Vitamin D levels and human sperm DNA fragmentation: a prospective, cohort study

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Abstract

Background: Intracytoplasmic sperm injection (ICSI) has revolutionized the treatment of couples with male factor infertility but results remain suboptimal and suggest the need for further investigation into the molecular biology of spermatozoa. Vitamin D has been implicated in spermatogenesis and sperm function. Hypovitaminosis D has been associated with abnormal testicular function, including elevated sperm DNA fragmentation in a murine model. This study’s objective was to evaluate if there is a correlation between Vitamin D sufficiency and human spermatozoa DNA fragmentation index % (DFI%) in infertile couples.

Results: A prospective cohort study using a consecutive, convenience sample of subjects with infertility. The primary endpoint was the effect of Vitamin D sufficiency on human spermatozoa DFI%, and secondary outcomes included Vitamin D’s effect on moderate DFI%, high DFI%, High DNA stainability % (HDS%), sperm density (million/mL), sperm total motility (% total) and sperm strict morphology (% total). Of the 111 participating, 9 were excluded, leaving 102 subjects. The subjects were stratified by vitamin D levels: deficient (< 20 ng/mL; n = 24), insufficient (20–30 ng/mL; n = 43), and sufficient (> 30 ng/mL; n = 35). There were no statistical difference between the categories of serum vitamin D levels and sperm DFI% as well as the secondary outcomes. An increased BMI was associated with low serum vitamin D levels (p = 0.0012).

Conclusion: Vitamin D deficiency was not associated with sperm DFI% or routine sperm parameters. Previous animal and human studies have demonstrated conflicting results between sperm parameters and Vitamin D levels. Redundant pathways in Vitamin D and calcium homeostasis in the human male reproductive tract may maintain essential reproductive processes during Vitamin D insufficiency or deficiency.

Trial registration: Trial Registration Number: MOD00002311 (ClinicalTrials.gov).

Keywords: Sperm chromatin structure assay, Sperm DNA fragmentation %, Vitamin D, Male infertility, Hypovitaminosis D

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Introduction

Idiopathic male factor infertility is common and defined as abnormal semen parameters with no determined underlying etiology [1]. Current treatments for unexplained male factor are often empiric, based on tenuous data at best [2]. The development of intracytoplasmic sperm injection (ICSI) has allowed infertile males to become biologic fathers. However, in vitro fertilization (IVF) with ICSI is a complex, expensive procedure in which healthy women undergo ovulation induction and surgical retrieval of oocytes with attendant risks, highlighting the importance of continued investigation into potential causes and treatments of idiopathic male factor [3]. Improvements in our understanding of spermatozoa molecular biology may improve treatments and successful conception through natural or assisted reproduction.

The semen analysis has been the gold standard for determining male fertility [4]. While important in the evaluation of the male, a semen analysis by itself cannot assess the capacity of the sperm to fertilize an ova and does not give a complete understanding of the health and function of the male reproductive organs [5]. One area of investigation that shows promise in evaluating male subfertility is sperm DNA fragmentation tests including the TUNEL assay, the Comet assay, the Sperm Chromatin Dispersion test, and the Sperm Chromatin Structure Assay (SCSA) [6–8]. The Sperm Chromatin Structure assay (SCSA) is a precise and repeatable flow cytometry analysis, which is used to measure acid-induced DNA fragmentation. The primary benefit of the SCSA is when the DNA Fragmentation Index % (DFI%) is > 25% IVF with ICSI is more likely to be successful than intrauterine insemination. An elevated DFI% also allows the patient the opportunity to make lifestyle changes and consider medical therapies to improve the value [9].

Vitamin D has an established role in calcium metabolism and skeletal health, as well as pleiotropic genomic and non-genomic effects in various organs throughout the body, including the reproductive system [10]. A study in Denmark found samples with more than two thirds of the sperm staining positive for the Vitamin D inactivating enzyme, Résumé

Contexte: L’injection intracytoplasmique de spermatozoïdes (ICSI) a révolutionné le traitement des couples avec infertilité masculine, mais les résultats restent sous-optimaux et suggèrent la nécessité d’aller plus loin dans l’étude de la biologie moléculaire des spermatozoïdes. La vitamine D a été impliquée dans la spermatogenèse et les fonctions des spermatozoïdes. Dans un modèle murin, l’hypovitaminose D a été associée à une fonction testiculaire anormale, y compris une fragmentation élevée de l’ADN des spermatozoïdes. L’objectif de cette étude était d’évaluer l’existence d’une corrélation entre un taux suffisant en vitamine D et l’indice de fragmentation de l’ADN (DFI) des spermatozoïdes humains chez les couples infertiles.

Résultats: Une étude de cohorte prospective utilisant un échantillon consécutif et approprié de sujets atteints d’infertilité. Le critère d’évaluation principal était l’effet d’un taux suffisant en vitamine D sur le DFI des spermatozoïdes humains. Les critères de jugement secondaires comprenaient l’effet de la vitamine D sur un DFI modéré, un DFI élevé, un taux élevé de coloration de l’ADN (HDS), le nombre de spermatozoïdes (millions/mL), la motilité totale des spermatozoïdes et sur la morphologie stricte des spermatozoïdes. Sur les 111 participants, 9 ont été exclus, laissant 102 sujets. Les sujets ont été stratifiés par niveau de vitamine D : déficient (<20 ng/mL ; n=24), insuffisant (20-30 ng/mL ; n=43) et suffisant (>30 ng/mL ; n=35). Il n’y a eu aucune différence statistique entre les catégories de taux sériques de vitamine D et le pourcentage de DFI des spermatozoïdes ainsi qu’avec les critères de jugement secondaires. Une augmentation de l’indice de masse corporelle était associée à de faibles taux sériques de vitamine D (p = 0,0012).

Conclusion: L’absence en vitamine D n’est pas associée au pourcentage l’indice de fragmentation de l’ADN des spermatozoïdes ou aux paramètres de routine des spermatozoïdes. Des études antérieures chez les animaux et les humains ont montré des résultats contradictoires entre les paramètres des spermatozoïdes et les niveaux de vitamine D. Les voies redondantes dans l’hémostase de la vitamine D et du calcium dans l’appareil reproducteur masculin humain peuvent maintenir les processus essentiels à la reproduction lors d’insuffisance ou de carence en vitamine D.

Mots-clés: Test de structure de la chromatine des spermatozoïdes, fragmentation de l’ADN des spermatozoïdes, vitamine D, infertilité masculine, hypovitaminose D.
CYP24A1 had four times greater likelihood of achieving pregnancy with intrauterine insemination \[11\]. A 2020 review found that vitamin D may have a positive effect on fertility potential, possibly due to an impact on sperm motility \[13\]. However, animal and human studies on Vitamin D’s effect on the male reproductive system have been inconsistent \[13–16\].

The purpose of this study was to evaluate Vitamin D sufficiency on human spermatozoa DNA fragmentation \%(DFI\%)\. Secondary endpoints included effect of Vitamin D levels on High DNA Stainability \%(HDS\%) and other sperm parameters including density \(\text{million/mL}\), total motility \%(total\), and morphology \%(total\).

**Materials and methods**

This was a convenience sample with consecutive recruitment of subjects, who presented to a Midwestern mid-size Reproductive Endocrinology and Infertility Clinic for infertility; both primary or secondary between March 26, 2019 and April 1, 2020. The inclusion criteria were male patients between 21 and 55 who presented with a history of infertility \(\text{attempted pregnancy for greater than or equal to one year, or greater than or equal to six months if the female partner was over 35 years of age}\)\. Exclusion criteria were severe oligozoospermia \(\leq 1 \text{ million/mL}\) in which there were inadequate numbers of spermatozoa to perform the SCSA. All subjects signed a written consent for participation. Our primary outcome was the Vitamin D level and Total DFI\% measured with the SCSA\%. Secondary outcomes included moderate DFI\% and High DFI\% and High DNA Stainability \%(HDS\%), sperm density \(\text{million/mL}\), total motility \%(total\), and strict morphology \%(total\). When gated the raw SCSA data can be separated into normal, moderate and high DFI\% \[9\]. Previous studies evaluating sperm morphology after cell sorting, demonstrate that normal and moderate DFI\% have normal morphology, while high DFI\% have elongated nuclei and signs of apoptosis. Currently, Total DFI\% is usually reported, but in the future with advances in cell sorting and ICSI one may be able to select sperm with moderate DFI\% and improve outcomes \[8, 9\].

The demographic and laboratory data was extracted from the patients’ electronic medical records \(\text{EMR}\) \(\text{(Epic Systems Corporation, Verona, Wisconsin)}\) and independently recorded by 2 investigators \(\text{eb,kh}\) onto a piloted data sheet. Also the meteorological season of the year when the blood sample for Vitamin D was obtained was recorded: Spring-March 1 to May 31, Summer-June 1 to August 31, Fall-September 1 to November 30, and Winter-December 1 to February 28. The two investigators independently entered patient data from the data sheet into separate, de-identified spreadsheet files using Microsoft Excel. After completion, the two Excel spreadsheet files were electronically compared and identified differences were resolved by comparison to original data in the EMR. Body Mass Index \(\text{BMI}\) was calculated from \(\text{weight (in kilograms)}\) and \(\text{height (in meters squared)}\) using the NIH BMI calculator \(\text{BMI} = \frac{\text{kg}}{\text{m}^2}\).

Semen samples were obtained by masturbation after 2–4 days of abstinence. The samples were ejaculated into a nontoxic specimen container and placed in a 37 °C water bath for 20–30 min to allow for liquefaction. The semen analysis was performed manually by two trained technicians in the Andrology laboratory after liquefaction using the fifth edition of the World Health Organization \(\text{WHO}\) guidelines. The normal semen parameters included semen volume of \(\geq 1.5 \text{ mL}\), sperm density of \(\geq 15 \text{ million/mL}\), total sperm motility of \(\geq 40\%)\, and morphology of \(\geq 4\)% using Kruger’s strict criteria \[17\]. Oligozoospermia was defined as \(< 15 \text{ Million sperm per mL}\), asthenozoospermia was defined as a total sperm motility of \(< 40\%)\, and teratozoospermia defined as \(< 4\%)\, normal forms by strict morphology.

A 0.2–0.5 mL sample of the raw semen was placed in a 1 mL cryovial and snap frozen in liquid nitrogen, which was then shipped to SCSA\%\, Diagnostics for assay. The Sperm Chromatin Structure assay \(\text{SCSA}\) which is a highly precise and repeatable, flow cytometry analysis was used to measure acid-induced DNA fragmentation. The semen sample is treated with acidic buffer solution \(\text{pH} = 1.2\) to allow the DNA to open at sites of DNA fragmentation and then treated with Acridine Orange \(\text{AO}\) staining solution composed of 0.20 M \(\text{Na}_2\text{HPO}_4\), 0.1 M citric acid buffer \(\text{pH} 6.0\), 1 mM \(\text{EDTA}\), 0.15 M \(\text{NaCl}\) and 6.0 \(\text{ug/mL}\) chromatographically purified \(\text{AO}\). After AO treatment the sample was run through the flow cytometer where it is exposed to a 488 nm wavelength excitation beam from a 15–35 mW laser. Red \(\text{630–650 nm}\) and green \(\text{515–530 nm}\) filters collect the fluorescent signal from the excited, AO stained sperm cells. AO is a metachromatic dye that fluoresces green when associated with native, double-stranded DNA and red when associated with single-stranded DNA. An increase in red/green fluorescence is consistent with increased DNA fragmentation. Parameters are collected based on red/green fluorescence intensity of the sperm sample. The raw data is sent to SCSAsoft\%\, for analysis. SCSAsoft\%\, analyzes the raw data and calculates the DNA Fragmentation Index \%(DFI\); moderately elevated DFI\% and high DFI\% as well as HDS\% \[8, 18\].

All semen samples were assayed in duplicate with about 5,000 sperm cells in each measurement. Prior to using the flow cytometer alignment is determined using standard fluorescent beads. An AO buffer must pass through the instrument lines for at least 15 min prior to establishing settings with reference samples. The reference sample is chosen for heterogeneity of DNA integrity \(\text{eg. DFI}\%\, of around 15\%) and are diluted to 1–2 Million sperm/
mL for use. Clinical Laboratory Improvement Amend-
ments (CLIA) certification, which ensures quality labo-
ratory testing in the USA, also requires reference samples with low and high DFI% be run for improved quality of analysis. During the use of the SCSA a fresh reference sample is run every 5 to 10 subject samples to exclude drift.

Blood
Blood was collected in the non-fasting state by standard venipuncture technique into a serum separator test tube, immediately centrifuged and the serum collected. The serum 25-OH Vitamin D level was assayed using a chemilumi-
nescent microparticle immunoassay (Architect, Abbott, Longford, Ireland). This assay was standardized using National Institute of Standards and Technology Standard Reference Material 2971. The limit of quantification with ≤ 20% Coefficient of Variation (CV) was 2.4 ng/mL, with a correlation coefficient of r = 0.99 (95% CI: 0.99,0.99) for liquid chromatography-tandem mass spectrometry. The CV for within-run and between-run assays for low control (20 ng/mL) was 2.2–2.4% and 2.7–3.6%, medium control (40 ng/mL) was 1.8–2.1% and 2.6–3.2%, and high control (75 ng/mL) was 2.2–2.8% and 2.4–4.1%, respectively. Vitamin D deficiency was defined as 25-OH Vitamin D ≤ 20 ng/mL, insufficiency 21–29 ng/mL, and sufficiency as ≥ 30 ng/mL [19].

Statistics
Sample size determination. Previous studies have dem-
onstrated a statistically significant 8% reduction in sperm motility in subjects with low Vitamin D levels [20]. An alpha of 0.05 and a power of 80% was used to calculate ‘n’. The sample size was determined to be 79 in the Vitamin D deficient group and 79 in the Vitamin D sufficient group. A previous study in this region demonstrated up to 75% Vitamin D deficiency in healthy working, South Dakota males, so the ‘n’ was adjusted to 105 in both groups [21]. Estimating that 10% would decline the SCSA® the ‘n’ was increased to 115 in each group for a total of 230.

An interim analysis was obtained after 111 subjects were recruited and their data were imported into the sta-
tistical program, SAS V 9.5. The relationship between the dichotomous variables: alcohol use, tobacco use, and BMI were examined using a chi-square test of independence. The relationship between deficient, insufficient and suf-
icient Vitamin D levels and sperm parameters were ana-
lyzed using an analysis of variance. Correlations between 25-OH-Vitamin D as a continuous variable and semen variables were examined using Pearson correlations. P < 0.05 was considered significant. The interim analy-
sis demonstrated no difference in primary or secondary endpoints. A repeat sample size determination using the

Results
Of the 111 subjects recruited, exclusions included 3 with no SCSA performed: one azoospermia, one severe oligo-
zoospermia, and one declined SCSA and six with no Vita-
min D determination. Of the remaining 102 subjects, 52 had primary and 50 had secondary infertility. Forty (39%) had male factor infertility of which 30 had teratozo-
spermia, 5 oligoteratozoospermia, 2 asthenozoospermia, 2 oligoasthenoteratozoospermia and 1 with oligospermia. The remaining 61% had normal semen analysis. Vitamin D levels in the 102 subjects demonstrated 24 had defi-
cient Vitamin D (15.28 ± 1.23 ng/mL (mean ± 95% CI)), 43 insufficient Vitamin D (24.87 ± 0.92 ng/mL), and 35 sufficient Vitamin D levels (42.27 ± 5.01 ng/mL). Three subjects had no BMI recorded, and the average BMI of 29.5 was imputed for these cases. The average age was 31.5 with a range of 23 to 50 years and BMI ranged from 18.3 to 52.4 kg/m², with increasing BMI significantly
associated with lower Vitamin D levels (Table 1).

There was no significant difference in our primary endpoint of Total DFI% and Vitamin D levels; deficient, insufficient, and sufficient. There were also no differ-
ces between Vitamin D sufficiency and our secondary outcomes including moderate DFI%, High DFI%, HDS%, sperm density, motility, and morphology (Table 2). Also using the Institute of Medicine’s more restrictive defini-
tion of Vitamin D deficiency (Deficient < 13 ng/mL) there was also no significant difference in our primary or sec-
condary endpoints (data not shown).

Vitamin D levels varied across the seasons as one would expect with varied sun exposure with the highest levels in Summer (30.45 ± 4.10 ng/mL, mean ± 95% confidence interval) and Fall (32.14 ± 6.29 ng/mL), and lowest in Winter (27.86 ± 5.65 ng/mL) and Spring (26.24 ± 5.48 ng/mL). However, these seasonal differences in Vitamin D did not reach statistical significance. Tobacco con-
sumption was not associated with changes in Vitamin D levels, but abstinence from alcohol resulted in a sig-
nificant increase in HDS, from 8 ± 0.92% to 12 ± 3.56% (mean ± 95% confidence interval; p = 0.0042).

Discussion
In this prospective, cohort study in males with deficient or insufficient circulating Vitamin D levels there was no difference detected in sperm DFI% compared to those with sufficient Vitamin D. There was also no correlation between Vitamin D levels and HDS%, as well as other semen parameters determined by the routine semen
analysis. Results confirmed previous studies which demonstrated decreasing Vitamin D levels with increasing obesity [22, 23]. This study did not demonstrate significant seasonal differences in Vitamin D levels, possibly because of the number of individuals with primarily indoor occupations, which could decrease seasonal variability in sun exposure [22–24].

The role of Vitamin D in the male reproductive system remains controversial with inconsistent results from both animal and human studies. In murine models, hypovitaminosis D has been associated with low circulating testosterone levels, through an indirect genomic effect on calcium metabolism in the Leydig cell affecting testosterone synthesis [25]. In Vitamin D deficient animals circulating Vitamin D levels are positively associated with expression and activity of aromatase, impacting estrogen metabolism. Vitamin D deficient rats with hypocalcemia have impaired spermatogenesis with decreased pregnancy rates, which improved with normalization of calcium levels suggesting that Vitamin D’s role in the reproductive tract is through calcium metabolism [12]. Vitamin D deficient rats have also been discovered to have an elevated DFI% [26]. Our study in the human demonstrated no difference in sperm DFI%, HDS% or other routine semen analysis parameters with varying Vitamin D levels.

In the majority of human studies, Vitamin D deficiency has been associated with increased Sex Hormone Binding Globulin, but no change in circulating testosterone, resulting in a net decrease in biologically active,

### Table 1  Demographic characteristics of male patients based on Vitamin D levels in Infertile couples

|                         | Total (n = 102) | Deficient Vitamin D (≤ 20 ng/mL) (N = 24) | Insufficient Vitamin D (21–29 ng/mL) (N = 43) | Sufficient Vitamin D (≥ 30 ng/mL) (N = 35) |
|-------------------------|----------------|-------------------------------------------|-----------------------------------------------|-------------------------------------------|
| Mean Age (range)        | 31.5 (23–50)   | 32.3 (26–47)                               | 31.4 (23–45)                                  | 31.1 (23–50)                              |
| BMI kg/m² (range)       | 30.5 (18.3–52.4) | 33.9 (22.8–52.4)*                         | 31.2 (20.8–51.2)*                            | 27.3 (18.3–38.2)                         |
| Race (number(%))        |                |                                           |                                               |                                           |
| Caucasian               | 92 (90.2)      | 19 (18.6)                                 | 42 (41.2)                                    | 31 (30.4)                                |
| African American        | 2 (1.9)        | 1 (98)                                    | 1 (98)                                       | 0 (0)                                    |
| American Indian         | 5 (4.9)        | 2 (1.9)                                   | 0 (0)                                        | 3 (2.9)                                  |
| Other                   | 3 (2.9)        | 2 (1.9)                                   | 0 (0)                                        | 1 (98)                                   |
| Tobacco Use (number(%)) |                |                                           |                                               |                                           |
| Using smokeless or smoking tobacco | 22 (21.6) | 7 (6.9)                                   | 10 (9.8)                                     | 5 (4.9)                                  |
| Alcohol Use (number(%)) |                |                                           |                                               |                                           |
| Using smokeless or smoking tobacco | 88 (86.3) | 19 (18.6)                                 | 39 (38.2)                                    | 30 (29.4)                                |
| Occupation (number(%))  |                |                                           |                                               |                                           |
| Inside                  | 32 (31.4)      | 3 (2.9)                                   | 15 (14.7)                                    | 14 (13.7)                                |
| Outside                 | 70 (68.6)      | 21 (20.6)                                 | 28 (27.5)                                    | 21 (20.6)                                |

* using smokeless or smoking tobacco
** p = 0.0012, Deficient and Insufficient Vitamin D were significantly different when compared to Sufficient levels
** Not significant

### Table 2  Comparison of Vitamin D levels and Sperm parameters

| Semen Variable            | Deficient Vitamin D (≤ 20 ng/mL) (N = 24) Mean (± 95% CI) | Insufficient Vitamin D (21–29 ng/mL) (N = 43) Mean (± 95% CI) | Sufficient Vitamin D (≥ 30 ng/mL) (N = 35) Mean (± 95% CI) | p* | p** |
|---------------------------|------------------------------------------------------------|---------------------------------------------------------------|-----------------------------------------------------------|----|-----|
| Total DFI%                | 10 (7.13)                                                  | 10 (9.8, 12)                                                 | 11% (8.8, 13)                                             | 0.7148 | 0.4561 |
| Moderate DFI%             | 6 (4.8)                                                   | 6 (5.8, 7.8)                                                 | 6 (4.9, 7.1)                                             | 0.9764 | 0.9976 |
| High DFI%                 | 3 (1.6, 4.6)                                              | 4 (3.5)                                                      | 5 (3.6, 6.4)                                             | 0.1503 | 0.0982 |
| HDS%                      | 8 (6.10)                                                  | 9 (7.11)                                                     | 9 (7.2, 11)                                              | 0.6776 | 0.6961 |
| Sperm Density (Millions/mL)| 86.5 (47.1, 125.4)                                         | 93 (76.8, 118.8)                                                 | 75 (54.8, 96.5)                                         | 0.5969 | 0.5981 |
| Motility (%)              | 65 (59.1, 70.9)                                           | 65 (62.6, 8)                                                 | 64 (60.6, 8)                                             | 0.7918 | 0.8522 |
| Morphology (%)            | 4 (3.2, 4.8)                                             | 5 (4.4, 6.6)                                                  | 6 (3.3, 8.7)                                             | 0.6023 | 0.3818 |

* There was no statistical difference between deficient, insufficient and sufficient Vitamin D levels and any of the Semen and sperm variables
** Combining deficient and insufficient Vitamin D compared to sufficient Vitamin D demonstrated no statistical significant difference

DFI% = DNA Fragmentation Index %; HDS% = High DNA Stainability %
free testosterone [26]. Vitamin D could impact sperm quality by lowering biologically active testosterone and through non-genomic effects on intracellular calcium and lipid metabolism. In some studies, inadequate Vitamin D has been associated with significant impairment in sperm motility, capacitation, and the acrosome reaction [16, 27, 28]. Similar to our study, other studies have not demonstrated significant changes in sperm parameters with deficient Vitamin D. In particular, one case series described four fertile males with 1,25-dihydroxy-vitamin D-resistant rickets due to a non-functioning VDR. These four males had normal serum calcium and phosphorous levels in adulthood, normal semen parameters, and 15 pregnancies resulting in 9 healthy children [29]. Our results combined with previous studies and the knowledge of the integral role of calcium homeostasis in human physiology supports the presence of redundant or local systems to maintain adequate calcium in the male reproductive system [10]. Similar to testosterone metabolism, the presence of Vitamin D metabolizing enzymes in the male reproductive tract could result in higher local levels compared to circulating levels, maintaining normal homeostatic levels during hypovitaminosis. Jueraite-baik K et al. previously demonstrated no association between seminal and serum Vitamin D levels, supporting different levels within the testicles to enhance spermatogenesis [30].

Limitations of this study include a small sample size. A power analysis using the extreme results from our interim analysis demonstrated a need for a tenfold increase in each group to have a power of 80% and alpha of 0.05. Thus, this study was underpowered to detect a statistical difference. Another limitation is using circulating Vitamin D levels as a surrogate for intratesticular levels. Further studies evaluating seminal plasma levels of Vitamin D could enhance these results [30]. This study is also limited by performance in a single, mid-size Reproductive Endocrinology and Infertility clinic.

Conclusion
This prospective cohort study demonstrated that human sperm DFI% was not associated with circulating Vitamin D levels; deficient, insufficient, and sufficient. This study also demonstrated no association between circulating Vitamin D levels and HDS% as well as classic sperm parameters as measured by routine semen analysis. This lack of association between these Vitamin D categories and sperm parameters may be related to insufficient difference in levels between deficient, insufficient and sufficient. To further investigate if a more dramatic difference between Vitamin D levels would demonstrate a difference in sperm parameters we used the Institute of Medicine’s more restrictive definition of Vitamin D deficiency (< 13 ng/mL) and there was no statistically difference. Further studies evaluating Vitamin D levels locally in the male reproductive tract are needed to further evaluate the role Vitamin D may play in male reproduction.

Abbreviations
AO: Acridine Orange; BMI: Body Mass Index; CI: Confidence Interval; CV: Coefficient of Variation; DFI%: DNA Fragmentation Index%; DNA: Deoxyribonucleic acid; EMR: Electronic Medical Record; EDTA: Ethylenediaminetetraacetic acid; HDS%: High DNA Stainability %; ICSI: Intracytoplasmic Sperm Injection; IVF: In Vitro Fertilization; mL: Milliliters; mW: Milli-Watts; nm: Nanometers; ng: Nanograms; n: Sample size; Na2HPO4: Disodium Phosphate; NaCl: Sodium Chloride; SCSA: Sperm Chromatin Structure Assay; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling; VDR: Vitamin D Receptor; WHO: World Health Organization.

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Author contributions
All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Elise Blaseg and Keith Hansen. The first draft of the manuscript was written by Elise Blaseg and Keith Hansen and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets generated and analyzed during the current study will be available at ClinicalTrials.gov.

Declarations

Previous Presentation
This study was presented at the Proceedings of the American Society of Andrology Annual Conference 2021; 2021 Apr 11–14; teleconference. This study was approved by the Institutional Review Board at Sanford Health (IRB00001575) and was registered with ClinicalTrials.gov (MOD000002311) prior to recruiting patients. All patients received oral and written consent prior to participation.

Consent for Publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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