Can Vitamin D Supplemental Therapy improve the Disturbed Follicular Fluid Milieu and affect the Outcome of ICSI?

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Abstract

Background: Vitamin D (VD) receptor is widely distributed in reproductive systems thus hypovitaminosis D can impair the response to ovarian stimulation.

Objectives: Determination of the effect of VD supplemental therapy (VD-ST) on the ovarian follicular fluid (FF) milieu in infertile women undergoing intracytoplasmic sperm injection (ICSI).

Patients and Methods: 103 infertile women were evaluated clinically and gave blood samples for estimation of serum and FF levels of 25-hydroxy vitamin D (25-OH-VD), and FF levels of tumor necrosis factor-α (TNF-α) and total antioxidant capacity and underwent a cycle of ICSI using the antagonist protocol before and after receiving 3-month VD-ST. The study outcome is the impact of VD-ST on the clinical pregnancy rate.

Results: Serum (P=0.0004) and FF (P=0.040) levels of 25-OH-VD were significantly increased, while FF levels of TNF-α were significantly (P<0.001) decreased after VD-ST. Number of retrieved oocytes (P=0.036) and embryos showing >95% fragmentation (P=0.019) rates were significantly increased with doubling of number of high-grade embryos and clinical pregnancy rate. These changes were correlated positively with increased in serum and FF levels of 25OH-VD and decrease of TNF-α level. Statistical analyses defined the higher increase in serum and FF levels of 25OH-VD as the important predictors for the number of oocytes and clinical pregnancy, while the higher decrease in FF levels of TNF-α was the important predictor for the rate of high-grade embryos.

Conclusion: VD-ST may play an important role for success of ICSI through increasing serum and FF levels of VD and decreasing FF levels of TNF-α.

Keywords: Follicular fluid; ICSI; Vitamin D supplemental therapy; Tumor necrosis factor-α; Total antioxidant capacity

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Introduction

The follicular fluid (FF) is formed when plasma constituents pass through the blood follicular barrier and combine with the secretions from the ovarian granulosa and theca cells **(Poulsen et al., 2019)**. Thus, FF constitutes the microenvironment required for oocyte development and subsequently the oocyte quality which later determines the embryo quality **(Bednarska-Czerwińska et al., 2019)** is a significant factor impacting the outcome of assisted reproductive technology (ART) procedures **(Klobučar et al., 2020)** and influences the following embryonic development up to healthy newborns **(Lazzarino et al., 2021)**.

Moreover, the analysis of FF components could be used to provide information about the biochemical milieu surrounding the growing oocyte and reflects changes in levels of multiple plasma proteins required for normal oocyte development **(Liu et al., 2020)**.

Vitamin D (VD) is a steroid hormone with an important role in human physiology and pathology and its receptor regulates 0.5-5% of the human genome **(Paffoni et al., 2019)**. The hormonally active form of VD is 1,25-dihydroxyvitamin D and its physiological actions are facilitated by the VD receptor (VDR) **(Christakos et al., 2020)**. VDR are widely distributed in reproductive systems of both sex, suggesting that VD is essential for fertility **(Chen & Zhi, 2020)**, thus hypovitaminosis D (HVD) down to any level can increase the risk of insulin resistance, metabolic diseases, polycystic ovary syndrome, and impaired ovarian responsiveness during ART procedures **(Rehman et al., 2021)**. Additionally, various immune cells such as B and T cells, macrophages, and dendritic cells are responsible for synthesizing active VD. In return, VD plays a role in regulating the differentiation and proliferation of immune system regulators, expression of interleukins, and antimicrobial responses **(Schröder-Heurich et al., 2020)**.

Reactive oxygen species (ROS) are produced in cells through mitochondrial and enzymatic sources **(Tejero et al., 2019)**. If not countered, these reactive species have the potential to induce oxidative damage to DNA **(Li et al., 2013)**, proteins, and lipids through peroxidation of membrane lipids and oxidation of carbonated proteins. This process also reduces the total antioxidant capacity (TAC), ultimately resulting in decreased cellular lifespan and/or cell necrosis **(Silwal et al., 2020)**.This study aimed to determine the effect of vitamin D supplemental therapy (VD-ST) on the ovarian FF milieu in infertile women undergoing intracytoplasmic sperm injection (ICSI).

Patients and Methods

Design: Prospective interventional study

Setting: Department of Obstetrics & Gynecology, Faculty of Medicine, Tanta University and multiple private IVF centers

All women attending the infertility outpatient clinic since Jan 2019 were eligible for evaluation. Women underwent clinical evaluation for collection of demographic and clinical data; age, weight, height, type of infertility; either primary or secondary, history of previous treatment for infertility medical, surgical or previous attempts of ART. Tran-vaginal ultrasonography (TVU) was performed to exclude organic obstacles for getting pregnant, polycystic ovaries, endometrial lesions. Blood samples were collected for

hormonal assay and estimation of random blood glucose.

Inclusion criteria: Infertile women secondary to tubal lesion, had history of previously failed ART, younger 45 years, had antral follicle count of >7 and serum anti-Müllerian hormone (AMH) level >1.1 ng/ml **(Younis et al., 2015)** and free of exclusion criteria were included in the study.

Exclusion criteria: Women had no previous attempts of ART, women had infertility secondary organic disease, hormonal disturbances, anatomical aberrations, women had high risk for ovarian hyperstimulation, women had premature ovarian dysfunction or poor ovarian response to ovarian stimulation (OS), body mass index >35 kg/m^2, women had diabetes mellitus, kidney or liver diseases, women had serum 25-hydroxy vitamin D (25OH-VD) more than 75 nmol/l and women refused to participate in the study.

Study protocol: Using the fixed antagonist protocol, the GnRH antagonist was administered continuously until the day of HCG administration, starting 5 days after stimulation with gonadotropins. On the day of oocyte retrieval, after oocyte recovery blood free FF was collected by under TVU-guidance using a 17G oocyte aspiration needle (Cook Medical, IN, USA) and a closed vacuum system tube. After the oocyte collection, using hyaluronidase solution (25 IU/ml), the corona radiata was removed by repeated pipetting. Metaphase II oocytes were selected, incubated in LGGF medium (Fertilization, Global, CooperSurgical, Trumbull, CT, USA), and injected with sperms using an inverted microscope (Nikon Eclipse TE 200, Singapore, Japan) at 400x magnification. Following fertilization, the cultured oocytes underwent assessment of their embryo cleavage on days 2 and 3. Additionally, embryo quality was evaluated on days 2, 3, and 5 using the alpha scoring system for classification **(De Placido et al., 2002)**. On the 2nd day of the cycle estradiol valerate (Progynova, 2 mg, Bayer Schering Pharma, UK) was given in dose- 2 mg and then incremental doses starting till endometrial thickness was 8 mm, then progesterone therapy was started and two day later, embryo transfer was commenced. Luteal support was provided as daily intramuscular injection of progesterone 100 and pregnancy was diagnosed at 14 days after embryo transfer according to levels of serum β-human chorionic gonadotrophin and was ascertained by US to detected viable fetal sac.

Laboratory investigations

Sample collection: The obtained FF after all oocyte retrieval was collected into sterile tube and stored at -20^{o}C till being assayed for 25-hydroxy vitamin D (25OH-VD), Human tumor necrosis factor-α (TNF-α) and total antioxidant capacity (TAC).

Two blood samples were collected during the study - one before the administration of the vitamin D suppression test (S1 sample), and one after (S2 sample). A total of 5 ml of blood was drawn under sterile conditions, clotting was allowed to occur, and then the blood was centrifuged at 3000 rpm for 10 minutes. This process separated the serum, which was carefully collected in a sterile Eppindorff tube and stored at -20^{o}C until further analysis for serum 25OH-VD. The blood samples were collected and labeled by a lab assistant who was unaware of the purpose or reason for the investigations.

Studied lab parameters: The levels of 25OH-VD and TNF-α in the serum and FF, as well as the levels of 25OH-VD and TAC in FF, were assessed using

ELISA kits and read using a 96 well microplate ELISA reader (Dynatech. MR 7000) according to the manufacturer's instructions.

The serum levels of 25OH-VD were measured using an ELISA kit (catalog no. ab213966 Abcam Inc., San Francisco, USA) through a quantitative sandwich enzyme immunoassay technique **(Heidari & Mirghassemi, 2012)**.

The serum levels of TNF-α were measured using an ELISA kit (catalog no. ab46087 Abcam Inc., San Francisco, USA) through a quantitative sandwich enzyme immunoassay technique **(Coughlan et al., 2001)**.

The serum levels of TAC were measured using an ELISA kit (catalog no. ab65329 Abcam Inc., San Francisco, USA) through a quantitative sandwich enzyme immunoassay technique **(Wan et al., 2016)**.

Study outcome

1. Primary outcome is the impact of VD-ST on the FF milieu as regards the inflammatory mediators and anti-oxidative markers.
2. Secondary outcomes include
   - The extent of change of outcomes of ovarian stimulation and ICSI procedure after VD-ST as regards the number of retrieved oocytes, and the embryo grading and chemical and clinical pregnancy rate.
   - The relation between the percentages of changes in the estimated lab variables and ICSI outcomes.

Statistical analysis

The acquired data were presented in various forms including mean, standard deviation, numerical values, percentages, median, and interquartile range. Paired t-test was utilized to analyze the differences between the estimated biomarkers before and after VD-ST, while Chi-square test (X2 test) and Mann-Whitney test were employed for the analysis of non-parametric data. Pearson's correlation analysis was conducted to assess the relationships between the percentage of change in the levels of estimated biomarkers and ICSI outcomes. The Automatic Linear Modeling (ALM) analysis was utilized to identify the predictors of ICSI outcome parameters among the estimated biomarkers. The statistical analysis was carried out using IBM® SPSS® Statistics (Version 22, 2015; Armonk, USA) for Windows statistical package. A p-value of less than 0.05 was considered statistically significant.

Results

During the duration of the study, 157 women were eligible for evaluation, 23 women were excluded for not fulfilling the inclusion criteria, 9 women were excluded for having sufficient VD serum level and 22 women were missed after giving the blood sample for estimation of serum 25OH-VD, while the remaining 103 women completed the study protocol (**Fig. 1**). Patients' enrolment data are shown in (**Table. 1**).

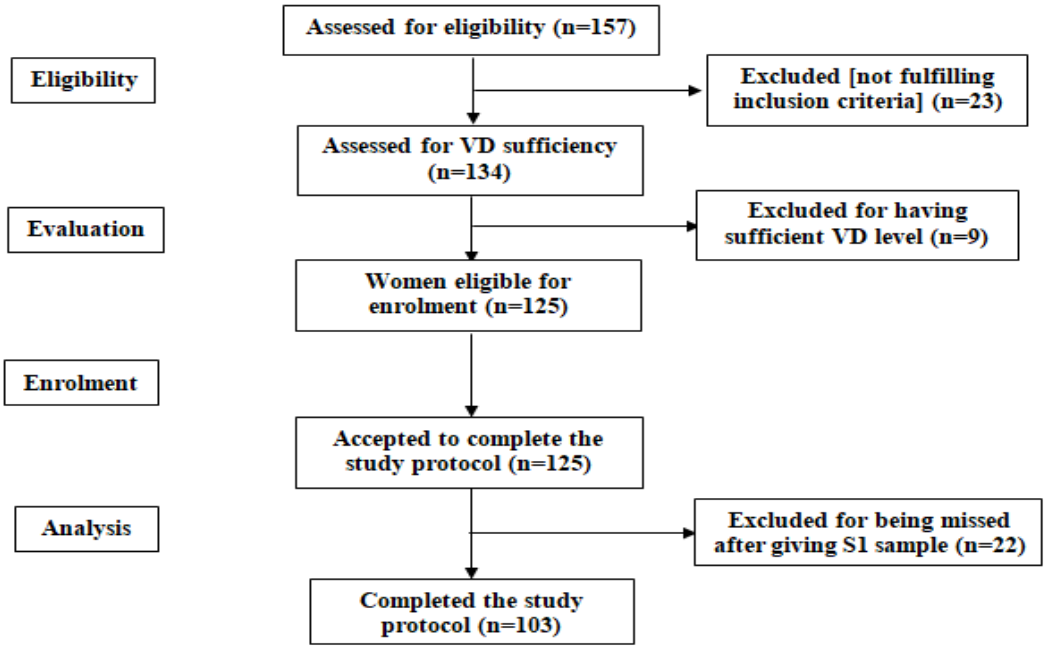

Fig.1. Consort flow sheet

Table 1. Enrolment data of studied women

Data	Findings
Age (years)	37.2 (3)
Weight (kg)	87.8 (4.9)
Height (cm)	167.2 (2.8)
Body mass index (kg/m^2)	31.4 (1.7)
Number of previous cycles	2 [2-3]
Number of offspring	0 [0-1]
Number of antral follicle count	10 [9-12]
serum anti-Müllerian hormone level	10.6 (2.2)

Data are presented as mean, standard deviation (SD), median, interquartile range [IQR]

At time of enrolment estimated serum 25OH-VD levels were insufficient in 25 S1 samples (24.3%) and were deficient in 78 S1 samples (75.7%) of which 34 S1 samples (33%) showed mild, 26 S1 samples (25.2%) showed moderate and severe VD deficiency was detected in 18 S1 samples (17.5%). At the end of VD-ST course, there were 13 S2 samples (12.6%) showed sufficient 25OH-VD serum level, 33 S2 samples (32%) showed insufficient levels and 57 S2 samples (55.4%) showed VD deficiency with 32 S2 samples (31.1%) showed mild, 18 S2 samples (17.5%) showed moderate and only 7 S2 samples (6.8%) showed severe deficiency with significantly (P=0.0002) lower frequency of VD deficiency in S2 serum samples in comparison to that detected in S1 serum sample. Moreover, mean serum levels of 25OH-VD in S2 samples were significantly (P=0.0004) higher than mean serum levels estimated in S1 serum sample with median increase by 29.35 [15.8-43.6]. Estimated levels of 25OH-VD in FF increased significantly (p=0.04) after VD-ST in S2 samples in comparison to levels estimated in S1 sample with median increase by 9% [IQR= 5.2-12.4]. On contrary, mean level of TNF-α estimated in S2 sample of FF was significantly (P<0.0001) lower in comparison to mean level estimated in S1 sample with median decrease by 33.3% [IQR= 22-46.2]. Regarding FF content of TAC, estimated levels after

VD-ST in S2 sample were non-significantly (P=0.082) higher on comparison to levels estimated in S1 sample with median increase by 7.2% [IQR= 4.5-11] (**Table. 2**).

Table 2. Laboratory findings in serum and FF samples obtained before (S1 sample) and after (S2 sample) VD-ST for studied women

Parameters			S1 samples	S2 Samples	P-value
Serum 25OH-VD levels	Frequency according to VD sufficiency	Sufficient	0	13 (12.6%)	0.0002
		Insufficient	25 (24.3%)	33 (32%)	
		Deficient	78 (75.7%)	57 (55.4%)	
	Frequency according to extent of deficiency	Mild	34 (33%)	32 (31.1%)	0.206
		Moderate	26 (25.2%)	18 (17.5%)	
		Severe	18 (17.5%)	7 (6.8%)	
	Mean (±SD) level (nmol/L)		34.8 (18.2)	44.8 (21.4)	0.0004
	Percentage of change		29.35 [15.8-43.6]		
FF 25OH-VD levels	Mean (±SD) level (nmol/L)		16.8 (5.2)	18.4 (5.7)	0.040
	Percentage of change		9 [5.2-12.4]		
FF TNF-α levels	Mean (±SD) level (ng/ml)		6.77 (1.4)	4.4 (1.16)	<0.0001
	Percentage of change		33.3 [22-46.2]		
FF TAC	Mean (±SD) level (mM of trolox)		10 (3.5)	10.9 (3.8)	0.082
	Percentage of change		7.2 [4.5-11]		

Data are presented number, percentage, mean, standard deviation, median, interquartile range, 25OH-VD: 25-hydroxy vitamin D; FF: Follicular fluid; TNF-α: Tumor necrosis factor-α; TAC: Total antioxidant capacity; P-value: indicates the significance of difference between levels estimated in the two samples; P<0.05 indicates significant difference; P>0.05 indicates non-significant difference

The percentage of increase of serum and FF levels of 25OH-VD were positively correlated (**Fig. 2**) and both showed positive significant correlation with the percentage of decreased FF TNF-α, while showed positive non-significant correlation with the percentage of increase of FF TAC level (**Table.3**)

Table 3. Pearson's correlation analysis of the percentage of change between S1 and S2 serum and FF levels of studied laboratory markers

Variables	% of increase of serum 25OH-VD level		% of increase of FF 25OH-VD level	
	r	p	r	p
% of increase of FF 25OH-VD level	0.240	0.015	-	-
% of increase of FF TNF-α level	0.252	0.010	0.401	<0.001

% of increase of FF TAC level	0.133	0.181	0.127	0.216

Data are presented as "r" Pearson's correlation coefficient, 25OH-VD: 25-hydroxy vitamin D; FF: Follicular fluid; TNF-α: Tumor necrosis factor-α; TAC: Total antioxidant capacity; P-value: indicates the significance of the correlation; P<0.05 indicates significant difference; P>0.05 indicates non-significant difference

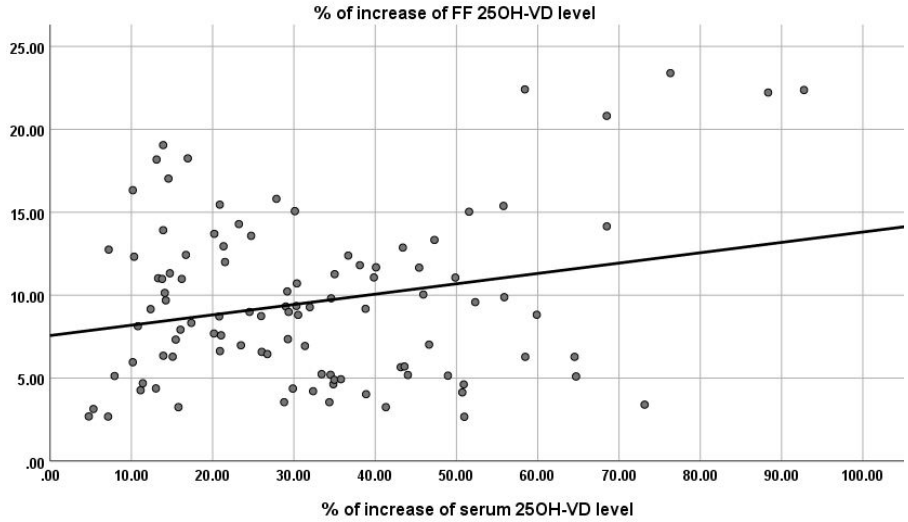

Fig. 2. Correlation between the % of increased serum and FF levels of 25OH-VD after VD-ST

All parameters of OS and ICSI outcome were significantly improved after VD-ST with significant increase of number of retrieved oocytes and number of embryos showing >95% fragmentation rate. Moreover, number of embryos of grades III and IV and clinical pregnancy rate were doubled after VD-ST in comparisons to rates reported before VD-ST as shown in (**Table.4**).

Table 4. Parameters of outcome of two sessions of ICSI; before (Session 1) and after (Session 2) VD-ST

Parameters		**Session 1**	**Session 2**	**P-value**
Number of retrieved oocytes	**≤10**	62 (60.2%)	47 (35.9%)	0.036
	11-20	41 (39.8%)	56 (49.5%)	
Embryo fragmentation rate	**<95%**	36 (35%)	21 (20.4%)	0.019
	>95%	67 (65%)	82 (79.6%)	
Embryo grading	**Grade I & II**	91 (88.3%)	80 (77.7%)	0.041
	Grade III & IV	12 (11.7%)	23 (22.3%)	
Clinical pregnancy	**No**	94 (91.3%)	84 (81.6%)	0.041
	Yes	9 (8.7%)	19 (18.4%)	

Data are presented as number, percentage; P-value: indicates the significance of difference between the two sessions; P<0.05 indicates significant difference; P>0.05 indicates non-significant difference

After VD-ST, the number of retrieved oocytes, embryo fragmentation rate, embryo grading and clinical pregnancy rate after session 2 of OS and ICSI showed positive significant correlation with the percentage of change in serum levels of 25OH-VD and in FF levels of 25OH-VD and TNF-α, while the correlation was positive non-significant with the percentage of change in the FF levels of TAC (**Table. 5**).

Table 5. Pearson's correlation analysis between the percentage of change between S1 and S2 serum and FF levels of studied laboratory markers

Outcome of OS & ICSI % of change in		Number of retrieved oocytes	Embryo fragmentation rate	Embryo grading	Clinical pregnancy
Serum 25OH-VD	r	0.478	0.251	0.309	0.411
	P	<0.001	0.011	0.002	<0.001
FF 25OH-VD	r	0.427	0.325	0.468	0.431
	P	<0.001	0.001	<0.001	<0.001
FF TNF-α	r	0.344	0.353	0.401	0.342
	P	<0.001	<0.001	<0.001	<0.001
FF TAC	r	0.210	0.156	0.168	0.207
	P	0.109	0.081	0.089	0.036

Data are presented as "r" Pearson's correlation coefficient, OS: Ovarian stimulation; ICSI: Intracytoplasmic sperm injection; 25OH-VD: 25-hydroxy vitamin D; FF: Follicular fluid; TNF-α: Tumor necrosis factor-α; TAC: Total antioxidant capacity; P-value: indicates the significance of the correlation; P<0.05 indicates significant difference; P>0.05 indicates non-significant difference.

The Automatic Linear Modeling (ALM) analysis for the importance of the variables for prediction of the outcome of OS and ICSI session after VD-ST, defined the higher percentages of increase in serum and FF levels of 25OH-VD as the important predictors for the number of oocytes will be retrieved by 65% and 35%, respectively (**Fig. 3**) and for the possibility of getting clinical pregnancy by 45% and 55%, respectively (**Fig. 4**), while the percentages of increase in FF levels of TNF-α and TAC were excluded. For prediction of the embryo fragmentation rate by >95%, ALM analysis defined higher percentages of change in FF levels of TNF-α (62%) and 25OH-VD (38%) as the important predictors, while the percentages of change in serum level of 25OH-VD and FF level of TAC were excluded (**Fig. 5**). Regarding the rate of embryos of III and IV, the higher the percentage of increase of FF level of 25OH-VD and the higher the percentage of decrease in the FF levels of TNF-α are the important predictors by 72% and 28%, respectively (**Fig. 6**).

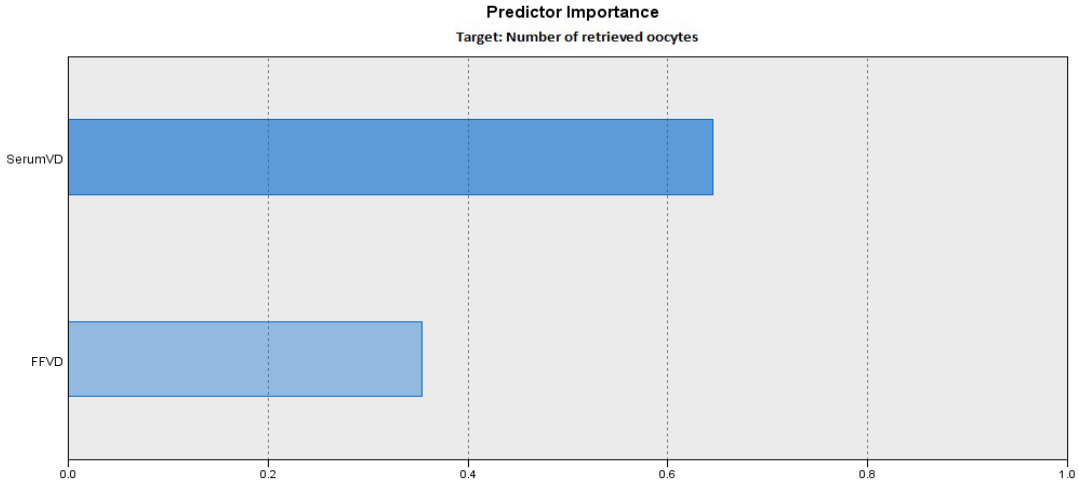

Fig. 3. The Automated Linear Modeling analysis of the % of change in serum and FF levels of estimated lab variables defined high % of increased 25OH-VD levels as predictors for number of the retrieved oocytes after VD-ST

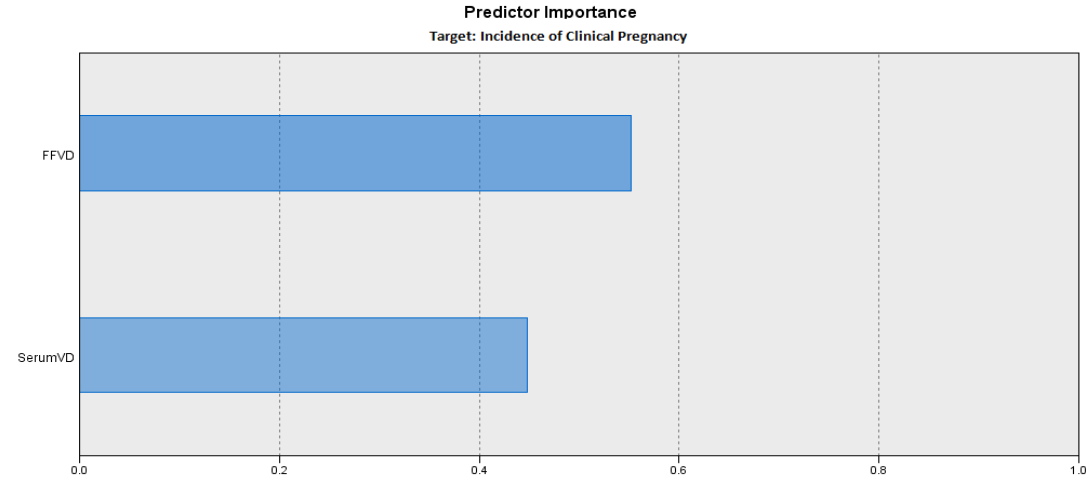

Fig. 4. The Automated Linear Modeling analysis of the % of change in serum and FF levels of estimated lab variables defined high % of increased 25OH-VD levels as predictors for oncoming clinical pregnancy rate after VD-ST

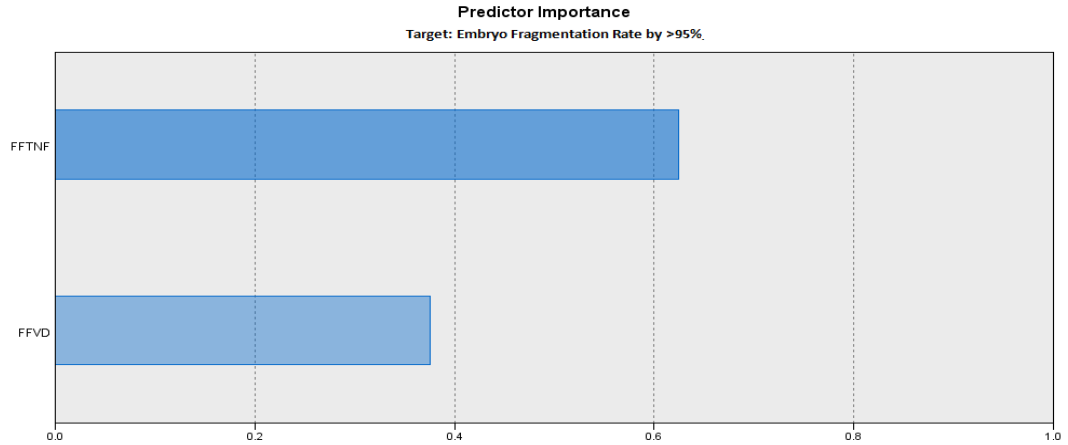

Fig. 5. The Automated Linear Modeling analysis of the % of change in serum and FF levels of estimated lab variables defined high % of decreased TNF-α levels and % of increased FF levels of 25OH-VD as predictors for the oncoming embryo fragmentation by >95% rate after VD-ST

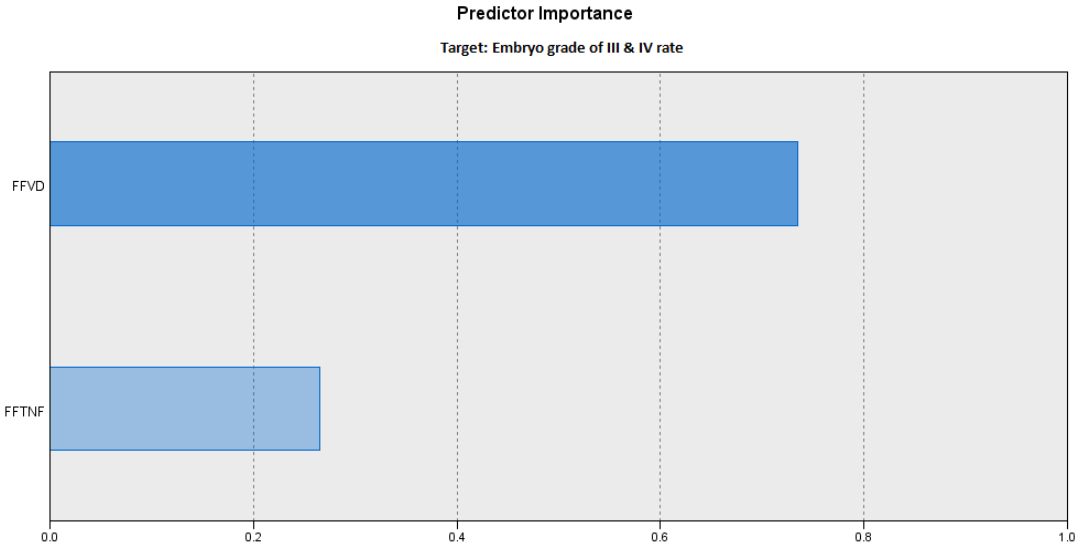

Fig. 6. The Automated Linear Modeling analysis of the % of change in serum and FF levels of estimated lab variables defined high % of decreased TNF-α levels and % of increased FF levels of 25OH-VD as predictors for the oncoming embryos of grades III & IV rate after VD-ST

Discussion

In the present study, vitamin D supplemental therapy (VD-ST) significantly increased serum and follicular fluid (FF) levels of 25OH-VD in all studied women had VD insufficiency or deficiency, with a positive significant correlation between serum and FF levels of 25OH-VD. These findings go in hand with multiple recent studies that detected a similar correlation **(Skowrońska et al., 2020; Neysanian et al., 2021; Jeremic et al., 2021)**.

Considering the percentage of embryo fragmentation could reflect the quality and the viability of the embryo, and so it could be considered as the most important in the embryo quality's morphological assessment. The current study detected significant correlation between the frequency of embryos had >95% fragmentation rate and the percentage of increase in serum and FF levels of 25OH-VD and FF level of 25OH-VD was found to be an important predictor for the embryo fragmentation rate by >95%. These findings are coincident with the previously detected correlation between FF levels of VD and the percentage of embryo fragmentation **(Skowrońska et al., 2020; Neysanian et al., 2021; Jeremic et al., 2021; Ciepiela et al., 2018)**. Moreover, **(Skowrońska et al., 2022)** found the FF levels of VD vary according to the oocytes developmental stage and correlate with embryo development status on day 3.

This study also detected a positive significant correlation between the percentage of increase of serum and FF levels of 25OH-VD and embryo grade of III and IV with the incidence of positive chemical and clinical pregnancy and the increased level of 25OH-VD could predict these outcomes. Similarly, **(Chu et al., 2019)** documented that the crude live birth rate was associated with serum VD in women undergoing ART and **(Abedi et al., 2019)** also detected improved quality of endometrium, rate of chemical and clinical pregnancy in women who received VD-ST for 6 weeks before ICSI. Recently, (**Neysanian et al., 2021**) found serum

and FF levels of VD significantly correlated with biochemical and clinical pregnancy.

The obtained results, the reported positive significant correlation between the percentage of increased serum and FF levels of 25OH-VD after VD-ST and number of retrieved oocytes and the high predictability of these increases, especially in FF, for the ongoing number of retrieved oocyte indicated that VD levels are important for determination of number and quality of oocyte and the outcome of ICSI. These data are in accordance with **(Muyayalo et al., 2021)** who documented low serum and FF levels of VD levels as a common character among infertile patients and found FF levels of VD were associated with embryo quality, normal fertilization, implantation rates, and clinical pregnancy rates.

The obtained results showed a positive significant correlation between the percentage of increased serum and FF levels of 25OH-VD and both were positively correlated with the percentage of decrease in FF levels of TNF-α. These findings may illustrate the effective anti-inflammatory effect of VD that may underlie the reported improved ICSI outcome. In support of this attribution, statistical analyses documented that the higher the percentage of decreased FF levels of TNF-α, the higher the embryo fragmentation rate and the incidence of embryos of grades III and IV.

In line with these findings, **(Piccinni et al., 2021)** detected that both hormonal and cytokine profile of FF influence the follicle size and development, also **(Wyse et al., 2021)** demonstrated that TNF-α can predict oocyte maturation rate. Moreover, **(Qasemi et al., 2021)** found dysfunction of mitochondrial DNA (mtDNA) packaging in granulosa cells, which are triggered by increasing FF levels of TNF-α and interleukin-6, induces elevated FF levels of cell-free mitochondrial DNA, and affects outcomes of ART.

Despite the lack of a significant correlation between the number of oocytes, fragmentation, and embryo grade II and IV rates, there was a positive and significant correlation observed between high TAC levels in FF and the diagnoses of chemical and clinical pregnancy. This is consistent with the findings of **(Ferreira et al., 2019)**, who discovered that serum levels of TAC could predict clinical pregnancy and live births following ICSI in women with stage I or II endometriosis. Additionally, **(Terao et al., 2019)** proposed that the balance between oxidative stress and antioxidant capacity in FF during oocyte retrieval is a crucial factor in fertilization and embryo division processes, and could potentially serve as predictive biomarkers in assisted reproductive technology (ART).

The lack of a significant correlation between the percentage increase in serum and FF levels of 25OH-VD and FF levels of TAC indicates that the positive impact of VD-ST on ART outcomes cannot be attributed to the antioxidant activity of VD. This supports the findings reported by **(Fatemi et al., 2017)**, who concluded that there is no evidence to suggest that VD plays a role in the success rate of IVF through an antioxidant mechanism

Conclusion

Hypovitaminosis D is prevalent among infertile women seeking for ART and VD-ST may play an important role for success of ICSI through increasing serum and FF levels of VD and decreasing FF levels of TNF-α. The reported weak improvement of FF levels of TAC indicated that VD did not improve ICSI outcome through an antioxidant

action. The relation between increased TAC levels in FF indicated the need for antioxidant supplementing therapy for infertile women prior to ART cycles.

Limitations

The study included only women had infertility secondary to tubal factor only and FF levels of anti-inflammatory cytokines need to be evaluated to ascertain of the mechanism of action of VD-ST.

Recommendations

Estimation of serum 25OH-VD prior to ART cycle and women with hypovitaminosis D must receive a course of VD-ST for 12 weeks prior to the cycle. Supplemental antioxidant therapy is also mandatory to improve the outcome of the ART cycle.

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