



The Effects of Vitamin D Levels on Pregnancy Outcomes in Patients Receiving Frozen Embryo Transfer

Donmuş Embriyo Transferi Uygulanan Hastalarda Serum D Vitamini Düzeylerinin Gebelik Sonuçlarına Etkisi

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ABSTRACT

Aim: The literature reports conflicting findings regarding the role of vitamin D in female fertility and assisted reproductive techniques. In this context, the present study aimed to evaluate the potential association between baseline serum 25-hydroxyvitamin D (25-OH vitamin D) levels and pregnancy outcomes in infertile patients undergoing frozen embryo transfer (FET).

Material and Method: In this retrospective study conducted at the Center for Assisted Reproductive Techniques, Kocaeli University Faculty of Medicine Hospital, a total of 276 infertile women scheduled for FET were screened. Among these patients, 92 women with available baseline serum 25-OH vitamin D measurements and complete pregnancy outcome data were included in the final analysis.

Results: When patients with serum 25-OH vitamin D levels <20 ng/mL (Group 1, n=48) were compared with those with levels ≥20 ng/mL (Group 2, n=44), demographic characteristics were similar between the groups. Serum Anti-Müllerian hormone (AMH) levels were significantly higher in Group 1 compared to Group 2 (p=0.014). There were no statistically significant differences between the groups in terms of pregnancy rate (41.6% vs 31.8%; p=0.328), clinical pregnancy rate (35.4% vs 25%; p=0.278), ongoing pregnancy rate (25% vs 18.2%; p=0.428), live birth rate (20.8% vs 18.2%; p=0.749), pregnancy loss rate (18.8% vs 13.6%; p=0.507), or twin pregnancy rate (4.2% vs 9.1%; p=0.421).

Conclusion: No statistically significant association was observed between pregnancy outcomes following frozen embryo transfer and baseline serum 25-OH vitamin D levels measured at the start of treatment. These findings suggest that, within the scope of this study, serum vitamin D levels were not indirectly associated with fertility through effects on endometrial receptivity or the implantation process.

Keywords: Frozen embryo transfer, pregnancy outcome, 25-OH vitamin D

ÖZ

Amaç: Vitamin D'nin kadın fertilitesi ve yardımcı üreme tekniklerindeki rolüne ilişkin literatürde çelişkili sonuçlar bulunmaktadır. Bu bağlamda, donmuş embriyo transferi (DET) yapılan infertil hastalarda tedavi başlangıcında ölçülen serum 25-OH vitamin D düzeylerinin gebelik sonuçları ile olası ilişkisinin değerlendirilmesi amaçlanmıştır.

Gereç ve Yöntem: Kocaeli Üniversitesi Tıp Fakültesi Hastanesi, Üremeye Yardımcı Teknikler Merkezinde retrospektif olarak yürütülen bu çalışmada, DET yapılması planlanan 276 infertil olgu taranmış, bu olgular arasından tedavi başlangıcında serum 25-OH vitamin D düzeyi ölçülmüş ve gebelik sonucuna ilişkin verileri eksiksiz olan 92 hasta nihai analize dahil edilmiştir.

Bulgular: 25-OH vitamin D düzeyi <20 ng/mL olan olgular (Grup 1, n=48) ile ≥20 ng/mL olan olgular (Grup 2, n=44) karşılaştırıldığında, grupların demografik özellikleri benzerdi. Serum Anti-Müllerian Hormon (AMH) düzeyleri Grup 1'de Grup 2'ye kıyasla istatistiksel olarak anlamlı derecede daha yüksekti (p=0,014). Gruplar arasında gebelik (%41,6 vs %31,8; p=0,328), klinik gebelik (%35,4 vs %25; p=0,278), devam eden gebelik (%25 vs %18,2; p=0,428), canlı doğum (%20,8 vs %18,2; p=0,749), gebelik kaybı (%18,8 vs %13,6; p=0,507) ve ikiz gebelik (%4,2 vs %9,1; p=0,421) oranları açısından istatistiksel olarak anlamlı bir fark saptanmadı.

Sonuç: Donmuş embriyo transferi sonrası elde edilen gebelik sonuçları ile tedavi başlangıcında ölçülen serum 25-OH vitamin D düzeyleri arasında istatistiksel olarak anlamlı bir ilişki saptanmamıştır. Bu bulgular, çalışmamız kapsamında serum vitamin D düzeylerinin fertilité üzerindeki olası etkilerini endometriyal reseptivite ve implantasyon süreçleri aracılığıyla ortaya koyan dolaylı bir ilişki bulunmadığını düşündürmektedir.

Anahtar Kelimeler: 25-OH vitamin D, donmuş embriyo transferi, gebelik sonucu

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INTRODUCTION

The current data of the World Health Organization show that 10-15% of married couples are affected by infertility. Assisted reproductive techniques are the only way to achieve pregnancy for most infertile couples. In-vitro fertilization (IVF) is the ideal treatment method for couples who are unable to conceive naturally or by in-utero insemination, or for infertile patients who are not suitable for these methods. Pregnancy success in freeze-thaw cycles is still low in cases when embryos cannot be transferred in a fresh state, and novel strategies are being tested to improve success. One of these evaluations is whether the serum 25-OH-D vitamin levels of a female patient have an effect on pregnancy outcome in frozen embryo transfer (FET) cases (1).

Vitamin D, produced mainly in the skin in the body, is a steroid hormone that is fat-soluble. Ergocalciferol, which is contained in plants, and cholecalciferol, which is prevalent in animal foods, are the main sources of exogenous vitamin D intake (2). The effects of vitamin D on many systems in the body have been shown in numerous studies (3,4). Studies both with humans and animals are conducted to clarify the potential role of vitamin D in female fertility (5).

The purpose of this study is to compare the success of pregnancy outcomes between the cases where the baseline serum 25-OH-vitamin D level obtained from female patients at the beginning of the frozen embryo cycle is above (≥ 20 ng/mL) and below (< 20 ng/mL) the insufficiency. Accordingly, it is aimed to reveal whether the serum vitamin D level affects endometrial receptivity and implantation success.

MATERIAL AND METHOD

This study was carried out by screening 276 infertile women who were scheduled to undergo frozen embryo transfer (FET) at Kocaeli University Medical Faculty Hospital, Center for Assisted Reproductive Techniques. Among these patients, 92 women with available baseline serum 25-OH vitamin D measurements and complete pregnancy outcome data were included in the final analysis. All participants were included in the study after obtaining informed consent. This study was approved by Kocaeli University Non-interventional Clinical Research Ethics Committee (Decision number: KÜ GOKAEK 2017/834, Date: 07.06.2017).

Patients aged between 24-42 years who were scheduled to undergo FET and had at least one good quality frozen embryo with the diagnosis of single or combined unexplained infertility, male factor infertility, anovulation, low ovarian reserve, bilateral tubal factor and endometriosis were included in the study. Cases who did not want to participate in the study, who had

endometrial polyps, submucous myomas, uncorrected uterine anomalies, hydrosalpinx, or uncontrolled systemic diseases, were excluded from the study.

Following the confirmation of ovulation in the luteal phase of the previous cycle, a suppressed FET cycle was performed in all cases by starting leuprolide acetate (Lucrin 5mg/ml/2.8ml vial, 14 Syringe Kit SC /Abbot) with a daily dose of subcutaneous 10 IU for pituitary suppression. On the third day of the menstrual cycle, the patients were then called for an ultrasound and blood tests. The patients were called for the measurement of serum 25-OH vitamin D levels, Thyroid Stimulating Hormone (TSH), Anti-mullerian Hormone (AMH), estradiol, and progesterone by drawing 3-5 cc blood daily on the third day of the menstrual cycle. The study continued using the blinded method by preventing the researcher and patient from knowing the 25-OH vitamin D levels. Cases whose TSH levels were not in the 0.5-4.5 mIU/L range were excluded from the study. Patients with an estradiol level of > 50 and a progesterone level of > 1 ng/mL continued to take Lucrin at a dose of 10 IU/day until complete suppression was achieved. Estrogen therapy was not started in these cases until it was determined by drawing blood every three days that suppression had been achieved. Estrogen treatment was not initiated in cases who had endometrial thickness of > 5 mm or had a follicle cyst larger than > 14 mm in the adnexal area in the ultrasound examination which was performed concurrently. Estrogen therapy was started in these cases when the endometrial thickness was ≤ 5 mm and no cyst was detected in the adnexal area in the ultrasonographic follow-up performed every three days.

In cases who met the criteria (estradiol < 50 ng/mL, progesterone < 1 ng/mL, endometrial thickness ≤ 5 mm, no cysts in the adnexal area) in the blood tests and ultrasound examinations performed, 6 mg oestradiol ng/mL (Estrofem tablet 2 mg 28 tablets /Novo Nordisk) was started orally divided into three equal doses daily while Leuprolid acetate was continued with a daily dose of 5 IU. The patients were called for the first ultrasonographic evaluation at the earliest on the 10th day of the menstrual cycle and on day 7 of the estrogen therapy. In this evaluation, while the estrogen therapy was continued with the same dose in cases with an endometrial thickness of ≥ 8 mm and a blood progesterone level of < 1 ng/mL, leuprolide acetate therapy was discontinued, and twice-a-day vaginal progesterone therapy was initiated (Crinone 90 mg gel 8% /Merck). Cases with an endometrial thickness of less than 8 mm were examined every other day to monitor for an increase in the endometrial thickness. Estrogen therapy was administered at a dose of 8 mg/day to the cases whose endometrial thickness did not increase sufficiently in 2 consecutive follow-ups. If available, two thawed embryos were transferred, and if not, one

thawed embryo was transferred on the 4th day of vaginal progesterone in cases with a third-day embryo, on the 6th day of vaginal progesterone in cases with a 5th-day embryo, and on the 7th day of vaginal progesterone in cases with a 6th-day embryo.

The embryo transfer was performed under the supervision of transabdominal ultrasonography with a full bladder. After the visualization of the cervix with a speculum, the cervix was purified from drug residues with saline and cleared of mucus by aspiration with a mucus-attracting catheter. First, a mock transfer was performed to determine the cervical canal and the uterine position. Then, with a full echo soft catheter (Prodimed) the cervix was passed by using a stylet only in patients that necessitated it, and the embryo transfer was completed by applying the lowest pressure possible on the Hamilton syringe without approaching the middle portion of the uterine cavity by more than 15 mm and without a fundal contact. No teneculum was used or no cervical dilatation was performed in any patient. The position of the air balloon was clearly observed in all cases. The embryo transfer catheter was slowly removed, and no bed rest was recommended for the patients after the transfer. Daily doses of 6 mg oral estrogen and 180 mg vaginal progesterone were continued after the embryo transfer. No vitamin treatments were recommended. On the 12th day after the embryo transfer, blood Human chorionic gonadotrophin (hCG) levels were measured to confirm pregnancy.

The main outcomes that were aimed to achieve in this study were the pregnancy rate revealed by hCG positivity, the clinical pregnancy rate obtained by ultrasonographic confirmation of the embryonic heartbeat, and the ongoing pregnancy rate confirmed by exceeding the 10th week of pregnancy. Additionally, the secondary aim of the study was to obtain the rates of multiple pregnancy and rates of abortion. After achieving the primary aims of the study, the study was unblinded, and the pregnancy success as a result of the FET cycle was compared between the cases with blood 25-OH-D vitamin levels above the deficiency level (≥ 20 ng/ml) and the cases at the insufficiency level (20 ng/ml).

The data analysis was performed with SPSS for Windows 20.0 package program. A Kolmogorov-Smirnov test was completed to check if the continuous variables were normally distributed. Descriptive statistics were presented as mean \pm standard deviation or median (minimum-maximum) for continuous variables, while categorical variables were presented as number and percentage (%) of cases. The student's t-test was used to determine the significance of the difference between the groups in terms of means. The nonparametric Mann-Whitney U Test was used for the data whose means could be calculated as the groups did not fit the normal distribution. The Pearson's Chi-Square test was used for

the analysis of categorical variables. The results were considered statistically significant for a p value of <0.05 .

RESULTS

A total of 92 patients were included in the final analysis, comprising 48 patients with baseline serum 25-OH vitamin D levels <20 ng/mL (Group 1) and 44 patients with levels ≥ 20 ng/mL (Group 2). The demographic characteristics and baseline clinical features of the two groups were comparable. The comparison of demographic and cycle-related parameters between the groups is presented in **Table 1**.

Table 1. Comparison of important characteristics of groups			
Property	Group 1 Vitamin D <20 ng/mL (n= 48)	Group 2 Vitamin D \geq 20 ng/mL (n= 44)	P value
Age (year)*	30.48 \pm 4.36	32.00 \pm 4.30	0.144
Partner age (year)*	34.27 \pm 4.16	35.10 \pm 6.47	0.939
Marriage duration (year)*	7.62 \pm 4.55 (n=24)	5.95 \pm 4.39 (n=20)	0.210
Gravida (n)*	0.52 \pm 1.24	0.55 \pm 0.82	0.307
Parity (n)*	0.10 \pm 0.31	0.14 \pm 0.38	0.636
Abortion (n)*	0.35 \pm 1.06	0.41 \pm 0.76	0.243
BMI (kg/size2) *	25.94 \pm 4,94 (n=24)	24,99 \pm 3,99 (n=33)	0.518
Smoking (n, %)	3 (6.3%)	5 (11.4%)	0.473*
Chronic medical disease (n, %)	4 (8.3%)	9 (20.5%)	0.095
Previous uterine surgery (n, %)	9 (18.75%)	11 (25.0%)	0.468
Number of previous fresh IVF	1.08 \pm 1.13	1.14 \pm 0.98	0.443
Number of previous FET	0.75 \pm 0.81	0.91 \pm 0.74	0.728
Genetic (n, %)	1 (2.1%)	0 (0.0%)	0.522*
Advanced age (n, %)	0 (0%)	2 (4.5%)	0.226*
Endometriosis (n, %)	1 (2.1%)	2 (4.5%)	0.467*
Bilateral Tubal factor(n, %)	2 (4.2%)	4 (9.1%)	0.298*
Low ovarian reserve (n, %)	4 (8.3%)	7 (15.9%)	0.263
Azospemia(n, %)	5 (10.4%)	2 (4.5%)	0.255*
Anovulation(n, %)	11 (22.9%)	6 (13.6%)	0.252
Unexplained infertility(n, %)	4 (8.3%)	5 (11.4%)	0.444*

Values are given as mean \pm standard deviation *p value was calculated by Fischer Chi Square Test **Abbreviations; BMI: Body mass index, ICSI: Intracytoplasmic sperm injection, FET: Frozen embryo transfer, PCOS: Polycystic ovary syndromeNote: For some variables, sample sizes differ due to missing data in the retrospective records

While the comparison of the characteristics of the FET cycle between the groups is presented in **Table 2**, the comparison of the rates of pregnancy, clinical pregnancy, ongoing pregnancy, live birth, abortion, and twin pregnancies is presented in **Table 3**.

Table 2. Comparison of FET cycle characteristics between groups

Property	Group 1 Vitamin D <20 ng/mL (n= 48)	Group 2 Vitamin D ≥20 ng/mL (n= 44)	P value
AMH level (ng/ml) *	9.73±7.79 (n=26)	4.67±4.21 (n=23)	0.014
TSH level (miu/l) *	1.96±1.24 (n=41)	1.84±0.77 (n=38)	0.702
AFC*	23.46±16.09 (n=12)	14.38±9.36 (n=16)	0.113
Estrogen used time (day)*	9.79±2.93	9.98±2.42	0.379
Endometrial thickness (mm)*	9.94±1.86 (n=47)	10.06±1.69 (n=43)	0.487
Progesterone level at the end of proliferation phase (ng/ml) *	0.53±0.3 (n=20)	0.43±0.27 (n=24)	0.094
Distribution of embryo transfer days (n)*			
Day 3 embryos (n, %)	18 (37.5%)	13 (29.5%)	0.420
Day 5 embryos (n, %)	21 (43.8%)	22 (50.0%)	0.548
Day 6 embryos (n, %)	9 (18.8%)	9 (20.5%)	0.837

*Values are given as mean±standard deviation, ** Abbreviations; AMH: Anti-mullerian Hormone, AFC: Antral Follicle Count, TSH: Thyroid Stimulant Hormone, Note; For some variables, sample sizes differ due to missing data in the retrospective records

Table 3. Comparison of pregnancy, clinical pregnancy, ongoing pregnancy, live birth, abortion, and twin pregnancy rates between groups

Property	Group 1 Vitamin D <20 ng/mL (n= 48)	Group 2 Vitamin D ≥20 ng/mL (n= 44)	P value
Pregnancy rate	20 (41.6%)	14 (31.8%)	0.328
Clinical pregnancy rate	17 (35.4%)	11 (25.0%)	0.278
Ongoing pregnancy rate	12 (25.0%)	8 (18.2%)	0.428
Live birth rate	10 (20.8%)	8 (18.2%)	0.749
Abortion rate	9 (18.8%)	6 (13.6%)	0.507
Twin pregnancy rate	2 (4.2%)	4 (9.1%)	0.421*

* p value was calculated by Fischer Chi-Square Test

The serum AMH value of the group with a serum 25-(OH) Vitamin D level higher than 20 was statistically significantly lower than the group with a low serum 25-OH vitamin D level. No significant difference was found between the groups in terms of both the characteristics of the FET cycle and the rates of pregnancy, clinical pregnancy, ongoing pregnancy, live birth, pregnancy loss, and twin pregnancy.

The comparison of the rates of pregnancy, clinical pregnancy, ongoing pregnancy, live birth, abortion, and twin pregnancy between cases with 25-OH vitamin D levels of <20 ng/mL and cases with 25-OH vitamin D levels of ≥20 ng/mL is presented in Table 3. There was no significant difference in pregnancy achievement and pregnancy outcomes between the two groups (p>0.05).

The retrospective analysis of the means of 25-OH vitamin D levels of cases who got pregnant, achieved clinical pregnancy, had an ongoing pregnancy, and gave live birth revealed no significant differences in the means of vitamin D levels between the groups, and the results are presented in **Table 4**.

Table 4. Comparison of mean vitamin D levels in pregnant women

Vitamin D levels (ng/mL) (mean±SD)			
Parameter	Negative	Positive	P value
Pregnancy	21.69±11.44	20.59±11.72	0.596
Clinical pregnancy	21.36±11.26	21.11±12.22	0.832
Ongoing pregnancy	21.51±11.33	20.46±12.31	0.649
Live birth	21.28±11.26	21.29±12.73	0.961
Abortion	21.49±11.63	20.20±11.08	0.657
Multiple pregnancy	20.85±10.94	27.51±17.87	0.351

In the study, we did not find any relationship between the levels of serum vitamin D and achieving pregnancy in the FET cycle.

DISCUSSION

There is no agreement on the ideal vitamin D levels for female reproductive health and fertility at the moment. Although the possible effect of vitamin D on the outcomes of assisted reproductive therapy (clinical pregnancy and live birth) has been evaluated in a limited number of studies, the data are inconsistent (6-10).

Studies examining the relationship between serum vitamin D levels and the effectiveness of IVF cycles reported that the clinical pregnancy rate is associated with vitamin D deficiency. According to a study measuring the 25-OH vitamin D levels in follicular fluid instead of serum, high vitamin D levels are associated with significantly higher clinical pregnancy and implantation rates, and follicular fluid vitamin D levels are an independent predictor of IVF cycle success (11). Similarly, research done over various IVF cycles has suggested a relationship between vitamin D levels and pregnancy outcome (12,13).

In contrast to these studies, which discovered a significant correlation between vitamin D levels and the success of IVF cycles using fresh embryos, Anifandis et al. reported a negative correlation between follicular fluid 25-OH vitamin D level, embryo quality, and clinical pregnancy rate (14).

We aimed to evaluate the data obtained from FET cycles of patients with good and very good quality embryos frozen in the previous cycle to rule out ovarian factors and reveal whether vitamin D has an effect on endometrial receptivity and the implantation process. As good quality embryos are already frozen, standardizing FET is easier. As a result, many concomitant variables associated with the patient and her partner in new cycles are eliminated, and the true effect on receptivity can be assessed. In this prospectively designed, single-blind study, no statistically significant relationship was found between the serum 25-OH vitamin D levels measured at the start of the FET cycle and pregnancy success. In this study, we evaluated case groups with

vitamin D levels both below and above 20 ng/mL. Furthermore, unlike many other studies, all samples were examined immediately without being frozen, but neither the researchers nor the patients were aware of their vitamin D levels. This reflects the strength of our work in minimizing bias. Similar to our study, Van de Vijver et al. in their prospective cohort studies, evaluated 280 infertile cases whose DET cycle was planned, in two separate groups as cases with 25-OH vitamin D levels below and above 20 ng/mL on the day of embryo transfer, the pregnancy rate in the vitamin D deficient group was found to be similar when compared to the vitamin D sufficient group (respectively; 40.9% vs. 48.3%, $p=0.2$). Similarly, no difference was found between the clinical pregnancy rates (32.2% vs 37.9%, respectively, $p=0.3$). Clinical pregnancy rates in this study were similar in cases of insufficiency, deficiency, and normal levels of vitamin D, and the multivariate logistic regression analysis revealed that vitamin D status was not associated with pregnancy outcomes. Moreover, in their study, which involved randomizing 114 infertile cases with 25-OH vitamin D levels of <30 ng/L into two groups with and without vitamin D replacement, Aflatoonian et al. reported that the results of the two groups had similar results in terms of ongoing FET cycle pregnancy and clinical pregnancy. Our findings suggest that baseline serum vitamin D levels may not play a major role in reproductive outcomes through endometrial receptivity or implantation in frozen embryo transfer cycles

The fact that the study was not designed to evaluate the effects of vitamin D at the tissue level represents an important limitation. In addition, the statistically significant difference in serum AMH levels between the groups should be considered a potential confounder, given the prognostic importance of AMH in assisted reproductive techniques; however, the lack of multivariate analysis limited the ability to assess this effect independently. Furthermore, the retrospective design of the study and the relatively limited sample size constitute additional important limitations.

CONCLUSION

In the present study, no statistically significant association was observed between pregnancy outcomes following frozen embryo transfer and baseline serum 25-hydroxyvitamin D levels measured at the start of treatment. Although this finding may suggest that vitamin D does not exert its effects on fertility through endometrial receptivity or the implantation process, such an interpretation should be made with caution, considering the sample size and study design. Therefore, larger-scale, prospective, and randomized controlled studies are warranted to more clearly elucidate the potential role of vitamin D at the endometrial level and its relationship with reproductive outcomes.

ETHICAL DECLARATIONS

Ethics Committee Approval: This study was approved by Kocaeli University Non-intrventional Clinical Research Ethics Committee (Decision number: KÜ GOKAEK 2017/834, Date: 07.06.2017).

Informed Consent: Because the study was designed retrospectively, no written informed consent form was obtained from patients.

Referee Evaluation Process: Externally peer-reviewed.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

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