Vitamin D Supplemental Therapy might improve the outcome of Intracytoplasmic Sperm Injection for Women had Diminished Ovarian Reserve

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Abstract

Objectives: Evaluation of the relation between serum levels of 25-hydroxy vitamin D (25-OHVD), tumor necrosis factor-α (TNF-α) and interleukin (IL)-4 and outcome of intracytoplasmic sperm injection (ICSI) for infertile women secondary to diminished ovarian reserve (DOR).

Patients & Methods: 82 infertile DOR women, defined according to Bologna Criteria, were evaluated and gave blood samples for estimation laboratory parameters before and after receiving 12-w VD supplemental therapy (VDST). All women received the ovarian stimulation flexible antagonist protocol, ICSI was performed, and the day-3 embryos were graded. All women who had no fertilization or had embryos of grade P-G2 underwent another session of ICSI after VDST.

Results: Pre-VDST, 77 had VD deficiency (VDD) and 5 had insufficiency, Post-VDST 65 women had VDD, 14 had insufficiency and 3 women had sufficient level. Post-VDST AFC was increased by 85.3% and serum levels of 25-OHVD, IL-4, and anti-Müllerian hormone were increased by 31.7%, 18.66%, and 6.18%, respectively, while TNF-α levels were decreased by 12.8% with significantly lower frequency of embryos of poor and fair grades. ROC curve analysis defined pre-VDST high serum levels of 25-OHVD and IL-4, and high AFC as positive, while high levels of TNF-α and high BMI as negative predictors for the high quality embryo.

Conclusion: VDD, obesity, and deregulated immune milieu in direction of inflammation may predispose or aggravate poor ovarian response to OS. VDST improved ovarian function and quality of oocytes with subsequent improvement of embryo quality most probably through equalization of immune milieu to the direction of anti-inflammatory.

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INTRODUCTION

Menopause represents the definite end of a woman's reproductive life and the onset of a persistent hypoestrogenic state and despite the increased mean menopausal age, a significant individual variation in age at natural menopause is evident [(1)].

The term diminished ovarian reserve (DOR) is applied clinically to infertile women who were predicted or thought to have a poor ovarian response (POR) to ovarian stimulation (OS) [(2)]. POR is accepted as a manifestation of DOR and early ovarian aging and a woman was diagnosed to have POR if she fulfilled at least two of the Bologna Criteria [(3)] and retrieval of ≤3 oocytes was applied as the cutoff to discriminate women with POR [(3)].

The balance between T helper 1 (Th1) and T helper 2 (Th2) cytokines plays a critical role in the immune response and multiple clinical processes [(4)]. The shift of the ratio between the pro-inflammatory (Th1) cytokines, as tumor necrosis factor-α (TNF-α) and interleukin (IL)-6, and anti-inflammatory (Th2) cytokines as IL-10 and IL-4 toward a predominance of Th1 may represent more severe inflammatory status [(5)].

Vitamin D (VD) is a fat-soluble vitamin involved primarily in calcium metabolism [(6)]. However, experimental studies assured that VD is a promising agent with a remarkable ability to decrease the severity of inflammation [(7)]. Moreover, based on growing evidence, a role of VD was postulated in reproductive health in both animals and humans [(8)].

Vitamin D deficiency (VDD) is a significant public health concern all over the world especially for being associated with many immune-mediated diseases [(9)]. VD supplemental therapy (VDST) was found to improve general and orthopedic health after bariatric surgeries [(10)], to have significant effects on blood pressure, abdominal obesity, and insulin and glucose metabolism in patients with metabolic syndrome [(11)], and of potential importance in women with or at a high risk of uterine fibroid development [(12)].

Objectives

This study targets to evaluate the relation between VD sufficiency status and serum inflammatory milieu, and the outcome of assisted reproductive technologies (ART) for infertile women secondary to DOR.

Design

Prospective interventional double-blinded study.

Setting

Departments of Obstetrics and Gynecology, and Medical Biochemistry, Faculty of Medicine, Benha University, and multiple private centers in Benha and Cairo; Egypt.

Ethical consideration

The study protocol was approved by the Local Ethical Committee at Benha Faculty of Medicine; RC2-4-2021. The enrolled women must sign their consent to participate in the study, give blood samples for required investigations and undergo receive the prescribed therapies. Blindness means that enrolled women will be blinded about the type of investigations and the biochemist will be blinded about the indication for studying these parameters and about the demographic data of studied women.

Patients & Methods

All women attending the Infertility Units seeking assisted pregnancy due to primary or secondary infertility were eligible for evaluation. All women underwent clinical and US evaluation to determine the possible cause of infertility and gave blood samples for routine and study parameters' evaluation.

Exclusion criteria

Age older than 40 years, male factor infertility, infertility secondary to endocrinopathy, congenital malformation, exposure to radio- or chemotherapy, ovarian or uterine diseases, obesity grade II with body mass index >35 kg/m2, patients had serum 25-hydroxy vitamin D (25-OHVD) level >75 nmol/l, or refusal to participate in the study.

Inclusion criteria

Infertile women aged <40 years, had BMI <35 kg/m^2, free of causes of infertility other than DOR, and signed the written fully informed consent to participate in the study and undergo the assigned investigation and receive the appropriate therapies were included in the study. Ten fertile women with age- and BMI cross-matched with DOR women and free of inclusion and exclusion criteria were enrolled as a control group for lab parameters.

Evaluation tools

1. Evaluation of ovarian reserve (OR) according to Bologna criteria including antral follicle count (AFC) and anti-Müllerian hormone (AMH) levels within ranges of <5–7 follicles &/or <0.5–1.1 ng/ml, respectively [13].
2. Evaluation of VD sufficiency status: women who had serum 25-OHVD level of ≥75 nmol/L were considered to have sufficient VD level and were excluded from the study, while women who had serum levels in the range of 50-75 nmol/L were considered to have insufficient VD level and women had serum level of <50 nmol/L were considered to have VDD and were categorized as mild, moderate and severe VDD if 25-OHD serum levels were 25-50 nmol/L, 12.5-25 nmol/L and <12.5 nmol/L, respectively [14].

Investigations

1. **US evaluation:** AFC was determined on the day 3 of the cycle by trans-vaginal ultrasound (TVU)
2. **Laboratory investigations**

   Two blood samples were obtained before (Pre-VDST) and after VDST (Post-VDST); 5 ml blood were withdrawn under complete aseptic conditions, allowed to clot, and then centrifuged at 3000 rpm for 10 minutes to separate serum that was collected in a sterile Eppindorff tube and stored at -80oC till be assayed. Blood samples were collected and numbered by an assistant who was blinded about the indication for investigations.

Studied lab parameters

Serum levels of anti-Müllerian hormone (AMH), 25-hydroxy vitamin D (25-OHVD), human tumor necrosis factor-α (TNF-α), and interleukin 4 (IL-4) were measured using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions and were read using a 96 well microplate ELISA reader (Dynatech. MR 7000)

1. Human serum AMH level was measured with an ELISA kit (catalog no. ab267629 Abcam Inc., San Francisco, USA) by quantitative sandwich enzyme immunoassay technique [15].
2. Human serum 25-OHVD level was measured with an ELISA kit (catalog no. ab213966 Abcam Inc., San Francisco, USA) by quantitative sandwich enzyme immunoassay technique [16].
3. Human serum TNF-α level was measured with an ELISA kit (catalog no. ab46087 Abcam Inc., San Francisco, USA) by quantitative sandwich enzyme immunoassay technique [17].
4. Human serum IL-4 level was measured with an ELISA kit (catalogue no.

ab100570 Abcam Inc., San Francisco, USA) by quantitative sandwich enzyme immunoassay technique [18].

Study protocol

ll women who fulfilled the inclusion criteria received the ovarian stimulation protocol according to hospital guidelines using the gonadotrophin-releasing hormone (GnRH) flexible antagonist in the form of subcutaneous injection of Gonal F in a dose ranging between 300-650 IU (75 IU; 5.5µg, Merck Serono Ltd, UK) daily from the 2nd day of the cycle in conjunction with cetrorelix (Cetrotide®, Merck, Germany) 250 µg daily starting when the dominant follicle reached 14 mm till the day of Human chorionic gonadotrophin (hCG) injection. The hCG injections using choriomon, (10000 units; IBSA; Switzerland) was injected in addition to two ampoule triptoreline acetate (Decapeptyl, Ferring Pharmaceuticals Ltd., Wittland, Germany; 0.1 mg, subcutaneous injection) as co-triggering when the mean diameter of the leading follicle reached ≥18 mm or >3 follicles reached a mean diameter of ≥16 mm followed by TVU-guided oocyte retrieval 36 hours later and fertilization was carried out by intracytoplasmic sperm injection (ICSI).

Embryo grading

The day-3 embryos are graded as good (G grade) if it contains 6-9 symmetric cells with no fragmentation, as fair (F grade) if cells are symmetric but there is only minor fragmentation and as poor (P grade) if cells are asymmetric with no or moderate fragmentation (19). Women had embryos of grade G1 were excluded from the study and those had no fertilization or embryos of P, F or G2 grade received the 12-wk VDST and were re-evaluated for AFC and the previously estimated lab parameters levels and undergo another session of OS and ICSI.

Protocol for VDST

All patients were provided with 12-wk vitamin D3 ST as a once-daily oral dose of 5000 IU soft gels (Sunvite Mega Potency Vitamin D3 5000 IU, Puritan's Pride, Inc., Oakdale, NY, USA), which was proved to be safe for correction of VDD [20]. VD soft gels must be taken with a meal to aid in the absorption of this fat-soluble vitamin [21].

Study outcomes

1. The primary outcome is the effect of VDST on the quality of the embryo on the 2nd session of ICSI for women with POR.
2. The secondary outcomes included:
   - The relation between the extent of change of serum 25-OHVD and embryo quality grade
   - The predictability of Pre-VDST serum levels of studied lab parameters and the Pre-VDST embryo grade and between the extent of change in serum levels of studied parameters and the Post-VDST embryo grade.

Statistical analysis

Obtained data were presented as mean, standard deviation, numbers, and percentages, median and interquartile range. Results were analyzed using paired t-test for analysis of differences between Pre- and Post-VDST variables and Chi-square test (X2 test) and Mann-Whitney test for analysis of non-numeric data. Pearson's correlation analysis was applied to evaluate correlations between variables and the grade of embryos. A receiver characteristic curve was used to determine the predictors of embryo grades among the correlated variables. Statistical analysis was conducted using IBM® SPSS® Statistics (Version 22, 2015; Armonk, USA) for Windows statistical package. P-value <0.05 was considered statistically significant.

Results

During the study duration from June 2019 till April 2021, 416 infertile women attended the ART Unit and were evaluated for inclusion criteria, 311 women were excluded mostly for having infertility due to other causes than POD or out of the enrolment age range, 17 women refused to sign the written consent to attend two session of ICSI if the 1st had failed and 6 women were excluded for having sufficient VD serum level (>75 nmol/ml), while 82 women with mean age of 36±1.8 (range:33-39 years) and BMI of 31.6±1.7 (range: 26.6-34.6 kg/m2) were enrolled in the study and completed the study protocol (Fig. 1).

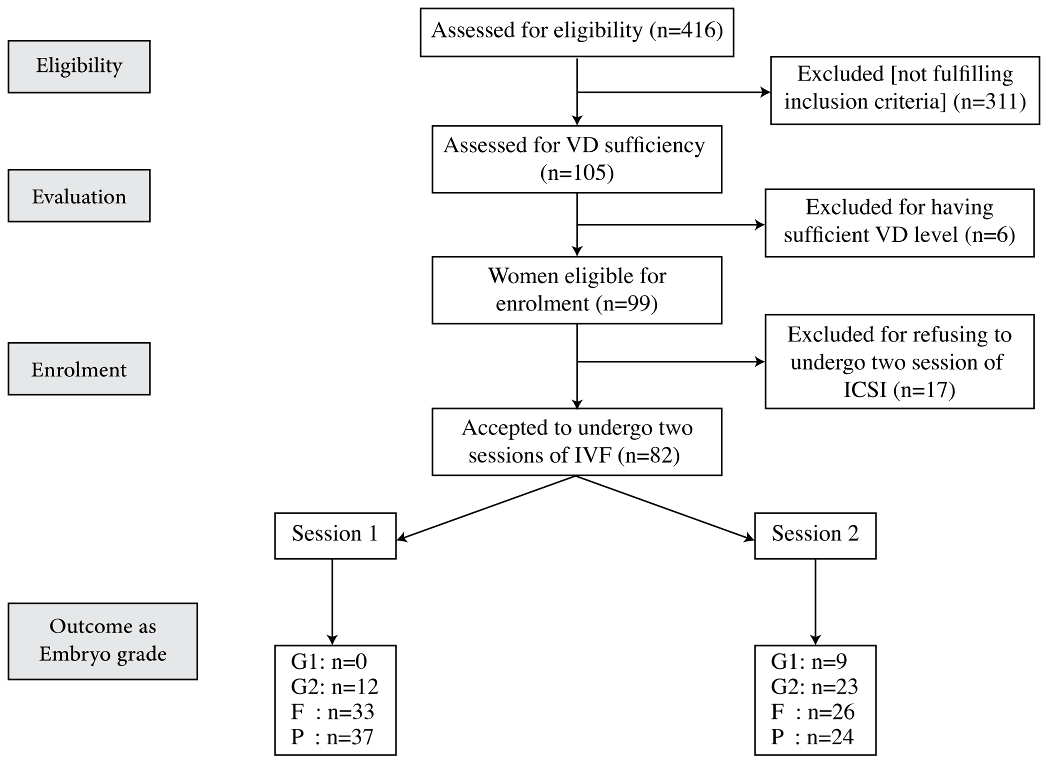

Figure 1: Consort Flow sheet

At the time of enrolment, 77 women (93.9%) had VDD, while only 5 women (6.1%) had insufficient VD levels. After the end of 12-wk VDST, no woman had severe VDD, 65 women (79.2%) had VDD, 14 women (17.1%) had insufficient VD level and 3 women (3.7%) had sufficient VD level with significantly (P=0.0002) lower frequency of women had VDD after VDST in comparison to Pre-VDST. Moreover, the mean value of 25-OHVD estimated Post-VDST was significantly (P=0.0007) higher than its Pre-VDST level with a mean percentage of increase of 31.7 (±10.8%). There were non-significant (P=0.055) differences between studied women as regards the Pre- and Post-VDST frequency of women who had serum TNF-α levels higher than the median of TNF-α level estimated in control samples. However, the mean value of serum TNF-α level estimated Post-VDST was significantly (P=0.0006) lower in comparison to the mean value of Pre-VDST estimated levels with the percentage of decrease of 12.8 (±7.4). On contrary, women's frequency according to serum IL-4 levels estimated Post-VDST showed a significant (P=0.00001) difference in comparison to Pre-VDST frequency in relation to the median value of IL-4 estimated in control samples. Also, Post-VDST levels of IL-4 were significantly (P<0.0001) higher in comparison to Pre-VDST levels with the percentage of increase of 18.66 (±6.28) as shown in table 1 & figure 2.

Table (1): Pre- and Post-VDST laboratory data of studied women

Data			Control (n=10)	Pre-VDST (n=82)	Post-VDST (n=82)	P-value
Serum 25-OHVD (nmol/ml)	Frequency according to level of VD sufficiency	Sufficient (>75)		0	3 (3.7%)	0.0002
		Insufficient		5 (6.1%)	14 (17.1%)	
		Mild VDD		45 (54.8%)	40 (48.8%)	
		Moderate VDD		18 (22%)	25 (30.4%)	
		Severe VDD		14 (17.1%)	0	
	Mean (±SD) level		78.4±2.8	26.7±13.1	34.7±16.6	0.0007
	Percentage of increased level			31.7±10.8		
Serum TNF-α (ng/ml)	Relation to median of control level (2.1)	<2.1		5 (6.1%)	14 (17.1%)	0.055
		2.1-3.15		69 (84.1%)	64 (78%)	
		(≥4.2)		8 (9.8%)	4 (4.9%)	0.0006
	Mean (±SD) level		1.9±0.47	3.07±0.77	2.67±0.7	
	Percentage of decreased level			12.8±7.4		
Serum IL-4 (ng/ml)	Relation to median of control level (1.4)	<1.4		16 (19.5%)	1 (1.2%)	0.00001
		1.4-2.1		66 (80.5%)	72 (87.8%)	
		≥2.8		0	9 (11%)	
	Mean (±SD) level		1.44±0.34	1.85±0.4	2.27±0.42	<0.0001
	Percentage of increased level			18.66±6.28		

Data are presented as mean; standard deviation (SD); numbers and percentages; Pre-VDST: before VDST; Post-VDST: after VDST; VDST: Vitamin D supplemental therapy; VDD: Vitamin D deficiency; 25-OHVD: 25-Hydroxy VD; TNF-α: Tumor necrosis factor-α; IL-4: Interleukin 4; P-value indicates the significance of difference between Pre- and Post-VDST levels; P<0.05 indicates the significant difference; P>0.05 indicates the non-significant difference

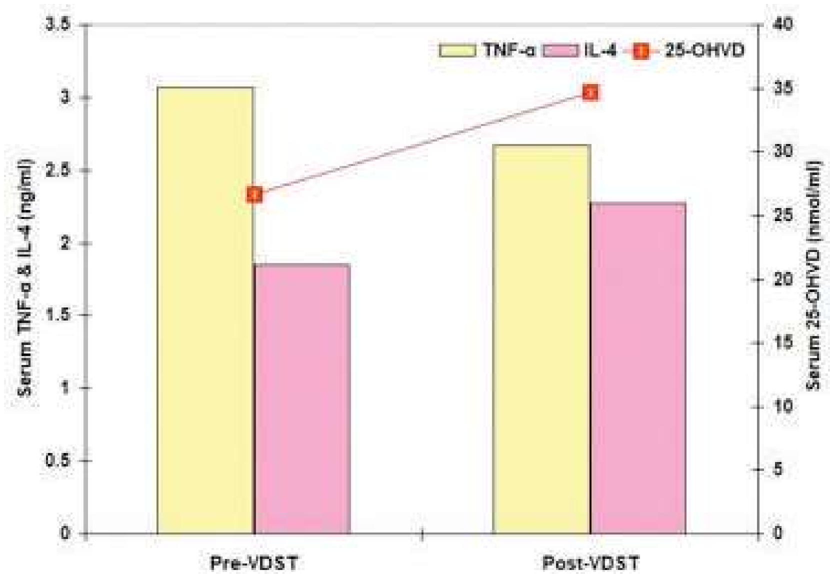

Fig. (2): Mean serum TNF-α, IL-4 and 25-OHVD levels estimated Post-VDST compared to its Pre-VDST levels

Interestingly, serum AMH estimated Post-VDST showed a non-significant (P=0.155) difference in comparison to Pre-VDST levels with the percentage of increase of 6.18 (±3.23). However, the median number of AFC increased significantly (P<0.0001) after VDST in comparison to AFC before VDST with a mean percentage of increase of 85.3 (±69%) and 16 women (19.5%) had AFC >7 (Table 2). Unfortunately, embryo grading before VDST defined 37 embryos of poor quality, 33 of fair quality, and only 12 embryos of G2 quality; on contrary, embryo grading after VDST defined 24 embryo of poor grade, 26 embryos of fair grade, 23 embryos of G2 grade and 9 of G1 grade with significantly (P=0.0011) lower frequency of embryos of poor and fair grades after VDST in comparison to before VDST.

Table (2): Serum AMH and AFC and grading of the obtained embryos of studied women Pre- and Post-VDST

			Pre-VDST (n=82)	Pre-VDST (n=82)	P-value
AMH (ng/ml)	Mean (±SD) level		0.65±0.17	0.69±0.18	0.155
	Percentage of increased level		6.18±3.23		
AFC	Frequency according to AFC	<5	79 (96.3%)	42 (56.1%)	<0.0001
		5-7	3 (3.7%)	24 (29.3%)	
		>7	0	16 (14.6%)	
	Median [IQR] level		3 [2-3]	4 [3-7]	<0.0001
	Percentage of increased level		85.3±64.1		
Embryo grading	Good	G1	0	9 (11%)	
		G2	12 (14.6%)	23 (28%)	
	Fair		33 (40.2%)	26 (31.7%)	0.0011
	Poor		37 (45.2%)	24 (29.3%)	

Data are presented as mean; standard deviation (SD); numbers and percentages; Pre-VDST: before VDST; Post-VDST: after VDST; VDST: Vitamin D supplemental therapy; VDD: Vitamin D deficiency; 25-OHVD: 25-Hydroxy VD; TNF-α: Tumor necrosis factor-α; IL-4: Interleukin 4; AMH: Anti-Müllerian hormone; P-value indicates the significance of the difference between Pre- and Post-VDST levels; P<0.05 indicates the significant difference; P>0.05 indicates the non-significant difference

Pre-VDST embryo grading showed a negative significant correlation with woman's age, and serum levels of TNF-α, while showed negative non-significant correlation with women's BMI and showed positive significant correlation with both AFC and serum levels of 25-OHVD and IL-4. Moreover, AFC before VDST showed a positive significant correlation with serum levels of 25-OHVD and IL-4, while showed negative significant correlation with serum levels of TNF-α. Also, serum levels of 25-OHVD showed a negative significant correlation with BMI, and serum levels of TNF-α, which showed a positive significant correlation with serum levels of IL-4. ROC curve analysis for these variables as predictors for the grading of the oncoming embryo excluded age and AMH and defined high serum levels of 25-OHVD and IL-4, and high AFC as significant positive predictors for high quality embryo and high serum levels of TNF-α and high BMI as significant negative predictor for the high-quality embryo (Fig. 3). Verification of these predictors using Regression analysis defined high AFC and serum levels of 25-OHVD and IL-4 levels as the significant positive predictors for high quality embryo (Table 3). To identify the pre-VDST important laboratory marker for prediction of quality of the oncoming embryo, The Automatic Linear Modeling analysis defined vitamin

D status as manifested by the 25-OHVD serum level as the most important predictor by 65% and improved anti-inflammatory status as defined by serum IL-4 as an important predictor by 35%, but excluded serum TNF-α level as a predictor (Fig. 4).

Table (3): Statistical analyses of clinical and laboratory findings at the time of enrolment and embryo grading of the Pre-VDST ICSI session

Variables	Embryo grade		VD		TNF-α		IL-4	
	Spearman's correlation analysis							
	r	P	r	p	r	p	r	P
Age	-0.338	0.002	-0.129	0.249	0.099	0.374	0.175	0.116
Body mass index	-0.208	0.061	-0.225	0.042	0.362	0.001	0.02	0.861
Antral Follicular Count	0.693	<0.001	0.474	<0.001	-0.247	0.026	0.312	0.004
Anti-Müllerian hormone	0.175	0.115	0.024	0.828	-0.027	0.807	0.155	0.164
25-OHVD serum level	0.514	<0.001						
TNF-α serum level	-0.309	0.005	-0.474	<0.001				
IL-4 serum level	0.435	<0.001	0.307	0.005	0.218	0.049		
	ROC curve analysis					**Regression analysis**		
Variables	AUC	±SE	p	95% CI		β		P
Age	0.345	0.090	0.078	0.168-0.522		Excluded		
Body mass index	0.268	0.072	0.008	0.126-0.410		Excluded		
Antral Follicular Count	0.843	0.066	<0.001	0.715-0.972		0.535		<0.001
Anti-Müllerian hormone	0.596	0.075	0.267	0.451-0.744		Excluded		
25-OHVD serum level	0.857	0.057	<0.001	0.745-0.969		0.196		0.026
TNF-α serum level	0.172	0.059	<0.001	0.055-0.288		Excluded		
IL-4 serum level	0.213	0.083	0.001	0.050-0.376		0.208		0.011

VD: Vitamin D; VDST: Vitamin D supplemental therapy; TNF-α: Tumor necrosis factor-α; IL-4: Interleukin 4; 25-OHVD: 25-Hydroxy VD; "r": Pearson's correlation coefficient; AUC: Area under the curve; SE: Standard error; CI: Confidence interval; β: Standardized coefficient; P-value indicates the significance of the result; P<0.05 indicates the significant difference; P>0.05 indicates the non-significant difference

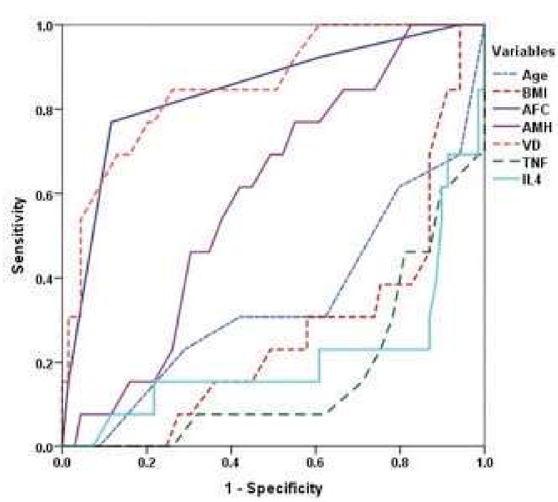

Fig. (3): ROC curve analysis of Pre-VDST variables as predictors of Pre-VDST embryo grade

Post-VDST embryo grading showed a positive significant correlation with Post-VDST AFC and percentage of increased serum level of 25-OHVD and IL-4 serum and ROC curve analysis defined increased number of AFC and a higher percentage of serum 25-OHVD level as the significant predictor for having good quality embryo (Table 4, Fig. 4).

Table (4): Statistical analyses of clinical and laboratory findings after VDST and embryo grading of the Pre-VDST ICSI session

Variables	Embryo grade		**ROC curve analysis**			
	r	P	AUC	±SE	p	95% CI
Post-VDST antral follicular count	0.777	<0.001	0.795	0.054	<0.001	0.689-0.901
% of increase of AMH level	0.160	0.152	0.467	0.068	0.643	0.333-0.601
% of increased 25-OHVD serum level	0.369	0.001	0.663	0.061	0.021	0.542-0.783
% of decreased TNF-α serum level	0.107	0.339	0.419	0.062	0.252	0.297-0.542
% of decreased IL-4 serum level	0.375	0.001	0.520	0.063	0.775	0.396-0.644

VDST: Vitamin D supplemental therapy; 25-OHVD: 25-Hydroxy VD; TNF-α: Tumor necrosis factor-α; IL-4: Interleukin 4; "r": Pearson's correlation coefficient; AUC: Area under the curve; SE: Standard error; CI: Confidence interval; P-value indicates the significance of the result; P<0.05 indicates the significant difference; P>0.05 indicates the non-significant difference

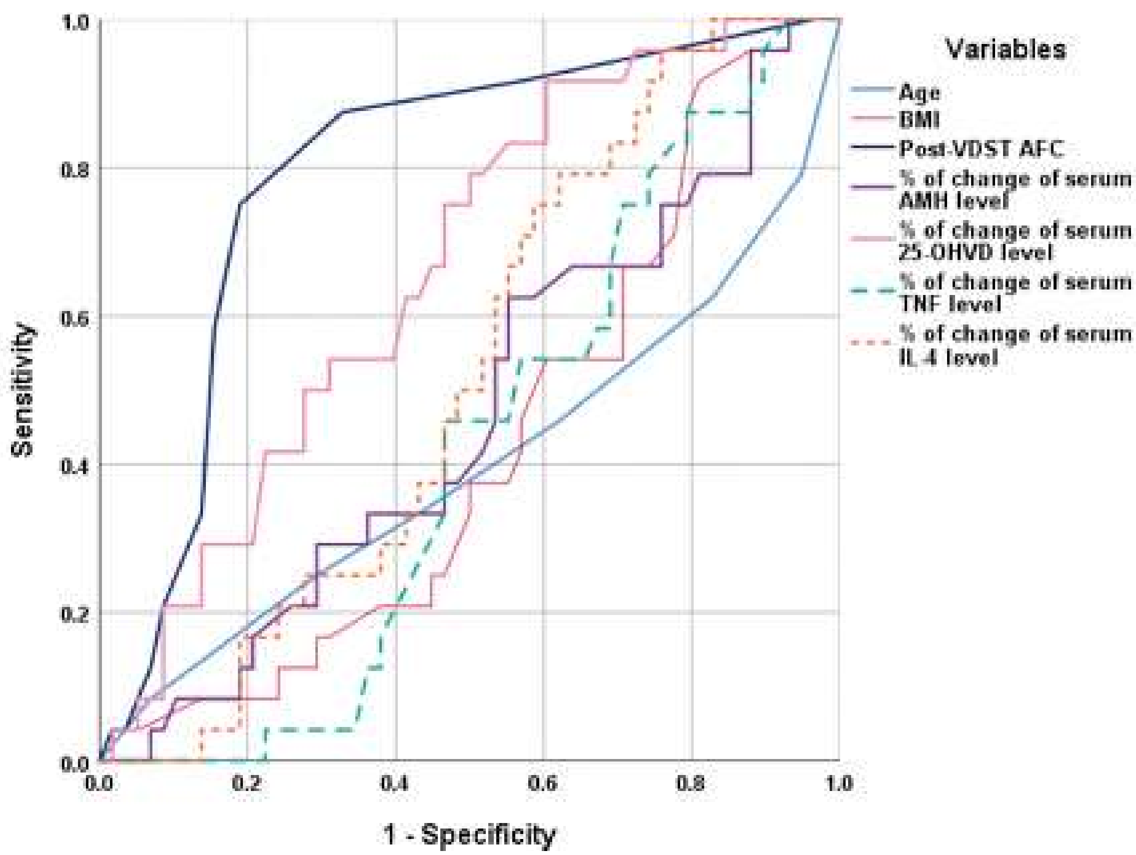

Fig. (4): ROC curve analysis of Post-VDST variables as predictors of Post-VDST embryo grade

Discussion

Throughout the period for case collection, 103 women younger than forty years had POD for a prevalence of 24.6%, a figure which is coincided with that previously reported [22] and 82 were had vitamin D deficiency-to-insufficiency. These findings spotlight on important coincident events including POD, vitamin D deficiency (VDD), and age; this triad could be attributed to the delayed age of marriage, weak exposure to ultraviolet rays to help to synthesize VD and sedentary life that induced obesity. In support of these assumptions, statistical correlation analysis detected a negative significant correlation between obesity and antral follicular count (ANF), serum level of 25-OHVD, and embryo grading after the initial ICSI trial.

In line with these data, Moreno-Santos et al. [23] found the active form of VD can modify adipose tissue physiology via its receptor, decreasing the expression of pro-inflammatory cytokines in adipose tissue and regulation of activation of insulin growth binding proteins in visceral adipose tissue and so VDD was associated with obesity and diabetes. Thereafter, Xu et al. [24] documented that the cumulative live birth rates for women with the poor ovarian response (POR) declined with increasing age and very low rate was associated with women aged >43 years old, so natural cycle IVF is of no benefit for these patients.

Recently, Bennouar et al. [25] found severe VDD was positively related to obesity with a higher risk of metabolic syndrome was in women than men. Moreover, Shea et al. [26] found women who had premature ovarian insufficiency were more likely to be obese and have decreased physical activity and de Sales Souza et al. [27] found VDD showed a high prevalence in adolescents with overweight living and is associated with increased cardiometabolic risk factors.

In a trial to explore the underlying mechanisms for this triad, Zanol et al. [28] experimentally detected strong negative correlations between adipocyte hypertrophy in animals maintained on a high carbohydrate diet and ovarian reserve, AMH levels and corpora lutea number with an increased number of atretic follicles. Also, Kang et al. [29] experimentally suggested that diet-induced obesity may lead to impaired function of T cells with inhibition of its autophagy, thus inducing the deregulation of T cell homeostasis, which underlie aggravation of inflammation that is commonly observed with obesity.

Vitamin D supplemental therapy (VDST) induced a significant improvement of AFC, but a non-significant increase in AMH serum levels in comparison to that reported before receiving VDST. Moreover, embryo quality grading was significantly improved in these women on receiving a second ICSI trial after receiving VDST and these grades were correlated with the new AFC and serum levels of 25-OHVD, but not with AMH levels. In line with these findings, Zhang et al. (22) documented that the AFC is better than AMH for predicting POR, the AFC had to be the preferred indicator for predicting ovarian response to subsequently develop an optimal individualized controlled ovarian hyperstimulation protocol.

The obtained results indicated a necessity for having within normal VD serum levels for maintenance and/or improvement of ovarian function and outcomes of ART and go in hand with experimental studies that supported a potential role for VD in follicular development; where VD receptor was detected in goat granulosa cells and VDST was found to seriously affect its proliferation by regulating cellular oxidative stress and cell cycle-related genes, and steroidogenesis [30] and a recent study documented that antral follicle is a target tissue for direct VD action that could regulate follicular development and function through increasing hormonal secretion by small and medium-sized follicles [31].

As another support for the efficacy of VDST to improve ovarian function, Refaat & El-

Boshy [32] experimentally treated VD sufficient female rats with supra-physiological VD3 supplements and detected improved ovarian functions by regulating the hypothalamic-pituitary-ovarian hormones with subsequent increased hormonal levels, increased counts of large follicles and corpora lutea and levels of mRNAs and proteins of targeted molecules.

Interestingly, the current study detected a reciprocal relation between the ratio of a pro to anti-inflammatory serum cytokines' levels; TNF-α/IL-4 ratio, and follicle development and improvement of embryogenesis, and such relation most probable was through vitamin D as evidenced by the negative significant correlation between AFC, serum 25-OHVD and embryo grade and serum levels of TNF-α, while the relations were positive with pre-VDST serum IL-4 levels and statistical analyses defined post-VDST percentage of increased IL-4 serum levels as a significant predictor for embryo grading and correlated with that of serum 25-OHVD.

These findings point to the possibility that improved pro/anti-inflammatory milieu after VDST may be behind the improvement of ovarian function and support that documented by Wu et al. [33] who found women with POR and VDD had significantly higher peripheral blood natural killer cell levels and cytotoxicity, CD19 + B and CD19+/5+ B-1 cell levels and significantly higher Th1/ Th2 cell ratio due to increased serum TNF-α levels and decreased serum IL-10 levels on comparison to women with POR and normal VD and women with normal OR and VD levels. Also, Chu et al. [34] detected inverse correlations between both total and free 25-OHVD levels with high-sensitivity C-reactive protein and leukocyte count in healthy reproductive-age women. In support of this assumption, the current study could not detect improved levels of AMH despite the increased AFC with the non-significant correlation between the extent of increased AMH levels and other variables. In line with this assumption, Wong et al. [35] found serum 25-OHVD level is significantly associated with AMH level in women with PCOS but not in ovulatory women. Recently, Lawal [36] detected no relationship between serum VD and AMH levels in infertile and fertile women. Moreover,

In support of the relation between the sufficiency of VD status and ovarian tissue and function through its impact on inflammatory milieu, multiple recent studies detected an inverse relationship between development and severity of ovarian cancer and vitamin D status and epidemiologic evidence indicates that VDST is associated with decreased cancer mortality [36, 37].

Conclusion

The diminished ovarian reserve may lie behind unexplained infertility in women younger than 40 years old. VDD, obesity, and deregulated immune milieu in direction of inflammation may predispose or aggravate poor ovarian response to ovarian stimulation for ICSI procedures. VDST improved ovarian function and quality of oocytes with subsequent improvement of embryo quality most probably through equalization of immune milieu to the direction of anti-inflammatory.

Limitations

Lack of exercise as an adjuvant to VDST to help to reduce obesity with its associated inflammatory mediators is a limitation of this study. Also, repeated courses of VDST were to be evaluated as management policy.

Recommendations

Estimation of serum 25-OHVD for infertile women especially those who had DOR and/ or obesity before attempts of ART and for women with VDD or insufficiency VDST is mandatory to improve the chance to get embryos of good quality and to save resources.

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