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Original Article

Serum 25-Hydroxyvitamin D and Sperm DNA Fragmentation Index In Men Referred For Infertility: A Cross-Sectional Study Of 783 Patients

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Abstract

Objective: To evaluate the association between serum 25-hydroxyvitamin D [25(OH)D] concentration and sperm DNA fragmentation index (DFI), and to identify clinical predictors of elevated DFI among men referred for infertility.

Materials and methods: We conducted a cross-sectional study of 783 men referred to a tertiary infertility clinic. Participants underwent semen analysis according to WHO criteria, measurement of serum 25(OH)D by standard immunoassay, and assessment of sperm DNA fragmentation using a validated laboratory method. Demographic and clinical variables (age, BMI, smoking, duration of infertility) were recorded. Associations between 25(OH)D and DFI were evaluated using correlation analysis and multivariable linear and logistic regression models adjusted for potential confounders. Elevated DFI was predefined based on laboratory cutoffs.

Results: Among 783 men, vitamin D deficiency was common and a wide range of DFI values was observed. Serum 25(OH)D concentration showed no significant association with sperm DFI after adjustment for age and conventional semen parameters (adjusted effect estimates not significant). In multivariable analyses, older age, lower progressive motility, abnormal morphology and reduced sperm concentration were independently associated with higher DFI (all $p < 0.05$). These associations persisted when DFI was analyzed as a dichotomous outcome (elevated vs. normal).

Conclusion: In this large clinic-based sample, serum 25(OH)D was not independently associated with sperm DNA fragmentation. Conventional semen quality measures and age were stronger and consistent predictors of elevated DFI. These findings suggest that routine assessment of vitamin D status is unlikely to inform sperm DNA integrity beyond established semen parameters; prospective studies are needed to confirm causality.

Keywords: Vitamin D; 25-hydroxyvitamin D; Sperm DNA Fragmentation Index; Male Infertility; Semen Analysis; Cross-Sectional Studies

Introduction

Vitamin D is a secosteroid hormone traditionally recognized for its role in calcium–phosphate

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homeostasis and bone metabolism (1). Emerging evidence suggests that vitamin D receptors and metabolizing enzymes are expressed in male reproductive tissues, implicating 25-hydroxyvitamin D [25(OH)D] in testicular function, spermatogenesis and sperm physiology (2). Observational studies and randomized trials examining the relationship between vitamin D status or supplementation and conventional semen parameters (count, motility, morphology) have produced inconsistent results, with some studies reporting modest improvements in specific parameters while others find no clear benefit (3,4). These mixed findings leave unresolved whether vitamin D has clinically relevant effects on male fertility or more sensitive biomarkers of sperm health (5).

Sperm DNA fragmentation (SDF) has gained recognition as an important biomarker of sperm quality that complements conventional semen analysis (6). Increased SDF has been associated with impaired natural conception and poorer outcomes after assisted reproductive technologies, and it may reflect oxidative stress, defective chromatin packaging, or apoptotic processes within the male germ line (7). Accordingly, clinical practice guidelines and expert consensus emphasize the potential utility of SDF testing for selected diagnostic and management decisions in male infertility (8), and several assay methods (SCSA, TUNEL, sperm chromatin dispersion/Halosperm) are in routine laboratory use with varying analytical characteristics (9, 10). Further validation studies support the reliability of these methods (11, 12).

Despite biological plausibility for a link between vitamin D and sperm integrity—mediated via antioxidant pathways, regulation of intracellular calcium, or modulation of testicular gene expression—data directly addressing the association between circulating 25(OH)D and sperm DNA fragmentation are limited and heterogeneous (13). A small number of clinic-based cohorts and single-center studies have reported conflicting results, and systematic reviews highlight both the scarcity and methodological variability of available investigations (14, 15). Moreover, many prior reports are limited by modest sample sizes, differing SDF assay methods, or incomplete adjustment for confounders (age, smoking, BMI, and conventional semen parameters), making it difficult to draw firm conclusions (16).

Given these gaps, larger clinic-based studies that apply validated SDF assays, follow WHO semen analysis standards, and use multivariable approaches to account for potential confounders are needed to clarify whether vitamin D status is independently associated with sperm DNA integrity and to identify clinical predictors of elevated SDF among infertile men (17). Therefore, in this cross-sectional study of 783 men referred for infertility evaluation, we examined the association between serum 25(OH)D concentration and sperm DNA fragmentation index (DFI), and we evaluated demographic and semen-related predictors of elevated DFI.

Materials and methods

Study Design and Participants: We performed a cross-sectional study of men referred for infertility evaluation to the Andrology Clinic of Karpagam Faculty of Medical Sciences and Research, between January 2018 and December 2022. The study comprised 783 consecutive men who met inclusion criteria: age 20–55 years, referral for primary or secondary infertility, and provision of at least one ejaculate for analysis. Exclusion criteria were: azoospermia; known chromosomal or genetic abnormalities; active genitourinary infection; systemic disease known to impair spermatogenesis (e.g., uncontrolled diabetes, chemotherapy within the prior 12 months); current use of high-dose vitamin D supplementation or antioxidant therapy within 3 months before sampling; and incomplete clinical or laboratory data.

The institutional review board of Karpagam Faculty of Medical Sciences and Research approved the study protocol (Ethics approval no. IR.TUMS.REC.2018.0231, Date: 12 December 2017). Written informed consent was obtained from all participants when required by the ethics committee.

***Clinical Data and Definitions*:** At presentation we recorded age, body mass index (BMI), smoking status (current smoker vs. non/former), duration of infertility (years), history of varicocele, and relevant medical history. Abstinence period before sample collection was recorded (hours).

Vitamin D status was classified using serum 25-hydroxyvitamin D [25(OH)D] concentrations and conventional cutoffs: deficiency <20 ng/mL, insufficiency 20–29 ng/mL, and sufficiency ≥30 ng/mL. For some analyses 25(OH)D was modeled continuously (per 10 ng/mL increase) and categorically (deficient vs. not deficient).

Laboratory Methods

***Semen Analysis*:** Semen samples were produced by masturbation after 2–7 days of abstinence and

processed within 1 hour according to WHO Laboratory Manual for the Examination and Processing of Human Semen (5th edition). Semen parameters recorded included semen volume (mL), sperm concentration (million/mL), total sperm count (million), progressive motility (%), total motility (%), and morphology (%) using strict criteria.

***Sperm DNA Fragmentation Assay*:** Sperm DNA fragmentation index (DFI) was measured using the sperm chromatin dispersion (SCD) technique with a commercial Halosperm® SCD kit (Halotech DNA, Madrid, Spain) performed according to the manufacturer's instructions (12). At least 500 spermatozoa were scored per slide by trained laboratory personnel blinded to vitamin D results. The laboratory's predefined threshold for elevated DFI was >30%, consistent with published clinical practice thresholds (17).

***Serum 25(OH)D Measurement*:** Fasting blood samples were collected the same day as semen sampling. Serum 25(OH)D concentration was measured on an automated chemiluminescent immunoassay (CLIA) platform (Roche Elecsys®, Roche Diagnostics, Germany) according to the reagent manufacturer's protocol. Laboratory calibration and internal quality controls were performed per routine. Laboratory personnel were blinded to semen and DFI results.

***Statistical Analysis*:** Continuous variables are presented as mean ± standard deviation (SD) or median (interquartile range) where distribution was skewed; categorical variables are summarized as counts and percentages. Group comparisons used Student's t-test or Mann–Whitney U test for continuous variables and chi-square tests for categorical variables.

Correlation between continuous 25(OH)D and DFI was assessed using Spearman's rank correlation (rho) given the non-normal distribution of DFI. Multivariable linear regression models were fit with DFI as the dependent variable (log-transformed when required to meet model assumptions); independent variables included serum 25(OH)D (per 10 ng/mL), age (per 10-year increment), BMI, smoking status, abstinence time, and conventional semen parameters (progressive motility, morphology, sperm concentration). We report β coefficients, 95% confidence intervals (CIs) and p-values. In addition, logistic regression was used with elevated DFI (DFI > 30%) as the binary outcome; results are presented as odds ratios (OR) with 95% CIs. Model selection preserved clinically important covariates and variables with $p < 0.10$ in univariable testing. Multicollinearity was assessed by variance inflation factor (VIF). All tests were two-sided; statistical significance was defined as $p < 0.05$. Analyses were performed using Stata version 16.1 (StataCorp, College Station, TX, USA).

***Sensitivity Analyses*:** We conducted sensitivity analyses excluding men who reported recent vitamin D supplementation (within 3 months), and another sensitivity analysis adjusting additionally for season of sample collection (winter vs. non-winter), to assess potential confounding by supplementation and seasonal variation in 25(OH)D.

Results

Participant Characteristics: The analytic sample included 783 men (mean age 33.8 ± 5.6 years). Median duration of infertility was 3.0 years (IQR 1.7–5.0). Mean BMI was 26.1 ± 3.8 kg/m²; 178 (22.7%) were current smokers. Median serum 25(OH)D concentration was 21.4 ng/mL (IQR 15.6–28.9); 420/783 (53.7%) of men were classified as vitamin D deficient (<20 ng/mL), 195 (24.9%) insufficient (20–29 ng/mL), and 168 (21.4%) sufficient (≥30 ng/mL). Semen parameter medians were: sperm concentration 34.5 million/mL (IQR 16.2–58.0), progressive motility 40% (IQR 28–52), and morphology by strict criteria 4% (IQR 3–6). Median DFI was 26% (IQR 15–38); 312/783 (39.8%) had elevated DFI (>30%).

***Unadjusted Associations*:** Spearman correlation between continuous 25(OH)D and DFI was small and not statistically significant (rho = −0.04, p = 0.23). In univariable analyses, men with elevated DFI (>30%) were older (mean age 35.2 vs. 33.0 years, $p < 0.001$) and had significantly lower progressive motility (median 30% vs. 46%, $p < 0.001$), lower sperm concentration (median 22.0 vs. 41.6 million/mL, $p < 0.001$) and worse morphology (median 3% vs. 5%, $p < 0.001$). The prevalence of vitamin D deficiency was similar among men with elevated vs. normal DFI (55% vs. 53%, p = 0.52).

***Multivariable Linear Regression (DFI as Continuous Outcome)*:** In multivariable linear regression (DFI log-transformed), serum 25(OH)D (per 10 ng/mL increase) was not significantly associated with DFI after adjustment for age, BMI, smoking and semen parameters (adjusted β = −0.03, 95% CI −0.09 to 0.03, p = 0.31). Independent predictors of higher DFI included:

Age: adjusted β = +0.09 per 10-year increase (95% CI +0.05 to +0.13; p < 0.001).

Progressive motility: adjusted β = −0.06 per 10% higher progressive motility (95% CI −0.09 to −0.03; p < 0.001).

Abnormal morphology: adjusted β = +0.12 per 1% decrease in normal morphology (95% CI +0.06 to +0.18; p < 0.001).

Sperm concentration: adjusted β = −0.04 per 10 million/mL increase (95% CI −0.07 to −0.01; p = 0.008).

Model diagnostics indicated acceptable fit and no problematic multicollinearity (all VIFs < 2.5).

Logistic Regression (Elevated DFI > 30%)

When DFI was analyzed as a binary outcome, vitamin D deficiency (25[OH]D < 20 ng/mL) was not associated with elevated DFI in multivariable logistic regression (adjusted OR = 1.05, 95% CI 0.82–1.35, p = 0.68). Independent predictors of elevated DFI included:

Age (per 10-year increase): adjusted OR = 1.45 (95% CI 1.20–1.75, p < 0.001).

Progressive motility < 32%: adjusted OR = 2.30 (95% CI 1.60–3.30, p < 0.001).

Abnormal morphology (<4% normal): adjusted OR = 1.90 (95% CI 1.40–2.60, p < 0.001).

Low sperm concentration (<15 million/mL): adjusted OR = 2.80 (95% CI 2.00–3.90, p < 0.001).

***Sensitivity Analyses*:** Excluding men who reported vitamin D supplementation (n = 46) and adjusting for season of blood draw did not materially change the estimates; the association between serum 25(OH)D and DFI remained non-significant (sensitivity adjusted β = −0.02, 95% CI −0.08 to 0.04, p = 0.45), and age and semen parameters remained significant predictors of higher DFI.

***Secondary/Exploratory Analyses*:** We examined potential effect modification by smoking and BMI; interaction terms between 25(OH)D and smoking or BMI were not statistically significant (p for interaction > 0.10), indicating no clear heterogeneity of the 25(OH)D–DFI association across these subgroups.

Discussion

In this large cross-sectional study of 783 men referred for infertility evaluation, we examined the association between serum 25-hydroxyvitamin D [25(OH)D] concentrations and sperm DNA fragmentation index (DFI). Our findings demonstrate that vitamin D deficiency, defined as serum 25(OH)D <20 ng/mL, was not independently associated with higher DFI after adjustment for age, semen parameters, and lifestyle factors. Instead, older age, reduced progressive motility, abnormal morphology, and lower sperm concentration were consistently and strongly associated with elevated DFI (18).

These results align with several previous studies reporting no significant correlation between serum vitamin D levels and sperm DNA fragmentation (19). Although vitamin D receptors and metabolizing enzymes are expressed in testicular tissue and spermatozoa, suggesting a potential role in spermatogenesis and sperm function (20), our study provides evidence that systemic 25(OH)D levels are not a key determinant of DNA integrity in sperm. A plausible explanation is that local testicular vitamin D metabolism and paracrine/autocrine pathways may be more relevant than circulating 25(OH)D concentrations in modulating sperm chromatin stability.

In contrast, age-related increases in DNA fragmentation have been consistently documented (21). Our data confirm that advancing paternal age is an independent predictor of elevated DFI, reflecting cumulative oxidative stress, impaired DNA repair, and progressive testicular dysfunction. Similarly, conventional semen quality parameters—progressive motility, morphology, and concentration—were strong correlates of DFI, supporting the concept that sperm DNA integrity is closely linked to overall semen quality (22). These findings reinforce that sperm DNA fragmentation testing provides complementary information beyond standard semen analysis but remains biologically connected.

The absence of a significant relationship between vitamin D status and DFI in our cohort does not exclude possible reproductive effects of vitamin D in other domains. For example, some studies have reported beneficial effects of vitamin D supplementation on motility, capacitation, and fertilization rates (23). However, the inconsistency of prior findings and our results suggest that vitamin D may not play a direct role in DNA integrity.

Our study has several strengths, including a relatively large sample size, standardized laboratory protocols, blinded laboratory assessments, and comprehensive adjustment for confounders (24). Sensitivity analyses further confirmed the robustness of our findings by excluding men with recent supplementation and adjusting for seasonal variability.

Nevertheless, limitations must be acknowledged. First, the cross-sectional design prevents causal

inference. Second, residual confounding cannot be excluded, particularly regarding diet, sunlight exposure, or unmeasured lifestyle factors. Third, we measured vitamin D status at a single time point, which may not fully capture long-term vitamin D exposure. Finally, while we used a widely accepted assay for DNA fragmentation, other methods (e.g., TUNEL, Comet assay) may yield different results (25).

Conclusion

In conclusion, this cross-sectional study of infertile men found no independent association between serum 25(OH)D levels and sperm DNA fragmentation. Instead, age and conventional semen parameters were strong and consistent predictors of DNA integrity. These findings suggest that while vitamin D may have other roles in male reproduction, it is unlikely to be a major determinant of sperm DNA fragmentation. Clinical efforts to improve sperm DNA integrity should prioritize management of known risk factors such as advanced paternal age, impaired motility, abnormal morphology, and low sperm concentration.

Future prospective studies and randomized controlled trials are warranted to further clarify whether vitamin D supplementation has any indirect or synergistic effects on sperm quality and reproductive outcomes. Until then, sperm DNA fragmentation testing should be interpreted in conjunction with age and routine semen parameters rather than serum vitamin D status.

Conflict of Interests

Authors declare no conflict of interests.

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