The Effect Follicular Fluid Vitamin A, E, D and B6 on Embryo Morphokinetics and Pregnancy Rates in Patients Receiving Assisted Reproduction

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ABSTRACT

OBJECTIVE: To evaluate the associations among levels of vitamins A, E, D, and B6 in follicular fluid embryo morphokinetics and quality, and clinical pregnancy rates.

STUDY DESIGN: A total of 58 patients with unexplained infertility admitted to the in vitro fertilisation center of Izmir Medical Park Hospital were included in this prospective clinical study. For each patient, vitamin levels were assayed using high-performance liquid chromatography. After intracytoplasmic sperm injection, for each oocyte, the relationships between each vitamin and subsequent embryo quality, embryo morphokinetics, and clinical pregnancy rates were investigated. Embryos were classified as grade A, B, C, or D according to morphokinetic parameters using t5 - t2 and t5 - t3 (cc3).

RESULTS: There was no significant correlation between embryo morphokinetic parameters (tpnf, t2, t3, t4, t5, t6, t7 and t8) and follicular fluid vitamin (A, B6, D, and E) levels (p >0.05). There was a significant positive correlation between t5 optimal and follicular fluid vitamin A levels (p <0.05). There was a significant positive correlation between cc2 optimal and follicular fluid vitamin B6 levels (p <0.05). Levels of vitamins A and B6 were significantly higher in grade A and B embryos than in grade C and D embryos. There were no significant relationships between vitamins E or D and embryo quality or between any vitamin and clinical pregnancy rates.

CONCLUSION: High levels of vitamins A and B6 in follicular fluid are significantly associated with high-quality embryos and optimal morphokinetics. However, none of the vitamins considered showed a significant relationship with clinical pregnancy rates.

Keywords: Embryomorphokinetics, Vitamin A, Vitamin B6, Vitamin D, Vitamin E

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Introduction

The microenvironment of the oocyte is an important factor in oocyte maturation; thus, the biochemical characteristics of the follicular fluid (FF) may play a critical role in oocyte quality, fertilisation, and subsequent embryonic development (1). The formation of reactive oxygen species (ROS) is a natural consequence of aerobic metabolism in oocytes, and is integral to maintaining oxygen homeostasis during oocyte maturation (2,3). When the balance between oxidants and antioxidants is not maintained, the cellular environment becomes oxidatively stressed (3). ROS are associated with mitochondrial changes, meiotic arrest in oocytes, embryonic block, and cell death (4-7).

Vitamins and minerals are micronutrients that play important roles in many steps of antioxidant reactions. They may directly cause non-enzymatic antioxidant activity or may be required for enzymatic antioxidant activity (4,8,9). Vitamins E and A are potent non-enzymatic antioxidants that protect the integrity of cell membranes and promote reproductive func-

tion (10). Beta-carotene functions as an antioxidant in lipid phases by quenching singlet oxygen and scavenging peroxyl radicals (11,12). Thus, vitamin A and carotenoids protect cells from superoxide radicals; in fact, low concentrations of these vitamins are correlated with anovulation (10,13). Vitamin E is a major chain-breaking antioxidant that destroys free radicals generated during ferrous ascorbate-induced lipid peroxidation (4,14). Vitamin E protects mouse embryos against oxidative damage (15). In addition, in vitro administration of vitamin E improves the development of bovine embryos (16). Vitamin B6 is a coenzyme mainly involved in the metabolism of amino acids, nucleic acids, and lipids, and plays a role in the glutathione antioxidant defence system (17). It may also react directly with peroxy radicals, scavenge radicals, and inhibit lipid peroxidation (17,18). Vitamin D in its biologically active form (1.25-dihydroxyvitamin D, or calcitriol) has antioxidant and anti-inflammatory effects in vitro (19-21). Some studies have suggested an impact of vitamin D on circulating oxidative and inflammatory markers (21,22).

Treatment with additional micronutrients before in vitro fertilisation (IVF) cycles protect the follicular microenvironment against oxidative stress, thus increasing the number of good-quality oocytes (10). Higher intakes of antioxidants β-carotene, vitamin C, and vitamin E are also associated with a shorter time to conception among couples undergoing treatment for unexplained infertility (23).

These findings suggest the importance of antioxidant vitamins in infertility patients. Although commercial supplements including these vitamins are commonly preferred in daily clinical practice in infertility clinics, interestingly, there is paucity of information on their exact roles in embryo quality and pregnancy rates. In addition, there are no data on the relationships between these vitamins and embryonic developmental kinetics. Therefore, we evaluated the associations among levels of vitamins A, E, D, and B6 in FF, embryo morphokinetics and quality, and clinical pregnancy rates.

Material and Method

A total of 58 patients with unexplained infertility who underwent intracytoplasmic sperm injection (ICSI) cycles were included in this prospective cohort study. The study was approved by the Institutional Review Board (Approval number:1454). The study was conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from all patients. The exclusion criteria were patient age > 41 years old, severe oligoasthenoteratospermia, polycystic ovary syndrome, diminished ovarian reserve, endometriosis, the presence of hydrosalpinx, and any endocrinological disease. All patients underwent a gonadotropin-releasing hormone (GnRH) antagonist protocol for ovarian stimulation. Daily recombinant gonadotropin (150-300 IU, Gonal-F; Merck, Turkey) doses were started on the second menstrual day according to the patient's characteristics. When the leading follicle exceeded 14 mm, a gonadotropin antagonist (Cetrotide, 0.25 mg; Merck) was started and human chorionic gonadotropin (hCG) (Ovitrelle, 250 µg; Merck) was administered when there were at least two follicles >18 mm. Transvaginal ultrasound-guided oocyte pick-up was performed 36 h after hCG injection.

Individual aspiration was applied for oocyte pick-up, and each follicle was recovered in a different tube. To avoid contamination from blood and flush medium or mixtures, only the FF from the first retrieved follicle, which contains a single oocyte-cumulus cell complex, was collected. After oocyte pick-up, oocytes isolated from FF samples were evaluated individually. One follicular sample per patient was used for analyses. FF samples were centrifuged at 2000xg for 10 min and the supernatants were stored at -80°C until further analyses. Analyses of FF samples were performed only in patients with single-embryo transfer, for which an embryo was obtained from the oocyte of that follicle.

A time-lapse monitoring system (Embryoscope; Unisense FertiliTech, Denmark) in an atmosphere of 5% O_2 and 7% CO_2 at 37°C was used to incubate embryos until transfer. Images of each embryo were taken every 20 min in seven different focal planes over 72 h of culture. Embryo Viewer software (Unisense Fertilitech Aarhus) was used to analyse embryonic images. The times from fertilisation to the following events were analysed: pronuclear fading (Pnf), when both pronuclei disappear; the first cleavage, when the zygote divides into two cells (t2); and when cleavage giving rise to 3 to 9 cells were observed for the first time (t3, t4, t5, t6, t7, t8, and t9, successively). The intervals between two consecutive cleavages were defined in the following manner: the duration of the second cell cycle (cc2=t3-t2) was the time from the division into a two-blastomere embryo to the time of the division into a three-blastomere embryo, and second synchrony (s2=t4-t3) was the time from this division to a four-blastomere embryo. The duration of the third cell cycle (cc3=t5-t3) was the time from the division into a three-blastomere embryo to the time of division into a five-blastomere embryo. The embryos were graded according to the time-lapse parameters previously described by Basile et al. (24). The classification was based on the t5-t2 and cc3 intervals as follows: if the value of t5-t2 was greater than 20.5 h, the embryo was categorised as A or B, and if the value fell outside the optimal range, the embryo was categorised as C or D. If the value of cc3 was between 11 and 18 h, the embryo was categorised as A or C, and if the value fell outside the optimal range, the embryo was categorised as B or D, depending on the t5-t2 value. Each FF analysis was performed for a single oocyte of a single embryo that was transferred to the patients. The relationship between vitamin A, E, D, and B6 content of each FF sample and subsequent embryo quality and morphokinetics were investigated. The relationships between vitamin levels optimal time-

lapse parameters, including t5, s^2, and cc2, were also analysed, as were FF vitamin levels and pregnancy rates. Vitamin levels in each patient were determined by reversed-phase high-performance liquid chromatography (HPLC; Shimadzu Prominence, Japan).

Embryo transfer was performed on day 3 of development. For luteal support, all patients received 90 mg progesterone gel (8% Crinone gel, Merck) and 200 mg vaginal micronised progesterone (Progestan caps, 200 mg; Kocak Farma, Turkey) daily. Biochemical pregnancy was determined when serum levels of β-hCG were >20 IU/I on Day 12 after embryo transfer, and clinical pregnancy was confirmed by ultrasound as the presence of foetal heart activity 8 weeks after embryo transfer.

Statistical Analyses

SPSS software (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Comparisons between two groups were performed using the independent t-test for parametric conditions and the Mann-Whitney U test for non-parametric conditions. Pearson and Spearman correlation analyses and receiver operating characteristic (ROC) curves were used. All values are expressed as the mean ± standard deviation (SD). P <0.05 was considered to indicate statistical significance.

Results

The baseline characteristics of patients with high- and low-quality embryos according to embryo morphokinetic parameters are shown in table I. Group 1 consisted of patients with grades A and B (high-quality) embryo and group 2 consisted of patients with grades C and D (low-quality) embryos according to embryo morphokinetic parameters. There were no significant differences between groups according to age, body mass index (BMI), infertility duration, total gonadotropin dose, number of oocytes, number of MII oocytes, number of embryos, or serum levels of anti-Müllerian hormone (AMH), Day 3 follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol, and vitamin D (Table I). Also there was not any correlation between baseline parameters and follicular fluid vitamin A, B6, E and D levels (p >0.05).

Both normal (tpnf, t2, t3, t4, t5, t6, t7, and t8) and optimal (t5, s2, cc2) embryomorphokinetic parameters were evaluated. There was no significant correlation between normal embryomorphokinetic parameters (tpnf, t2, t3, t4, t5, t6, t7 and t8) and follicular fluid vitamin (A, B6, D, and E) levels (p >0.05). There was a significant positive correlation between t5 optimal and follicular fluid vitamin A levels (p <0.05). There was a significant negative correlation between s2optimal and follicular fluid vitamin D levels (p <0.05). There was a significant negative correlation between cc2optimal and follicular fluid vitamin E levels (p <0.05). There was a significant positive correlation between cc2 and follicular fluid vitamin B6 levels (p <0.05). The embryos were graded according to optimal embryo morphokinetic parameters. When the relationship between follicular fluid vitamin levels and embryomorphokinetic grades were evaluated; vitamin A and B6 levels were significantly higher in grade A and B embryos than in grade C and D embryos (Table II). However, there were no significant correlations between vitamin E and D levels and embryo quality, or between any vitamin and clinical pregnancy rates (Table II).

Receiver operating characteristic curve analyses also indicated that higher embryo quality (grades A and B) was significantly associated with vitamin A and B6 levels (Figure 1). The area under the curve (AUC) for vitamin A was 70.4 (95% confidence interval [CI], 0.536-0.872; p=0.025); that for vitamin B6 was 0.679 (95% CI, 0.503-0.855; p=0.050). Higher embryo quality was associated with vitamin D levels, but this difference was not significant. The AUC for vitamin D was 0.677 (95% CI, 0.495-0.859; p=0.052). There was no association between embryo quality and vitamin E levels. The AUC for vitamin E was 0.586 (95% CI, 0.406–0.767; p=0.344). Finally, clinical pregnancy rates were not associated with vitamin levels.

Table I: *Baseline characteristics of patients with high- and low-quality embryos according to embryo morphokinetic parameters*

	Group 1	Group 2	*p*
Age (years)	31.15±4.56	33.75±4.46	0.066
Infertility duration (months)	54.41±37.15	59.50±40.71	0.710
BMI (kg/m^2)	25.03±5.70	28.83±5.68	0.093
AMH (ng/mL)	2.30±1.11	1.91±1,20	0.326
Day3 FSH (mLU/mL)	5.98±2.26	5.35±2.05	0.412
E2 (pg/mL)	43.42±47.71	37.20±12.21	0.661
Total gonadotropin dose (IU)	2367.59±1011.82	2647.91±778.43	0.400
Duration of induction (days)	9.48±1.90	9.08±1.24	0.513
Trigger Day E2	2122.77±1163.77	1855.18±1441.87	0.588
Number of oocytes (n)	14.51±6.97	13.25±7.26	0.608
Number of MII oocytes (n)	11.96±6.08	8.91±4.62	0.131
Number of embryos (n)	8.55±4.89	6.66±3.91	0.247
Vit D (Plasma)(µg/L)	16.40±8.17	21.13±10.01	0.200

Group 1: Patients with grades A and B (high-quality) embryos according to embryo morphokinetic parameters.
Group 2: Patients with grades C and D (low-quality) embryos according to embryo morphokinetic parameters.

Table II: *Associations between levels of vitamins A, E, D, and B6 in follicular fluid and embryo quality and clinical pregnancy rates.*

	Vitamin		n	Mean ± SD	*p*
Embryo quality	Vitamin A (µg/L)	Grade A-B	34	343.099±102.919	**0.017***
		Grade C-D	24	263.716±100.653	
	Vitamin E (mg/L)	Grade A-B	34	2.117±0.642	0.505
		Grade C-D	24	2.452±2.691	
	Vitamin D (µg/L)	Grade A-B	34	17.535±13.340	0.999
		Grade C-D	24	17.537±29.144	
	Vitamin B6 (ng/mL)	Grade A-B	34	19.602±15.034	**0.049***
		Grade C-D	24	12.521±9.927	
Clinical Pregnancy	Vitamin A (µg/L)	Non-pregnant	26	309.398±120.441	0.830
		Pregnant	32	315.438±92.879	
	Vitamin E (mg/L)	Non-pregnant	26	2.249±2.059	0.673
		Pregnant	32	2.086±0.652	
	Vitamin D (µg/L)	Non-pregnant	26	17.655±14.892	0.971
		Pregnant	32	17.833±21.484	
	Vitamin B6 (ng/mL)	Non-pregnant	26	16.960±14.702	0.685
		Pregnant	32	18.603±15.683	

**: p<0.05*

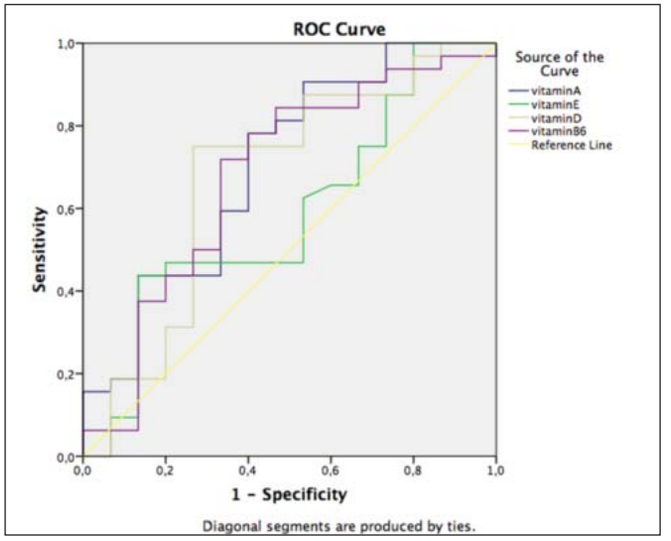

Figure 1: *ROC curve analysis of vitamin A,E,D, and B6 levels for the prediction of embryo quality*

Discussion

High FF vitamin A and B6 levels were significantly associated with high embryo quality and optimal morphokinetics. However, there were no significant associations between vitamin D and E levels and embryo quality, or between any vitamin and pregnancy rates. To the best of our knowledge, this is the first study to evaluate the relationships between levels of vitamins A, B6, E, and D in FF and embryo developmental kinetics. In addition, oocytes and embryos were continuously evaluated via embryoscope images, and embryo grading was performed according to morphokinetic parameters in an objective manner.

The role of vitamin A and its active metabolite retinoic acid during normal embryonic growth and development has been reported in previous animal studies. In addition to its antioxidant effects, vitamin A and its metabolites affect ovarian follicular growth, quality, and steroidogenesis (25-27). Currently, many gene products related to reproduction are modulated by vitamin A (28). It promotes cytoplasmic maturation in bovine oocytes via its modulatory effects on the gene expression of gonadotropin receptors (27). It also improves embryo quality after in vitro maturation of bovine and porcine oocytes (25,26). However, there are very limited data on the relationship between vitamin A and human follicular development. Tiboni et al. reported that smokers have significantly lower levels of FF beta-carotene and lower fertilisation rates compared to non-smokers (29). In addition, FF vitamin A levels are associated with high-quality oocytes (30). Our results support these findings and the fundamental role of vitamin A in embryo quality.

We also found a positive correlation between FF vitamin B6 (pyridoxine) levels and embryo quality. The antioxidant properties of pyridoxine have recently been reported (31,32). Because pyridoxine is essential for the metabolism of homocysteine into the amino acid cysteine, pyridoxine deficiency is correlated with elevated homocysteine levels and coronary artery disease (33). Vitamin B6 deficiency is also associated

with problems in defence mechanisms against lipid peroxidation and exercise-induced oxidative stress (34,35). Vitamin B6-deficient diets are correlated with increased plasma and tissue lipid peroxidation in rats (36). Despite these pertinent findings in animal studies, interestingly, there is no information regarding the relationship between vitamin B6 and embryonic development in humans in the literature. Our findings support a possible beneficial effect of vitamin B6 on oocyte and embryo quality.

Current data on vitamin D status and assisted reproductive technology outcomes are conflicting, but the majority of studies support vitamin D supplementation in deficient patients (37). Vitamin D deficiency is correlated with female infertility and poor IVF outcomes (38,39). However, the exact mechanism of the positive effect of vitamin D is unclear. In a previous study, pregnancy rates were higher among women with high levels of vitamin D than among women with low levels, but there was no association between embryo quality and vitamin D status (40). The authors concluded that the effects of vitamin D on pregnancy rates may be related to the endometrium rather than oocytes. This hypothesis was supported by another study that reported lower pregnancy rates in vitamin D-deficient recipients of egg donation (41). In addition, in another study, FF vitamin D levels were not associated with oocyte quality and fertilisation, independent of pregnancy outcomes (42). Our study supports these reports, as we did not find any associations between vitamin D levels in FF and embryo quality or morphokinetic parameters.

We also did not find a relationship between vitamin E levels and embryo quality. Some animal studies have suggested that vitamin E improves in vitro maturation, fertilisation, and blastocyst development (43). However, some studies have not found any association between vitamin E and reproductive success [27,30]. In addition, in a previous study, the active form of vitamin E during oocyte maturation impaired the acquisition of oocyte developmental competence and also significantly diminished the percentage of blastocysts (44). The authors suggested that physiological concentrations of ROS may be required during oocyte maturation (44). Therefore, there may be upper and lower limits to the optimal antioxidant vitamin levels in FF for oocyte maturation.

Although dietary regulation or vitamin supplementation before IVF cycles is a very common management strategy, interestingly, there is a paucity of information on the subject. Therefore, we investigated the effects of FF levels of vitamins that are commonly found in multivitamin supplements. We did not find any associations between pregnancy rates and vitamin levels. However, the positive correlations between levels of vitamins A, D, and B6 and embryo quality suggest the importance of these vitamins to IVF outcomes. Thus, vitamin A and B6 supplementation before the start of IVF treatment cycles may be beneficial for improving embryo quality.

The limitations of our study were that we did not evaluate the plasma levels of these vitamins (except vitamin D), and did not evaluate the correlations between plasma and FF levels. Therefore, we cannot draw an absolute relationship between blood levels of these vitamins and embryo development. Another limitation of the study is small number of patients and larger number of studies are needed in this issue.

In conclusion, we found positive correlations between vitamin A and B6 concentrations in FF and subsequent embryo quality and morphokinetic parameters. Our results support the roles of vitamin A and B6 in early embryogenesis and embryo morphokinetics. Further research is necessary to elucidate the clinical effects of these vitamins on IVF outcomes.

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