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RESEARCH ARTICLE

Oral Supplementation of Vitamin D3 Regulates Estrous Cycle Due to its Estrogenic Effects in Rats

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ABSTRACT

Vitamin D3 is a steroid hormone, like estrogens, and could regulate diverse female reproductive processes, including estrous cycles (EC) and uterine growth. Eighteen albino rats (138.33±8.37g BW) with regular EC, assigned into three groups (1-3, n=6), were used to investigate effects of vitamin D3 (vitD3) on EC length. Group 1 rats orally received 5 ml/kg BW distilled water (DW). Groups 2 and 3 orally received 0.025 mg/kg and 0.125 mg/kg of vitD3 dissolved in DW respectively for four weeks. Another 25 pre-pubertal rats (67.75±6.54g; 5 intact and 20 ovariectomized), assigned into five groups (A-E, n=5), were used to determine estrogenic activity of vitD3. Groups A (intact rats), B, C, D and E subcutaneously received 5 ml DW, 5 ml paraffin oil, 0.1 mg stilbesterol, 0.025 mg vitD3 and 0.125 mg vitD3 per kg BW, respectively for four days. Absolute uterine weights (AUW), relative uterine weights (RUW) and histomorphometric parameters of the uterine horns were recorded for rats of each group. The results showed that oral administration of 0.025 mg/kg BW vitD3 significantly ($P<0.05$) shortened proestrus and diestrus phases, but prolonged estrus phase of EC when compared with controls and 0.125 mg/kg BW group. However, total cycle length and metestrus phase were not affected. Similarly, 0.125 mg/kg BW vitD3 had no effect on EC or its phases. VitD3 and stilbesterol increased ($P<0.05$) AUW, RUW, endometrial thickness and uterine luminal folds when compared to the paraffin oil-treated and intact rats. Moreover, the endometrial thickness was higher ($P<0.05$) in paraffin-treated rats compared with the controls. It was therefore concluded that vitD3 altered EC of rats due to its estrogenic activity.

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INTRODUCTION

Vitamin D3 is a steroid hormone, synthesized mainly by the skin on exposure to ultraviolet light (Keeney *et al*., 2013). As a steroid hormone, this vitamin is related to estrogen, progesterone, testosterone, mineralocorticoids, and glucocorticoids; and substantive cross-reactivity with steroid and nuclear hormones receptors have been established (Sirajudeen *et al*., 2019). In recent times, Vitamin D3 deficiency has been linked with infertility (Zanatta *et al*., 2011) and consumption of ergocalciferol normalized the reproductive cycles and resulted in pregnancy in patients with fertility problems (Lerchbaum and Obermayer-Pietsch, 2012).

Estrogens are female sex steroids that play significant role in female reproductive physiology (Amenyogbe *et al*., 2020). Estrogens regulate several processes of female reproduction, for instance, estrous cycle, uterine growth, endometrial glands secretion, ovulation and activities of the hypothalamic-pituitary-gonadal axis (Hamilton *et al*., 2017). Previous reports have also associated estrogens with increased protein synthesis and uterine weight accompanied by increased water uptake, fluid retention and ballooning of the uterus (Zhao *et al*., 2019). Histologically, estrogens also induce uterotrophic changes such as increase in diameter of the uterus, thickness of endometrium and height of endometrial epithelium (Marquardt *et al*., 2019). Estrogens are also involved in non-receptivity of embryos for implantation and opening of the vagina, which are among the qualitative measure of estrogenic potency (Mardanshahi *et al*., 2019). It has been previously demonstrated that vitamin D3 and 17β-estradiol

upregulated each other's receptors, including vitamin D3 receptor (VDR), estrogen receptor-α (ERα), and estrogen receptor-β (ERβ) in the hippocampus of ovariectomized rats (Zhang *et al*., 2020).

Identification of the estrous cycle phases in rats is achieved through vaginal cytology (Ajayi and Akhigbe, 2020). This is due to the fact that the composition, thickness and structure of the stratified squamous and mucosal epithelium, as well as endocrine regulation in their reproductive tracts show more variations across the estrous cycle as compared to other animal species (Mahjabeen *et al*., 2018).

For more than two decades, the standard model usually employed in investigating the estrogenicity of a substance is the use of pre-pubertal female ovariectomized rats. It has been documented that while some non-gonadal organs synthesize estrogens, the ovary is the major organ shouldered with the bulk responsibility of producing estrogens (Cui *et al*., 2013). Ovariectomy therefore leads to diminished level of estrogen (a major female sex hormone) (Kulkarni *et al*., 2012). Bearing in mind that vitamin D3 is a steroid hormone, the present research was designed to determine its effects on estrous cycle length in normal cycling pubertal rats; and estrogenic activity in ovariectomized pre-pubertal albino rats.

MATERIALS AND METHODS

Experimental rats: For the estrous cycle experiment, 18 sexually mature female albino rats with regular estrous cycles, 11-13 weeks of age, weighing 138.33±8.37g, were used. For the estrogenic activity experiment, 25 sexually immature female albino rats, 3-4 weeks of age, weighing 67.75±6.54g, were used. The rats were housed in fly-proof metal cages at room temperature (27-32^{o}C), in a dark room devoid of direct or reflective ultraviolet rays from the sun. Fresh clean water and commercial feed (Vital® Growers feed - Grand Cereal Oil Mill Limited, Jos, Nigeria, containing 14.5% crude protein) were provided *ad libitum*. The research protocol was approved by the Institutional Animal Care and Use Committee of the faculty of Veterinary Medicine, University of Nigeria, Nsukka (FVM-UNN-IACUC-2019-1126) and the standard guidelines for use of laboratory animals for experimental purposes were strictly followed (NRC, 1996).

Vaginal cytology and estrous cycle monitoring: For monitoring effects of vitamin D3 on estrus cycle, 18 cycling rats were randomly assigned to 3 groups (1-3) of 6 rats each. Group 1 rats (control) received 5ml/kg body weight (BW) of distilled water. Groups 2 and 3 received 0.025 and 0.125mg/kg BW vitamin D3 (Valupak® Vitamins Ltd, Leeds, UK) dissolved in distilled water, respectively. The treatments were given orally twice weekly for four consecutive weeks (Gbotolorun *et al.*, 2008). Vaginal smears were examined once each day between 9.00 and 10.00 am and the phase of the estrous cycle recorded on daily basis (Abu and Uchendu, 2011). Normal saline (10 µl) was used as vaginal lavage, using microtitre pipette inserted approximately 1 cm deep, to avoid excessive cervical stimulation and a consequent pseudo-pregnancy (Goldman *et al*., 2007). The fluid aspirated from the vagina was then placed on the glass slide, covered with a cover slip and viewed under light microscope (x40) to identify the phase of estrous cycle based on the predominance of cell types. Proestrus was recognized by preponderance of epithelial (round and nucleated) cells, estrous was characterized by predominance of cornified (irregular and anucleated) cells, metestrus was marked by uniform mixture of epithelial cells, cornified cells and leukocytes, while diestrus was identified by prevalence of leukocytes (Marcondes *et al.*, 2002).

Ovariectomy and determination of estrogenic activity: Twenty-five (25) sexually immature female rats were anaesthetized for 20-50 minutes using 5-10 mg/kg body weight of xylazine and 80-100mg/kg body weight of ketamine. Thereafter, 20 rats were ovariectomized using standard surgical procedure, as previously described (Mbegbu *et al*., 2017), while five immature rats were sham-operated.

Fifteen days after ovariectomy, 25 immature female rats were randomly assigned to five equal groups (A-E). Group A comprised of five rats which were sham-operated, but not ovariectomized and received 5 ml/kg body weight of distilled water (normal 'intact' rats). Rats in Group B received 5 ml/kg body weight of paraffin oil. Group C rats were given 0.1 mg/kg body weight of stilbestrol suspended in paraffin oil. This group served as the positive control. Group D and E rats were administered 0.025 and 0.125 mg/kg body weight of vitamin D3 respectively. All the treatments were given subcutaneously on daily basis for four consecutive days. On the 5th day, the body weights of the rats were recorded. They were subsequently sacrificed humanely. The uterine horns were removed following laparotomy, blotted on a filter paper and weighed on a digital weighing balance. The respective uterine weights (absolute uterine weight; AUW) were then expressed as percentage of the body weight of each rat (relative uterine weight; RUW).

Tissue collection and processing for histology: About 3-5 mm thick sections of the uterine horns were fixed overnight in Bouin's fixative composed of 75 ml saturated aqueous picric acid, 25 ml of formalin (40% formaldehyde) and few drops of acetic acid. The tissues were processed for haematoxylin and eosin (H&E) staining, using standard technique (Suvarna *et al*., 2019). The stained slides were examined under light microscope (X100) and the height of the epithelial lining of the endometrium was measured using the line measurement tool of ImageJ computer software (version 1.48; NIH).

Statistical analysis: The computer software, statistical package for social sciences (SPSS) version 23.0 (IBM Corp, Armonk, NY, USA) was used for the statistical analysis. Data obtained were analyzed using one-way analysis of variance (ANOVA). The means were compared using Duncan's New Multiple Range Test and differences were considered significant at $P<0.05$.

RESULTS

Effect of vitamin D3 on estrous cycle: Administration of vitamin D3 at 0.025 mg/kg BW significantly ($P<0.05$) shortened the duration of proestrus and diestrus phases but prolonged the duration of estrus phase of the cycle when

compared to the control group rats and rats treated with 0.125mg/kg BW of vitamin D3. However, the total cycle length and metestrus phase of the cycle did not differ among three groups (Table 1). Moreover, there was no difference in the duration of proestrus, estrus and diestrus phases in rats of control group and those given vitamin D3 at 0.125mg/kg BW.

Uterotrophic effect of vitamin D3: Investigation of the estrogenic activity of vitamin D3 based on absolute uterine weights (AUW) and relative uterine weights (RUW) revealed that the rats treated with stilbesterol and vitamin D3 (low as well as high dose) had higher (P<0.05) AUW and RUW when compared to the paraffin oil-treated and intact control rats (Fig. 1). However, differences in AUW and RUW among rats of former three groups were non-significant. Similarly, there was no difference in these parameters between parafin-treated and intact control rats.

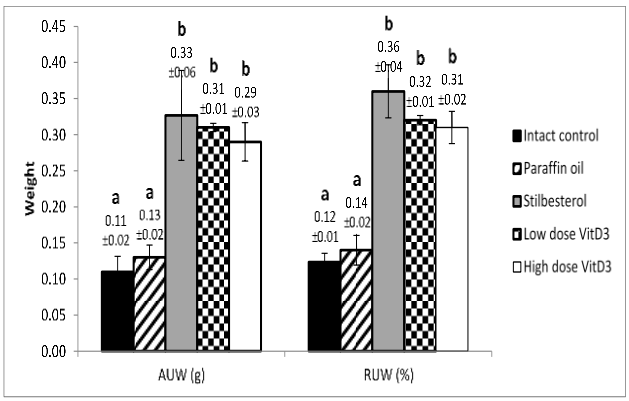

Fig. 1: Uterotrophic effect (Estrogenicity) assessed using absolute uterine weights (AUW) and relative uterine weights (RUW) in immature female rats of different treatment groups: Values with different letters indicate significant differences among five treatment groups for each parameter (P<0.05).

Effect of Vitamin D3 on uterine histomorphometry: The rats treated with stilbesterol (Fig. 2C) showed the highest endometrial thickness, followed by low dose vitamin D3-treated rats (Fig. 2D), high dose vitamin D3-treated rats (Fig. 2E), paraffin oil-treated rats (Fig. 2B) and intact control rats (Fig. 2A). Moreover, intact control rats showed the least uterine luminal convolutions or folds, while stilbesterol treated and low dose vitamin D3-treated rats showed the highest uterine luminal convolutions or folds. The high dose vitamin D3-treated rats also showed points of sloughed off epithelium (Fig. 2E).

Measurement of the height of the epithelial lining of the endometrium (endometrial thickness), using the line measurement tool of ImageJ computer software (version 1.48; NIH), showed significantly higher (P<0.05) value in stilbesterol-treated rats compared to the rest of the four groups (Fig. 3). Rats given 0.025 and 0.125 mg/kg BW of vitamin D ranked second (P<0.05), with non-significant difference between high and low dose treated rats, while the paraffin oil-treated group ranked third (P<0.05) with respect to the thickness of endometrium. The intact control rats had the least (P<0.05) endometrial thickness (Fig. 3).

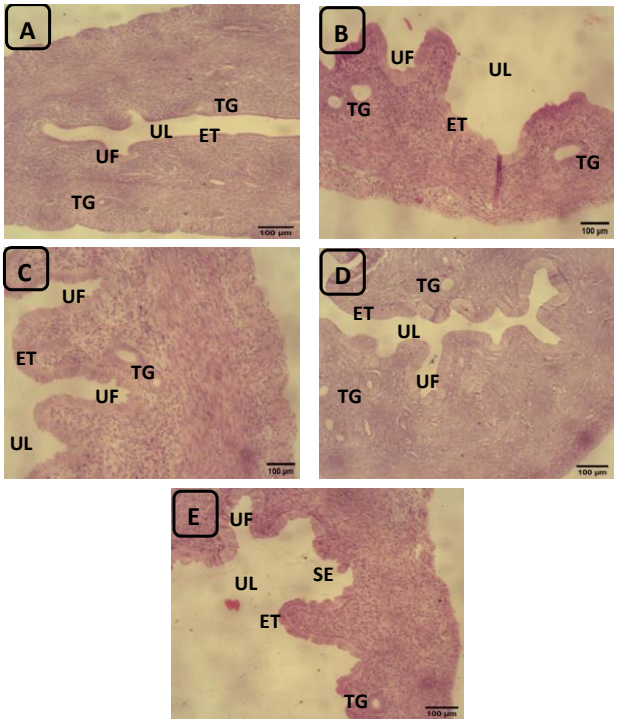

Fig. 2: Photomicrographs of uterine horns from: (A) intact control rats showing very thin endometrium and absence of uterine folds; (B) paraffin-oil-treated rats showing moderate increase in endometrial thickness and uterine folds; (C) stilbesterol-treated rats showing very high increase in endometrial thickness and uterine folds; (D) vitamin D3 low dose-treated rats showing high increase in endometrial thickness and uterine folds; (E) vitamin D3 high dose-treated rats showing greater increase in endometrial thickness and uterine folds. ET=Endometrial thickness, UL=Uterine lumen, UF=Uterine folds or convolutions, TG=Tubular gland, SE=Sloughed off epithelium. H&E (X100).

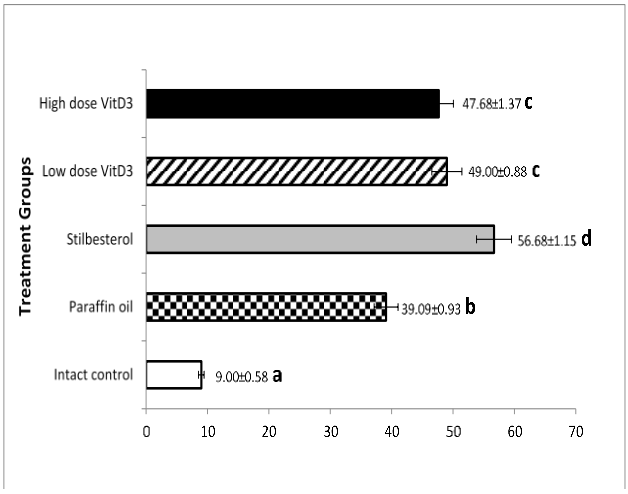

Fig. 3: Endometrial thickness (height of endometrial epithelium) of immature female rats of different treatment groups: Values with different letters indicate significant differences among groups (P<0.05).

Table 1: Effects of vitamin D3 supplementation on estrous cycle length (days) in sexually mature female albino rats

Groups	Proestrus	Estrus	Metestrus	Diestrus	Cycle length
1 (control)	0.75±0.09[a]	0.91±0.21[b]	2.39±0.08[a]	0.74±0.23[a]	4.80±0.23[a]
2 (0.025 mg/kg)	0.37±0.09[b]	1.23±0.18[a]	2.61±0.28[a]	0.52±0.07[b]	4.73±0.29[a]
3 (0.125 mg/kg)	0.56±0.14[a]	0.83±0.13[b]	2.45±0.21[a]	0.62±0.04[a]	4.46±0.23[a]

Means with different superscript letters in a column are significantly different (P<0.05).

DISCUSSION

Besides mineralization of bones, the role of vitamin D3 in other body organs, including the reproductive tissues, is becoming increasingly glaring (Christakos *et al.*, 2013). In this study, vitamin D3 administration at 0.025mg/kg body weight in adult rats significantly shortened the proestrus and diestrus but prolonged the estrous phases of the cycle when compared to the control group and the group treated with 0.125mg/kg body weight of vitamin D3. However, there was no variation in the total cycle length and metestrus phase of the cycle among rats of three groups. The proestrus phase of the estrous cycle is predominantly under the influence of FSH and the shortened proestrus is most likely to be associated with the decline in FSH secretion and activity (Shahrokhi *et al.*, 2018). The observed increase in the duration of estrous could be due to the fact that vitamin D3 supplementation precipitates uterotrophic activity and stimulates the estrogenic receptors through the stimulation of selective estrogen receptor modulation (Sbisa *et al.*, 2018). It implies that administration of vitamin D3 can increase chances of mating and conception in farm animals. It is noteworthy that the shortened diestrus may not directly translate to altered diestrus of pregnancy and progesterone secretion. In consonance with the findings of this study, Dicken *et al*. (2012) discovered that peripubertal vitamin D3 deficiency disrupts hypothalamic-pituitary-ovarian physiology, leading to significant delay in vaginal opening, arrested follicular development and prolonged estrous cycles characterized by extended periods of diestrus. However, high dose of vitamin D3 (0.125 mg/kg BW) did not show any beneficial effect compared with the controls, which could be due to possible toxicity associated with fat soluble vitamins (Mbegbu and Obidike, 2020).

Estrogenicity is the ability of several steroid hormones, such as estradiol, to stimulate the development and maintenance of female secondary sex characteristics, exerting systemic effects such as increase in uterotrophic activity (uterine wet weight, uterine luminal convolutions and cornification of endometrial epithelium) typical of estrous phase in many female mammals (Sun *et al.*, 2016). Selective estrogen receptor modulators have been proposed as suitable alternatives to 17β-estradiol (Sbisa *et al.*, 2018). Owing to the fact that vitamin D3 is regarded as a steroid hormone, the estrogenic activity was investigated. In this study, rats treated with stilbesterol and vitamin D3 (both low and high dose) showed significantly higher ($P<0.05$) AUW and RUW when compared to the paraffin oil-treated and intact control rats. This might have been an indication that vitamin D3 has estrogenic or like activity. During the proliferative phase of the uterus, when estradiol is the dominant steroid hormone in the female animals, there is increase in proliferation of uterine stroma cells, uterine glands and leukocyte infiltration (Yang *et al*., 2015). This could be the basis for the increase in AUW and RUW in rats given stilbesterol or vitamin D3. Further evidence for the estrogenic activity of vitamin D3 was obtained from the histology of the endometrium. The rats treated with stilbesterol had the highest endometrial thickness ($P<0.05$), followed by low dose or high dose vitamin D3-treated rats, paraffin oil-treated rats and intact control rats. Moreover, intact rats showed the least uterine luminal convolutions or folds, while stilbesterol treated and low dose vitamin D3-treated rats showed the highest uterine luminal convolutions or folds. The high dose vitamin D3-treated rats also showed points of sloughed off epithelium. It is likely that vitamin D3 is as potent as stilbesterol in the induction of estrogenicity owing to the fact that paraffin oil with some aromatic hydrocarbons might have potentiated the estrogenic effect exhibited by stilbesterol (Tarnow *et al*., 2014). This idea is also supported by the findings that rats treated with paraffin oil showed significantly higher endometrial thickness compared to controls. However, there was no difference in AUW and RUW between paraffin-oil treated and intact control rats.

In consonant with our finding, in a porcine model, it was reported that vitamin D3 altered the expression of some enzymes involved in the steroidogenic pathway and increased the synthesis of estrogens (Hong *et al.*, 2017). The pathophysiology of the observed estrogenic activity of vitamin D3 can be deduced from previous reports which have incriminated vitamin D3 as a steroid hormone having the capacity to stimulate estrogen receptors (Sbisa *et al.*, 2018). Vitamin D3 is known to modulate different organs and energy metabolism through the induction of creatine kinase activity (Choi *et al*., 2013). According to Krishnan *et al.* (2012), treatment of intact and ovariectomized female rats with vitamin D3 analogs up-regulated estrogen-induced response to creatine kinase activity and estrogen receptors modulation. The well documented increase in 3β-Hydroxysteroid dehydrogenase (3β-HSD) and production of estrogen could be among the mechanisms involved in the estrogenic effect of vitamin D3 on the uterine tissues of the rats. In another study involving pregnant rats, vitamin D3 exhibited progesterone like activity (Monastra *et al*., 2018). This shows that vitamin D3 is a potent stimulator of progesterone receptors, especially during gestation when estrogenic receptors (ERα and ERβ) could be masked (Cheng *et al*., 2005).

Conclusions: supplementation of adult rats with low dose of vitamin D3 (0.025mg/kg BW) altered estrous cycle by shortening proestrus and diestrus phases, but elongated estrus phase. These effects seem to be due to the observed estrogenic activity of this vitamin.

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Authors contribution: The results presented and discussed in the present work were extracts from PhD thesis of ECM supervised by IRO and LOA. ECM, IRO and LOA conceptualized and set out the methodology of the experiment. ECM and CTM performed the laboratory and data analysis; and wrote the original draft. All authors read and finally approved the manuscript.

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