

Impact of Vitamin D Deficiency on ICSI Outcomes

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Abstract

Vitamin D deficiency is widespread: 50 to 80% of the world population. In reproduction medicine, the literature remains discordant about the influence of vitamin D on intracytoplasmic sperm injection outcomes. In this context, a study was conducted whose, its main objective was to evaluate the association between the vitamin D levels and pregnancy rates in a large population of patients followed for *in vitro* fertilization. Our findings suggest that women with sufficient levels of vitamin D are significantly more likely than those with insufficient levels to achieve a pregnancy following intracytoplasmic sperm injection. We suggest making determining vitamin D status as part of a routine infertility assessment and before assisted reproductive treatment, especially in women with high body mass index.

Keywords: Vitamin D; Infertility; Intracytoplasmic sperm injection

Abbreviations

ART: Assisted Reproductive Technology; IVF: *In Vitro* Fertilization; ICSI: Intracytoplasmic Sperm Injection; VDR: Vitamin D Receptor; BMI: Body Mass Index; FF: Follicular Fluid; COC: Cumulus-Oocyte Complexes; VDBP: Vitamin D Binding Protein; CPR/ET: Clinical Pregnancy Rate per Embryo Transfer; IOM: The US Institute of Medicine

Introduction

In the past few years, knowledge about the role of vitamin D has grown, and its physiology has made considerable progress. Vitamin D has been well-known to be involved in bone metabolism, and calcium-phosphate homeostasis. Emerging data identifies critical roles for vitamin D and many areas of medicine are involved: regulation of cellular growth, immunity and autoimmunity, central nervous system, cardiovascular diseases, infections, malignancies and reproduction [1]. Thus, vitamin D has passed from a vitamin with a limited role in bone health [2] to a hormone playing a role in general health.

Currently, active research about vitamin D tends to show an increasing number of its benefits, especially on women's reproductive health. The link between the vitamin D status and reproduction has been largely shown in murine models, but data in humans is still less convincing [3].

The existence of vitamin D receptors (VDR) in critical reproductive organs as the uterus, endometrium, ovaries and placenta, has given evidence to the positive physiological role of Vitamin D in women's reproductive health [2].

In fact, the role of Vitamin D in ovarian steroidogenesis is well established [4] and recent evidence has shown an effect of vitamin D on uterine receptivity [5]. In addition, vitamin D has also been shown to be involved in the pathophysiology of some disorders among women of childbearing age that are most commonly encountered among infertile women undergoing *in vitro* fertilization (IVF) procedures [6].

On the other hand, vitamin D deficiency is highly prevalent in the general population and especially in women in reproductive age. Over a billion people around the world are vitamin D deficient or insufficient [7]. However, the classification of the vitamin D status varies in the literature, and because the relation between vitamin D and fertility has been investigated only recently, no specific cut-off values have been referenced in the literature.

Worldwide, natural dietary sources of vitamin D are limited. Therefore, vitamin D is mainly obtained by cutaneous production from exposure to the sun. However, many factors influence the amount of ultraviolet B (UVB) from sunlight that reaches the skin and its effectiveness. These include time of day, season, latitude, altitude, clothing, sunscreen use, pigmentation, and age. Even those who normally reside in sunny climates are commonly found to be deficient in Vitamin D, probably due to cultural habits and dress.

Realizing the pandemic of vitamin D deficiency and to further elucidate the role of vitamin D in women's reproduction, our objectives were to determine the prevalence of vitamin D deficiency among infertile women living in a sun-rich environment and to determine whether serum and follicular fluid vitamin D were predictive of IVF outcomes.

Materials and Methods

Study design

A prospective cohort study was undertaken in the IVF center at Aziza Othmana Hospital. Ninety two infertile women undergoing Intracytoplasmic Sperm Injection (ICSI) were enrolled between July 2013 and July 2014.

The main objective of the study was to evaluate the association between the vitamin D levels and pregnancy rates in a large population of patients undergoing ICSI.

In agreement with general recommendations, the patients were divided into two groups according to the serum Vitamin D level [8]:

-Group A (n=61): vitamin D level <30 ng/ml

-Group B (n=31): vitamin D level ≥ 30 ng/ml

Then, the cohort study was also divided into three groups rather

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than two according to the serum vitamin D levels: (<20 ng/ml), (21-29 ng/ml) and (\geq 30 ng/ml).

The study protocol was approved by the Ethics Committee of the hospital, and included written consent from the participants.

Patients

Stimulation by standard long agonist protocol, FSH \leq 10 (IU/L), age \leq 38 years and first or second ART cycle were the inclusion criteria. Severe male factor, endometriosis, short agonist protocol, white puncture and cycles with no embryo transfer were the exclusion criteria.

IVF cycles

After complete desensibilization (long agonist protocol), using the gonadotrophin-releasing hormone agonist, ovarian stimulation with Gonal F (Serono), or Menopur (Ferring) was started on day 2 or 3 of the following cycle. The starting dose of FSH was selected on the basis of age, day 3 FSH level and the number of antral follicles. The dose was then adjusted according to the patient's response.

Monitoring of the ovarian response was assessed by transvaginal ultrasound and serum E2 assays, on the fifth day and repeated as needed. When 3 or more follicles reached 17 mm, HCG (human chorionic gonadotropin) was administered. After 36 h, transvaginal ultrasound-guided ovum pick-up was performed, under local anesthesia.

The examination of the follicular liquid looking for cumulus-oocyte complexes (COC) was performed using a binocular magnifying glass. The collected COC were rinsed and placed in an incubator at 37°C under 5% CO₂ in a suitable culture medium covered with mineral oil for 1-2 h.

Oocytes must be freed from cumulus cells and corona around them to facilitate microinjection. This denudation was both enzymatic with a brief exposure to hyaluronidase (HYASE-10X, Vitrolife) and mechanic by repeated pipetting using micropipettes. The denuded oocytes were then rinsed and placed in a culture medium (G1, Vitrolife) and incubated at 37°C under 5% CO₂. All semen samples were taken in the laboratory in a sterile container by masturbation after a period of sexual abstinence from 3 to 5 days. The sperm preparation allowed removing seminal plasma and selecting a population of mobile and morphologically normal spermatozoa. The sperm preparation was carried out by a density gradient (SpermGrad, Vitrolife, Sweden) in order to retain the dead and abnormal spermatozoa, the germs and the round cells. After removal of the supernatant, the pellet was washed with a washing solution (Spermrinse, Vitrolife, Sweden).

After the preparation of the oocyte and a good selection of the sperm, the microinjection of sperm into the oocyte cytoplasm was performed under the inverted microscope (Nikon Eclipse TE 300) fitted with a hotplate thermostatted at 37°C and a micromanipulation system (Narishige).

Meanwhile, 16 to 18 h after ICSI, the fertilization rate, expressed as the ratio between oocytes with two pronuclei (2PN) and the total number of injected oocytes, was assessed. Following the cleavage of all normal fertilized oocytes, the morphological grade of all embryos was assessed 48 h after ovum pick-up. It was scored according to the BLEFCO classification, which considered the number of blastomeres, the fragmentation rate and the regularity of blastomeres.

Fresh embryos were transferred on day 2 or on day 3 after intracytoplasmic injection with Frydman catheter (CDD, France). The number of embryos transferred depended on the embryo quality, the number of embryos available and the patient's history. Generally two or

three embryos were transferred. The remaining good quality embryos were frozen after the consent of the couple for possible future transfer.

The luteal phase was supported by 200 mg of vaginal progesterone (Utrogestan) twice a day and by an injection of Progesterone[®] every three days. The administration started on the day of the oocyte retrieval. In case of pregnancy, Utrogestan was continued until the tenth gestation week.

Vitamin D status

On the day of the ovum pick-up, the follicular fluid was collected from follicles \geq 14 mm. Following the oocytes isolation, the FF for each patient was pooled, centrifuged at 1500x g for 10 min and the supernatant was stored at -20°C until assayed. It must be noted that the FF used should be clear and free of blood contamination.

Serum samples were also obtained to determine the vitamin D level. The serum was separated by centrifugation, and the samples were frozen at -20°C until assayed.

Vitamin D was assayed by ELISA 25-OH Vitamin D total, DRG Diagnostics, according to the manufacturer's protocol.

The Serum vitamin D levels were defined as deficient (<10 ng/ml), insufficient (10-29 ng/ml) or sufficient (30-100 ng/ml). The follicular fluid was defined based on previously determined serum criteria.

Pregnancy diagnosis

The serum β hCG was checked 15 days after the embryo transfer. Pregnancy was detected by serum β -hCG analysis (β hCG>50 IU/ml). Clinical pregnancy was confirmed by ultravaginal ultrasound with at least one gestational sac in the uterine cavity.

Parameters studied

The parameters analyzed and compared between the groups investigated were:

- Clinical parameters: male and female ages, infertility etiology, infertility duration, body mass index (BMI), the evaluation of the ovarian reserve (FSH and LH).
- Ovarian stimulation parameters: E2 level on the day of HCG administration, the endometrial thickness, the total oocytes retrieved and the number of mature oocytes.
- ICSI parameters: the fertilization rate, the cleavage rate, the number and status of the embryos, the presence of frozen embryos, the pregnancy rate and the implantation rate.
- The prevalence of vitamin D sufficiency, insufficiency and deficiency, and the effect of vitamin D on IVF cycle parameters.

Statistical analysis

The data were analyzed using the SPSS version 20.0. The continuous variables were determined as the mean \pm standard deviation or median values, and categorical data were reported as percentage (%). The Chi-squared test and Fisher's exact test were used to compare the qualitative variables. Univariate analyses determined the association between the FF and the serum Vitamin D levels with patients and cycle parameters (Student's t test, Mann-Whitney U test as appropriate).

Spearman's correlation test was utilized to determine the relationship between the serum and follicular fluid Vitamin D. p values<0.05 were considered significant.

The sample size was calculated based on an expected clinical

pregnancy rate per embryo-transfer in patients with sufficient vitamin D levels of 40% and stating a 60% reduction in women with insufficient vitamin D levels. With 80% power and a significant level of 5%, the calculated number of patients to be recruited was at least 102. This assumption was based on our institution's pregnancy rate and previous vitamin D IVF studies. A one year recruitment period was planned.

Results

Overall, 102 infertile women were initially considered for enrollment. 10 patients were excluded for the following reasons: three for 100% immature oocytes, one for white puncture, five for lack of embryo to transfer, and one for sperm sample failure, keeping only 92 patients for data analysis.

The mean age of patients was 31, 8 years, the mean level of serum vitamin D was 26, 01 ng/ml and the mean level of follicular fluid vitamin D was 18,87 ng/ml.

Characteristics	Group A n=61 (66,3%)	Group B n=31 (33,7%)	p-value
Female age (Years)	31,31 ± 4,0	32,87 ± 3,5	0,072
Male age (Years)	37,67 ± 4,7	37,52 ± 4,7	0,882
BMI (kg/m ²)	28,53 ± 5,8	26,0 ± 4,2	0,03
Type of infertility, n (%)			
Primary	50 (82)	27 (87,1)	0,37
Secondary	11 (18)	4 (12,9)	
Duration of infertility (Years)	5,88 ± 2,7	5,76 ± 2,8	0,85
FSH (mIU/ml)	6,3 ± 1,5	6,45 ± 1,9	0,68
LH (mIU/ml)	4,19 ± 1,9	4,38 ± 1,8	0,66
Day of HCG injection (days)	11,11 ± 1,3	10,9 ± 1,5	0,50
Mean number of FSH ampoules per patient (75 IU each)	29,84 ± 9,4	30,98 ± 7,9	0,56
Peak oestradiol (ng/ml)	2314,7 ± 1170,8	2246,2 ± 966,6	0,78
Endometrial thickness (mm)	10,09 ± 1,7	11,55 ± 2,5	0,002
Mean number of oocytes retrieved	11,4 ± 5,4	11,8 ± 6,9	0,72
Number of mature oocytes per patient	7,9 ± 4,3	8 ± 4,1	0,91
Fertilization rate (%)	62,2	59,5	
Mean number of embryos transferred per patient	2,0 ± 0,5	2,13 ± 0,5	0,30
Number of patients with frozen embryos, n (%)	22 (36)	12 (38,7)	0,46
Implantation rate n (%)	4,9 % (6)	36,3% (24)	0,0001
CPR/ET, n (%)	9 (14, 7)	23 (74,1)	0,000
Live birth rate n (%)	5 (55,5)	20 (86,9)	0,000

*(CPR/ET: Clinical Pregnancy Rate per Embryo Transfer)

Table 1: Patient and IVF cycle characteristics by the vitamin D status.

Characteristics	Vitamin D <10 ng/ml n=13 (14%)	Vitamin D 10-29 ng/ml n=48 (52%)	Vitamin D ≥ 30 ng/ml n=31 (34%)	p-value
Female age (Years)	31,15 ± 3,1	31,35 ± 4,2	32,87 ± 3,5	0,19
BMI (kg/m ²)	28,6 ± 3,9	28,5 ± 6,3	26,0 ± 4,2	0,19
Follicle Stimulating Hormone (mIU/ml)	5,97 ± 1,4	6,40 ± 1,5	6,45 ± 1,8	0,66
Day of HCG injection (days)	11,54 ± 1,4	11,0 ± 1,2	10,9 ± 1,59	0,38
Endometrial thickness (mm)	9,8 ± 2,06	10,15 ± 1,6	11,54 ± 2,5	0,007
Mean number of oocytes retrieved	11,23 ± 4,5	11,46 ± 5,7	11,87 ± 6,9	0,93
Mean number of top quality embryos per patient	1,62 ± 1,4	1,98 ± 1,7	3,06 ± 2,5	0,035
CPR/ET, n (%)	0 (0)	9 (18,7)	23 (74,1)	0,000
Live birth rate n (%)	-	5 (83,4)	15 (71,4)	0,000

Table 2: Characteristics of the three groups identified by vitamin D status.

The characteristics of the two groups A and B are shown in Table 1. The comparison between them showed a statistically significant difference concerning the BMI and endometrial thickness (p<0, 05). The BMI was higher in the deficient group and the endometrial thickness was higher in the sufficient group.

The clinic and paraclinic characteristics, the stimulation parameters, the ovarian response, the embryo development, and the number of embryos transferred were similar in both groups.

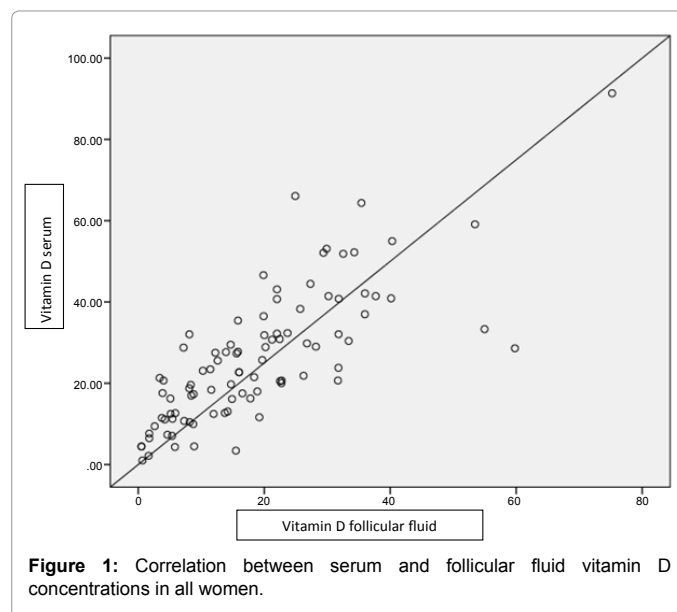
Women with 25 OH vitamin D ≥ 30 ng/ml had a significantly higher clinical pregnancy rate compared to those with deficient 25 OH vitamin D levels.

The data analysis was repeated by distributing the women into three rather than two categories according to the serum vitamin D levels (<20 ng/ml), (21-29 ng/ml) and (≥ 30 ng/ml).

Women with sufficient 25 OH vitamin D had a significantly higher chance of obtaining top quality embryos (Table 2).

In addition, chances of pregnancy were observed to increase in accordance with the rise in categorized level of 25 OH vitamin D.

Otherwise, there was a significant linear correlation between the levels of vitamin D in serum and follicular fluid (r=0,7). This means that levels of vitamin D in the follicular fluid provide a reliable reflection of the serum levels. The correlation of the two parameters is illustrated in Figure 1.



Discussion

The aim of this study were to clarify the prevalence of vitamin D among a population of infertile women living in a sun-rich environment, to study the correlation between FF vitamin D and body stores of vitamin D and to analyse the effect of vitamin D deficiency on the IVF outcome. To date, this is the first study of its kind in Tunisian population.

Vitamin D is described as “the sun vitamin” [9]. Seasonal changes of human natural reproduction have been demonstrated for a long time, showing a spike in birth rates during hot seasons [10]. Actually, the role of vitamin D in ovarian steroidogenesis is now well established [11]. Several experiments through murine demonstrate the importance of vitamin D on female infertility. Indeed, the first experiments have shown that female rats with deficiency in vitamin D had lower fertility rates [12]. On the other hand, the VDR (vitamin D receptor) knockout mice had developed hypogonadism hypergonadotropic and impaired folliculogenesis [3].

Despite the abundance of sunshine in North Africa allowing vitamin D synthesis all the yearlong, in this study, very low levels of vitamin D have been recorded in Tunisia. The extent of prevalent vitamin D insufficiency in this cohort of infertile women can be explained by limited sun exposure and/or by using sunblock, by a relatively dark skin color, obesity, lack of government regulation for vitamin D fortification of food and cultural clothing habits.

The first study dealing with vitamin D in infertile women undergoing *in vitro* fertilization (IVF) procedures, was conducted in 1992, and included only 10 patients and it demonstrated the presence of metabolites of vitamin D in the follicular fluid for the first time [11]. Then, several studies were published in this area with sometimes contradictory results. In their study, Estes et al. found a decrease in the expression of the free form of the carrier protein of vitamin D (VDBP) in follicular fluid of patients who's IVF resulted in pregnancy [13].

Importantly, vitamin D has also been involved in the physiopathology of diseases leading to infertility [9]. It has also been suggested that vitamin D deficiency contributes to metabolic disorders found in the metabolic syndrome, namely obesity and insulin resistance [14]. Indeed, our results also revealed a positive correlation between vitamin D levels and BMI, which was higher among the group of women with vitamin D deficiency.

Some randomized controlled clinical trials have shown the beneficial effects of vitamin D supplementation on body-weight regulation [15]. It should be noted that vitamin D deficiency and high BMI are still among the rare modifiable risk factors before starting an IVF, which may hold beneficial therapeutic implications.

Our results also showed a positive correlation between FF vitamin D levels and body vitamin D stores. Although our study was limited by the small sample size, we found significantly higher pregnancy rates and a higher live birth rate in patients with higher FF and serum levels of vitamin D. It is unclear why the clinical pregnancy rates among vitamin D deficiency group were so low. It is possible that though statistically significant, this is a chance finding. The number of patients in the study was relatively small, and we could not identify any clinically important differences in patient characteristics or cycle parameters (including embryo quality) contributing to the very low pregnancy rates. It is possible that the influence of vitamin D status on pregnancy outcomes was overshadowed by other factors that contribute to the lower pregnancy rate observed. However, the correlation between pregnancy rates and vitamin D deficiency is similar to this reported in some studies.

In agreement with our results, Ozkan et al. [16] prospectively studied a population of 84 infertile women followed by IVF. This study also found a strong correlation between serum and FF Vitamin D assays. The vitamin D sufficiency was also found to be a successful IVF predictor. It was also demonstrated that each gain of 1 ng/ml of vitamin D follicular fluid would increase the probability of a clinical pregnancy by 6% ($p=0.030$).

Anifandis et al. [17] also found an association between vitamin D levels and IVF outcome.

However, Aleyasin et al. [18], in a study on a small population of women did not found similar results to ours. Indeed, in his prospective study of a cohort of 82 women undergoing IVF, there was no significant association between pregnancy rates and vitamin D levels.

Finally, the retrospective study of Rudick et al. [19] included 188 patients followed by IVF. The clinical pregnancy rate in “non-Hispanic Whites” patients were 4 times higher in the group of patients with normal vitamin D level (>30 ng/mL) compared to women with deficiency of vitamin D (20-30 ng/mL) or deficiency (<20 ng/mL). Conversely, Asian patients with insufficient vitamin D had better pregnancy rates than Asian patients with normal vitamin D dosage, noting different results depending on the ethnicity of the patients ($p<0.01$).

The main hypothesis of this author was a direct effect of vitamin D on endometrial vitamin D levels and not correlated with ovarian stimulation parameters and embryo quality markers.

The results obtained suggest that without affecting the number and quality of oocytes, vitamin D can independently improve implantation. Indeed, our study showed positive correlation of vitamin D with the endometrial thickness. So, in the absence of any significant relationship with ovarian response, and with the significantly higher endometrial thickness found in this study, our observations may identify endometrial receptivity as the potential target for positive influences of higher vitamin D levels. This is further corroborated by studies showing an increased expression of mRNA and protein of HOXA 10, a determining factor of embryo implantation, in stromal endometrial cell after vitamin D supplementation [20,21].

Recent studies have demonstrates that vitamin D is important throughout gestation as well, not just at the time of implantation. There are varying levels of vitamin D metabolites and HOXA 10 expression throughout pregnancy in the endometrium [22]. There is also growing evidence that vitamin D supplementation may improve the birth outcome and prevent some obstetrics complications [23].

These conclusions are consistent with prior reports which confirm that the association between vitamin D levels and the number and quality of oocytes and embryos has not been found [19,23].

Finally, it has been noted that the different studies carried out did not use the same diagnostic tests for vitamin D deficiency.

Indeed, the methods of vitamin D assay have differences in terms of specificity with respect to the two forms of vitamin D: OH D2 and OH D3. The dosage of vitamin D is still not performed in routine in Tunisia, and it is not available in all medical analysis laboratories. In fact, it is an assay that is not easy to develop, even in developed countries [24] due to the fact that 25 OH D is a highly hydrophobic molecule and that there are two forms to be assayed, 25 OH D2 and 25 OH D3.

Nowadays, there is no reference method for the determination of 25 OH D. In this study, the ELISA technique was used in order to assess the vitamin D status of our patients, which determines the two fractions of vitamin D, for better accuracy.

As matter of fact, the thresholds chosen to define hypovitaminosis D are not consensual according to authors and learned societies.

A 25 OH D dosage of less than 20 ng/mL is unanimously considered insufficient. The US Institute of Medicine (IOM) maintains this level of sufficiency at 20 ng/mL [25]. This threshold was at the same time challenged by the Endocrine Society which fixed it at 30 ng/mL [26].

Other medical bodies and experts have taken a position on the vitamin D sufficiency threshold. The American Academy of Pediatrics recommended a threshold of 32 ng/mL [27]. The French National Academy of Medicine, in a May 2012 report, defined the vitamin D adequacy threshold as a 25 OH D dose above 30 ng/mL [28]. In total, if we disregard the advice given by the IOM, there appears to be a global consensus on the definition of vitamin D adequacy for serum assays.

Conclusion

In conclusion, vitamin D deficiency is epidemic worldwide, Tunisia and many other sunny countries are no exceptions. Higher vitamin D levels are associated with better pregnancy rates after ICSI. This study shows evidence that this association is the result of the effect of vitamin D in the improvement of endometrial thickness.

Given our results, assessment of vitamin D status might be considered as a part of a routine infertility assessment and before artificial reproductive treatment, especially in women with higher BMI. In addition, vitamin D supplementation may be an easy and costly effective way of improving pregnancy rates and deserves further investigation.

Ethics Approval and Consent

The study protocol was approved by the Ethics Committee of Aziza Othmana hospital, and included written consent from the participants.

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