STEREOLOGICAL EVALUATION OF VOLUME DENSITIES OF OVARIAN FOLLICULAR COMPONENTS IN HYPERPROLACTIN RATS TREATED WITH IMMATURE COCONUT WATER.

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ABSTRACT

Background: Hyperprolactinaemia distorts ovarian structural and functional development, causing anovulation and infertility. The evaluations of volume densities (VDs) of structural components of the ovary such as; granulosa cells, theca cells and antrum have been previously used to measure folliculogenesis and determine the physiological status of ovaries. Immature coconut water (ICW) has been found to contain β-sitosterol, a compound capable of synthesizing steroid hormones *in-vivo*. Hence, this study aimed to determine the effects of ICW on VDs of follicular components in hyperprolactin rats. **Methods:** Mature female Sprague-Dawley rats were induced with hyperprolactinaemia and withdrawn to check for recovery status of an experimental induction and another set of rats were post-treated with ICW for 8, 16 and 28 days. The animals were sacrificed at the end of administration, ovarian tissues were harvested and histologically processed for the stereological evaluations of follicular components. **Results:** VDs of the antrum, granulosa cells and theca cells in the 16 and 28 days post-treated groups (19.25±7.46 ;43.22± 5.10;15.22±3.40 at16 days and 17.70±3.56; 42.75±5.23;15.10±3.60 at 28 days for the antrum, granulosa cells and theca cells respectively) were statistically comparable with the control group (19.76 ± 6.15; 43.75 ±5.71; 15.10± 3.81 for the antrum, granulosa cells and theca cells respectively) while the 16 and 28 days recovery groups (38.57±4.00*;27.20±6.32*;13.66±2.54 at 16 days and 36.58±4.00 *;24.39±5.77*;14.71±2.49 at 28 days for the antrum, granulosa cells and theca cells respectively) were not comparable with the control. **Conclusion:** These indicate that ICW stimulates follicular growth in hyperprolactin post-treated rats in the same structural pattern as the control group, which can depict a physiological approach.

Keywords: Ovary, Follicle, Immature coconut water, Hyperprolactin, Stereology, Volume densities

INTRODUCTION

Hyperprolactinemia is defined as a persistent increase in the blood level of prolactin (PRL) >25ng/ml in non-lactating and non-pregnant people. It is a frequent endocrine contributor to reproductive issues (Thapa et al., 2021). According to reports, hyperprolactinaemia affects ovarian function, which results in irregular menstruation, anovulation and

consequently infertility. High prolactin inhibits follicular maturation which prevents the formation of mature eggs, by the decreasing the secretions of follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Ramadan et al., 2021). These subsequently reduce the mitoses of granulosa cells and follicular estradiol productions by altering FSH-receptor binding sites (Misty et al., 2000). Therefore, changes in follicular components have been highlighted in the forecast of the effects of hyperprolactinaemia on folliculogenesis, hence a valuable tool in evaluating ovarian physiology and follicular growth and developmen pattern. Moreso, stereological evaluations of ovarian follicles integrated with endocrine regulations are being used to provide information on the site of action in folliculogenesis (Zahra, 2018).

Hyperprolactinaemia has been reported in disorders relating to infertility. In Nigeria, hyperprolactinaemia is a common hormonal alteration leading to ovulatory disorders and endometriosis (Olooto et al., 2012). A clinical study observed 36.2% of hyperprolactinaemia in infertile women in north-west Nigeria (Akande *et al.,* 2009).

Immature coconut water (ICW) was found to have properties similar to estrogen as estrogen levels were similar to animals with intact ovaries in postmenopausal rats (Nisaudah et al., 2009). β-sitosterol and sterols like stigmasterol, α-spinasterol and fucosterol may be accountable for the strong estrogenic effect of ICW by enhancing the biosynthesis of estrogen (Punghmatharith, 1998).

Natural non-steroid plant chemicals called phytoestrogen, often known as dietary estrogen, have both estrogenic and antiestrogenic effects. Depending on the situation, plant estrogen has been utilized as a viable alternative to hormone replacement treatment to improve fertility or not (Yildiz and Fatih, 2005). However, therapeutic approach is evolving and has proven to be quite effective (Gupta et al., 2005). Therefore, this study was designed to find out the estrogenic effects of ICW on volume densities of follicular components in hyperprolactin-induced animals using a stereological evaluation tool to measure the follicular growth and development rate.

MATERIALS AND METHODS

This study was carried out at the Department of Anatomy, Faculty of Basic Medical Sciences, College of Medicine, University of Lagos. This study was conducted per the internationally accepted principles for laboratory guidance on animal usage and care under the Institutional Animal Care and Use Committee (IACUC) guidelines. All research protocols were conducted under the guiding principles of animal experimentation.

A total of fifty-five cyclic female Sprague-Dawley (SD) rats weighing 145-170g were obtained from a breeding stock of the Nigerian Institute of Medical Research (NIMR), Yaba Lagos, Nigeria. A taxonomist in the Department of Zoology of the University of Lagos authenticated the experimental

animals. The animals were kept in standard ventilated plastic cages in the animal house of the Department of Anatomy and allowed to acclimatize for two weeks under standard laboratory conditions with a photoperiodicity of twelve hours of light alternating with twelve hours of darkness. The animals were fed with commercially available rat chow from Pfizer Nigeria Plc., Ikeja, Lagos and had free access to tap water.

Determination of the phases of estrous cycle in the animals
Puberty in the female rats was measured by estrous cyclicity. The phases of estrous cycle of the rats were established by daily histological examination of fresh vaginal smear each morning between 8:00 and

10:00 am. A small amount (approximately 0.2 ml) of normal saline was drawn into the plastic suction pipette. The tip of the pipette was pushed gently into the entrance of the vagina canal to a depth of 5mm and the fluid was flushed into the vagina canal and back up into the pipette two or three times by gently squeezing and releasing the bulb of the pipette. The smear was gently dropped onto the glass slide and viewed under a light microscope with a 40x objective lens (Marcondes et al., 2002). Staging the estrous cycle phases was done based on the cellular components of the viewed vagina smear. The metestrus phase was established with a collected smear showing leukocytes amidst remnants of large squamous cells. The diestrus phase showed a predominance of leukocytes and a few large nucleated cells. The smear with large nucleated cells with the leukocytes projections was designated as the proestrus phase. The estrus phase showed large flakes of squamous cells (McLean et al., 2012).

Hyperprolactin induction
Metoclopramide hydrochloride (MCH) was used to induce hyperprolactinaemia experimentally. The drug was purchased from Pfizer Pharmaceutical, Ikeja Lagos. MCH was administered at a dose of 0.2mg/100gbw daily for 28days. MCH administration was calculated based on the animals' weekly weights (Raffaele et al., 2009; Vinícius et al., 2013).

Collection of immature coconut fruits
The immature coconut fruits were harvested from a coconut farm owned by Chief William Kudofoke in Ajara -Topa, Badagry, Lagos, Nigeria. The fruit was authenticated at the federal institute of forestry research in Ibadan. It was assigned the ascension No FHI 109665.

Extraction and storage of the immature coconut water
The immature coconut fruits underwent washing and dehusking. Through the germinal hole, the water content (liquid endosperm of coconut fruits) was extracted. The gathered water was immediately poured into a clean, sealed bottle, kept in the refrigerator and changed every three weeks. Caution was taken in order to prevent particles from getting into the coconut water and metal contact with the water was avoided during extraction (Bustamante, 2002; FAO, 2007).

Treatment protocol
A total of fifty-five animals with approximately 4 days of cyclicity were used for this study. They were randomly divided into four major groups, 1 to 4 and further divided into eleven sub-groups of five animals each. Each rat was served with the extract according to the weight using a simple percentile faction and administration was achieved by the use of an oropharyngeal canula. The administration of substances was as shown in table 1.

Animal sacrifice and ovarian tissue collection
Sacrifices were done using cervical dislocation and incisions were made on the ventral surface of the pelvic region to extract the ovary for histological processing. The animals were sacrificed during the proestrous phase of estrous cycle following the end of administration. A midline incision on the anterior abdominal wall was made to access the abdominal cavity. The ovaries were carefully dissected out and trimmed of fat. The ovaries were weighed and fixed in 10% formal saline for histological processing.

Preparation of histological slides
The fixed tissues were transferred into ascending grades of alcohol. On day 1, the tissues were placed in 70% alcohol for 7

hours, then transferred into 90% alcohol. On day 2, the tissues were passed through three grades of absolute alcohol for one hour each and finally cleared in xylene. The tissues were then infiltrated in molten paraffin wax at 58°C and blocked out. Serial sections of 5 µm thick cut by the rotary microtome were obtained from a solid block of tissue and floated in a water bath. The sections were picked with clean slides onto which egg albumin had been coated and dried on the hot plate at 52°C. The mounted sections were dewaxed in xylene and then hydrated in descending grades of alcohol. The sections were then stained with Haematoxylin and counter-stained in Eosin and dehydrated. The sections were finally cleared in xylene and a drop of mountant was placed on the section and covered with a coverslip (Mohamed and Laurence 2011).

Stereology evaluations of follicular components using the counting grid method
The stereological evaluation of the volume densities of the antrum, granulosa cell and theca cell were carried out only on sections showing mature follicles according to the method described by Gundersen et al., 1998. A restricted artificial boundary was marked on the section limited to the boundary of the follicle. A counting grid with a set of regularly spaced points was placed on the field. All points hitting profiles (disregarding their relationship to the frame) were counted.
A point was considered a hit if a profile (including its boundary) covers the upper right corner in the cross-section where two lines cross each other. The volume density is calculated as the proportion of volume by simple percentile fraction; VD (profile counted/total specimen profile X100) (Gundersen *et al.,* 1988; Lars et al., 2020).

Statistical analysis
Results were expressed as mean ± standard deviation. Analysis was carried out using analysis of variance (ANOVA) with Scheffe's post hoc test. The level of significance was considered at $p < 0.05$

Table 1: Showing administrations and durations of test substances

GROUP/SUBGROUPS	ADMINISTRATION	DURATION (Days)
1 (Negative control)	Distilled water	28
2 (Induction and Recovery)		
a. **Induction**	0.2mg/100g of MCH	28
b. **Withdrawal short**	0.2mg/100g of MCH 28 days + withdrawal 8 days	36
c. **Withdrawal medium**	0.2mg/100g of MCH 28 days + withdrawal 16 days	44
d. **Withdrawal long**	0.2mg/100g of MCH 28 days + withdrawal 28 days	56
3 (Post-treated)		
a. **Post-treated short**	0.2mg/100g of MCH 28 days + 5ml/100g ICW 8 days	36
b. **Post-treated medium**	0.2mg/100g of MCH 28 days + 5ml/100g ICW 16 days	44
c. **Post-treated long**	0.2mg/100g of MCH 28 days + 5ml/100g ICW 28 days	56
4 (Positive control)		
a. **ICW short**	5ml/100g of ICW	8
b. **ICW medium**	5ml/100g of ICW	16
c. **ICW long**	5ml/100g of ICW	28

RESULTS

Stereological evaluations of VDs of follicular components were done only on section with mature follicles. Mature ovarian follicles were seen in the histological sections of all the ICW-treated and the 16- and 28-days recovery and post-treated groups while the induced and the 8 days withdrawal and post-treated groups did not show mature follicles in their histological sections. Stereological measurements of the follicular components in the 16 and 28 days post-treated and ICW groups(19.25±7.46;43.22±5.10;15.22±3.40

at 16 days and 17.70±3.56; 42.75±5.23;15.10±3.60 at 28 days for the antrum, granulosa cells and theca cells respectively) demonstrated comparable results in the volume densities with the control group (19.76 ± 6.15; 43.75 ±5.71; 15.10± 3.81 for the antrum, granulosa cells and theca cells respectively). This shows that immature coconut water post-treated groups demonstrated comparable stereological evaluation of volume densities of mature follicles with the control groups and the groups treated with immature coconut water only.

However, the 16 and 28 days withdrawal groups, which were the recovery groups, the volume densities (38.57 ± 4.00*;27.20 ± 6.32*; 13.66 ± 2.54 at 16 days and 36.58 ± 4.00 *;24.39 ± 5.77*;14.71 ± 2.49 at 28 days for the antrum, granulosa cells and theca cells respectively) were not statically comparable with the control group (19.76 ± 6.15; 43.75 ±5.71; 15.10± 3.81 for the antrum, granulosa cells and theca cells respectively at $p < 0.05$) even though the

histological sections present mature ovarian follicles.

Table 2: Stereological evaluations of volume densities of the antrum, granulosa cells and theca cells of mature ovarian follicles of hyperprolactin rats treated with immature coconut water

GROUPS	SUB-GROUPS	ATR_{VD}(%)	$GRAC_{VD}$(%)	TAC_{VD}(%)
Control	$DSTL 2_{8days}$	19.76 ± 6.15	43.75 ±5.71	15.10± 3.81
Recovery	$MCH_{28\ days}$-WD $_{16\ days}$	38.57± 4.00*	27.20± 6.32*	13.66± 2.54
	$MCH_{28\ days}$-WD $_{28\ days}$	36.58± 4.00 *	24.39± 5.77*	14.71± 2.49
Post-treated	$MCH_{28\ days}$-ICW_{16days}	17.70± 3.56	42.75± 5.23	15.10± 3.60
	MCH_{28days}-ICW_{28days}	19.25± 7.46	43.22± 5.10	15.22± 3.40
Positive control (ICW-treated)	ICW $_{8days}$	18.00 ± 4.77	49.55 ±5.19	15.13± 3.31
	ICW $_{16days}$	19.09 ± 5.15	44.55 ±7.14	16.13± 3.00
	ICW $_{28days}$	18.07 ± 7.70	46.55 ±5.10	16.13± 3.33

All values are expressed as mean ± standard deviation *Significant differences; $p < 0.05$. DSTL: Distilled water; ICW: Immature coconut water; MCL: Metoclopramide hydrochloride; WD: Withdrawal; VD: Volume density; ATR: Antrum; GRAC: Granulosa cell; TAC: Theca cell.

DISCUSSION

According to mounting evidences (Lecomte et al., 1997; Susan et al., 2004; Zhong et al., 2011), PRL has a direct inhibitory effect on gonadotropin actions in the ovary and subsequently inhibits follicular development, inhibiting the formation of a mature egg.
Studies have revealed that ICW exhibits estrogen-like qualities that can control endocrine secretions. ICW was given to many groups of postmenopausal rats in a previous study, and the rats showed estrogen levels that were equivalent to those of rats that still had their ovaries (Nisaudah et al., 2009). It has been hypothesized that the presence of β-sitosterol in GCW, along with other sterols like stigmasterol, frucosterol, and β-spinasterol (plant sterols known to be involved in the synthesis of steroid hormones in-vivo), is what gives it such a potent estrogenic effect by promoting

the production of endogenous estrogens that target the development of granulosa and theca cells. According to Punghmatharith (1998), the β-Sitosterol is structurally linked to animal cholesterol and may serve as a precursor to sex hormones. For measuring follicular growth and development, researchers have examined changes in microscopic components (Myers et al., 2004). An essential indicator of the process of folliculogenesis with regard to the endocrine signals and paracrine/autocrine mechanisms that control the growth and maturation of oocytes and their supporting follicular cells are accurate stereological estimations of ovarian follicles at various stages of development (West, 1990). The ovarian follicle is a three-dimensional structure, made up of theca and granulosa cells that emit hormones to regulate the

reproductive cycle and generate fertile eggs at ovulation. As follicular cells are unique in their hormone productions and modulations, with consequent indices in folliculogenesis, volume density evaluation of ovarian follicles is used to measure ovarian development and generate information on the physiological status of the ovaries (Yi-Xun et al., 2019). The stereological analyses revealed that 16 and 28 days after receiving ICW following experimental induction of hyperprolactinaemia, the mean volume densities of the antrum, granulosa and theca cells were statistically equivalent with that of the control group. The mean volume densities of the antrum, granulosa and theca cells in the recovery groups (16- and 28-days withdrawal groups), where folliculogenesis were restored following induction, were not comparable to that of the control group. Folliculogenesis is physiologically influenced by the granulosa cells. According to reports, the granulosa cells are in charge of producing estrogen and growth hormones that aid the development of oocyte (Jingqiang et al., 2021). Due to their vascular nature, theca cells communicate with the circulating blood network and give vital nutrients to the growing follicle. The fact that they create androstenedione, which the granulosa cells employ to make estradiol is more significant (Chen et al., 1993; Ashkenazi et al., 2005). In summary, the ovarian follicles will expand as a result of the granulosa and theca cells' proliferative activity and revert to abnormalities brought on by hyperprolactinaemia.

The results from this study further strengthen the effects of ICW on follicular growth and reproductive hormone profile in hyperprolactin rats (Bakare et al., 2019a; Bakare et al., 2019b). Through stereological evaluations of volume densities of follicular components, this study further revealed granulosa and theca cell proliferations as target sites of actions of ICW in hyperprolactin rat.

CONCLUSION
It is evident from this study that ICW demonstrates ameliorative effects on follicular growth and development in hyperprolactin rats by enhancing the propagation of granulosa mitoses and theca cell proliferation. This study's findings clearly depict that ICW is a promising candidate in the reversal of infertility caused by high prolactin in female Sprague-Dawley rats. The stereological evaluation employed in this study allowed for an unbiased and quantitative assessment of the changes occurring within the ovarian follicles. The use of stereology provided reliable and accurate estimations, enhancing the validity of our findings. This research outcome provides a foundation for future research and may have implications for the development of alternative treatment options for reproductive disorders. Further exploration of the underlying mechanisms and long-term effects is essential to fully understand the therapeutic potential of immature coconut water in reproductive health.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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