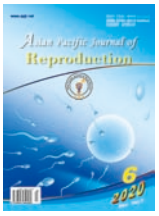

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Vitamin D3 supplementation influences ovarian histomorphometry and follicular development in prepubertal albino rats

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ABSTRACT

Objective: To evaluate the development of ovarian follicles in female albino rats following vitamin D3 supplementation.

Methods: Eighteen prepubertal female albino rats, aged 3-4 weeks, weighing (70.25±9.16) g, were assigned to three groups (n=6 in each group). Group A was treated with 5.00 mL/kg of distilled water and served as the control group, group B was treated with 0.025 mg/kg of vitamin D3 dissolved in distilled water, and group C was treated with 0.125 mg/kg of vitamin D3 dissolved in distilled water. All treatments were administered orally, twice weekly for 28 days. Blood and ovaries were harvested under anaesthesia. Serum vitamin D3 levels were determined by using spectrophotometric method. Ovaries were processed for histology and every10th hematoxylin and eosin stained-section was selected for histomorphometry. The number of follicles at each developmental stage was estimated.

Results: Both 0.025 mg/kg and 0.125 mg/kg of vitamin D3 significantly increased serum concentrations of vitamin D3 and calcium (P<0.05), but did not alter inorganic phosphorus concentration (P>0.05). The control group had fewer growing follicles (primary, secondary and antral follicles) and more non-growing follicles (primordial and atretic follicles) when compared with the vitamin D3-supplemented groups (P<0.05). Vitamin D3 at 0.025 mg/kg significantly increased antral follicles and corpora lutea counts (P<0.05). Vitamin D3 at 0.125 mg/kg significantly increased total, primordial and atretic follicles counts (P<0.05), but significantly decreased primary, secondary, antral follicles count, ovarian weight, relative ovarian weight, and ovarian surface area when compared with the control group and rats treated with 0.025 mg/kg of vitamin D3 (P<0.05).

Conclusions: Vitamin D3 supplementation at 0.025 mg/kg can enhance optimal ovarian follicle recruitment and development in female rats.

KEYWORDS: Folliculogenesis; Histomorphometry; Ovary; Rats; Vitamin D3

1. Introduction

Folliculogenesis or maturation of the ovarian follicle, involves phasic progression of primordial follicles previously arrested at the diplotene stage of oocyte development into large pre-ovulatory follicles and ultimately female gametes[1]. The characteristic developmental features of different follicular types in most mammalian species have been documented[2-5]. In the course of folliculogenesis, atresia of growing follicles could occur, and granulosa cells undergo apoptosis and replaced by fibrous tissues[6]. Unlike male mammals, the reproductive capacity of female mammals is determined prior to or around the time of birth[7]. As the female animal advances in age, from neonatal life to puberty, a proportion of these germ cells undergo phasic recruitment and are lost through atresia; and upon attainment of puberty, follicular recruitment and development during each cycle further depletes the primordial follicles pool[8]. Some intrinsic and extrinsic factors are known to influence this process of ovarian follicles recruitment and development[9]. The role of vitamin D in ovarian follicles development and fertility has been documented[10-13], and most female reproductive organs like the endometrium, myometrium, ovary, mammary gland, cervix, and placenta, are well furnished with vitamin D receptor[14]. It has also been reported that consumption of ergocalciferol normalized the reproductive cycles and resulted in

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pregnancy in patients with fertility problems[15]. It has been proposed that vitamin D deficiency could reduce the germ cells reserve[16].

Vitamin D is a steroid hormone, synthesized mainly by the skin on exposure to ultraviolet light; however, low percentage of vitamin D (up to 20%) is provided by diet[17]. The active form of vitamin D, 1,25-dihydroxyvitamin D3 mediates its action through vitamin D receptor[18]. Vitamin D deficiency is a global health challenge, affecting all continents of the world, with variation in prevalence among different age groups and gender[19]. For example, up to 42% of US adult population is vitamin D deficient with vitamin D serum levels <50 nmol/L or <20 ng/mL[20]. In the Middle East, a large population of adolescent girls, up to 70 % in Iran[21], and about 80% in Saudi Arabia[22], had serum 25-hydroxyvitamin D [25(OH) D] levels below 25 nmol/L. The awareness of the risk of vitamin D deficiency is becoming more glaring due to the small content of vitamin D in diets as well as other socio-cultural factors[23-25]. This study was therefore designed to evaluate the effects of vitamin D on development of ovarian follicles in female albino rats with vitamin D3 supplementation.

2. Materials and methods

2.1. Acquisition of vitamin D

Vitamin D tablets used for this study were procured from Valupak® Vitamins Ltd, Leeds, UK. Each tablet contained 1 000 IU of vitamin D3 as the main component in the excipient. The tablets were ground into fine powder and reconstituted in distilled water as required.

2.2. Experimental animals

Eighteen prepubertal female albino rats aged 3-4 weeks, weighing (70.25±9.16) g, were used for this study. They were acclimatized for one week prior to commencement of the experiment. The rats were procured from the laboratory animal breeding unit of Department of Veterinary Anatomy, Faculty of Veterinary Medicine, University of Nigeria, Nsukka. The rats were housed in fly-proof metal (aluminium) cages at room temperature (27 ℃ – 32 ℃), in a dark room devoid of direct or reflective ultraviolet rays from the sun, in the laboratory animal unit of Department of veterinary Physiology and Pharmacology, Faculty of Veterinary Medicine, University of Nigeria, Nsukka. 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*.

2.3. Experimental design

The rats were assigned to three groups of six rats per group. Group A (the control group) received 5.00 mL/kg body weight of distilled water. Group B received 0.025 mg/kg body weight of vitamin D3 dissolved in distilled water. Group C received 0.125 mg/kg body weight of vitamin D3 dissolved in distilled water (0.025 mg/kg wass the recommended supplementation dose, and 0.125 mg/kg was five times the recommended supplementation dose given to assay for possible toxicity)[19,23]. All the treatments were given orally twice weekly for four consecutive weeks (8-9 weeks of age, just at the point of attainment of puberty) when blood samples were collected for determination of serum vitamin D, calcium and phosphorus. Each (5 mL) of blood samples was collected from the retro bulbar plexus in the median canthus of the eye, allowed to stand for about 30 min and centrifuged for 5 min at 2 000 ×*g* to obtain the serum. The sera were stored in labeled plastic tubes at -20 ℃ until biochemical analyses.

2.4. Spectrophotometric assay for serum 25(OH)D, calcium and phosphorus

With strict adherence to the manufacturer's instructions, quantitative spectrophotometric determination of serum 25(OH)D, calcium and phosphorus was done based on the principal of competitive antibody binding. A standard curve was obtained by plotting a graph of the concentration of standards against the absorbance. A corresponding equation for calculating the concentrations of 25(OH)D, calcium and phosphorus was deduced from the respective graphs. The color intensities were inversely proportional to the amount of 25(OH)D, calcium and phosphorus in the sample.

2.5. Tissue collection and processing for histology

Using laparotomic incision, the ovaries were harvested under anesthesia. The ovaries were weighed by using sensitive balance, and the ovarian weights were expressed in relation to the body weights of the rats. About 3-5 mm thick sections of the ovaries 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 successively dehydrated in ascending grades of alcohol, cleared in xylene and infiltrated with molten paraffin wax. The tissues were embedded in a fresh molten paraffin wax to form hard blocks which were mounted on a microtome and serially sectioned at 5 μm thick. The tissue sections were mounted on glass slides coated with 20% albumin, deparaffinised, rehydrated in descending grades of alcohol and stained with haematoxylin and eosin (H & E)[26].

2.6. Morphological classification and quantification of ovarian follicles

The number of ovarian follicles in serially sectioned H & E stained ovary of each rat was calculated by using ratio estimation method[27].

Briefly, every 10th section was selected for morphometric evaluation of the follicles under the microscope. Images were collected by using a DM4000 Bright field upright microscope (Leica Microsystems, Milton Keynes, UK), and DC500 color camera (Leica Microsystems, Milton Keynes, UK). Both the microscope and the camera were connected to desktop computer installed with Leica Application Suite software version 2.8.1 for windows. The objective used was 10× HC PL FLUOTAR PH1 (NA=0.3). All the images captured for each section were merged by using Adobe Photoshop CC (Adobe Systems). The number of follicles at each developmental stage was identified and counted with cell counter tool on ImageJ software (version 1.48; NIH). All healthy follicle types were defined by using an established follicular classification system[28].

Follicles of various classes were quantified in each section to obtain the total number of follicles at each developmental stage. A true estimate of the total number of follicles at each maturational stage within the entire ovary was calculated by using the formula: Nt = Nf × St × ts / (So × do)[27,28]; where Nt = total calculated number of follicles of one type within the ovary; Nf = total number of follicles counted from every section observed; St = total number of sections in the ovary; ts = thickness of the section (in μm); So = total number of sections observed; and do = mean oocyte diameter of that follicular type. This calculation allowed raw counts of follicular number in relation to the total number of sections and section thickness, hence accounting for the proportion of the ovary not included in the sampling analysis.

Using line and area (free hand) measurement tools of ImageJ software, oocyte diameters for each follicle type and ovarian surface areas were estimated respectively, on H & E stained section from five randomly selected rats in each group and average deduced.

2.7. 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 by using one-way analysis of variance. The means were separated by using Duncan's new multiple range test and differences in the means less than probability values of 0.05 (P<0.05) were considered significant. The data were expressed as mean±standard deviation (mean±SD).

2.8. Ethics statement

The research proposal 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 adhered to strictly.

3. Results

3.1. Serum vitamin D, calcium and phosphorus concentrations

Vitamin D3 supplementation at both 0.025 mg/kg and 0.125 mg/kg significantly increased serum levels of vitamin D and calcium when compared to the group A (the control group) (P<0.05). The rats in the control group had vitamin D and calcium levels of [(4.66±0.14) $\times 10^1$] nmol/L, and [(0.86±0.08)$\times 10^1$] mg/dL, while the rats treated with 0.025 mg/kg and 0.125 mg/kg of vitamin D3 had vitamin D and calcium levels of [(5.63±0.18)$\times 10^1$] nmol/L, and [(1.16±0.13) $\times 10^1$] mg/dL, and [(5.85±0.32)$\times 10^1$] nmol/L, and [(1.25±0.08)$\times 10^1$] mg/dL, respectively. However, there was no significant difference in serum phosphorus concentrations across the groups (P>0.05) (Figure 1).

3.2. Differential and total ovarian follicles counts

Rats treated with 0.025 mg/kg of vitamin D3 significantly increased antral follicles and corpora lutea counts when compared with the control group (P<0.05) (Table 1).

Vitamin D3 supplementation at 0.125 mg/kg vitamin D3 significantly increased total, primordial and atretic follicles counts (P<0.05), but significantly decreased secondary and antral follicles counts as compared with the control group (P<0.05). However, treatment with 0.025 mg/kg of vitamin D3 also decreased total, primordial and atretic follicles counts (P<0.05), but increased primary, secondary, antral and corpora lutea counts when compared with the rats treated with 0.125 mg/kg of vitamin D3 (P<0.05) (Figure 2).

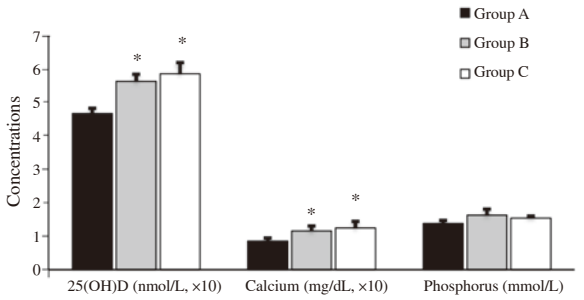

Figure 1. Effect of vitamin D supplementation on serum levels of vitamin D [25(OH)D], calcium and phosphorus in female prepubertal rats. Group A (the control group) receives 5.00 mL/kg of distilled water; Group B receives 0.025 mg/kg of vitamin D3; Group C receives 0.125 mg/kg vitamin D3. *: significantly different at P<0.05 *versus* the control group.

Table 1. Effect of vitamin D supplementation on differential and total follicles counts in female prepubertal rats.

Parameters	Group A	Group B	Group C
Total follicles	2168.79±131.04[a]	2361.91±139.38[a]	2851.76±150.27[b]
Primordial follicles	1860.45±126.99[a]	2010.57±133.54[a]	2680.94±135.08[b]
Primary follicles	131.56±19.94[ab]	143.78±16.75[b]	117.33±15.23[a]
Secondary follicles	40.83±7.06[b]	49.24±7.56[b]	25.03±6.22[a]
Antral follicles	26.04±6.16[b]	36.52±5.14[c]	14.67±4.03[a]
Corpus luteum	15.18±4.32[a]	24.35±6.02[b]	11.08±3.78[a]
Atretic follicles	3.15±1.18[a]	2.89±1.26[a]	7.02±2.15[b]

Means with different superscript letters in a column are significantly different at $P<0.05$.

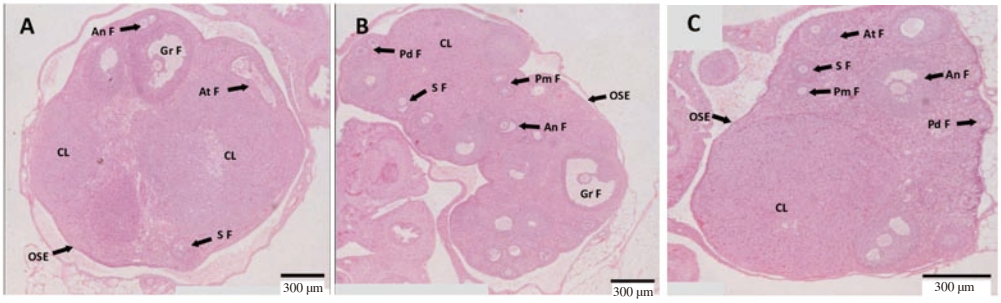

Figure 2. Photomicrograph of sections of the ovaries from the rats of groups A, B and C (H & E staining; magnification ×100). Pd F=Primordial follicle, Pm F=Primary follicle, SF=Secondary follicle, An F=Antral follicle, Gr F=Graffian follicle, At F=Atretic follicle, CL=Corpus luteum, OSE=Ovarian surface epithelium. A: Group A (the control group) has fewer primordial, primary and antral follicles, and some atretic follicle. B: Group B (0.025 mg/kg vitamin D supplementation) reveals a good number of primordial, primary and antral follicles, and fewer atretic follicle. C: Group C (0.125 mg/kg vitamin D supplementation) shows the highest number of primordial follicles atretic follicles, but the least number of primary secondary and antral follicles.

3.3. Ovarian weight, relative ovarian weight, and ovarian surface area

Vitamin D3 supplementation did not alter the body weights when compared with the control group ($P>0.05$). However, it was observed that 0.125 mg/kg vitamin D3 significantly decreased the ovarian weight [$(3.37\pm0.12)\times10^{-2}$ g], relative ovarian weight [$(2.86\pm0.18)\times10^{-4}$ g], and ovarian surface area [$(2.25\pm0.34)\times10^{-2}$ µm] when compared to the control group {[$(3.66\pm0.08)\times10^{-2}$ g]; [$(3.43\pm0.14)\times10^{-4}$ g]; [$(4.15\pm0.49)\times10^{-2}$ µm]} and rats treated with 0.025 mg/kg vitamin D3 [$(3.76\pm0.11)\times10^{-2}$ g]; [$(3.54\pm0.22)\times10^{-4}$ g]; [$(4.38\pm0.38)\times10^{-2}$ µm], respectively ($P<0.05$) (Figure 3).

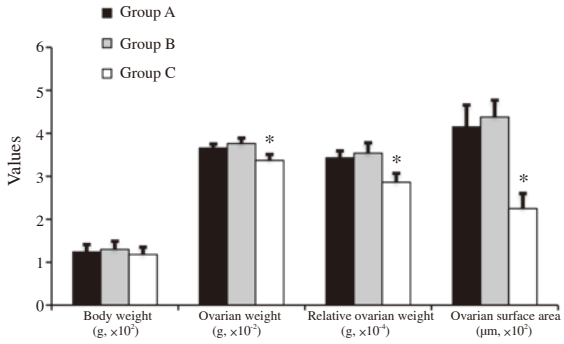

Figure 3. Effect of vitamin D supplementation on body weight, ovarian weight, relative ovarian weight and ovarian surface area in female prepubertal rats. *: $P<0.05$.

4. Discussion

Based on the current trend of vitamin D deficiency across the globe, supplementation with vitamin D for combating this health challenge is on the increase[29]. This has always helped to elevate the level of vitamin D in the blood for optimal physiological and biochemical processes. However, toxicity has been recorded with vitamin D supplementation especially at higher doses and under chronic conditions[30]. In the present study, the observed increase in serum 25(OH)D following supplementation could be attributed to increased bioavailability and absorption through the enterocytes brush-border membrane, possibly through passive diffusion and membrane carriers like cholesterol transporters. It has also been proposed that consumption of vitamin D with fatty diets improves its bioavailability and absorption[31]. From the work of Trautvetter *et al*, supplementation with daily 10 µg vitamin D significantly increased plasma 25(OH)D concentration after 4 weeks of treatment[32].

It is well established that vitamin D acts to increase blood concentrations of calcium. This is generated through the activity of parathyroid hormone found within the renal tissues. Parathyroid hormone preserves blood calcium by stimulating production of vitamin D3 within the kidney and facilitating the mobilization of calcium and phosphate from bone. It also maximizes tubular reabsorption of calcium within the kidney. This activity results in

minimal losses of calcium in urine. Most importantly, vitamin D facilitates absorption of calcium from the small intestine. However, due to detrimental effects associated with increases in phosphate, parathyroid hormone also has a potent effect on the kidney to eliminate phosphate which is otherwise known as phosphaturic effect[32]. This may explain why there was no significant variation between the treated and control rat groups.

The process that regulates the initiation of follicular growth needs further elucidation, but not likely to be entirely dependent of gonadotrophins, since exogenous gonadotrophin treatment is unable to alter the number of follicles recruited into growing ovarian pool. An intra-ovarian mechanism is therefore assumed to regulate folliculogenesis[33]. Vitamin D level has been known to influence follicle stimulatine hormone (FSH) release and recruitment of primordial follicles[34]. Our previous work indicated that vitamin D3 is required for normal expression of vasa gene, a marker for competence to resume meiotic cell division by primordial follicles[35]. The ovarian sections in the current research showed that vitamin D3 supplementation preserved a good number of primordial and growing follicles, while decreasing the rate of follicular atresia. The scanty primordial follicles in the control group rat ovaries could be dueto previously reported increase in serum FSH associated with vitamin D deficiency, resulting in recruiting greater number of primordial follicles into the growing follicles pool[36]. The growing follicles might have secreted anti-mullerian hormone in a relatively greater quantity, leading to inhibition of FSH activity required for continued growth of the antral follicles unto dominance and ovulation. However, the observed relatively higher number of follicles undergoing atrophy in the rats treated with 0.125 mg/kg body weight of vitamin D3 could be attributed to the possible toxicity associated with high supplementation dose. The increase in the primordial follicles count with 0.125 mg/kg vitamin D3 supplementation may not be beneficial because it can lead to recruitment below normal the minimum number of follicles required for optimal fertility[37].

In other words, normal supplementation dose of 0.025 mg/kg body weight gave the best result as it permitted optimal follicle recruitment and development (increased the number of antral follicles and corpora lutea). This shows that recruited primordial follicles in the normal supplementation dose group have less anti-Mullerian hormone-induced follicular growth inhibition, hence a higher number of follicles develop into antral follicles and ultimately ovulate with corpus luteum formation. The decrease in the primary follicles count justifies the observed increase in the primordial follicle number[38]. The total number of ovarian follicles increased with vitamin D3 supplementation at 0.125 mg/kg body weight as compared with the control and low dose groups due to the high numbers of non-recruited primordial germ cells, owing to the fact that this class of follicles is usually the highest in number at any given time under normal cyclical condition[37]. But the increased number of atretic follicles points to the fact that the toxicity at high dose hindered normal follicle development.

The ovarian surface area is a standard measure of gonadotoxicity[39]. From this study, while there was no difference in the mean body weights of the rats, vitamin D3 supplementation caused a dose-dependent decrease in the absolute ovarian weight, relative ovarian weight and ovarian surface area of the rats. This could be attributed to the sub-chronic toxic effect of high dose vitamin D supplementation which might have led to ovarian atrophy, and degeneration of the ovarian stromal and follicular cells. High dose vitamin D supplementation also causes down-regulation of the gonadotropin secretion which is needed to maintain integrity of the ovarian tissue[40]. Necrosis of the granulosa cells and the oocyte, shown by a greater number of atretic follicles at 0.125 mg/kg vitamin D supplementation, could as well be responsible[41]. The significant reduction in the ovarian surface area also lends credence to the reduction in ovarian weight, relative ovarian weight. Lipolytic action of vitamin D on abdominal visceral organs which has been reported[42]. The high dose of vitamin D3 may have adversely affected the ovarian stromal cells and increased the inherent lipolytic activity; but may not be gonadotoxic to the primordial follicles owing to the high count recorded[42].

Some limitations which the present study faced however, may include the fact that the follicles counts were subjective to the judgment of the independent individuals employed to manually view and count the follicles. Again, the first tissue sections which usually contained the ovarian surface epithelium and no observable follicles were excluded in the estimation. Nevertheless, we maintained the same pattern throughout the experiment.

In conclusion, as the impact of vitamin D beyond bone mineralization is becoming increasingly glaring, the present study has shown that vitamin D plays a significant role in reproductive physiology of prepubertal female rats through regulation of folliculogenesis (ovarian follicles development) and has the tendency to directly affect the fertility outcomes. From all indications, the recommended dose (0.025 mg/kg body weight) vitamin D supplementation in rats gave the optimal folliculogenesis and prospects for enhanced fertility.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Authors' contributions

This work was an aspect of PhD thesis of the first author and supervised by the second author. Edmund Chidiebere Mbegbu and Ikechukwu Reginald Obidike designed the experiment and performed the laboratory procedures. All authors took part in writing and final approval of the manuscript.

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