

Vitamin D And Male Reproduction

Haiam A Mohammed, Nora E Abd el-Hamid, Hamada MMZ
and El-Badry AA

Department of Physiology, Fac. of Vet. Med., Zagazig University

ABSTRACT

To study the relationship between vitamin D and male reproduction, fifty male albino rats were obtained as weanlings (21 days-old) and divided into 2 groups, vitamin D-deficient group fed vitamin D-deficient diet and vitamin D-replete group fed the same diet but received 2 µg cholecalciferol per week in 0.1 ml propylene glycol by a single intraperitoneal injection. The animals in both groups were maintained till became adult. The results revealed detection and expression of vitamin D receptor (VDR) gene in the testis of young rats at 2 months of age, a significant increase in acrosome reaction percentage, sperm motility and sperm count in the vitamin D-replete group while sperm abnormalities were significantly higher in the vitamin D-deficient group. The results of this study suggest an important physiological role played by vitamin D in male reproductive biology.

Key words: Vitamin D, Reproduction, Male rat.

INTRODUCTION

Vitamin D is a highly regulated steroid hormone system. It has been well-known for its function in maintaining calcium homeostasis and bone health. However, the spectrum for vitamin D mediated effects have expanded in recent years (1). The biologically most active form of vitamin D, 1 α ,25-dihydroxyvitamin D₃ or calcitriol, exerts its biological activities through both genomic and non-genomic effects. The classic genomic responses are generated by regulating gene transcription through binding with vitamin D receptors, while, the rapid or non-genotropic responses include rapidly activating a variety of signal transduction pathways at or near the plasma membrane (2).

Vitamin D receptor (VDR) is expressed in calcium-regulating tissues as intestine, skeleton and parathyroid gland as well as in the ovary and testis (3). Indeed, it is widely distributed in the female and male reproductive tissue of rodents suggesting an important local role

played by vitamin D in such tissues (4). VDR has been expressed in the smooth muscles of the epididymis, spermatogonia and Sertoli cells of rodents (5). VDR is also found in similar amounts in isolated seminiferous tubules and interstitial tissue of adult rats (6).

Acrosome reaction happens only in capacitated sperm and is a prerequisite for a sperm to fuse with an egg. It was reported that vitamin D increases the intracellular calcium in human spermatozoa as it induced a significant higher proportion of motile spermatozoa to undergo the acrosome reaction (under capacitating conditions) (7). A high concentration of vitamin D in the vicinity of the cumulus-oocyte complex could be important since the vitamin D mediates Ca²⁺-rise and subsequent acrosome reaction facilitating penetration of the cumulus cells, allowing binding and subsequent fertilization of the oocyte (8). In addition, It was recorded that the percentage of motile spermatozoa declined in VDR null mutant male mice from 15% to less

than 1% at 10 weeks of age and there was also a transient increase in testicular weight (4).

It had been recorded that there was a significant reduction in testicular and epididymal sperm count in vitamin D deficient male rats (9). Moreover, the sperm count in testis and epididymis was significantly decreased at 120th and 150th day of age in vitamin D deficient male rats (10). Further investigation on VDR null mutant male mice revealed that the number of functional sperms was markedly decreased (4). The cross-sectional study on 300 men from the general population found that men with vitamin D deficiency had a lower proportion of morphologically normal spermatozoa compared with men with sufficient vitamin D status (7).

Therefore, this study aimed to investigate the relation between vitamin D and reproduction in male rats through: a- Detection of vitamin D receptor gene in the testis of young rats. b- Effect of vitamin D deficiency and supplementation on acrosome reaction percentage of spermatozoa. c- Effect of vitamin D deficiency and supplementation on epididymal sperm count, motility and abnormalities.

MATERIALS AND METHODS

Animals and diets: 50 male weanling albino rats weighing 30 gm average body weight were obtained from Laboratory Animal Unit in Faculty of Veterinary Medicine, Zagazig University and divided into 2 groups, vitamin D-deficient group fed vitamin D-deficient diet and vitamin D-replete group fed the same diet but received 2 µg cholecalciferol per week in 0.1 ml propylene glycol by a single intraperitoneal injection (11,12). The animals in both groups were maintained till became adult. All animals were housed in stainless steel cages and maintained on a 12/12 h light/dark cycle.

The vitamin D-deficient rats were prevented from all potential sources of ultraviolet light (as fluorescent light and sunlight) and were provided by incandescent lighting only to exclude the possibility of endogenous production of vitamin D by the skin.

Sampling: Testicular samples were taken from young male rats at the age of one and two months for detection of vitamin D receptor (VDR) gene using RT-PCR. The tail of epididymis was used for obtaining suspension of semen from adult male rats in both groups at the age of 4 months for measurement of acrosome reaction percentage in spermatozoa and semen analysis as follows: the cauda epididymis was excised and placed in a sterilized petri dish containing 2 ml warm normal saline (37°C). To obtain the epididymal contents, the cauda epididymis was macerated in the saline using sterilized scissors. The obtained suspension was handled exactly as the semen (13).

RT-PCR analysis for Detection of vitamin D receptor (VDR) gene in the testis.

RNA Extraction: RNA was extracted from testis of young rats using Biozol (Total RNA Extraction Reagent) Bioflux Cat# BSC51M1 according to manufacture instructions. Briefly, 100 mg of tissue was ground in 1ml of Biozol and incubated at room temperature for 30 minutes. 200 µl of chloroform was added and incubated on ice for 15 minutes then centrifugation at 15000 rpm for 20 minutes. The uppermost layer was taken into another tube. Equal volume of isopropanol was added and incubated at -20°C for 30 minutes then centrifuged at 12000 rpm for 10 minutes. The supernatant was discarded and the gel like pellet was washed once by 1 ml of 75% ethanol then the ethanol was discarded and the pellet was resuspended in 50 µl Diethylpyrocarbonate (DEPC) water.

cDNA synthesis: 5µl of RNA template was reverse transcribed to cDNA at 25°C for 5 minutes followed by incubation at 42°C for 60 minutes. Finally the reverse transcription reaction was stopped by heating the samples to 70°C for 5 minutes using Revert AidTM H

Minus First strand cDNA synthesis Kit (Fermentas Life sciences Lot: 00059751) according to manufacture instructions.

PCR: PCR was performed in Master Mix [Dream Taq Green MM (2X) Thermo Scientific] in a total volume of 25 μ l. In each

reaction 3 μ l of cDNA were added to 25 μ M of each oligonucleotide primers forward and reverse. The sequences and references were demonstrated in table (1).

Table 1. Sequences and references of House keeping gene or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and VDR gene

Target Gene	Sequence	Reference
House keeping gene (GAPDH)	Forward 5'-GGTGGAGGTCGGCGTCAACGGATT-3' Reverse 5'-GATGCCAAAGTTGTCATGGATGACC-3'	(14)
VDR	Forward 5'-CCCATGGCAAACCTCAAA-3' Reverse 5'-GCCCAAGCTATCTGAAGAACAA-3'	(15)

Gel electrophoresis: RT-PCR product of GAPDH and VDR genes were detected in 1.5% agarose gel in Tris-borate-EDTA (TBE) buffer and visualized by ethidium bromide staining (0.5 μ g/ml). The electrophoretic picture was taken by digital camera 12 mega pixels.

Table 2. Cycling protocol of PCR for amplification of GAPDH

Target	Amplicon size	Cycling condition			No. of cycles
		Step	Temp.	Time	
House keeping gene (GAPDH)	500bp	Initial denaturation	95°C	5 min	One cycle
		Denaturation	95°C	45 sec	35 cycles
		Anealing	55°C	45 sec	
		Extension	72°C	1 min	
		Final extension	72°C	10 min	One cycle

Table 3. Cycling protocol of PCR for amplification of Vitamin D receptor (VDR) gene

Target	Amplicon size	Cycling condition			No. of cycles
		Step	Temp.	Time	
Vitamin D receptor	844bp	Initial denaturation	94°C	4 min	One cycle
		Denaturation	94°C	60 sec	30 cycles
		Anealing	60°C	60 sec	
		Extension	72°C	90 sec	
		Final extension	72°C	10 min	One cycle

Acrosome reaction

The acrosome reaction in vitamin D replete and vitamin D deficient males was determined as follows: 100 μ l of semen suspension was transferred to clean sterile tube containing 1 ml

of Tyrodes albumin lactate pyruvate (TALP) medium which consists of the following ingredients (16): 100 mM NaCl, 3.1 mM KCl, 0.3 Mm NaH₂PO₄, 10 mM HEPES, 0.4 mM MgCl₂, 25 mM NaHCO₃, 1 mM Sodium

pyruvate, 21.6 mM Sodium lactate and 2 mM CaCl_2 at 36 °C and adjusted to pH = 7.4.

Determination of acrosome reaction percentage was carried out using silver nitrate stain as follows (17): 100 gm silver nitrate added to 200 ml distilled water, semen was smeared on clean slides and air dried. The slides were fixed in 70% ethanol for 2 minutes then in 95% ethanol for 2 minutes and stained with silver nitrate stain for 2 hours at 65 °C in a humidified air incubator. Rinsing the slides with distilled water and drying in air was done then microscopic examination was carried out using high power (40x).

Semen analysis

Sperm motility (%): Sperm motility within epididymal semen was assessed as soon as possible after extraction. A drop of the suspension was put on a clean glass slide and covered by a glass cover slide both pre-warmed at 37°C. Percentage of forward progressive motile spermatozoa was recorded through examination under high power (40x) of light microscope (18).

Sperm cell concentration: 200 µl of the suspension with 800 µl of normal saline previously added to it few drops of formaline 40% for killing spermatozoa were put together in a plastic tube and mixed well then the spermatozoa were counted in an improved hemocytometer counting chamber (19).

Sperm abnormalities (%): The percentage of abnormal spermatozoa was determined using Eosin-Nigrosin stain. The stain was prepared as follows: 5 gm Eosin was dissolved in 100 ml distilled water (5% solution), solution A, then 10 gm Nigrosin was dissolved in 100 ml distilled water (10% solution), solution B. The solution A & B have been observed stable at room temperature and were kept separated in sterilized bottles with rubber stoppers. A drop of diluted semen was mixed on a clean warm slide with a small drop of eosin stain (5%) and a drop of nigrosin stain (10%) and thin smears

were made on other slides. The stained semen smears were dried in air and then fixed by passing rapidly on flame. Microscopic examination was carried out using (100x) oil immersion lens (20). One hundred spermatozoa were counted and classified as either normal or abnormal specifying which defects are most common (21).

Statistical analysis: Data were collected and analyzed using Statistical Package for Social Sciences (22). To estimate the difference between the replete and deficient group, Independent sample t-test was used according to (Students-t). Data were presented as mean \pm SEM (standard error of mean) and significance was declared at (P-Value < 0.05).

RESULTS AND DISCUSSION

The present study investigated the relationship between vitamin D and fertility in male rats. The presence of specific vitamin D receptors in the testis of young rats as shown in figure (1) suggests a possible physiological role played by vitamin D in male reproduction. This result is in agreement with the previous reports (23) which recorded the immunolocalization of VDR in 30 day-old rat testis using confocal microscopy. It was proved by immunohistochemistry the localization of VDR on the head (nucleus) and mid-piece of human sperm (24). In addition, another study revealed the expression of VDR in human sperm by RT-PCR, Western blot and Immunofluorescence techniques (25). Furthermore, the marked expression of VDR and the vitamin D metabolizing enzymes in human testis, ejaculatory tract and mature spermatozoa is demonstrated (26).

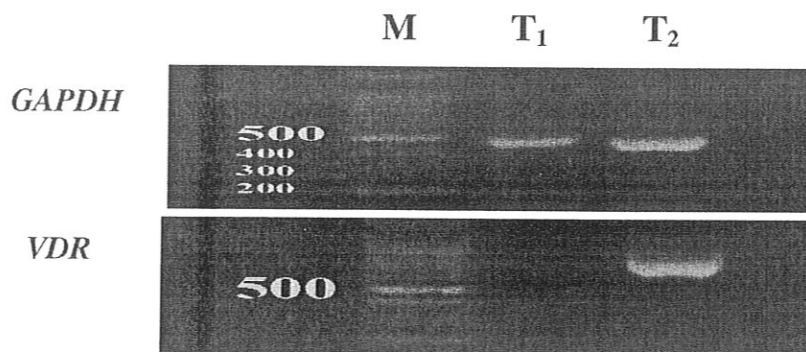


Figure 1. Bands of gel-electrophoresis for mRNA amplification of GAPDH and VDR gene in testis of one month-aged rat (T_1), testis of two month-aged rat (T_2), showing detection and expression of VDR gene in two month-aged testis (T_2).

Table 4. Effect of vitamin D deficiency and supplementation on acrosome reaction % and semen characters in male rats

Parameters	Replete group	Deficient group
Acrosome reaction %	62.8±4.32 ^a	37.5±5.32 ^b
Sperm motility %	75±2.89 ^a	35±6.45 ^b
Sperm cell count per ml×125×10 ⁴	52.33±4.33 ^a	32.33±1.45 ^b
Sperm abnormalities %	11.75±0.48 ^a	17.25±0.95 ^b

Means within the same row carrying different superscripts were significant at $P < 0.05$.

Regarding the percentage of acrosome reaction and motile spermatozoa, the presented data in table (4) revealed a significant reduction in the percentage of acrosome reaction as well as sperm motility in vitamin D deficient male rats. These results are parallel to the previous findings (7) in human spermatozoa where vitamin D induced the acrosome reaction in vitro and increased sperm motility. The results of sperm motility are also similar to that in VDR null-mutant male mice (4) which revealed that the percentage of motile spermatozoa declined from 15% to less than 1% at 10 weeks of age. This can be demonstrated by the ability of vitamin D to increase the intracellular calcium levels of spermatozoa through combining with its receptors which are confirmed to be found and expressed in testis and spermatozoa as mentioned before. The

increase in intracellular calcium induced hyperactivated motility and consequently changes associated to capacitation and acrosome reaction (25).

Concerning sperm count, the results in table (4) clarified the significant increase of this parameter in the vitamin D replete group while it was markedly decreased in the deficient group. This result is consistent with the previous reports in male rats (9,10) and VDR null-mutant male mice (4).

Vitamin D deficiency resulted in a significant increase in the percentage of sperm abnormalities as table (4) shows. This result agree with the cross-sectional study (7) which revealed that men with vitamin D deficiency had a lower proportion of morphologically normal spermatozoa compared with men with

sufficient vitamin D status. In contrast, another study found an association of high vitamin D levels with lower percentage of normal morphology sperm (27).

In conclusion, the results of this study provide evidence that vitamin D plays an important role in male fertility as its receptors were found in the testis as well as it improved the acrosome reaction and semen properties. These findings need further investigations to clarify the physiological role of vitamin D in male reproductive biology.

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المخلص العربى

فيتامين (د) والتكاثر فى الذكور

هيام أحمد محمد، نوره السيد عبد الحميد، محمد محمد زكى حمادة ، عادل عبد التواب البدرى

قسم الفسيولوجيا- كلية الطب البيطرى- جامعة الزقازيق

أجريت هذه الدراسة لبحث العلاقة بين فيتامين (د) والخصوبة فى ذكور الفئران. تم تقسيم عدد ٥٠ من ذكور الفئران عند عمر الفطام (٢١ يوم) إلى مجموعتين؛ مجموعة ناقصة العليقة فى فيتامين (د) وأخرى مزودة بفيتامين (د) عن طريق الحقن خلال الغشاء البريتونى بجرعة ٢ ميكروجرام من مادة الكوليكالسيفيرول مذابة فى ٠,١ من البروبلين جليكول أسبوعيا. تم مراعاة الحيوانات فى كلتا المجموعتين حتى عمر البلوغ. أثبتت الدراسة الوجود وكذلك التعبير الجينى لمستقبلات فيتامين (د) فى الخصية للفئران غير البالغة عند عمر شهرين وكذلك أسفرت الدراسة عن حدوث ارتفاع معنوى فى النسبة المنوية لتفاعل (الجسيم الطرفى) الأكروسوم فى الحيوانات المنوية و لحركة وعدد الحيوانات المنوية للمجموعة المزودة بفيتامين (د) بينما كان هناك ارتفاع معنوى فى نسبة الحيوانات المنوية غير الطبيعية فى المجموعة التى تفتقد إلى فيتامين (د). تشير نتائج هذه الدراسة إلى الدور الفسيولوجى الهام لفيتامين (د) فى تكاثر الذكور.