European Review for Medical and Pharmacological Sciences 2022; 26: 499-505

Relationship between serum vitamin D levels semen parameters and sperm DNA damage in men with unexplained infertility

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Abstract. – OBJECTIVE: The aim of the study was to investigate the relationship between serum level of vitamin D, semen analysis parameters and sperm DNA damage in men with unexplained subfertility.

PATIENTS AND METHODS: Fifty-eight men diagnosed with unexplained infertility and 50 age and BMI matched fertile men were included in the study. A participant whose semen parameter is normal but pregnancy is not achieved was accepted as unexplained male infertility. Blood samples were taken from all participants following three-day abstinence for measurement of vitamin D. Sperm DNA damage was assessed by Aniline Blue staining of the collected samples.

RESULTS: Compared with the fertile men, male patients with unexplained infertility had significantly lower vit D levels (27.00 ng/mL (12.63-39.30) *vs.* 23.66 ng/mL (7.50-55.00), $p<0.004$). While the number of patients with vitamin D levels lower than 20 ng/mL was 26 (44.8%) in the infertile group, it was recorded as 5 (10.0%) in the fertile group ($p<0.001$). DNA damage was found in 31.50% (9.0-71.0) of the infertile men and 26.00% (11.0-54.0) of the fertile men. DNA damage was found to be significantly higher in the unexplained infertile group ($p<0.002$). In men with unexplained male infertility, serum vit D levels were positively correlated with total sperm count ($r = 0.527$, $p<0.001$), total motility ($r = 0.527$, $p<0.001$) and sperm morphology ($r = 0.416$, $p = 0.001$). There was a negative and significant correlation between vit D levels and sperm DNA damage ($r = -0.605$, $p<0.001$). In the logistic regression analysis, serum vit D > 20 ng/mL led to an improvement in fertility outcome.

CONCLUSIONS: Men with unexplained infertility exhibit decreased serum vit D levels and increased sperm DNA damage.

Key Words:
Unexplained male infertility, Semen, Sperm DNA damage, Vit D.

Introduction

The inability of couples under the age of 35 to conceive despite one year of unprotected intercourse is called infertility and affects approximately 10-12% of couples in the general population. It covers 60 to 80 million pairs when compared to the general population[1-4].Problems related to male factor are responsible for approximately 40-50% of all infertile couples[1,2].Men with lower sperm parameters according to the criteria determined by WHO as normal are considered as male factor infertility[5]. The problem encountered in approximately 90% of the men is low sperm count (oligospermia), sperm motility defects (asthenospermia) or abnormality in sperm morphology (teratospermia)[6]. Despite advances in semen analysis methods, the underlying etiology cannot be determined in one out of four men with male factor infertility. Patients in this categoryare called male factor infertility of unknown origin. They are classified under two subheadings as idiopathic male infertility and unexplained male infertility, according to semen analysis results. Although these two definitions are often used interchangeably, actually these are two completely different entities[7,8]. A decrease in semen quality for an unknown reason is accepted as idiopathic male infertility. Since patients in this group often

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exhibit morphological problems in addition to decreased sperm count and motility, they are also called idiopathic oligoasthenoteratozoospermia. Unexplained male infertility, on the other hand, defines patients whose semen parameters are normal but pregnancy is not achieved[7,8].

Semen analysis is an easy, inexpensive and reproducible method that evaluates male factor infertility with approximately 90% sensitivity[9]. However, while approximately 25% of men with abnormal sperm parameters become pregnant spontaneously, pregnancy cannot be achieved in 10% of couples with normal sperm parameters[10]. For this reason, it is thought that routine semen parameters do not provide clear information regarding sperm functions and their fertilizing abilities. Patients with unexplained male factor infertility who cannot achieve pregnancy despite normal semen parameters are a good example of this. The underlying reasons for not achieving pregnancy despite normal semen values are not clearly known. It has been suggested that socioeconomic and nutritional reasons, as well as the status related to the geographical region, may lead to deterioration in semen functions in this group of patient[11]. Since there are plenty of trace elements and vitamins in the seminal fluid, it is accepted that there is a relationship between nutrition and sperm parameters, and some studies[3,4] have been designed and published on the subject. The relationship between vit D and semen parameters gained even more importance after it was reported that vitamin D supplementation altered granulosa cell gene expression[12]. In this context, the possible relationship between vitamin D and semen parameters has been discussed by many researchers recently, but the results were significant in some studies[11,13] and no relationship could be shown in others[14]. The reasons such as the inhomogeneity of the participants, the variable age range, and the collection of all kinds of infertile men in the same pool were accepted as the main reasons for the differences in results between studies[14]. Men with unexplained male infertility constitute a homogeneous group in which pregnancy could not be achieved despite having normal semen parameters. Whether there is a relationship between serum Vit D level and sperm parameters in men with unexplained male infertility has not been demonstrated to date. Therefore, this study was planned to determine the possible relationship between serum vit D levelsemen parameters and sperm DNA damage.

Patients and Methods

Patient Selection

The study population consists of 58 infertile couples, both of whom were diagnosed with unexplained male infertility. Participants were selected among men who applied for infertility treatment at Şişli Memorial Hospital Urology Clinic and Bahçeşehir University Medicalpark-Göztepe Infertility Clinic between 1 April 2020 and 31 December 2020. To diagnose unexplained infertility for the male partner, we stipulated that the female partner should also have been diagnosed with unexplained infertility. Thus, we excluded other possible causes of female infertility. Females with the results of ovulatory function, tubal patency, and semen analysis tests did not reveal that any etiology was considered as unexplained infertility. Age, duration of marriage and infertility, body mass index (BMI) in both groups of participantswere recorded at the time of admission. Semen analysis was performed after three days of abstinence according to the fifth edition of the World Health Organization (WHO 2010) laboratory manual for the examination and processing of human semen criteria[15]. In semen analysis, men who could not achieve pregnancy despite semen volume of 1.5 mL, sperm concentration 15 million spermatozoa/mL, total sperm count 39 million spermatozoa per ejaculate, morphology 4%, vitality 58%, progressive motility 32% and total motility 40% are defined as unexplained infertile.

Detection of pathology in any of the semen parameters, presence of known etiological factors such as cryptorchidism or history of reproductive tissue surgery, history of chemotherapy or radiotherapy or severe oligoasthenoteratozoospermia, patients who received hormonal treatment or vitamin D supplementation at last six months were excluded. In addition, couples with IVF/ICSI decision were excluded from the study. Fifty age and BMI matched fertile menwith at least two children were taken as the control group. Blood samples were taken for measurement of vitamin D, cholesterol, triglyceride, high density lipoprotein [HDL], low density lipoprotein [LDL], follicle stimulating hormone [FSH], luteinizing hormone [LH], testosterone, estradiol[E2], and prolactin. For measurement of serum vit D levels, semen samples were taken following three-day of abstinence. Thus, we have objectively and clearly evaluated the change in vit D levels during active spermatogenesis. Serum levels of vitamin D were measuredby using an electrochemiluminescence

immunoassay (ELC) kiton an Elecsys 2010immunoassay analyzer and were given as ng/mL. Sperm DNA damage was assessed by Aniline Blue staining in infertile and fertile groups. The study was performed according to the guidelines of the Helsinki Declaration on human experimentation and was approved by the Local Ethics Committee of Medeniyet University (KNO:2021/0192).

Aniline Blue Staining for Sperm DNA Damage

The semen samples collected from the patients and controls were washed and 10 µl of sample was dropped on the slide and a smear was made. The smeared preparations were dried in air and fixed with 4% formalin at room temperature. The slides were rinsed twice for 1 minute and stained with 5% aniline blue in 4% acetic acid (pH 3.5) solution for 10 minutes. After staining, the slides were rinsed with distilled water 3 times for 2 minutes each, dried in air and covered with Ecomount. A hundred sperm cells were counted by examining on the 100x objective with immersion oil under a phase contrast microscope. The number of damaged sperm DNA as a result of aniline staining was given as a percentage.

Statistical Analysis

Data analysis was performed on SPSS 21 (SPSS Inc., IBM, Armonk, NY, USA). Normality of distribution was evaluated with Shapiro-Wilk test. Normally distributed variables were analyzed with the independent samples *t*-test. Non-normally distributed variables were analyzed with the Mann-Whitney U test. Spearman correlation coefficients were calculated for the assessment of relationships between continuous variables. The distributions of categorical variables were evaluated using Pearson Chi-square tests or Fisher's exact tests. Logistic regression analysis (backward conditional method) was performed to determine risk factors affecting-fertility status. Data were given as mean ± standard deviation or median (minimum-maximum) for continuous variables according to normality of distribution, and as frequency (percentage) for categorical variables. Differences were considered statistically significant if the *p*-value <.05.

Results

The laboratory and demographic characteristics of each group of participants areshown in Table I. Since the groups were matched by age and BMI, there was no significant difference between the groups in terms of these parameters. Serum FSH and triglyceride levels of the infertile group were significantly higher than the control group. Sperm parameters were similar in both groups, and interestingly, morphological findings were worse in the fertile group. Other demographic and hormonal parameters of the patients were similar.

Compared with the fertile group, male patients with unexplained infertility had significantly lower vit D levels (27.00 ng/mL (12.63-39.30) *vs.*23.66 ng/mL (7.50-55.00), $p<0.004$). While the number of patients with vitamin D levels lower than 20 ng/mL was 26 (44.8%) in the infertile group, it was 5 (10.0%) in the fertile group, and the difference was statistically significant ($p<0.001$).When the groups were evaluated in terms of sperm DNA damage, it was found in 31.50% (9.0-71.0) of infertile men and 26.00% (11.0-54.0) of fertile men. DNA damage was found to be significantly higher in the unexplained infertile group ($p<0.002$). In men with unexplained infertility, serum vit D levels were positively correlated with total sperm count ($r = 0.527$, $p<0.001$), total motility ($r = 0.527$, $p<0.001$) and sperm morphology ($r = 0.416$, $p = 0.001$). However, there was a negative and significant correlation between vit D levels and sperm DNA damage ($r = -0.605$, $p<0.001$). There was no significant correlation between total testosterone and vitamin D. In the fertile group, there was no significant correlation between vit D levels and sperm DNA damage and other sperm parameters (Table II).

Backward stepwise multiple logistic regression analysis was performed to determine factors affecting the fertility status of participants. The model established for this purpose included vit D, DNA damage, male age, smoking status, sperm count and sperm morphology. We found that a vitamin D level greater than 20 ng/mL (OR: 6.50, 95% CI: 1.20-35.13, *p*: 0.030) and total motility (OR: 1.04, 95% CI: 1.01-1.08, *p*: 0.022) were associated with improved fertility ($p<0.001$). Other variables included in the model did not have positive effects on fertility ($p>0.05$, for each). When subgroup regression analysis was performed on 26 patients in the infertile group with serum vit D levels <20 ng/mL, it was found that sperm DNA damage had a negative effect on fertility (OR: 6.12, 95% CI: 1.10-28.20, *p*: 0.010).

Discussion

The relationship between vit D levels and semen parameters has been written by many re-

Table I. Demogarphic and laboratory characteristics of both groups.

	Fertile Group (n=50)	Unexplained infertility (n=58)	*p*-value
Male age (year)	33.18±4.14	34.67±4.01	0.061
Female age (year)	29.56±3.45	30.55±4.29	0.194
Duration of marriage (year)	4.5(1.5-18.0)	6.0(1.0-18.0)	0.040
Duration of infertility (year)	3.0(1.0-10.0)	3.0(1.0-13.0)	0.977
FSH (mIU/mL)	4.35(2.44-21.70)	5.90(2.09-22.10)	0.019
LH (IU/L)	4.39(2.10-16.70)	4.21(1.40-11.33)	0.332
Total testosterone (ng/dL)	4.27(2.98-7.35)	4.53(2.00-12.21)	0.192
Estradiol (pg/mL)	14.83(11.85-44.80)	16.30(11.34-44.80)	0.062
Prolactin (µg/L)	13.94(10.70-21.28)	13.70(5.45-24.35)	0.228
Vitamin D level (ng/mL)	27.00(12.63-39.30)	23.66(7.50-55.00)	0.004
Vitamin D<20 ng/mL	5 (10.0%)	26 (44.8%)	<0.001
Body mass index (kg/m^2)	24.85(20.94-31.91)	24.85(20.19-32.87)	0.904
Cholesterol (mg/dL)	197.50(155.0-288.0)	197.00(143.0-311.0)	0.793
Triglyceride (mg/dL)	130.50(45.0-532.0)	146.00(45.0-216.0)	0.025
HDL (mg/dL)	50.32(36.00-64.00)	52.00(35.00-138.00)	0.832
LDL (mg/dL)	125.50(34.0-174.0)	130.00(42.0-181.0)	0.968
Comorbidity count	1 (2.0%)	6 (10.3%)	0.120
Smoking	19 (38.0%)	29 (50.0%)	0.290
Alcohol consumption	48 (96.0%)	47 (81.0%)	0.037
Semen volume (mL)	3.05(1.5-5.8)	3.00(0.8-5.0)	0.694
Sperm count (/mL)	27.50(4.30-95.00)	24.00(0.18-87.00)	0.259
Total motility	58.50(7.0-81.0)	58.00(0.0-80.0)	0.698
Sperm morphology	2(0-4)	2(0-3)	0.014
DNA damage (%)	26.00(11.0-54.0)	31.50(9.0-71.0)	0.002

DNA: Deoxyribonucleic acid, FSH: Follicle-stimulating hormone, HDL: High-density lipoprotein, IG: Infertile group, LDL: Low-density lipoprotein, LH: Luteinizing hormone, SPG: Spontaneous pregnancy group
Data are given as mean ± standard deviation or median (minimum - maximum) for continuous variables according to normality of distribution and as frequency (percentage) for categorical variables.

searchers, and in great majority of these studies[1-5], vit D levels of infertile men were found to be significantly lower than in fertile controls. Consistent with these studies, we also found that vit D levels in men with unexplained infertility-were significantly lower than those in the fertile group. To be more precise, while we detected vit D insufficiency (20-30 ng/ml) in all infertile men, we detected vitamin D deficiency (<20 ng/ml) in only five infertile men. Since vit D has a very short half-life, the amount of circulating vit D in the serum at the time of measurement may have decreased physiologically, so it should be emphasized whether the levels detected in the studies are insufficiency or deficiency[16]. Although the decrease in serum vit D levels in infertile men has been confirmed by many studies, the relationship between vit D levels and semen parameters is not very clear. Some studies[11,14] reported a positive correlation between vit D levels and sperm count and motility, while others reported no correlation. Results may be inconsistent as most of these studies were performed either in patient groups with abnormal semenparameters[17]or in nonhomogeneous patient groups consisting of idiopathic infertile men[18]. We found a positive and significant correlation between vit D and sperm count, motility and morphology. The main reason for this strong correlation we found between vitamin D levels and semen parameters may be due to the fact that our patients were selected from a homogeneous group. Indeed, the most important feature that distinguishes our study from others is that the patient group consisted of men with unexplained infertility.

Failure to achieve pregnancy despite normal semen analysis parameters in unexplained infertile men suggests the presence of a molecular or genetic defect that impairs sperm functions. All of the participants in our study had vit D insufficiency and some of them had vit D deficiency. Despite normal number, motility and morphology, we can list the possible causes of inadequate function of sperm in unexplained infertile men as follows. Vit D is a fat-soluble molecule and plays an important role in calcium homeostasis in both male and female reproductive systems[12,19]. It shows its physiological effects through vita-

Table II. Correlation between vitamin D levels, semen analysis parameters and total testosterone levels.

	Unexplained infertility group (n=58)		Fertile group (n=50)		All patients (n=108)	
	R	p	r	p	r	p
Sperm count	0.527	<0.001	0.050	0.732	0.392	<0.001
Total motility	0.527	<0.001	0.241	0.091	0.409	<0.001
Morphology	0.416	0.001	-0.063	0.663	0.289	0.002
DNA damage	-0.605	<0.001	-0.177	0.220	-0.527	<0.001
Total testosterone	0.139	0.298	-0.015	0.917	0.050	0.609

DNA: Deoxyribonucleic acid.

min D receptors (VDR) and the functional active amount of vit D in male reproductive organs is close to serum levels[20]. The presence of VDR has been conclusively reported in testicular tissue, prostate and spermtazoa[21]. Additionally intense metabolization of vit D in the male reproductive system and increased expression of VDRs in the sperm neck are findings that strongly support the necessity of vit D for the sperm to be functionally active[22]. The presence of vit D metabolizing enzymes in mature spermatozoa is important evidence that sperms need vit D for their functions[22]. It is known that incubation of semen samples with vit D for 30 minutes causes a significant increase in sperm velocity parameters. This increase in progressive motility is due to vitamin D-dependent calcium release and subsequent cAMP/PKA activation and ATP production[23]. All these data are proof that physiological functions of sperm may not be realized in unexplained male infertile cases due to low serum and reproductive tract vit D levels.

Calcium supplementation to infertile animals with Vit D deficiency provides a direct improvement in the fertility status of the men, suggesting that the effect of Vit D on sperm functions is mediated by calcium[24]. Vit D may be involved in both progressive motility and the realization of the acrosome reaction in the zona pellucida by regulating calcium concentration in the neck and head of the sperm[25]. Insufficient fertilization and embryo development in round sperm without a tail or in ICSI procedures without a tail break may also be due to insufficient calcium release. The need for both vit D and calcium in the early stage of spermatogenesis and in the selection of germ cells[26] indicates the presence of a vit D dependent process at all stages of spermatogenesis.

Apart from all these mechanisms, we stained the samples with aniline blue dye to test whether vit D deficiency causes sperm DNA damage. We found a significant increase in sperm DNA damage in the unexplained infertile group compared to the fertile controls. In correlation analysis, we found a negative and significant correlation between vit D and sperm DNA damage. After binding to the VDR receptor, Vit D initiates a slow genomic effect by stimulating the release of ligand-activated transcription factor in the nucleus[27]. In unexplained infertile patients with Vit D deficiency, sperm DNA damage may occur because this slow genomic effect cannot be fully realized.Spermatozoa are transcriptionally inactive cells. For this reason, they function by nongenomic mechanisms during spermatogenesis or fertilization. This feature is critical for the protection of ejaculate sperm from genomic damage[28].However, we could not find a link between vit D levels and DNA damage in logistic regression analysis. When subgroup regression analysis was performed on 26 patients with serum vit D deficiency (<20 ng/ml) it was found that sperm DNA damage had a negative impact on fertility. This issue needs to be clarified with studies that will examine the relationship between Vit D and sperm DNA damage in more detail.

Conclusions

Despite the low number of participants, we found that serum vit D levels were significantly lower in men with unexplained infertility. In addition, we found a positive correlation between vit D and the number, motility and morphology of sperm, while we found a negative correlation with DNA damage. Since gonadal failure has been reported in mice with null mutants for the VDR, receptor polymorphism is as important as vit D levels in infertile men[29]. Although

our study did not evaluate VDR polymorphism or vit D binding protein levels, it is of clinical importance because it evaluated the relationship between vit D levels and sperm DNA damage. It is evident that there is a need for comprehensive studies investigating semen and serum Vit D levels, sperm DNA damage and clinical pregnancy rates.

Conflicts of interest

The authors declare no conflicts of interest.

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