and once per a week throughout the duration of the experiment, using an electronic analytical and precision balance (BA210S, d= 0.0001 g) (Satorius GA, Goettingen, Germany). Experimental procedures involving the animals and their care were conducted in conformity with International, National and institutional guidelines for the care of laboratory animals in Biomedical Research and Use of Laboratory Animals in Biomedical Research as promulgated by the Canadian Council of Animal Care. Further the animal experimental models used conformed to the guiding principles for research involving animals as recommended by the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals [14].

Animal Grouping and Exposure

The study was conducted in four groups (A, B, C and D) undisturbed cages with cross ventilation. Each group has a total number of five (5) animals per group. The rats in group A served as the negative control group while the rats in B, C and D were exposed via whole body inhalation to the commercially available mosquito coil smoke for 8 hours (7am-3pm), 12 hours (7am-7pm) and 16 hours (7am-11pm) every day for 3 weeks respectively.

Animal Sacrifice and Sample Collection

The rats were at the time of sacrifice first weighed and then anaesthetized by placing them in a closed jar containing cotton wool soaked in chloroform. Blood sample was collected from the heart of each rat immediately after sacrifice with the aid of a 21G needle mounted on a 5 ml syringe (Hindustan Syringes and Medical Devices Ltd., Faridabad, India). That was then inserted into the heart based on prior palpation of the apex beat. At least about 5 ml of blood was aspirated after which the thoracic cage was opened to allow direct access and more blood collection under adequate direct visualization of the heart. The blood samples was collected into tubes containing 2% sodium oxalate and centrifuged at 3000 rpm for 10 minutes using a table top centrifuge (P/C 03) and the serum extracted. The abdominal cavity was opened up through a middline abdominal incision to expose the reproductive organs. One of the ovaries of each animal was fixed in Bouin's fluid for histochemical and histological examination. The remaining ovary was homogenized by using plastic mutter with aid of phosphate buffer. Each homogenates and each blood samples were stored at 20 degree Centigrade for biochemical/hormonal assays.

Histological and Histochemical Analysis

This was done as essentially as described by Akunna *et al.;* in 2016 [15]. The organs were cut in slabs of about 0.5 cm thick and fixed in Bouin's fluid for a day after which it was transferred to 70% alcohol for dehydration. The tissues were passed through 90% alcohol and chloroform for different durations before they were transferred into two changes of molten paraffin wax for 20 min each in an oven at 57° C. Serial sections of 5 µ m thick were obtained from a solid block of tissue and were stained with haematoxylin and eosin stains, after which they were passed through a mixture of equal concentration of xylene and alcohol. Following clearance in xylene, the tissues were oven–dried. Light microscopy was used for the evaluations.

Histological slides were prepared from the formol-saline fixed testes. However, before embedding, it was ensured that the sections were orientated perpendicular to their long axes, and chosen as "vertical sections". For each ovary, five vertical sections from the polar and the equatorial regions were sampled [16] and an unbiased numerical estimation of the following morphometric parameters was estimated using a systematic random scheme [17] while Periodic Acid-Schiff (PAS) reaction with hematoxylin counterstaining, after which they were passed through a mixture of equal concentration of xylene and alcohol. Following clearance in xylene the sections were oven-dried between 35°C and 40°C [18].

Morphological assessment

Follicle Counts

Different categories of follicles were identified and classified according to Pederson and Peters in 1968 [19]. The primordial follicles were counted from every 4th section, and primary follicles (type 3a) from every 6th section were counted. A different counting procedure was followed for advanced primary follicles (type 3b) and pre-antral and antral follicles. Each section of the ovary was observed and only the follicles showing full size oocyte was included in counts of respective category and care was taken not to repeat the counting of the same follicle more than once.

Follicular atresia

Atreatic follicles were identified following morphological criteria described by Greenwald and Roy [20] in hematoxylin-eosin stained serial sections the ovary. The earliest sign of atresia was presence of 5% pyknotic granulose cells in the largest cross section of the follicle.

DETERMINATION OF BIOCHEMICAL PARAMETERS

Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH)

The assays were done according to the procedure adapted by Amballi *et al.;* in 2007 [21]. Briefly, the blood that was collected into plain containers was allowed to clot. Each sample was centrifuged at 1000 rpm for 10 min to achieve separation. The serum obtained was put into aliquots in each case, labeled and stored at -20^0C). One aliquot of each specimen was taken at a time, to avoid repeated freezing and thawing, and the samples were analyzed for hormone estimation using enzyme immunoassay (EIA), according to the World Health Organization (WHO) matched reagent programme protocol (manual) for EIA kits (protocol/ version of December 1998 for LH, FSH). The kids were supplied by NIADDK – NIH (USA). Serum progesterone was determined by ELISA using MAP LAB PLUS (Biochemical systems international, RM 2060) according to the manufacturer's direction.

Assay of ovarian non-enzymatic antioxidants

Estimation of lipid peroxidation (Malondialdehyde)

Lipid peroxidation level in the ovary was determined chemically according to the method described by Ruiz- Larnea *et al.;* 1994 [22] on the bases of MDA reaction with thiobarbituric acid (TBA) which forms a pink complex that can be measured photometrically. In this method 0.5 ml liver homogenate supernatant (1g liver tissue was homogenated in 10 ml phosphate buffer pH 7.4 and centrifuged at 5000 rpm for 10 minutes) was added to 4.5 ml TBA working reagent [0.8 g TBA was dissolved in 100 ml percloric acid 10%, and mixed with 20% trichloroacetic acid (TCA) in volume ratio 1 to 3, respectively). In a boiling and shaking water bath, the sample- reagent mixture was left for 20 minutes, then carried to cool at room temperature and centrifuged for 5 minutes at 3000 rpm. The absorbance of the clear pink supernatant was measured photometrically at 535 nm against reagent blank (0.5 ml distilled water + 4.5 ml TBA working reagent.

STATISTICAL ANALYSIS

All data were expressed as mean ± SD of number of experiments (n = 5). The level of homogeneity among the groups was tested using Analysis of Variance (ANOVA) as done by Snedecor and Cochran (1980). Where heterogeneity occurred, the groups were separated using Duncan Multiple Range Test (DMRT). A value of $p < 0.05$ was considered to indicate a significant difference between groups [23]. Analysis of data was done using both electronic calculator and Statistical Package for Social Sciences (SPSS)/ PC computer program (version 20.0 SPSS, Cary, NC, USA).

RESULTS

Body Weight

Result shows that animals in Group A had a significant ($p>0.05$) enhancement in body weight when compared to rats in other groups. Though the animals in all group shows increase in weight but the rate of the increase in weight was not pronounced when compared to the negative control group of rat.

Table 1: Showing the Statistical Analysis of the body weights of the rats before and during the experiment.

GROUPS	Initial Weight (g)	Weight for 1st Week (g)	Weight for 2nd Week (g)	Weight for 3rd Week(g)
A (Negative Control)	113.4±8.3	127.8±9.9	136.6±10.7	146.0±11.1
B (M. Coil for 8 hrs)	131.0±18.6	145.0±21.1	143.7±10.2	145.6±8.1
C (M. Coil for 12 hrs)	118.0±13.5	125.4±14.7	127.0±17.1	128.7±17.7
D (M. Coil for 16 hrs)	110.4±14.5	117.8±14.9	124.6±14.3	125.0±16.3

Values are means ± SD. n = 5 in each group.

Histomorphometry of the Ovary

The morphology of the ovaries was verified on the following: Primary follicle, Secondary follicle, Graffian follicle and Atretic follicle. There was a significant ($p<0.01$) decrease in primary follicle, graffian follicle and artretic follicle shows a significant ($p<0.01$) decrease in rats group B; group C and group D rats when compared to the rat in negative control (group A). Although there was a significant ($p<0.01$) decrease in secondary follicle of rats in group C and D, the secondary follicle in group B was significant at $p<0.05$

when compared to the rats in group A; as shown in (Table 2 and Fig. 1-8).

Table 2: Showing the effect of Esbiothrin-base mosquito coil on the Primary Follicles, Secondary Follicles, Graffian Follicles, and Atretic Follicles.

GROUPS	Primary Follicles	Secondary Follicles	Graffian Follicles	Atretic Follicles
A(Negative Control)	12.5±1.0	6.1±1.2	5.1±0.4	5.0±3.5
B (M. Coil for 8 hrs)	5.5±2.0**	4.0±1.1*	2.0±5.3**	19.0±1.0**
C (M. Coil for 12 hrs)	5.2±1.4**	3.0±0.5**	1.9±1.2**	20.1±3.0**
D (M. Coil for 16 hrs)	4.1±0.7**	2.8±1.2**	1.7±2.3**	24.0±7.3**

*,** represent significant increases or decreases at $p < 0.05$ and ($p < 0.01$) respectively when compared to negative control (Group A). Values are means ± SD. n = 5 in each group.

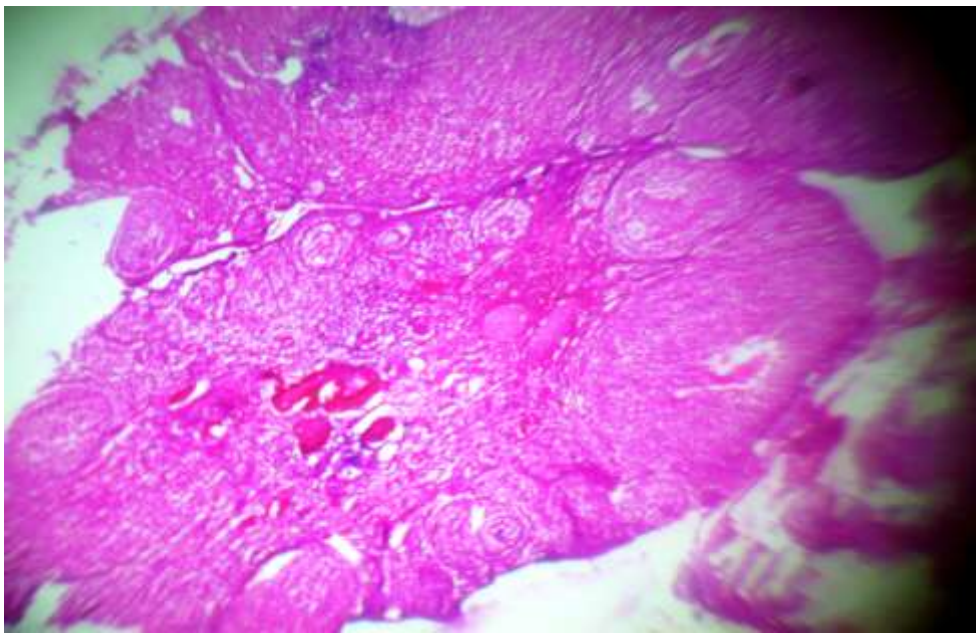

Fig 1: Histological micrograph of a cross-section of the ovary of group A rat (Negative Control). Slide showing the Primary Follicles, Secondary Follicles, Graffian Follicles, and Atretic Follicles. Stain: haematoxylin and eosin; Magnification: x 400.

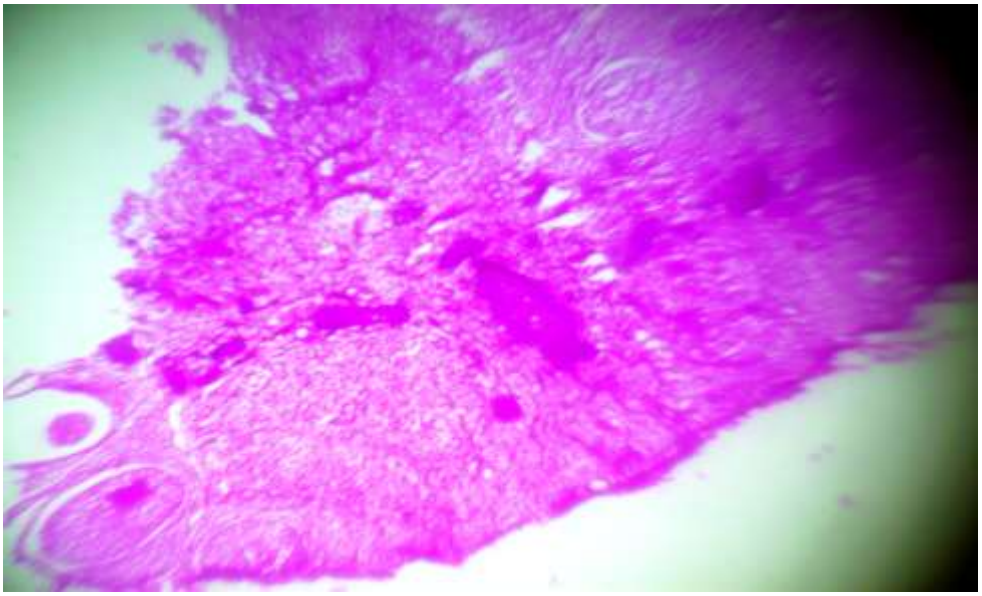

Fig 2: Histological micrograph of a cross-section of the ovary of group B rat (M. Coil for 8 hrs). Slide showing the Primary Follicles, Secondary Follicles, Graffian Follicles, and Atretic Follicles. Stain: haematoxylin and eosin; Magnification: x 400.

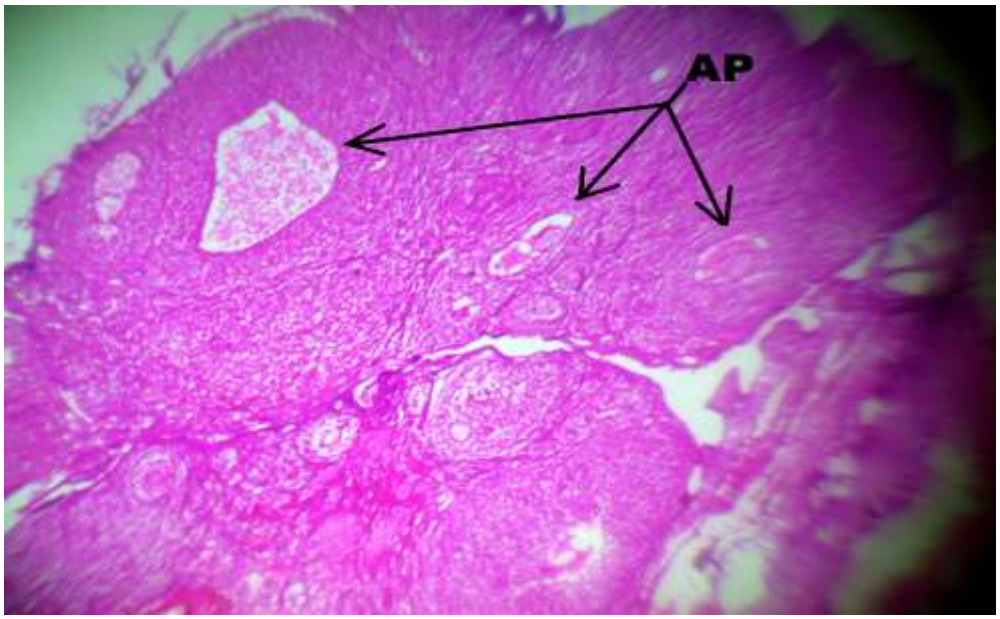

Fig 3: Histological micrograph of a cross-section of the ovary of group B rat (M. Coil for 12 hrs). Slide showing the Primary Follicles, Secondary Follicles, Graffian Follicles, and Atretic Follicles. Stain: haematoxylin and eosin; Magnification: x 400.

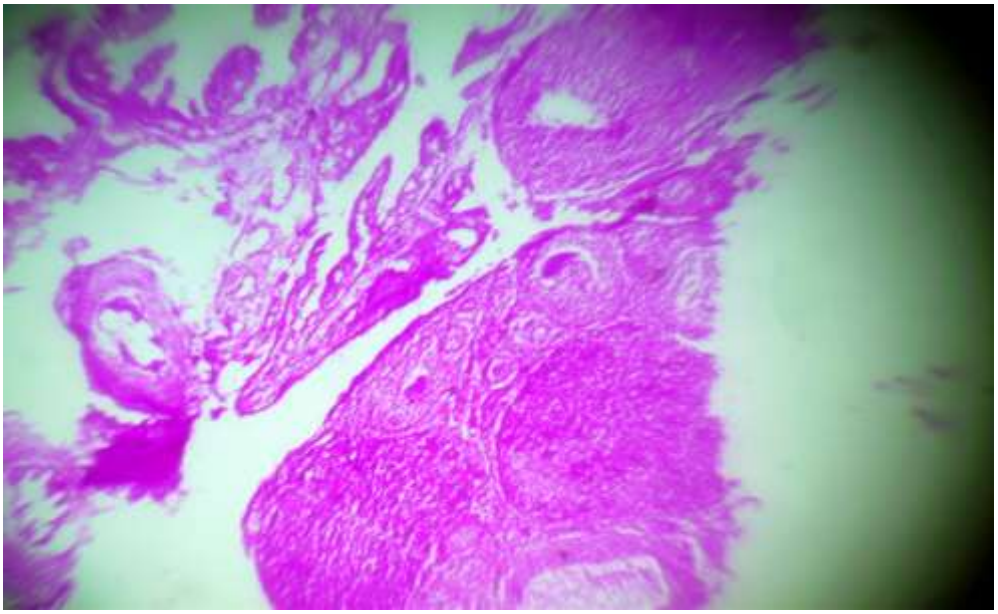

Fig 4: Histological micrograph of a cross-section of the ovary of group D rat (M. Coil for 16 hrs). Slide showing the Primary Follicles, Secondary Follicles, Graffian Follicles, and Atretic Follicles. Stain: haematoxylin and eosin; Magnification: x 400.

Biochemical Results

The effect of Esbiothrin-based mosquito coil on the two main female reproductive hormones namely FSH and LH activities and MDA levels were verified as shown in table 3. The rats in group B and C had a significant ($p<0.05$) decrease in the level of FSH and LH when compared with those in group A; while group also had decrease in the level of FSH and LH with a significant ($p<0.01$) decrease when compared to group A. In the level of MDA the animals in group B and C show a significant ($p<0.05$) increase when compared to animals in group A while the animals in group D show a significant ($p<0.01$) increase when compared to animals in group A.

Table 3: Showing the effect of the repellent of Esbiothrin-base mosquito coil on the FSH and LH activity and MDA level

GROUPS	FSH (mlU/ml)	LH (mlU/ml)	MDA (nmol/mg)
A(Negative Control)	3.4±0.04	2.4±0.1	0.5±0.04
B (M. Coil for 8 hrs)	2.4±0.07*	1.5±0.1*	2.9±0.8*
C (M. Coil for 12 hrs)	2.1±0.07*	1.1±0.2*	3.3±0.4*
D (M. Coil for 16 hrs)	1.9±0.03**	0.7±0.1**	3.8±0.1**

**represent significant increases or decreases at $p < 0.05$ and ($p < 0.01$) respectively when compared to negative control (Group A). Values are means ± SD. n = 5 in each group.

DISSCUSSION

General Consideration

According to the report of World Health Organization informal consultation on the evaluation of insecticides (1996), vector control is the major approach of dipping malaria in the community level, but for an individual, personal protection against mosquito bite is the first line of defense [24, 8]. This explains the drastic increase in the use of Esbiothrin-based mosquito coil. It has been largely reported that such xenobiotic chemicals have estrogenic and testiculotoxic effect [1, 12, 25]. Because mosquito coil consumers usually use mosquito coils for at least several months every year, cumulative effects from long-term exposure to the coil smoke may also be a concern.

Epidemiologic studies have shown that long-term exposure to mosquito coil smoke can induce asthma and persistent wheeze in children [26, 2]. Akunna *et al.;* in 2013 [27] reported its testiculotoxic nature in animal models. It is a common knowledge that ovary is principally vulnerable to oxidative damage by lipid peroxidation.

Gross Anatomical Parameters

Unlike the report of Akunna *et al.;* in 2013 [27] which stated a reduction of body weight post exposure to allethrin-based mosquito coil, we observed a non-significant decrease in body weight of the experimental rats exposed to Esbiothrin-based mosquito coil when compared to the control group of rats.

The increase in body weight observed herein could be as the result of level of food intake. It could also mean that our experimental animals are still their active growth phase. It will also be scientific if we attribute the weight gain to the sex of the animal. After all, most studies on mosquito coil smoke toxicity were on male models. Several studies have associated gonadal weight to infertility [27-30]. However, we were unable to determine the weight of the ovary as a result of the surrounding fats and adipose tissue. We believe this could create avenue for biasness.

The Morphological Effects

The decrease in the number of primary follicle in our work was also responsible for the decrease in the number of secondary follicle; which in-turn lead to the decrease in the number of graffian follicle. This was supported by the stage of follicular maturation. This may be responsible for lack/inadequate release of matured follicle which may lead to infertility.

In addition, Rajeswary *et al.;* in 2007 [31] recorded decrease in glucose-6-phosphate dehydrogenase in testes of rats treated with carbendazim (metabolite of Topsin). Mahadevaswami *et al.;* in 2000 [32] reported that mancozeb fungicide caused a significant decrease in the levels of protein, glycogen, total lipid, phospholipids, and neutral lipid in the liver, uterus, and ovary. In addition to the decrease in the compensatory ovarian hypertrophy, mancozeb treatment reduced the number of healthy follicles with a concomitant increase in the number of atretic follicles.

The Biochemical Effects

Maturation of pre-ovulatory follicles and ovulation are under the combined and balanced influences of ovarian and extra ovarian hormones. Imbalances or alterations in these hormones lead to irregularity in the ovarian functions and duration of estrous cycle [33]. Follicle stimulating hormone is the central hormone of mammalian reproduction, essential for gonadal development and maturation at puberty as well as gamete production during the fertile phase of life [33, 34]. It stimulates the growth and maturation of ovarian follicles by acting directly on the receptors located on the granulosa cells. The reduction in the levels of FSH by the Esbiothrin-based mosquito coil may hamper folliculogenesis and delay maturation of the follicle in the pre-ovulatory phase [5, 34]. This could explain the significant decrease in the number of primary follicle.

It is possible that the Esbiothrin-based mosquito coil might have exerted its effect on the anterior pituitary or the hypothalamus since the secretion of FSH is regulated by the gonadotropic releasing hormone secreted by the hypothalamus. The reduction in the levels of the hormone may adversely affect conception in the female animals. This study agreed with the work of Benie *et al.;* in 2003 [35] where administration of Afrormosia laxiflora, Pterocarpus erinaceus and Cola nitida stem bark decreased the release of the gonadotropins (LH and FSH) thereby affecting the follicular count [34].

Luteinizing hormone stimulates secretion of sex steroids from the gonads. In females, ovulation of mature follicles in the ovary is induced by a large surge of LH secretion during the pre-ovulatory periods. Several authors have demonstrated that LH release surges at the proestrous stage are responsible for ovulation [34, 36, 37]. Any substance capable of inhibiting this release could provoke disruption of ovulation by decreasing the number of mature follicles or induce an oestrous cycle disruption at rest [33, 35]. This review supports our line of thought that impairment in the release of LH might be chief progenitor in loss of follicles observed in our study.

Progesterone which is produced in the ovaries, placenta, and adrenal glands, helps to regulate the monthly menstrual cycle, prepare the body for conception and pregnancy as well as stimulate sexual desire [33]. The hormone also encourages the growth of milk-producing glands in the breast during pregnancy. High progesterone levels are believed to be partly responsible for symptoms of premenstrual syndrome (PMS), such as breast tenderness, feelings of bloat and mood swings. The feedback inhibition of GnRH secretion by estrogens and progesterone provides the basis for the most widely-used form of contraception. Such feedback inhibition of GnRH prevents the mid-cycle surge of LH and ovulation [33].

Free radicals are important for normal physiological processes. However, when the balance between reactive oxygen species and antioxidant defense system is compromised, oxidative stress, a state in which oxidation exceeds the antioxidant systems in the body secondary to a loss of the balance between them is ensued. It causes hazardous events such as lipid peroxidation and oxidative DNA damage, but also physiologic adaptation phenomena and regulation of intracellular signal transduction. This could explain the significant increase in the level of MDA observed in our study which is in accordance with that of Akunna *et al.;* in 2013 [27].

Malondialdehyde is considered a valuable indicator of oxidative damage of cellular components [38]. Oxidative stress is an important factor in the pathogenesis system that has high content of polyunsaturated membrane lipid [39]. The ovarian derangement indicated in the experimental model might have been as a result of active metabolites generated by Esbiothrin which could have aided the production of lipid peroxides, resulting in oxidative degenerative changes in the cell and inhibition of mitochondrial action and eventually causing cell death [40-43, 27].

CONCLUSIONS

Supported with evidences, this study extensively made known the degree of the ovarian derangement in Wistar rat as a result of Esbiothrin-based mosquito coil exposure. It has also implicated lipid peroxidation as a mechanism pathway for Esbiothrin-induced ovarian damage. Although the evidences from our study are clear, the findings may not be directly extrapolated in higher animals. However, based on the results from this work, studies aimed at producing alternative mosquito repellents with minimal toxicity should be an area of practical interest.

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