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ORIGINAL PAPER

The effect of vitamin D in vitro supplementation on sperm deoxyribonucleic acid fragmentation

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Summary ***Objective: This study aimed to identify the direct effect of vitamin D on sperm DNA integrity after swim-up preparation.***
Materials and methods: Normozoospermia samples were gathered from 12 men and assessed for their baseline characteristics, including DNA Fragmentation Index (DFI). Each sample was then prepared using the swim-up method. Half of the samples were incubated with vitamin D, while the other half were incubated with a standard sperm-washing medium.
Results: Vitamin D significantly reduced the DFI compared to the baseline (5.5 ± 3.4% versus 17.6 ± 4.2%; p < 0.001) and the swim-up-only group (5.5 ± 3.4% versus 12.0 ± 4.2%; p < 0.001). Microscopic examination reflected these results, showing a reduction in the number of small halos and no halos with an increased appearance of large to medium-sized halos.
Conclusions: These results suggest that vitamin D incubation is valuable in protecting sperm from DNA damage that develops during sperm preparation. However, additional investigation is warranted to explore other preparation methods and to elucidate the underlying mechanisms.

KEY WORDS: Assisted reproductive technology; DNA damage; Male infertility; Swim up.

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INTRODUCTION

Infertility is a significant global health issue, affecting approximately 1 in 7 couples, with contributions from both male and female factors equally distributed (1-3). Among the various factors contributing to male infertility, damage to sperm DNA is a critical concern, impacting 20-40% of subfertile men. This DNA damage can arise from several sources, including imperfections in the apoptosis process during spermatogenesis (4), protamination during spermiogenesis (5), and oxidative stress from various endogenous and exogenous factors (6). Excessive production of *reactive oxygen species* (ROS) beyond the body's antioxidant capacity can lead to significant damage to sperm function, causing lipid peroxidation, protein oxidation, mitochondrial dysfunction, and DNA damage (7, 8).

In the context of *assisted reproductive technology* (ART), sperm preparation aims to isolate spermatozoa with optimal morphology and motility for successful fertilization (9). However, the cellular structure and function of spermatozoa can be compromised during sperm preparation due to repeated centrifugation cycles, which induce ROS formation and result in the loss of seminal plasma antioxidants (10). Studies have shown that ROS production in normal spermatozoa can increase 2-5 times after indirect swim-up preparation, with exposure to ROS from 1-2 hours leading to a 2-4-fold increase in DNA damage (11). To mitigate these effects, sperm selection methods such as the swim-up technique are employed to reduce the main sources of intracellular ROS, namely leukocytes and immature sperm (12). According to the WHO laboratory manual, the swim-up method is a simple method that allows motile sperm to swim into an overlaid medium, separating them from non-motile spermatozoa. This method enhances sperm motility, average velocity, normal morphology and fertilization rates in vitro in mammals in compared to a lower fraction (13). However, simple washing or swim-ups from the pellet have been associated with a sudden burst of ROS production, reduced motility, and impaired sperm-oocyte fusion in the zona-free hamster oocyte penetration test (14).
Despite these challenges, the swim-up method has been shown to maintain better sperm DNA integrity compared to other procedures, such as density gradient centrifugation, demonstrating reduced DNA fragmentation and vacuolization (15, 16).
Antioxidant administration, including vitamins C, E, catalase and glutathione, has been suggested to protect sperm DNA integrity during preparation (17).
Specifically, serum vitamin D levels have been correlated with improvements in sperm number, motility, morphology, and DNA fragmentation (18). Vitamin D plays a crucial role in calcium regulation within the male reproductive system, and its activation in semen has been shown to enhance intracellular calcium levels, motility and acrosome reaction in spermatozoa (19). Additionally, vitamin D functions as a membrane antioxidant (20) and influences gene expression related to cell proliferation, differ-

entiation, apoptosis and oxidative stress through its receptors in the nucleus and post-acrosome of spermatozoa (21-23).

Although numerous studies have explored the role of vitamin D in the reproductive system, research specifically examining its impact on reducing the *DNA fragmentation index* (DFI) of human spermatozoa remains limited. The DFI is a crucial indicator of semen quality, reflecting the integrity and damage to sperm DNA, thereby detecting potential sperm damage (24). *Blaseg et al.* (2022) found no association between circulating vitamin D levels and human DFI. However, though the study was limited by using circulating vitamin D as a proxy for intratesticular levels (25). Additionally, many studies have not considered ART factors in their analyses (26, 27).

Therefore, this study aims to investigate the effect of vitamin D in vitro administration on sperm DFI in normozoospermic samples following sperm preparation using the swim-up method, addressing a critical gap in current reproductive research.

Materials and methods

A total of 12 semen samples (n = 12) were included in this study, each with a volume of 3 mL. The inclusion criteria for selecting samples were men aged 26-35 years who agreed to participate and signed the informed consent, had abstained from ejaculation for 2-7 days, and were diagnosed with normozoospermia, characterized by a semen volume greater than 2 ml. *Baseline* (BL) characteristics assessed for each sample included patient age, body mass index, semen volume, sperm concentration, progressive motility, non-progressive motility, and DFI.

Samples were collected using sterile, clean instruments maintained at the same temperature as the spermatozoa to prevent bacterial contamination, which can reduce sperm quality. All tools were sterilized, disinfected, wrapped in aluminium foil, and stored at 37°C until use. Following the WHO protocols (2021). Samples were collected via masturbation onto a sterile glass container and allowed to liquefy for 20-30 minutes. Semen volume, motility, and total sperm count were measured to ensure normal values before inclusion in the study.

After the initial assessment, sperm samples were prepared using the swim-up method. The prepared samples were then divided into two groups: one undergoing swim-up preparation with vitamin D incubation (SD) and the other undergoing *swim-up* preparation only (SU). Unprepared samples (BL) were also included in the evaluation. Spermatozoa from all groups (BL, SD, and SU) were evaluated for DNA fragmentation index.

Sperm preparation

Sperm preparation using the swim-up method was performed by mixing semen and *Sperm Rinse*™ medium (*Vitrolife, USA*) in a 1:1 ratio in a 5 ml tube. The mixture was then centrifuged at a speed of 400 G for 10 minutes. After centrifugation, the supernatant was discarded, and the pellet was resuspended with 2 ml of *Sperm Rinse*™ medium. The resuspended pellet was centrifuged again at a speed of 400 G for 5 minutes. The supernatant was discarded, and 2 ml of *SpermRinse*™ medium was gently added to the pellet along the tube wall. The tube was then placed at 45° angle and incubated at 37°C for 45 minutes. Finally, 1 ml of the upper medium fraction was carefully transferred to two new tubes, with 500 µl in each tube, for further analysis (13).

Vitamin D incubation

A solution of active vitamin D, 1.25(OH)2D3 (Calcitriol, molecular weight 416.64), was prepared in 100% ethanol with a stock concentration of 10 µg/ml (24 µM) and stored in a -20°C. To analyse the effect of in vitro vitamin D administration on DNA fragmentation, 500 uL post-preparation spermatozoa were incubated with 1 nM 1.25(OH)2D3 at 37°C, for 45 minutes (19). The working solution concentration was prepared in stages starting from 1 mM, 10 µM, 10 nM, and finally 1 nM. The control group without vitamin D was incubated with SpermRinse™ medium under the same conditions.

DNA fragmentation index

DNA Fragmentation was assessed using the DNA Fragmentation Kit (*SpermFunc®, BRED-002, BRED Life Science Technology Inc.*), following the manufacturer's protocol. Observations were made using a light microscope at 400X magnification to distinguish between unfragmented DNA (big and medium halos) and fragmented DNA (small halos, no halos, and damaged spermatozoa) in 500 spermatozoa cells. Normal sperm DNA presented

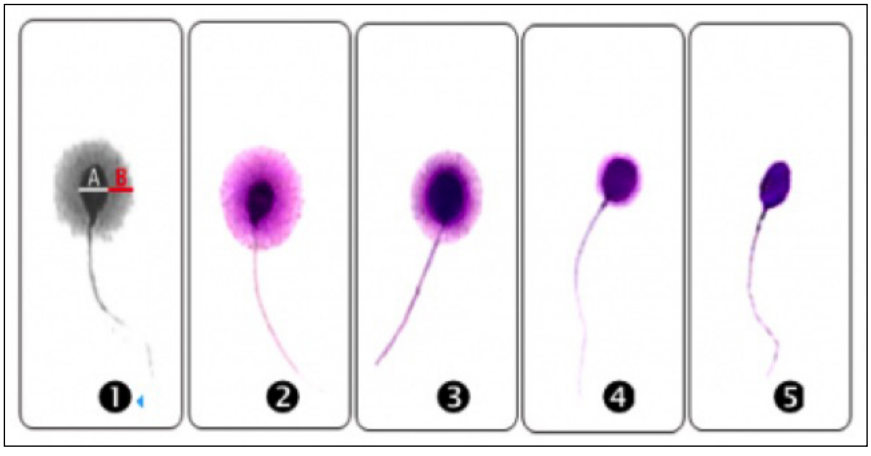


Figure 1.
Determination of DNA fragmentation based on halo size (28). 1) The diameter of the core as the determinant of DNA fragmentation. 2) Large halo and 3) medium halo show spermatozoa without DNA fragmentation. 4) Small halo and 5) no halo indicating spermatozoa DNA fragmentation.

as medium (halo thickness smaller than the length but greater than one-third of the core minor diameter) to big halos (halo thickness equal to or greater than the length of the core minor diameter). In contrast, damaged/fragmented sperm DNA presented as no halos or small halos (halo thickness equal to or less than one-third of the core minor diameter) (Figure 1). The percentage of spermatozoa with DNA fragmentation was calculated as the *DNA Fragmentation Index* (DFI).

Statistics

Data were recorded and analysed to compare the effect of vitamin D incubation on sperm DNA integrity between the vitamin D-treated group (SD), the control group (SU), and the unprepared samples (BL). This comparison aimed to determine whether vitamin D supplementation during sperm preparation could enhance DNA integrity in spermatozoa, thus potentially improving outcomes in ART.
Statistical analyses were performed using the *Statistical Package for the Social Sciences* (SPSS) version 26.0 for Windows. The Shapiro-Wilk test was used to determine whether the data were normally distributed ($p > 0.05$) or not normally distributed ($p < 0.05$). Differences in DFI values between groups were tested using paired t-test, and results were expressed as mean ± standard deviation. A p-value of less than 0.05 was considered statistically significant.

Ethical approval

The study was approved by the *Health and Humanities Research Ethics Committee, Faculty of Medicine, Universitas Airlangga* (Code: 242/EC/KEPK/FKUA/2023). Samples were collected between August-October 2023.

Results

Patient characteristics

A total of 12 volunteers participated during the study period. All DFI data between groups were found to be normally distributed ($p > 0.05$). The mean age of volunteers was 30.9 years (30.9 ± 2.6), reflecting the reproductive age of men. The *body mass index* (BMI) was 27.4 kg/m^2 (27.4 ± 4.1), classified as level 1 obesity; the mean baseline DFI value was 17.6 ± 4.2%, which falls within the normal range. Baseline characteristics are summarized in Table 1.

Table 1.
Age and semen profile of the study subjects.

Subject characteristics	Mean ± SEM (n = 12)
Patient age (in years)	30.9 ± 2.6
Body Mass Index (kg/m^2)	27.4 ± 4.1
Semen volume (ml)	3.4 ± 1.0
Sperm concentration (million/ml)	48.7 ± 25.1
Progressive motility (%)	52.4 ± 13.4
Non-progressive motility (%)	6.8 ± 3.7
DNA fragmentation index (%)	17.6 ± 4.2

Table 2.
Paired t-test results from each treatment group on DFI.

Variable	Group 1 (Mean ± SD)	Group 2 (Mean ± SD)	P-value
DFI	BL (17.6 ± 4.2)	SU (12.0 ± 4.2)	< 0.001
	SU (12.0 ± 4.2)	SD (5.5 ± 3.4)	< 0.001
	BL (17.6 ± 4.2)	SD (5.5 ± 3.4)	< 0.001

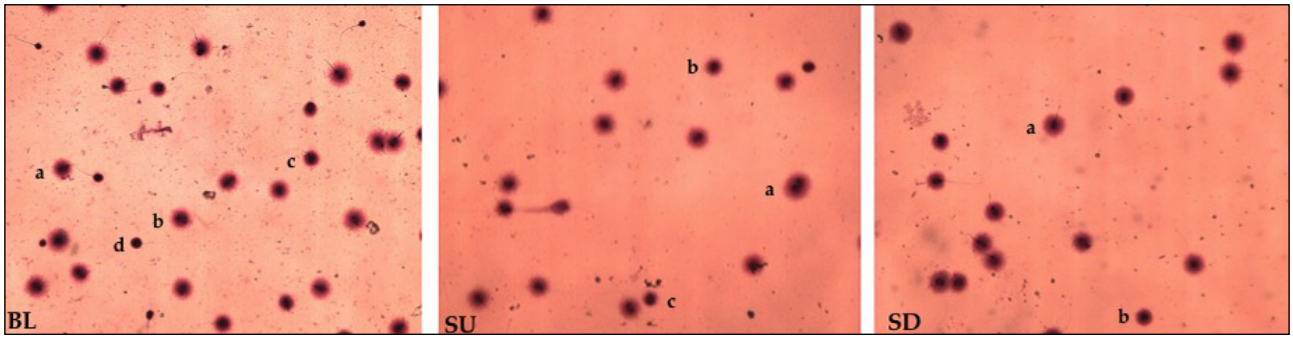
DFI between groups

A paired t-test was conducted to compare the DFI within each sample group (Table 2). The SU group exhibited a significantly reduced DFI (12.0 ± 4.2%) compared to the BL characteristics (17.6 ± 4.2%). Furthermore, the DFI was significantly decreased in the SD group (5.5 ± 3.4%) compared to both the SU group (12.0 ± 4.2%) and the initial BL (17.6 ± 4.2%), with mean reductions of 6.5% and 12.1% respectively. These findings suggest that vitamin D incubation during sperm preparation resulted in a significant improvement in sperm DNA integrity compared to the control groups.

Evaluation of spermatozoa DNA fragmentation examination

In each group, variations in the appearance of a large halos, medium halos, small halos and no halos were observed in semen samples before and after preparation. Specifically, the presence of small halos and no halos was noticeably reduced in the post-preparation samples of the SD group (Figure 2).

Figure 2.
Evaluation of spermatozoa's DNA fragmentation in the BL, SU and SD groups.
Image caption: a. Large halos; b. Medium halos; c. Small halos; d. No halos.



Discussion

The decline in sperm quality and function due to increasing reproductive disorders globally is a significant concern in reproductive health. A recent study highlighting the impact of vitamin D deficiency on mouse fertility and subsequent improvements in semen parameters has shed new light on the role of vitamin D in male reproductive function (29). Numerous studies have emphasized the role of vitamin D in male reproductive health, particularly in enhancing sperm quality and motility (30).

Infertility affects approximately 15-20% of couples of reproductive age, with male factors contributing to around 50% of cases (31, 32). In our study, the average age of the sample population was 30.9 ± 2.6 years, consistent with previous research (33). Additionally, the average BMI of the participants fell within the category of level 1 obesity. Obesity is known to increase the risk of sperm DNA damage in infertile men due to its association with increased oxidative stress (34, 35).

Sperm DNA integrity is critical to successful fertilization and embryogenesis (36). However, previous studies have been limited in evaluating the functional status of spermatozoa (37), indicating the need for further research. In our study, despite having normal semen parameters, semen samples exhibited a DFI of 17.6 ± 4.2%. This finding aligns with previous research conducted by *Halim et al.*, where the DFI before semen processing was 16.12% (13.48-19.04) (38). Oxidative stress levels that are insufficient to induce cell death can still disrupt sperm function, highlighting the importance of addressing DNA fragmentation in infertility (39). The extent of DNA fragmentation's impact on fertilization depends on both the level of DNA damage and the DNA repair capacity of the oocyte (40).

Our study revealed that supplementation of vitamin D, calcitriol [1.25(OH)2D3] at a concentration of 1 nM after sperm preparation, led to a significant reduction in sperm DNA fragmentation by 6.5%. Notably, there was a synergistic effect between sperm preparation and vitamin D administration in decreasing DNA fragmentation compared to baseline levels. These findings differ from a study by *Moghadam et al.* (2019), which reported no repair of DNA damage with vitamin D administration after swim-up (41). This discrepancy may be attributed to differences in the DNA damage examination methods used, with the sperm chromatin dispersion method proving more effective than the TUNEL method in diagnosing sperm DNA damage in unexplained infertility (40).

The protective mechanism of vitamin D on sperm DNA integrity can be explained in several ways. Firstly, the hydrophobic part of vitamin D binds to fatty acid residues on the spermatozoa membrane, thereby protecting membrane integrity (20). Additionally, vitamin D has been shown to enhance the integrity of spermatozoa membranes during cryopreservation by reducing intracellular ROS levels (41). VDR expression in testes and spermatozoa, along with cellular uptake of circulating vitamin D, play crucial roles in regulating spermatozoa motility and acrosome function (19). Moreover, administration of 20,000 nM of vitamin D has been associated with increased expression of heat shock protein 70 (HSP70), a marker of oxidative stress and lipid peroxidation, indicating a dose-response relationship of vitamin D as an antioxidant (42).

While our study supports the role of antioxidants in maintaining sperm chromatin integrity during sperm preparation, definitive conclusions cannot be drawn due to certain limitations. This study did not directly examine intraspermatozoal ROS levels and endogenous antioxidants. Future research should address these factors and analyse abnormal semen samples to further elucidate the role of vitamin D in infertility. Additionally, investigations into other variables, such as ROS levels, antioxidants, lipid peroxidation, and acrosome reactions, as well as their impacts on fertilization outcomes, pregnancy, and embryo development, are warranted. Comparative studies between swim-up and other sperm selection procedures, such as density gradient centrifugation, are also needed to evaluate their efficacy and drawbacks in reducing DNA damage with vitamin D administration.

Conclusions

This study demonstrated that DFI significantly decreased following sperm preparation using the swim-up method. Additionally, the DFI further decreased significantly in the group treated with vitamin D compared to both the pre-preparation and post-preparation without vitamin D administration groups.

These findings highlight the protective role of vitamin D against DNA damage incurred during sperm preparation. However, further studies are necessary to elucidate the underlying mechanism of this protective effect and to compare the efficacy of vitamin D supplementation with other sperm preparation methods. Future research should also consider examining different variables such as ROS levels, antioxidants, lipid peroxidation, acrosome reactions, and their impacts on fertilization outcomes, pregnancy, and embryo development.

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Conflict of interest: The authors declare no potential conflict of interest.