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# ACTIVITIES OF FSH RECEPTORS ON THE RESTORATION OF REPRODUCTIVE INDICES IN HYPERPROLACTIN RATS TREATED WITH GREEN COCONUT WATER

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## ABSTRACT

**Background of the study:** Green coconut water (GCW) exhibits fertility-enhancing properties. This study investigated the role of GCW on follicle stimulating hormone activation in understanding its mode of action.

**Aim:** We investigated the effect of GCW on the expression of FSH-receptors in the restoration of reproductive indices in hyperprolactin rats.

**Material and Methods:** Forty adult female Sprague-Dawley rats were randomized into four experimental study groups (A, B, C and D) of 10 rats each. The animals in group A received distilled water only. Group B, the positive control received GCW only, group C is the induction group and the animals received 5 mg/Kg B.W. metoclopramide. Group D is the post-treatment group and animals were treated with GCW following the experimental induction of hyperprolactinemia.

**Result:** The animals post-treated with GCW showed high expression level of FSH receptors at a molecular weight of 70 kDA that was comparable with the control and GCW-treated groups. However, low expression of the FSH receptors was seen in the induced group at a molecular weight of 10 kDA when compared with the control and GCW-treated groups. Additionally, there were comparable evaluations in the rate of pregnancy and number of fetuses in the animals post-treated with GCW and the control and GCW-treated groups. However the animals in the induced group recorded no pregenacy.

**Conclusion:** This study's outcome clearly depicts that GCW is an effective potential natural agent in the reversal of infertility caused by hyperprolactinemia through the activation of FSH receptors.


**Keywords**: Hyperprolactinemia, Female, Infertility, Green Coconut Water, Follicle Stimulating Hormone

## INTRODUCTION

Infertility is defined by the failure to achieve successful pregnancy after one year of non-contraceptive, regular, unprotected and active sexual intercourse[1,2]. It is a social and public health issue with significant medical, psychological and economic problems that possess negative impact on overall well-being and productivity[3]. Reproductive process is a neuroendocrine event where hypothalamic release of gonadotropin-releasing hormone (GnRH) into the hypophysial portal blood system targets the anterior pituitary to produce gonadotropins (FSH and LH) which exert their effects on the gonads to stimulate reproductive activities[4]. Apparently, endocrine regulations are critical driver of the hypothalamic pituitary gonadal (HPG) axis, a major signaling pathway that exert modulatory effects on the development of sexual characteristics, follicular development, ovulation, menstruation, pregnancy and overall reproductive functions. All hormones encompassing the endocrine system must be well- regulated

and coordinated, hence any alteration causes hormonal imbalances leading to infertility.

Hyperprolactineamia is a common endocrine cause of infertility. It is a disorder of the HPG-axis indicating persistently high level of prolactin (PRL) in non-pregnant and non-lactating individuals[5]. PRL is a lactating hormone that must be kept under tonic inhibitory control by prolactin-inhibiting factor in the absence of pregnancy and lactation[6]. Thus, high serum level of PRL is seen during lactation to reduce fertility thereby protecting the lactating mother from premature pregnancy. In the absence of lactation, when it becomes persistently high, it inhibits the secretion of GnRH from the hypothalamus, thereby suppressing the release of gonadotropins and its corresponding actions on the gonads causing hyprolactin-induced infertility[7]. The mechanisms that may account for the suppression of gonadal function in hyperprolactinemia include subsequent suppression of the levels of FSH and LH to become inappropriately low and/or inactive at receptor sites inhibiting folliculogenesis and ovulation[8]. Low serum estrogen level is mainly projected in hyperprolactinemia as excess PRL causes reduction in granulosa cell proliferation which will lead to the reduction of granulosa cell estradiol production. The positive estrogen feedback on LH pulsatility and surge needed for ovulation will be disrupted[9].

Green coconut water (GCW) is the liquid endosperm of an immature coconut fruit. It is a natural rich source of minerals and electrolytes that boost energy levels instantly, plays vital role in maintaining proper fluid balance in the body and alleviates some disease conditions[10]. It has been reported that GCW demonstrated estrogen-like property when administered in several groups of postmenopausal rats where estrogen levels at the end of administration were comparable with rats that still had their ovaries[11]. It has been suggested that GCW contains β-sitosterol in addition to other sterols, such as; stigmasterol, fucosterol and α-spinasterol which are plant sterols known to be involved in the synthesis of steroid hormones *invivo*[12]. These may be responsible for the estrogenic effect of GCW by facilitating the synthesis of endogenous estrogens. Previously, GCW has been shown to stimulate folliculogenesis by increasing the number of mature follicles in the ovary with corresponding balance in reproductive hormones[13,14]. It is important to know that hormones exert their effects at receptor sites where their actions are activated, therefore reproductive hormones that are released bind to specific receptors on the target organ to produce signaling effect that results in gene or protein expressions to exert reproductive functions[15]. This implies that hormone levels may be regulated but may not be active at their receptor sites to initiate actions. Therefore, this study investigated the effect of GCW on the actions of FSH receptors in the ovary to provide the understanding on how the established fertility enhancing effects manifest and consequently unraveling the mode of action of GCW particularly targeting hormone receptor sites at the gonadal level in stimulating the process of folliculogenesis.

## Materials and Methods

### Source of Green coconut fruits

Fresh green coconuts at immature stage of about 6 month old were purchased from Adeojo coconut farm in Badagry, Lagos. The average weight of the fruits was 500 g and authenticated at the Department of Botany, University of Lagos with plant's ascension no LUH: 10127 by Dr George Nodza.

**Green coconut water (GCW) extraction, preservation and oral dose estimation**

The green coconut fruits were be washed and dehusked. A sterile rod was used to open the germinal pore and through this opening, the water was extracted hygienically from the fruit. The GCW was poured directly into an airtight bottle and preserved in the refrigerator. There was avoidance of metal contact with the coconut water and caution was taken in preventing particles from entering into the water during the process of extraction[16]. The administration of an oral dose level of 5ml/kg of body weight of GCW daily for 21 days based on dosage used in previous studies [17,18].

**Animal Handling**

Ethical approval was obtained from the Ethics Committee of the College of Medicine, University of Lagos on the Use of Laboratory Animals for experiments with ethical approval number CMUL/ACUREC/01/24/1345. All procedures were carried out following the standard protocols and safety guidelines for Care and Use of laboratory animals for scientific investigations

**Experimental design (animal distributions, events and durations)**

Forty female Sprague-Dawley rats of weights between 90–160 g were used. The rats were obtained from a breeding stock named Priceless Test Animals in Ilogbo Eremi Oko-Afo, Badagry, Lagos and authenticated by the Department of Zoology of the University of Lagos. The rats were allowed to acclimatized in the animal house of the College of Medicine, University of Lagos for two weeks. The rats were housed in netted iron cages, fed with commercially available rat chow and provided water ad libitum. Standard laboratory conditions of temperature $32^0$C and 12hrs light-dark cycle were maintained. Subsequently, the animals were subjected to daily vaginal lavage for cytological depictions of the various stages of estrous cycle to confirm that they have attained reproductive stage with establish estrous cycling. The animals were divided into four study groups (A, B, C and D) of ten animals each. The animals in group A received normal saline for 28 days. Group B is the positive control group and the animals received GCW only for 28days. The animals in group C were treated with 0.5 mL of 5 mg/Kg of metoclopramide hydrochloride to induce hyperprolactinemia while group D, the post- treatment group received 5ml of GCW after induction. The first sub-sets of five animals were sacrificed for evaluating the expression of follicle stimulating hormone receptor (FSHR) following the end of experimental durations while the last sub-set of five were used for fertility assessment.

**Study of Oestrous cycle**

The phases of oestrous cycle of the experimental animals were established by daily cytological examination method of fresh vaginal smear in the morning between 8:00 and 10:00 am. A small amount (approximately 0.2 ml) of normal saline was drawn up into the suction pipette. The rat was held in place with one hand around its waist with the ventral surface downward to provide additional support and to prevent the animal from struggling whilst the other hand was used to hold the pipette. The tip of the pipette containing normal saline was pushed gently into the entrance of the vagina canal to a depth of 2mm[19]. The fluid was flushed into the vagina and back up into the pipette two or three times by gently squeezing and releasing the bulb of the pipette. The collected smear was dropped onto the surface of the glass slide in three points and over-lied with cover slips to ensure the smear is of uniform alignment, making it easier to focus and prevent smear coalescing. The smeared glass slide was viewed under a light microscope with 40x magnification objective lens by blind reading [20,21].

**Microscopic interpretation of collected vagina smear**

The estrous cycle of rats lasts an average of four days and was characterized by four phases which are; the estrus, proestrus, diestrus and metestrus. The cells lining the wall of the vagina of the female rat correspond to the levels of circulating hormones and this provides a valuable marker for studying estrous cycle. Therefore, the presence, absence or proportional distribution of cornified cells and leucocytes were used in staging estrous cycle. The first day of the estrous cycle was designated as the metestrus, the presence of leukocytes amidst remnants of large squamous cells in the smear histology was used to stage this phase. The second day showed smear presentation of small nucleated cells and designated as dioestrus phase. The third day showed numerous large nucleated cells and was designated as the proestrus phase. The fourth phase was designated as the estrus phase with histological evaluation of large flakes of squamous cells[21].

**Induction of Hyperprolactinemia**

Metoclopramide hydrochloride (MCH) was used as the hyperprolactin-inducing agent. MCH was administered at a dose of 0.5 mL of 5 mg/Kg body weight dissolved in distilled water daily for 28days to experimentally induce hyperprolactinaemia[22]. The dose was calculated by simple proportion based on the animal's weights and administered via oral route with the use of an oropharyngeal canula.

**Expressions of FSHR in the ovarian tissue**

The proteins were first separated using polyacrylamide gel to characterize individual protein in a complex sample. Subsequently, the separated molecules were transferred or blotted onto a second matrix from gel to membrane with the use of porous pads and filter paper to facilitate the transfer. The mixture was heated at 80$^{o}$C for 5 minutes on a heating block to denature the protein. A 10 ul volume was loaded on 10%

gel and electrophoresed at 80 volts (Stacking) for 1 hour and 120 volts (separating) for 2 hours. One of two gels was stained and the other was used for transfer on the Polyvinylidene fluoride (PVDF) membrane for 1 hour. The membrane was then blocked to prevent any nonspecific binding of antibodies to the surface of the membrane. Blocking buffers were used to block free sites on a membrane to reduce background interference and improving the signal ratio. The transferred protein was then probed with a combination of antibodies specific to the protein of follicle stimulating hormone which acted as the primary antibody. This was followed by 5 washes in TBST, a mixture of tris-buffered saline and Polysorbate 20 and then incubated in secondary antibody (Goat anti-rabbit IgG (H+L)-HRP) for 1 hour at room temperature with moderate shaking. The membrane was kept in a dark container, protected from light. 2ml of TMB (3, 3', 5, 5'-tetramethylbenzidine) a soluble substrate that yields a blue colour was added to the membrane to visualize protein. The chemiluminescent signal was captured as shown in figure 1 below[22]

**Fertility assessments**

The second sub-set of five animals in each group A to D were caged with age-matched adult male rats of proven fertility (ratio 1 male to 2 female rats) and monitored daily for signs of mating (the presence of mating plug in the vaginal os) and the interval between male presence and observed mating plug. The animals were allowed to carry foetuses to term for the determination of foetal survival rate.

**Statistical Analyses:** Data on steroidogenic protein expressions was analyzed with One-Way ANOVA and the differences among means was tested with orthogonal contrasts. The evaluations on mating and foetal survival (percentage) rate were compared by Chi-square.

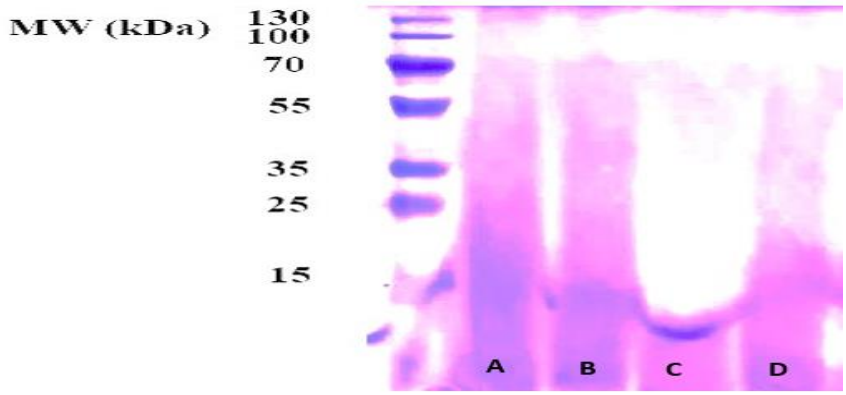


**FIGURE 1**: **Image showing the expression level of FSH proteins in the experimental and control groups in Sprague-Dawley rats. A: control; B: GCW only; C: induced MCH 5 mg/kg; D: post-treated MCH + GCW**

**TABLE 1:** The number of pregnancies and foetuses in the experimental and control groups in Sprague-Dawley rats. A: control; B: GCW only; C: induced MCH 5 mg/kg; D: post-treated MCH + GCW

| GROUPS | SUB-GROUP DETAIL | NO OF PREGNANT RATS | MEAN NO OF FOETUSES |
|---|---|---|---|
| Control (A) | DSTL | 5 | 9.24±1.31 |
| GCW (B) | GCW | 5 | 10.21±0.85 |
| Induction (C) | MCH | 0 | 0.00±0.00 |
| Post-treated (D) | MCH - GCW | 5 | 8.20±0.04 |

All values are expressed as mean ± standard deviation *Significant differences; $p < 0.05$

## RESULT AND DISCUSSION

The animals post-treated with GCW (group D) upon the induction of hyperprolactinemia have higher band of expression level of FSH proteins with molecular weight of 70kDa when compared with the induced group C with molecular weight of 15kDa. More so, the post-treated group with GCW showed comparable level of expression with the GCW-treated group B and the control group A. Additionally, the induced group C showed the lowest band of expression of FSH proteins with molecular weight of 15kDa. The animals that were post-treated with GCW also have high pregnancy rate and number of foetuses when compared with the hyperprolactin-induced group. The rate of pregnancy and number of foetuses in the post-treated group were comparable with the control and GCW treated groups. Finally, no congenital malformations were observed at the time of birth in both experimental and control groups.

Persistent prolactin level projected in hyperprolactinemia causes dysregulation of GnRH neurons in the hypothalamus leading to decrease pituitary FSH and LH secretions. The alterations of gonadotropins will suppress folliculogenesis and consequent the reduction of ovarian oestrogen below the regulated level due the inhibition of granulosa cell proliferation that is responsible for the production of steroid hormone[24]. However high oestrogen level can exert negative feedback on the HGP-axis disrupting the endocrine system and consequently causing infertility effects [25]. Hence hormones must be tightly regulated to enhance reproductive effects. More importantly, hormones exert their effects at receptor sites where their actions are activated, reproductive hormones that are released bind to specific receptors on the target organ to produce signalling effect that results in the receptor or protein expressions

and consequently exerting reproductive actions[15]. Therefore, the understanding the role of these hormones and their actions at receptor sites are essential for the development of treatment modalities. Phytoestrogen or dietary oestrogens are naturally occurring non-steroid plant compounds that have the ability to cause estrogenic or/and antiestrogenic effects[26]. Plant oestrogen has been used as alternative source of hormone replacement therapy. GCW is a rich source of phytohormones such as; cytokinin, auxin and diphenylurea [27]. The estrogenic effect of GCW has been reported to exert ameliorative action on brain damage that was induced by hormonal imbalances[11]. Additionally, GCW exhibited selective oestrogen moderating effect that was comparable with oestradiol benzoate in reducing skin atrophy and aging[28]. More specifically, the estrogenic property of GCW has been demonstrated as a fertility enhancing agent in our previous studies where it regulated reproductive hormones with consequent positive influence on ovarian follicles[17, 18, 29]. In this present work, evidence of its activities on FSHR unravelled the mechanism of action of GCW exerting its estrogenic effect on ovarian folliculogenesis. The oestrogen property of GCW relaunching the endocrine pathway in hyperprolactin-induced infertility may be attributed to the activation of FSHR in the ovaries to exert the action of FSH in stimulating folliculogenesis. The pregnancy assessment outcome from this study also further collaborates the fertility enhancing effects through pregnancy sustenance to full-term under regulatory hormonal balance.

## CONCLUSION

The phytoestrogenic property of GCW exert effects that revitalizes endocrine dysregulation caused by hyperprolactinemia to stimulate folliculogenesis through the activation of FSHR at its action sites. It is important to investigate GCW phytoestrogenic effects on the activities of other reproductive hormones at their receptor sites for the understanding of the comprehensive mechanism of action of GCW needed for its usage to benefit man.

**Conflicts of Interest**

The Authors declared no conflict of interest

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**Author Contributions**

Bakare AA: Study conception and design, Research execution, Protein expression aspect, Result interpretation, Discussion and Manuscript writing; Osiagwu DD: Protein expression aspect, Result interpretation and Discussion; Elemoso TT: Study design, Research execution and Manuscript writing. All authors read and approved the manuscript

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