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Effect of Vitamin D Supplementation on Follicular Fluid BMP-15 and Anti-Müllerian Hormone Production in Average Responder Women Undergoing Intracytoplasmic Sperm Injection

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ABSTRACT

Vitamin D (VD) is known to play a crucial role in various physiological processes, including reproductive health. This study investigates the impact of VD supplementation on follicular fluid BMP-15 protein levels and anti-Müllerian hormone (AMH) production in women undergoing Intracytoplasmic Sperm Injection (ICSI). To evaluate the effects of VD supplementation on ovarian reserve markers, and endometrial thickness in women undergoing ICSI. A total of 90 women aged 25-40 years, undergoing ICSI, were enrolled in the study. Participants were divided into a study group receiving VD supplementation and a control group. Baseline characteristics, hormonal profiles, and ovarian reserve markers (AMH and antral follicle count) were assessed. Serum and follicular fluid samples were collected on the day of oocyte retrieval, and VD levels were measured using an ELISA kit. Statistical analyses were performed to compare outcomes between groups. Ninety infertile women undergoing ICSI were allocated to either a control group (n=45) or a study group (n=45). Participants ranged in age from 20 to 40 years, with a mean age of 29.13 ± 3.42 years in the control group and 29.07 ± 2.99 years in the study group. The study found no significant difference in AMH levels between the study group (2.11 ± 0.61 ng/mL) and the control group (2.21 ± 0.61 ng/mL; p = 0.438). VD showed a weak and non-significant correlation with AMH in the study group (r = 0.124, p = 0.418), but a moderate and significant positive correlation in the control group (r = 0.331, p = 0.026). BMP-15 protein levels were weakly correlated with AMH in the study group (r = 0.160, p = 0.294) but showed a significant moderate positive correlation in the control group (r = 0.341, p = 0.022). VD supplementation did not significantly affect AMH levels but

was associated with a higher antral follicle count in women undergoing ICSI. These findings suggest that while VD may not influence ovarian reserve as measured by AMH, it could play a role in enhancing follicular development.

1. INTRODUCTION

Vitamin D (VD), a fat-soluble steroid hormone and micronutrient, is synthesized endogenously upon exposure to ultraviolet B (UVB) radiation through the cutaneous conversion of 7-dehydrocholesterol [1]. While approximately 80% of VD synthesis occurs through this process, the remaining 20% is derived from dietary sources [2]. Primarily, Vitamin D affects calcium-phosphorus balance and facilitates bone mineralization [3]. In the female reproductive system, VD performs several autocrine, paracrine, and endocrine roles, such as regulating ovarian and endometrial growth of cells and the development of genes related to endometrial receptivit [4].

The 2024 Institute of Medicine (IOM) guidelines and the 2011 Endocrine Society guidelines recommend a daily VD intake of 600 IU for individuals aged 1-70 years, including pregnant and lactating women [5]. However, the American College of Obstetrics and Gynecology (ACOG) recommends a higher daily intake of 1,000-2,000 IU of VD for pregnant women who are deficient. Additionally, they may recommend higher doses for individuals with specific health conditions or those at increased risk of deficiency [6].

While clinical primary infertility is relatively low at 3.8%, demographic infertility in the MENA region is significantly higher at 22.6% [7]. In Egypt, 12% of couples experience infertility, with 4.3% experiencing primary infertility and 7.7% experiencing secondary infertility [8]. VD may influence embryogenesis and follicle development [9]. Moreover, it appears to modulate primary follicle recruitment by regulating anti-Müllerian hormone production (AMH) [10]. VD deficiency is prevalent among infertile couples, with approximately 15% of infertile individuals exhibiting low VD levels, particularly female partners [11].

Bone morphogenetic protein-15 (BMP-15), a critical oocyte-secreted factor involved in follicle maturation and oocyte competence [12]. BMP-15 plays a pivotal role in ovarian function by regulating granulosa cell proliferation and follicular growth, both of which are essential for successful oocyte development [13]. Additionally, emerging evidence suggests that VD levels in follicular fluid may correlate with ovarian reserve markers, including AMH and antral follicle count (AFC), as well as endometrial thickness, a key determinant of implantation potential [14]. In this study, we aim to investigate the relationship between follicular fluid VD levels, BMP-15 protein, and key reproductive parameters, including ovarian reserve markers (AMH and AFC), and endometrial thickness, in women undergoing ICSI.

2. MATERIAL and METHODS

2.1 Study Design and Participants

This prospective case-control study was conducted at Amshag Hospital for IVF/ICSI, Sohag City, Sohag Governorate, Egypt. All participants provided written informed consent. Inclusion criteria were women aged 20-40 years who were diagnosed with non-

polycystic ovary syndrome (PCOS) but had no prior or current history of ovarian hyperstimulation syndrome (OHSS). They were selected based on specific criteria: normal prolactin levels, absence of other endocrinological abnormalities (including thyroid disorders and diabetes), normal ovarian reserve as indicated by anti-Mullerian hormone (AMH) levels within the 1-3 ng/ml range, and no evidence of endometriosis. All participants underwent a thorough medical evaluation, including a detailed history, physical examination, abdominal and vaginal ultrasonography, and routine laboratory tests to exclude any potential confounding factors. Exclusion criteria were women with age older than 40 years or younger than 20 years, diagnosis of PCOS according to the Rotterdam criteria, a history of endometriosis within the past year, a history of OHSS, severe male factor infertility characterized by immotile sperm, or poor response patient.

Ninety women eligible for ICSI cycles were enrolled and divided into two groups: a study group of 45 women aged 20-40 who received a daily supplement of 10,000 IU of VD3, starting on day 20 of the preceding cycle and continued daily until the day of oocyte retrieval, and a control group of 45 women of the same age range who received no supplementation.

2.2 Primary and Secondary Outcomes

The primary and secondary outcome relationship between follicular fluid VD levels, BMP-15 protein, and key reproductive parameters, including ovarian reserve markers (AMH and AFC), and endometrial thickness, in women undergoing ICSI.

2.3 Sample Size Calculation

Due to the lack of clear evidence for the primary outcome, a formal sample size calculation was not performed. Instead, patients were enrolled based on inclusion criteria to achieve comparable group sizes [15].

2.4 Ethical Considerations

The study protocol was approved by the Ethical Review Committee of the Faculty of Medicine, Assiut University (IORG0010947-MB-21-8-A). All participants provided written informed consent, including information about potential risks and complications associated with metabolic abnormalities [16].

2.5 Ovarian Stimulation and Oocyte Retrieval

A standardized protocol was implemented for ovarian stimulation in all patients. Prior to controlled ovarian hyperstimulation (COH), patients received daily subcutaneous injections of a gonadotropin-releasing hormone (GnRH) agonist, Decapeptyl (0.1 mg, Fering, Germany), and oral VD3 supplementation (D-Dep, 10,000 IU, Globe International Company) beginning on day 20 of the preceding cycle. COH was initiated on cycle day 3 with recombinant follicle-stimulating hormone (r-FSH), Gonal F, administered according to a center-specific protocol. The starting dose of r-FSH was individualized based on patient age and body mass index (BMI). Human chorionic gonadotropin (hCG, 10,000 IU, Choriomon, IBSA) was administered intramuscularly when at least one follicle reached a diameter of 17 mm or greater. Ovum pick-up was performed 34-36 hours post-hCG administration under transvaginal ultrasound guidance.

2.6 Sample Collection and Analysis

FF was collected during oocyte aspiration using a closed vacuum system. Blood samples were collected before COH initiation.

2.6.1 VD measurements

Serum and follicular fluid samples were collected from all participants at two time points: (1) prior to the initiation of vitamin D supplementation (baseline) and (2) on the day of oocyte retrieval. Serum was separated by centrifugation and stored at -80°C until analysis. Vitamin D (25-OH-D) concentrations were measured using a commercially available ELISA kit (SinoGeneClon Biotech Co., Ltd, Hangzhou, China), according to the manufacturer's instructions. Briefly, 50 μL of each serum or follicular fluid sample was loaded into microtiter plate wells pre-coated with a monoclonal anti-25-OH-D antibody. After incubation and washing steps to remove unbound material, an HRP-conjugated secondary antibody was added, followed by TMB substrate. The reaction was stopped, and absorbance was read at 450 nm using a microplate reader. Sample concentrations were calculated using a standard curve constructed from provided calibrators. All samples and standards were assayed in duplicate to ensure reliability, and intra- and inter-assay coefficients of variation were <10%.

All serum and follicular fluid samples were either assayed immediately or stored at -80°C until analysis to prevent degradation and avoid repeated freeze-thaw cycles. For the ELISA, standards and samples were prepared according to the manufacturer's protocol. Absorbance was measured at 450 nm using a microplate reader, and concentrations were calculated using a standard curve generated from the provided calibrators. All samples were analyzed in duplicate. The intra-assay and inter-assay coefficients of variation were both less than 10%, ensuring reliability of the results.

2.6.2 BMP-15 measurements

BMP-15 protein concentrations were measured in follicular fluid samples collected at the time of oocyte retrieval. Samples were centrifuged and stored at -80°C until analysis. We used a commercial ELISA kit specific for human BMP-15 (SinoGeneClon Biotech Co., Ltd, Hangzhou, China), following the manufacturer's protocol. Briefly, 50 μL of each follicular fluid sample was loaded into wells pre-coated with anti-BMP-15 antibody. After incubation and washing, an HRP-conjugated detection antibody and TMB substrate were sequentially added. The reaction was terminated with stop solution, and absorbance was read at 450 nm using a microplate reader. BMP-15 concentrations were calculated from a standard curve generated using kit calibrators. All measurements were performed in duplicate, and intra- and inter-assay coefficients of variation were less than 10%.

2.6.3 ICSI and Embryo Transfer (ET)

ICSI was performed on mature oocytes using standard techniques. Embryo quality was assessed using the Gardner grading system. ET was performed on 5 after oocyte retrieval, with the number of embryos transferred based on patient age and embryo quality.

2.7 Statistical Analysis

Data were analyzed using appropriate statistical methods, considering the non-normal distribution of the data. The Shapiro-Wilk test was used to assess normality. Chi-square and Fisher's exact tests were used to compare pregnancy and abortion rates between groups.

3. RESULTS

Ninety infertile women undergoing ICSI were allocated to either a control group (n=45) or a study group (n=45). Participants ranged in age from 20 to 40 years, with a mean age of 29.13 ± 3.42 years in the control group and 29.07 ± 2.99 years in the study group. The control group received no VD supplementation during induction, while the study group received a VD supplement. No significant differences in age were observed between the two groups (P=0.922).

AMH levels were comparable between the study group and the control group, with no statistically significant difference (p = 0.438). However, AFC was significantly higher in the study group compared to controls (p = 0.000*). The number of oocytes and M2 oocytes did not differ significantly between groups (p = 0.062 and p = 0.105, respectively) (Table 1).

Table 1: AMH, AFC, Number of Oocytes, and M2 in Study and Control Group.

Parameter	Study Group (n = 45)	Control Group (n = 45)	p-value
Age (years)	29.07 ± 2.99	29.13 ± 3.42	0.922
Baseline 25-OH-D (ng/mL)	18.5 ± 4.2	18.8 ± 4.0	0.812
AMH (Mean ± SD)	2.11 ± 0.61	2.21 ± 0.61	0.438
AMH Range	1.0–3.0	1.0–3.0	
AFC (Mean ± SD)	13.00 ± 2.95	8.69 ± 2.45	0.000*
AFC Range	10.0–22.0	5.0–15.0	
No. of Oocytes (Mean ± SD)	12.04 ± 3.15	13.51 ± 4.14	0.062
No. of Oocytes Range	8.0–21.0	7.0–22.0	
M2 (Mean ± SD)	9.09 ± 2.61	10.18 ± 3.62	0.105
M2 Range	5.0–16.0	5.0–19.0	

VD showed a weak and non-significant correlation with AMH in the study group (r = 0.124, p = 0.418), but a moderate and significant positive correlation in the control group (r = 0.331, p = 0.026*). Similarly, the relationship between VD and AFC was non-significant in both groups, with weak correlations (study: r = 0.229, p = 0.131; control: r = -0.014, p = 0.929). However, VD demonstrated a significant positive correlation with endometrial thickness in both groups (Figure 1), with a stronger association in the control

group (study: r = 0.365, p = 0.014*; control: r = 0.527, p = 0.000*) (Table 2).

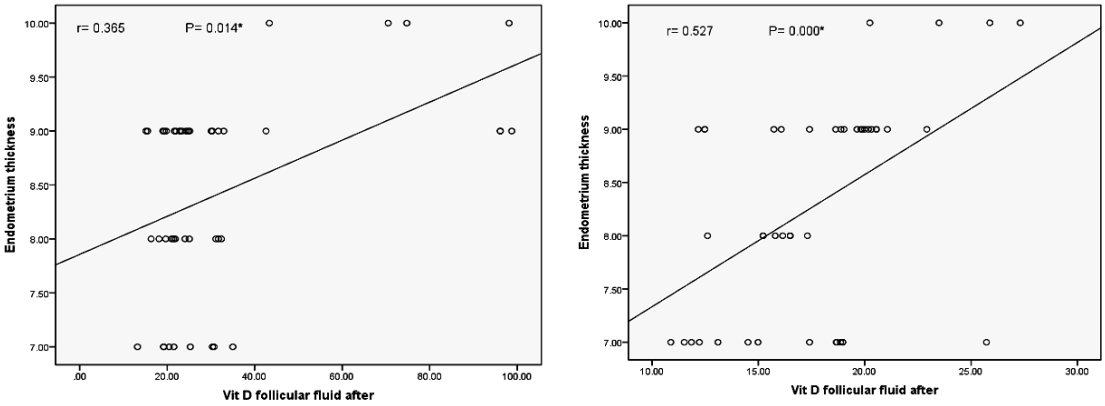

Figure 1: Correlation between follicular fluid vitamin D and endometrium thickness in study (A) and control (B) groups

Table 2: Correlation of Follicular Fluid Vitamin D with Various Parameters

Parameter	Group	r-value	P-value
AMH	Study	0.124	0.418
	Control	0.331	0.026*
AFC	Study	0.229	0.131
	Control	-0.014	0.929
Endometrium Thickness	Study	0.365	0.014*
	Control	0.527	0.000*

BMP-15 protein levels were weakly correlated with AMH in the study group (r = 0.160, p = 0.294) but showed a significant moderate positive correlation in the control group (r = 0.341, p = 0.022*) (Figure 2).

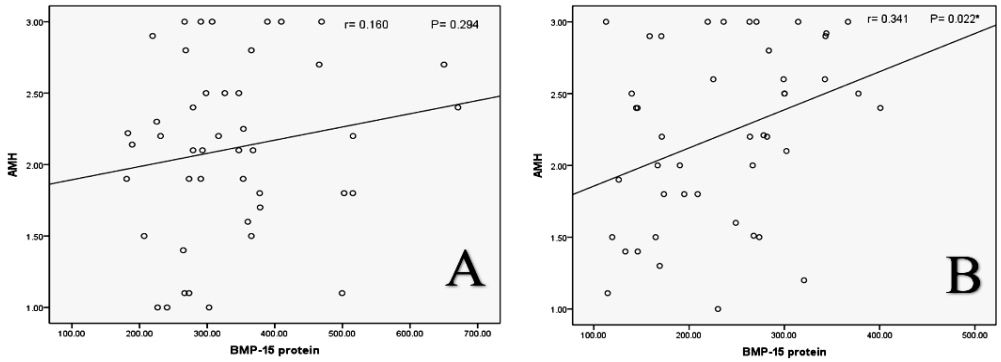

Figure 2: Correlation of BMP-15 protein AFC study (A) and control (B) group

Similarly, the correlation between BMP-15 and AFC was weak and non-significant in both groups (study: r = 0.196, p = 0.197; control: r = 0.048, p = 0.752). BMP-15 exhibited a stronger association with endometrial thickness in the control group (r =

0.377, p = 0.011*), compared to a non-significant trend in the study group (r = 0.271, p = 0.071) (Figure 3) (Table 3).

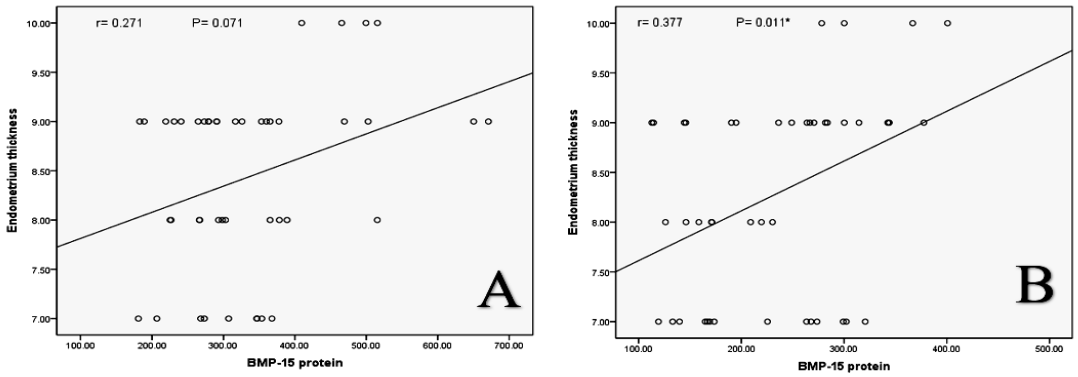

Figure 3: Correlation between BMP-15 protein and endometrium thickness between study (A) and control (B) groups

Table 3: Correlation of BMP-15 Protein with Various Parameters

Parameter	Group	r-value	P-value
AMH	Study	0.160	0.294
	Control	0.341	0.022*
AFC	Study	0.196	0.197
	Control	0.048	0.752
Endometrium Thickness	Study	0.271	0.071
	Control	0.377	0.011*

4. DISCUSSION

Several studies have demonstrated that VD plays an important role in the reproductive system due to the expression of VD receptors and VD metabolizing enzymes in many female reproductive tissues, including the pituitary, ovary, uterus, and placenta [17,18]. Moreover, VD has a role in ovarian steroidogenesis and is involved in the pathophysiology of some disorders in women of childbearing age [19].

This study aimed to evaluate the impact of VD supplementation on various In Vitro Fertilization (IVF) parameters, follicular fluid VD levels, BMP-15 protein, and key reproductive parameters, including ovarian reserve markers (AMH and AFC), and endometrial thickness, in women undergoing ICSI.

Our study revealed no significant differences between the study and control groups regarding AMH. The mean AMH level in the study group was recorded at 2.11 ± 0.61 ng/mL, while the control group exhibited a slightly higher mean AMH level of 2.21 ± 0.61 ng/mL. Statistical analysis yielded a p-value of 0.438, indicating that the difference in AMH levels between the two groups was not statistically significant. Both groups had AMH levels within the normal range, with values ranging from 1.0 to 3.0 ng/mL. These results suggest that VD supplementation did not have a measurable impact on AMH levels among the participants, indicating that the ovarian reserve, as assessed by AMH, remained comparable between those receiving VD and those who did not. This finding is

important for understanding the potential role of VD in reproductive health, particularly in relation to ovarian function and reserve.

Several studies have explored the association between VD and AMH levels, and some have reported similar findings to our study [20]. In contrast with our results, a study found a significant increase in AMH levels following VD supplementation [21]. Our study, which included a general population, may not have observed such a pronounced effect due to the absence of underlying hormonal imbalances.

Conversely, other studies have reported a positive association between VD levels and AMH. A study by Moridi *et al* found that women with higher VD levels had significantly higher AMH levels compared to those with lower levels [20]. This suggests a potential role for VD in maintaining ovarian reserve. However, it is crucial to consider the methodological differences between studies, such as sample size, duration of supplementation, and baseline VD levels, which may influence the observed results.

The results regarding AFC and endometrium thickness demonstrated notable differences between the study and control groups. The study group exhibited a mean AFC of 13.00 ± 2.95, significantly higher than the control group's mean AFC of 8.69 ± 2.45, with a p-value of 0.000, indicating a statistically significant difference. This suggests that VD supplementation may positively influence ovarian response, as reflected in the higher AFC in the study group. In terms of endometrium thickness, the correlation with VD levels was also significant; the study group showed a positive correlation ($r = 0.365$, $p = 0.014$) between follicular fluid VD and endometrium thickness, while the control group demonstrated an even stronger correlation ($r = 0.527$, $p = 0.000$). These findings indicate that VD may play a role in enhancing both ovarian reserve, as indicated by AFC, and endometrial receptivity, which are critical factors for successful implantation and overall reproductive outcomes.

Several studies have supported the positive association between VD and ovarian function, particularly in relation to AFC and endometrial thickness. For instance, a study demonstrated that women with higher VD levels had significantly higher AFC compared to those with lower levels [22]. This suggests a potential role for VD in stimulating follicular growth and development. Similarly, a study found a positive correlation between serum VD levels and endometrial thickness [23]. The authors concluded that VD supplementation may improve endometrial receptivity, which is crucial for successful implantation.

While the majority of studies have shown a positive association between VD and ovarian function, some studies have reported conflicting results. A study did not find a significant association between VD levels and AFC or endometrial thickness in a group of infertile women [24]. However, it is important to note that this study had a relatively small sample size and may have been underpowered to detect significant differences.

These results consistent with Bacanakgil and his collogues who found that there is an improvement in the ovarian reserve markers were obtained with VD supplementation, VD might be considered as a fertility treatment for patients with diminished ovarian reserve [21].

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These results consistent with Bacanakgil and his collugues who found that there is an improvement in the ovarian reserve markers were obtained with VD supplementation, VD might be considered as a fertility treatment for patients with diminished ovarian reserve [21]. Also, the current results are congruent with Zhao Jing et al., 2018 who stated that deficient VD was associated with decreased probability of live birth after IVF/ICSI [25], which proved in our study in control group. So, VD should be supplied to women with deficient level VD. Furthermore, Justin et al., 2019 study indicated that VD deficiency and insufficiency is highly prevalent among women undergoing assisted reproductive treatment. Consequently, serum VD status is associated with better and hopeful IVF outcomes [26].

Few old studies proved the negative relation of serum or follicular VD levels with IVF parameters such as van de Vijver et al., 2016 who demonstrated frankly in his study that VD deficiency does not significantly impair pregnancy rates in patients undergoing IVF [27]. Interestingly, the correlation results support the strong and positive relation between serum and follicular VD concentration with the quality of Oocytes, denoting the higher VD concentration, the better Oocyte quality and vitality. Basically, the good quality of the Oocytes makes them more valid for transfare and later fertilization process. A study by Ozyurt and his collogues study was very consistent to the current results and showed that FF 25-hydroxyvitamin D levels correlate positively with total and MII oocyte counts, positive pregnancy test [28].

5.CONCLUSION

The findings of this study highlight the multifaceted roles of VD and BMP-15 protein in reproductive physiology. While follicular fluid VD demonstrated significant correlations with endometrial thickness in both study and control groups, its relationship with ovarian reserve markers was more pronounced in the control group. Similarly, BMP-15 protein exhibited stronger associations with AMH and endometrial thickness in the control group compared to the study group.

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Authors' contributions

R.A.H, A.A.A, M.Y.A, A.A.Y contributed to the conceptualization, methodology, practical work, and writing of the original draft. **All authors** have read and approved the final manuscript and agree to its submission.

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Data Availability Statement

Not applicable

Conflicts of Interest

The authors declare no conflict of interest.

Consent to Participate

Not applicable

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