Vitamin D deficiency and infertility: insights from *in vitro* fertilisation cycles

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Abstract

Context: Vitamin D deficiency has been proven to affect fertility in mammals but data in human is less convincing. In particular, data on in vitro fertilisation (IVF), an attractive model to draw information on this topic, are sparse and conflicting.

Objective: To investigate IVF outcome in women with deficient 25-hydroxy-vitamin D [25(OH)D] serum levels (<20 ng/ml).

Design: Prospective cross-sectional study.

Setting: Infertility Unit of an academic setting.

Patients: The main inclusion criteria were as follows: 1) indication to IVF, 2) age 18-42 years, 3) BMI 18-25 Kg/m^2, 4) adequate ovarian reserve according to Bologna criteria. Eligible women provided a serum sample for 25(OH)D measurement at the time of cycle preparation. Subjects were subsequently excluded if the cycle was cancelled or if the attempt was excessively delayed.

Intervention: Quantitative detection of serum 25(OH)D.

Main outcome measure: Clinical pregnancy rate.

Results: The number of recruited women with serum 25(OH)D <20 ng/ml and ≥20 ng/ml was 154 and 181, respectively. The clinical pregnancy rates were 20% (30/154) and 31% (56/181), respectively (p=0.02); the adjusted Odds Ratio for clinical pregnancy in women with vitamin D ≥20 ng/ml was 2.15 (95%CI: 1.23-3.77). Subgroup analyses showed that the group of women with the highest serum levels (>30 ng/ml) had the highest chances of pregnancy.

Conclusions: Vitamin D is an emerging factor influencing female fertility and IVF outcome. Additional studies are pressingly needed to confirm a causal relationship and to investigate the potential therapeutic benefits of vitamin D supplementation.

Introduction

A critical role of vitamin D in the reproductive function of mammals has been known for more than three decades (1, 2). Mice that are deficient for 1α-hydroxylase, the enzyme converting 25-hydroxy-vitamin-D [25(OH)D], the storage form of the vitamin, into the biologically active form 1,25-di-hydroxy-vitamin-D, are infertile and exhibit uterine hypoplasia and absent corpora lutea (3). Accordingly, in rodent models of diet-induced vitamin D deficiency, fertility is severely impaired (1). The pathogenic mechanisms explaining these detrimental effects are still poorly clarified but available evidence suggests that they are presumably multifactorial, involving the regulation of the hypothalamus-pituitary axis, ovarian folliculogenesis and uterine implantation (2).

In contrast to the large body of evidence in animal models, the role of vitamin D in human reproduction has been less extensively investigated. The vitamin D receptor is expressed in most reproductive organs, including endometrium, myometrium, ovarian, cervical and breast tissues (4, 5). *In vitro* experiments suggest that, in contrast to rodents, vitamin D would be more involved in regulating embryo-implantation and to a lesser extent folliculogenesis (2, 6). However, *in vivo* data supporting a role for vitamin D in female fertility in general and embryo implantation in particular are not robust. In particular, evidence emerging from *In Vitro* Fertilisation (IVF), a valuable model to draw inferences on vitamin D insufficiency in some specific aspects of human fertility, is sparse and conflicting. Two studies evaluated IVF outcome based on the follicular fluid levels of 25(OH)D but results were inconsistent (7, 8). Moreover, four original articles investigated the association between 25(OH)D serum levels and pregnancy rates in IVF cycles. Rudick et al. (9) observed that the clinical pregnancy rate progressively decreases with declining vitamin D status in non-Hispanic whites, but not in women of Asian ethnicity. The same study group recently reported data on 99 recipients of egg donation of different ethnicities and showed a significant decline in clinical pregnancy rate with the decrease in vitamin D status (10). On the contrary, two recent Iranian studies failed to detect a statistically significant influence of serum 25(OH)D

levels on pregnancy rates (11, 12). However, both studies were performed in a population with an extremely low rate of subjects with sufficient serum levels of vitamin D.

We deem it important to definitely clarify whether vitamin D deficiency negatively affects clinical pregnancy rate, as this may potentially open new therapeutic scenarios for women scheduled for IVF and, more in general, to all women with infertility. To this aim, we set up a large prospective cross-sectional study comparing IVF outcome in women with 25(OH)D serum levels <20 ng/ml and ≥20 ng/ml.

Materials and Methods

Between January 2012 and December 2012, all women referred to the Infertility Unit of the Fondazione Ca' Granda for IVF were considered for study entry. Initial selection criteria were as follows: 1) indication for IVF, 2) age 18-42 years, 3) body mass index (BMI) between 18 and 25 Kg/m^2 and 4) adequate ovarian reserve according to Bologna criteria (13). Additional exclusion criteria were a history of malignancy, hypertension, diabetes, multiple sclerosis, need for chronic medical treatments, autoimmune disorders and coronary, hepatic or renal diseases. To prevent confounders, women were subsequently excluded if they did not undergo fresh embryo-transfer or if time between serum measurement of 25(OH)D and embryo transfer exceeded an arbitrary threshold of 90 days or if they had a summer break between the 25(OH)D assessment and the IVF attempt. The study was approved by the local Ethical Committee and all recruited patients gave specific written informed consent.

All patients selected for IVF in our Unit underwent a clinical assessment and a baseline transvaginal ultrasound prior to initiation of controlled ovarian hyperstimulation (COH). Eligible women accepting to participate provided a serum sample for 25(OH)D measurement at this time and were managed according to a standardised clinical protocol as reported elsewhere (14). In the case of hyper or hypo-response, the cycle could be cancelled before ovum pick up. Cycles could be also cancelled if the number of retrieved oocytes exceeded 15, in the presence of symptoms and signs suggestive of Ovarian Hyperstimulation

Syndrome or if serum progesterone exceeded 1,500 pg/ml at the time of human Chorionic Gonadotropin (hCG) administration. Serum hCG assessment to detect pregnancy was performed at +14/+16 days after hCG administration. If positive, women underwent a transvaginal sonography three weeks later. Clinical pregnancy was defined as the presence of at least one intrauterine gestational sac with viable foetus. The implantation rate was calculated as the number of viable embryos divided by the number of transferred embryos.

The quantitative detection of total 25(OH)D levels was performed using a commercially available kit based on a chemiluminescence technology (DiaSorin, Stillwater, USA). The intra and inter-assay coefficients of variations were 10% and 15%, respectively. The physicians and biologists engaged in the cycle management were blinded to the results of the 25(OH)D assessment.

Statistically significant differences were determined using Fisher's-Exact test, Chi-Square test, Student's *t*-test or the Wilcoxon test, as appropriate. According to WHO recommendations, women were separated into two different groups: 25(OH)D <20 ng/ml and ≥20 ng/ml (15). The primary outcome was the clinical pregnancy rate per embryo transfer. A binomial logistic regression model was used to adjust for variables known to be associated with pregnancy rate or 25(OH)D concentration and for those that were found to differ ($p<0.10$) between the two groups. The sample size was calculated based on an expected clinical pregnancy rate per embryo-transfer in patients with 25(OH)D ≥20 ng/ml of 30% and stating as clinically relevant a 50% relative reduction in women with deficient 25(OH)D. Setting type I and II errors to 0.05 and 0.10, the calculated number of women to be recruited was at least 120 per study group. Based on preliminary evidence in our population documenting deficient 25(OH)D peripheral levels in about 50% of women (16) and based on the main characteristics and cycle outcome of our population (17), we planned a one year recruitment period.

Results

Overall, 803 women were initially considered for enrolment. Three hundred and twenty-three of them were excluded for the following reasons: inclusion criteria not fulfilled (n=189), missing 25(OH)D results (n=10) and unsuitable time frame between 25(OH)D measurement and IVF cycle (n=124). Four hundred and eighty eligible women initiated the cycle. The number of women with 25(OH)D serum levels <20 ng/ml and ≥20 ng/ml was 222 (46%) and 258 (54%), respectively. The number of women who did not perform fresh embryo-transfer in the two study groups was 68 (31%) and 77 (30%), respectively (p=0.92), leaving 154 and 181 women, respectively, for data analysis.

The baseline clinical characteristics of the two study groups are shown in Table 1. A statistically significant difference emerged for ethnicity, BMI and duration of infertility. Ovarian responsiveness, oocyte competence, embryo development and number of embryos transferred were similar between groups, but women with sufficient 25(OH)D had a higher chance of obtaining top quality embryos; therefore, the frequency of embryo transfer at the blastocyst stage was higher (Table 2). Women with 25(OH)D ≥20 ng/ml had a higher clinical pregnancy rate compared to those with deficient 25(OH)D levels. The crude odds ratio (OR) was 1.85 (95%CI: 1.11-3.08) (p=0.02). The OR adjusted for age, ethnicity, BMI, parity, duration of infertility, number of retrieved oocytes, number of transferred embryos and study period was 2.15 (95%CI: 1.23-3.77) (p=0.007). A similar figure was observed when considering the implantation rate; the crude OR of implantation in women with 25(OH)D ≥20 ng/ml was 1.83 (95%CI: 1.19-2.83, p=0.006). The OR adjusted for the above mentioned variables and for the presence of at least one top quality embryo was 1.91 (95%CI: 1.20-3.05, p=0.006).

Data analysis was repeated by dividing women into three rather than two categories according to serum 25(OH)D levels: namely deficient (<20 ng/ml), insufficient (21-29 ng/ml) and sufficient (≥30 ng/ml) levels (18). The results are shown in Supplemental Tables 1 and 2. Of note, chances of implantation and pregnancy were observed to increase in accordance with the rise in categorized level of 25(OH)D. Crude and adjusted ORs for clinical pregnancy in women with 25(OH)D levels exceeding 30 ng/ml were 1.9

(95% CI 1.0-3.3; p=0.04) and 2.1 (95%CI: 1.1-4.0; p=0.03), respectively. Regarding implantation rate, the crude and adjusted ORs were 2.0 (95%CI: 1.2-3.2; p=0.006) and 2.2 (95%CI: 1.3-3.8; p=0.004), respectively.

Discussion

Based on this cross-sectional study, the clinical pregnancy rate and the implantation rate are significantly lower in women with vitamin D deficiency. Our results are mainly in line with those observed in the two studies from Rudick and co-workers (9, 10). They contrast with those from the two Iranian studies (11, 12), but the latter two contributions were exposed to a significant risk of type II error given the extremely low proportion of women with sufficient vitamin D levels (11, 12). Overall, we believe that it can be concluded that vitamin D insufficiency negatively affects clinical pregnancy rate in women undergoing IVF. This effect appears to be mediated at both the ovarian and endometrial level. Indeed, despite a similar responsiveness to COH, the number of top quality embryos and, as a consequence, the rate of women reaching the stage of blastocyst transfer is higher in women with serum 25(OH)D ≥20 ng/ml. Moreover, the chances of embryo implantation are also enhanced. The effect of vitamin D at uterine level may be exerted either via the regulation of target genes such as *HoxA10* or through the effects of the hormone on the local immune responses thought to be critical for the development of a normal pregnancy (6). Noteworthy, in our supplemental data analysis the best IVF outcome was observed in women with serum 25(OH)D ≥30 ng/ml, i.e. those with 25(OH)D sufficiency based on the most recent recommendations (18).

Some limitations of our study should be recognised. Firstly, albeit statistically significant, the 95%CI of the ORs are ample and further evidence is thus required to obtain a more precise estimation of the magnitude of the association. In this regard, it has to be pointed out that our study represents the largest available contribution on this topic.

Secondly, a causal relationship between low 25(OH)D and reduced pregnancy rate can be claimed but not proven. It also cannot be excluded that the two conditions are only associated. The large body of evidence available in animal models and data from *in vitro* studies in women strongly favour a causal relationship (2), but evidence from RCTs is required to definitely support a role for vitamin D in influencing the chances of pregnancy. The availability of such studies would potentially markedly influence clinical practice considering in particular that vitamin D supplementation is a simple and cheap intervention and is free of relevant side effects. Also, of particular note, there is growing and consistent evidence that vitamin D supplementation may improve birth outcome and prevent some relevant obstetrics complications such as preeclampsia or gestational diabetes (19, 20).

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Table 1. Baseline characteristics of selected women according to vitamin D status.

Characteristics	Vitamin D <20 ng/ml n=154	Vitamin D ≥20 ng/ml n=181	p
Age (years)	37.0 ± 4.3	36.8 ± 3.8	0.71
Race			0.04
Caucasian	140 (91%)	176 (97%)	0.02
Hispanic white	10 (6%)	1 (1%)	0.003
Other	4 (3%)	4 (2%)	1.00
BMI (Kg/m^2)	21.1 ± 2.0	20.4 ± 2.0	0.004
Duration of infertility (years)	4.6 ± 2.5	4.0 ± 2.5	0.03
Previous deliveries	14 (9%)	28 (16%)	0.08
Previous gynaecologic surgery	25 (16%)	25 (14%)	0.54
Previous IVF cycles	91 (59%)	112 (62%)	0.65
Day 3 serum FSH (IU/ml)	7.7 ± 3.0	8.1 ± 3.1	0.23
AMH (ng/ml)	2.3 ± 2.2	2.2 ± 2.1	0.67
Total AFC	10.8 ± 8.5	10.6 ± 6.7	0.81
Indication			
Unexplained / reduced ovarian reserve	50 (32%)	44 (24%)	0.11
Male factor	55 (36%)	67 (37%)	0.82
Tubal Factor / endometriosis	29 (19%)	33 (18%)	0.89
Mixed	20 (13%)	37 (20%)	0.08
Vitamin D serum levels (ng/ml)	14.1 ± 3.8	29.1 ± 8.3	0.001
Days between Vitamin D test and IVF cycle	36.7 ± 22.8	33.7 ± 21.3	0.22

Data is expressed as Mean ± SD or Number (Percentage)

AFC: Antral Follicle Count

Table 2. IVF cycle characteristics according to vitamin D status.

Characteristics	Vitamin D <20 ng/ml n=154	Vitamin D ≥20 ng/ml n=181	p
Hyperstimulation protocol			0.42
Long protocol	62 (40%)	60 (33%)	0.21
GnRH antagonist	54 (35%)	69 (38%)	0.57
Flare-up	39 (25%)	52 (29%)	0.543
Total dose of FSH used (IU)	2700 ± 1221	2655 ± 1339	0.75
Duration of treatment (days)	9.4 ± 2.1	9.4 ± 2.0	1.00
Number of oocytes retrieved	8.1 ± 4.2	7.9 ± 4.2	0.62
Number of oocytes used	5.5 ± 2.8	5.5 ± 2.7	0.94
Fertilization rate (%)	75 (56-100)	80 (63-100)	0.57
Number of embryos at cleavage stage	3.5 ± 2.1	3.8 ± 2.2	0.15
Number of Top Quality Embryos obtained			0.01
0	76 (49%)	74 (41%)	0.13
1-2	69 (45%)	78 (43%)	0.83
≥ 3	9 (6%)	29 (16%)	0.003
Embryo Transfer stage [a]			0.17
Cleavage stage (Day 2)	49 (32%)	53 (29%)	0.64
Cleavage stage (Day 3)	91 (59%)	99 (55%)	0.44
Blastocyst stage (Day 5)	14 (9%)	29 (16%)	0.07
Number of transferred embryos [b]			0.62
1	39 (25%)	51 (28%)	0,62
2	93 (60%)	110 (61%)	1.00
3	22 (14%)	20 (11%)	0.41
Clinical Pregnancy	30 (20%)	56 (31%)	0.02
Implanted embryos (Implantation Rate)	37 (13%)	70 (21%)	0.006

Data is expressed as Mean ± SD or Median (Interquartile Range) or Number (Percentage)

Top Quality Embryo was definied as 4-cells embryo on Day 2 or 8-cells embryo on Day 3, with a relative degree of fragmentation <10%.

For variables with ≥3 categories, both general and subgroup p values are reported

[a] Transfer was performed on day 2 if the number of viable embryos on day 2 was ≤ 2 and at blastocyst stage (day 5) if the number of good quality embryos on day 3 was ≥ 4. In the remaining situations, embryo transfer was performed on day 3.

[b] The number of embryos to be transferred was chosen on an individual basis taking into consideration prognostic factors and quality of the available embryos.

Supplemental Table 1. Baseline characteristics of selected women according to vitamin D status.

Characteristics	Vitamin D <20 ng/ml n= 154	Vitamin D 20-29 ng/ml n= 117	Vitamin D ≥30 ng/ml n= 64	p
Age (years)	37.0 ± 4.3	36.7 ± 3.7	37.0 ± 4.1	0.79
Race				0.15
Caucasian	140 (91%)	114 (97%)	62 (97%)	0.07
Hispanic white	10 (6%)	2 (2%)	2 (3%)	0.17
Other	4 (3%)	1 (1%)	0 (0%)	0.43
BMI (Kg/m^2)	21.1 ± 2.0	20.1 ± 2.1	20.3 ± 2.2	0.003
Duration of infertility (years)	4.6 ± 2.5	4.1 ± 2.3	4.0 ± 2.7	0.19
Previous deliveries	14 (9%)	15 (13%)	13 (20%)	0.07
Previous gynaecologic surgery	25 (16%)	15 (13%)	9 (14%)	0.79
Previous IVF cycles	91 (59%)	71 (61%)	36 (56%)	0.96
Day 3 serum FSH (IU/ml)	7.7 ± 3.0	7.9 ± 2.9	8.5 ± 3.4	0.21
AMH (ng/ml)	2.3 ± 2.2	2.2 ± 2.2	2.2 ± 1.8	0.91
Total AFC	10.8 ± 8.5	10.7 ± 6.1	10.6 ± 7.5	0.98
Indication				0.01
Unexplained / reduced ovarian reserve	50 (33%)	32 (27%)	12 (19%)	0.12
Male factor	55 (36%)	39 (33%)	28 (44%)	0.35
Tubal Factor / endometriosis	29 (19%)	16 (14%)	17 (27%)	0.11
Mixed	20 (13%)	30 (26%)	7 (11%)	0.01
Vitamin D serum levels (ng/ml)	14.1 ± 3.8	24.4 ± 2.8	37.9 ± 7.9	0.001
Days between Vitamin D test and IVF cycle	36.7 ± 22.8	34.6 ± 24.5	32.1 ± 13.4	0.36

Data is expressed as Mean ± SD or Number (Percentage)

AFC: Antral Follicle Count

Supplemental Table 2. IVF cycle characteristics according to vitamin D status.

Characteristics	Vitamin D <20 ng/ml n= 154	Vitamin D 20-29 ng/ml n= 117	Vitamin D ≥30 ng/ml n= 64	p
Hyperstimulation protocol				0.29
Long protocol	62 (40%)	43 (37%)	17 (27%)	0.16
GnRH antagonist	54 (35%)	39 (33%)	30 (47%)	0.17
Flare-up	39 (25%)	35 (30%)	17 (27%)	0.70
Total dose of FSH used (IU)	2700 ± 1221	2815 ± 1265	2492 ± 1118	0.23
Duration of treatment (days)	9.4 ± 2.1	9.6 ± 2.1	9.1 ± 2.1	0.31
Number of oocytes retrieved	8.1 ± 4.2	7.6 ± 4.3	8.4 ± 4.0	0.46
Number of oocytes used	5.5 ± 2.8	5.4 ± 2.8	5.8 ± 2.6	0.68
Fertilization rate (%)	75 (56-100)	83 (67-100)	75 (57-86)	0.13
Number of embryos at cleavage stage	3.5 ± 2.1	3.9 ± 2.3	3.8 ± 1.9	0.29
Number of Top Quality Embryo obtained				0.05
0	76 (49%)	47 (40%)	29 (45%)	0.32
1-2	69 (45%)	52 (44%)	24 (38%)	0.58
≥ 3	9 (6%)	18 (16%)	11 (17%)	0.01
Embryo Transfer stage [a]				0,12
Cleavage stage (Day 2)	49 (32%)	38 (33%)	15 (23%)	0,42
Cleavage stage (Day 3)	91 (59%)	64 (55%)	35 (55%)	0,71
Blastocyst stage (Day 5)	14 (9%)	15 (13%)	14 (22%)	0,04
Number of transferred embryos [b]				0.65
1	39 (25%)	30 (26%)	21 (33%)	0,49
2	93 (60%)	74 (63%)	36 (56%)	0.65
3	22 (14%)	13 (11%)	7 (11%)	0.67
Clinical Pregnancy	30 (20%)	33 (28%)	23 (36%)	0.03
Implanted embryos (Implantation Rate)	37 (13%)	40 (18%)	30 (26%)	0.004

Data is expressed as Mean ± SD or Median (Interquartile Range) or Number (Percentage)

Top Quality Embryo was defined as 4-cells embryo on Day 2 or 8-cells embryo on Day 3, with a relative degree of fragmentation <10%

[a] Transfer was performed on day 2 if the number of viable embryos on day 2 was ≤ 2 and at blastocyst stage (day 5) if the number of good quality embryos on day 3 was ≥ 4. In the remaining situations, embryo transfer was performed on day 3.

[b] The number of embryos to be transferred was chosen on an individual basis taking into consideration prognostic factors and quality of the available embryos.