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Original Article

In vitro Vitamin D Treatment Enhances Sperm Motility and Capacitation of Fertile and Infertile Men

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ABSTRACT

Background: Male infertility is associated with vitamin D deficiency, and much evidence suggests ways to correct this deficiency to restore fertility. Vitamin D supplementation in men significantly improved fertility and semen parameters. This study evaluated the effects of *in vitro* vitamin D treatment on various sperm function parameters, such as sperm motility and capacitation, in both fertile and infertile men. **Materials and Methods:** Semen samples from 20 fertile and 20 infertile men were collected and divided into control and vitamin D-treated groups. The treated samples were incubated with 1 nM vitamin D for 1 hr. Sperm motility, viability, and intracellular calcium levels were assessed. Capacitation status, mitochondrial membrane potential, and protein expression were evaluated in fertile and infertile men using immunofluorescence assays. **Results and Discussion:** *In vitro* vitamin D treatment improved sperm motility and increased sperm viability in fertile and infertile males. This leads to increased intracellular calcium release and hyperpolarization of mitochondrial membranes. The results of Chlortetracycline (CTC) and phosphotyrosine immunofluorescence tests revealed a higher capacitation status in the vitamin D-treated group. *In vitro* vitamin D treatment positively influenced the expression of sperm-oocyte interaction proteins. It also enhances calcium ion levels and stimulates acrosome reaction and membrane hyperpolarization, thereby enhancing sperm functionality. **Conclusion:** *In vitro* vitamin D treatment enhanced sperm motility, viability, and capacitation, suggesting its potential benefits in improving IVF outcomes.

Keywords: Vitamin D, Male Infertility, Sperm Motility, Capacitation, Phosphotyrosine Protein.

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INTRODUCTION

Infertility is defined as failure to conceive after at least 12 months of regular unprotected sexual intercourse (Cox *et al.*, 2022). The global infertility prevalence is reported around 17.5%, and male factors are estimated to account for approximately 30-50% of infertility cases (Eisenberg *et al.*, 2023). Infertility causes immense social and emotional distress and imposes a significant economic burden on both patients and the health care system (Agarwal *et al.*, 2021). Male fertility is often thought to rely on sperm concentration and motility, which enables sperm to effectively navigate the reproductive system effectively (Björndahl and Kirkman Brown, 2022). Capacitation and the acrosomal reaction are crucial processes for preparing sperm for events such as sperm-oocyte fusion, initiating complex signal transduction steps necessary for conception (Puga Molina *et al.*, 2018; Stival *et al.*, 2016). Upon encountering the oocyte's protective barrier, the Zona Pellucida (ZP), sperm penetration is facilitated by ZP glycoprotein, which acts as a catalyst and triggers the acrosomal reaction (Gao *et al.*, 2024). This initial interaction culminates in sperm-oocyte fusion, a transformative process that heralds the activation of oocytes and the initiation of zygote formation (Zafar *et al.*, 2021).

A group of proteins coordinates the complex fertilization process. V-ATPase regulates ATP hydrolysis (Carrasquel Martínez *et al.*, 2022), whereas phosphotyrosine facilitates the key processes during capacitation (Tian *et al.*, 2024). Another protein, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase 2 (SERCA 2), facilitates acrosomal reactions (Mata-Martínez *et al.*, 2021). Among these proteins, IZUMO1 is central to facilitating sperm and oocyte binding, as well as PLCζ and PAWP, which are essential factors for sperm and oocyte activation (Yelumalai *et al.*, 2015). Each of these components plays a crucial role in ensuring successful progression of fertilization. A defect in any of these steps may render the sperm unable to fertilize the oocyte.

Vitamin D has emerged as a versatile regulator of various biological processes, including its well-established role in bone health and immune modulation. Previous research has

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highlighted its potential reproductive effects, particularly on male fertility (Cito *et al*., 2020; C. de Angelis *et al*., 2017). Studies have shown an association between serum vitamin D levels and various sperm parameters in fertile and infertile men (Cristina de Angelis *et al*., 2017). Optimization of vitamin D levels may improve male fertility and address infertility.

Despite considerable research, gaps in our understanding of the relationship between vitamin D and sperm-derived proteins remain. Thus, our study aimed to evaluate the effects of *in vitro* vitamin D treatment on human semen samples and elucidate the mechanisms that influence sperm motility, capacitation, and fertility. By exploring these pathways, we seek to enhance our understanding of how vitamin D might improve male fertility outcomes and translate these findings into clinical interventions that benefit couples facing infertility.

MATERIALS AND METHODS

Participant Recruitment and Semen Collection

Ethical approval was obtained from the Medical Research Ethics Committee of the University of Malaya Medical Center (MREC No: 2021629-10288) to ensure adherence to the national and international ethical guidelines.

Sample Size Selection

A power analysis was conducted using G*Power software version 3.1 to determine the required sample size to detect a significant difference between fertile and infertile men. With a medium effect size of d=0.95, an alpha error probability of 0.05, Degrees of Freedom (df)=38, and a desired power (1-beta error probability) of 0.83, the analysis indicated that a sample size of 20 participants in each group (fertile and infertile) would be sufficient to detect a statistically significant difference. Thus, a total sample size of 40 was justified through power analysis. This sample size provides adequate statistical power to minimize the risk of Type II errors (false negatives) and ensures a reasonable chance of detecting a true effect if one exists. Therefore, this experimental study involved fertile and infertile men.

Semen samples were collected following written informed consent from 40 males (20 fertile and 20 infertile) aged between 20 and 45 years who visited the Rawatan Utama dan Klinik Am (RUKA) clinics and the Reproductive Unit at the Universiti Malaya Medical Centre for semen analysis. The inclusion criteria were as follows: Semen Analysis to assess sperm concentration, morphology, and motility, as well as an evaluation of medical and sexual history, along with a physical examination. Detailed exclusion criteria were established to specify the medical conditions that disqualified the participants from the study. Exclusion criteria were applied to prevent interference due to oxidative stress. Participants taking hormonal supplements or those with certain medical conditions, such as hormonal and genetic disorders, were excluded. A comprehensive questionnaire was administered to gather participants' relevant demographics and health information. All samples were collected according to World Health Organization (WHO) guidelines (World Health Organization, 2021).

Semen Processing

Semen samples were incubated at 37°C for 30 min for liquefaction, and parameters were assessed according to WHO 2021 guidelines (World Health Organization, 2021). Samples with all basic semen parameters above the lower fifth percentile were categorized as 'Fertile Group,' while samples with one of the basic semen parameters below the lower fifth percentile were categorized as 'Infertile Group.' Semen analysis was performed using a sperm counting chamber (Shukratara, India). Samples were centrifuged at 300× g for 10 min in a density gradient using specific percentages for the layers, with the upper layer consisting of 50% and the lower layer composed of 90% (90%-50%), to separate motile sperm using a sperm separation kit (Irvine Scientific, The Netherlands, Catalogue number 99264). The purified sperm were then washed twice with 1 mL sperm nutrition solution, PureSperm Wash (Nidacon, Catalogue ID: PSW-100 or Sperm Washing Medium, Irvine Scientific, Catalogue ID: 9983).

Determination of Vitamin D Dosage and Incubation Duration

A pilot study was conducted to determine the optimal dosage of vitamin D and duration of incubation of sperm with vitamin D for *in vitro* treatment. The washed sperm were incubated with 0.1 nM, 1 nM, and 10 nM of Calcitriol ($1,25(OH)_2D$, IU/mL CAS No: 3222-06-3, MedChem Express, Monmouth Junction, NJ, USA), which was adopted from previous study (Jueraitetibaike *et al*., 2019) for 45 and 60 min, and total sperm motility was measured. The concentrations and times were chosen based on preliminary research indicating the potential efficacy 1 nmol/L of $1,25(OH)_2D$ for treatment.

In vitro experimental Treatment

Purified samples from both fertile and infertile groups were aliquoted to serve as the control and vitamin D-treated groups. The control group was incubated in sperm nutrition solution for 1hr at 37°C, and the vitamin D-treated group was incubated with 1 nmol/L of $1,25(OH)_2D$ (selected from the pilot study) in sperm nutrition solution for 1 hr at 37°C. Following incubation, the samples were centrifuged at 500 × g for 6 min, and the sperm pellets were collected and resuspended in sperm nutrition medium. The sperm pellet was aliquoted for subsequent basic and advanced semen and immunofluorescence analysis. The pellets were resuspended in 4% paraformaldehyde and stored at 4°C until immunofluorescence analysis. A flowchart summarizing the research methodology was shown in Figure 1.

Basic Semen Analysis

Semen analyses involved measuring the sample volume, pH, and liquefaction. Sperm concentration and total motility were assessed using a sperm counting chamber (Shukratara, India). The Eosin Nigrosin (Sperm Processor, India, Catalogue No: SP/SFT/V-003-A) staining technique was used to measure the live-to-dead sperm ratio. All parameters were determined according to World Health Organization guidelines (World Health Organization, 2021).

Aniline Blue Staining for Sperm Nuclear Protein

Aniline blue staining (Sperm Processor, India, Catalogue No: SP/SFT/NP-008) was used to determine sperm nuclear protein levels. Briefly, after liquefaction, 1 mL of neat semen was washed twice in 0.2 M phosphate buffer (pH=7.2), centrifuged (1000 g for 10 min), smeared on a slide, air-dried, and then fixed with 3% glutaraldehyde in 0.2 M phosphate buffer (pH=7.2). The slides were stained with 5% aniline blue and observed under a microscope. Mature sperm heads stain red and immature sperm stain blue, allowing the calculation of the immature nuclear protein percentage.

Sperm Chromatin Dispersion (SCD) Method

The Sperm Chromatin Dispersion (SCD) method was used to assess the DNA fragmentation. A DNA Fragmentation kit (Sperm Processor, India; Catalogue no: SP/SFT/DNA-009) was used. Briefly, semen samples (15 million/mL) were diluted with the appropriate buffer and incubated with agarose. Slides were treated with acid denaturation (0.08 N HCl), neutralizing solution, and lysis solution (0.8 M Dithiothreitol (DTT), 1% sodium dodecyl sulfate (SDS), and 2 M NaCl), followed by Wright's stain. The DNA Fragmentation Index (DFI) was calculated as follows:

$$\mathrm{DFI} = \frac{(\text{Fragmented sperm} + \text{Degraded Sperm})}{\text{number of sperm evaluated}} \times 100\%$$

The usual range of DFI is less than 15%, equivocal is between 15% and 25%, and abnormal is greater than 25%. Additionally, the SCD method, using the same kit, was used to measure DNA fragmentation.

Measure of Intracellular Calcium

Intracellular calcium levels were assessed using Fluo-4 AM fluorescent calcium ion indicator (CAS No.: 273221-67-3, Catalogue No: HY-101896 from MedChemExpress, USA). Sperm samples from the control and treatment groups were incubated with 2 μM fluo-4 AM in sperm nutrition solution at 37°C for 1 hr. After washing with PBS, the samples were examined under a confocal microscope, and images of calcium-containing spermatozoa were analyzed for intensity using the ImageJ software.

Chlorte-Tracycline (CTC) staining of Sperm Capacitation and Acrosome Reaction

Chlortetracycline (CTC) (CAS No.: 64-72-2, Catalogue No: HY-B1327 from MedChem Express, USA) was used to assess sperm capacitation status. The samples were incubated in CTC solution (5.82 mM CTC, 130 mM NaCl, 20 mM Tris-HCl, pH 7.8) for 10 min and then fixed with 4% paraformaldehyde in PBS. The samples were evaluated using a fluorescence microscope (Evos Floid Imaging System; Thermo Scientific, USA).

Mitochondrial Membrane Potential

The MitoPT JC-1 Assay (ImmunoChemistry Technologies, USA) was used to assess the sperm mitochondrial membrane potential. Briefly, after treatment of the control group with sperm nutrients and the other group with vitamin D, working solutions were prepared and incubated in the dark at 37°C for 30 min. After centrifugation (1000 × g for 10 min) and washing, the samples were examined under a fluorescence microscope (Evos Floid Imaging System; Thermo Scientific, USA).

Immunofluorescence Assays

Immunofluorescence was performed on sperm samples by loading them into PAP molds and allowing them to air-dry. PAP molds were drawn onto slides pre-coated with 0.01% poly-L-lysine using an Imm-edge pen (Vector Laboratories, UK) and PARA-Tissuer pen (Agar Scientific, UK). Approximately 100 μL of the sperm suspension was loaded into PAP molds and air-dried for 30 min. The samples were then washed thrice with PBS and permeabilized overnight with 0.5% Triton X-100 in PBS. The blocking step was performed using Blocking One solution (Nacalai Tesque, Japan) for an hour. The slides were then incubated overnight at 4°C with antibodies against phosphotyrosine (Novus Biologicals, USA).

After washing three times with PBS for 5 min, the slides were incubated for 1 h with 1:200 dilution of the secondary fluorescent antibody (donkey anti-rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, DyLight™ 650; Invitrogen, UK) in Blocking One. The slides were washed thrice with PBS for 5 min and mounted with Vectashield H-1200 mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, UK). Imaging was performed using a confocal laser-scanning microscope (Leica DM400B; Leica Microsystems, Germany).

Statistical Analysis

Data were analyzed using a 2-tailed paired t-test for paired samples. Statistical analyses were conducted using GraphPad Prism. In addition, effect-size calculations were performed for a clearer interpretation of the results. Potential limitations of statistical analysis include the relatively small sample size and inherent variability, which may affect the robustness of the findings.

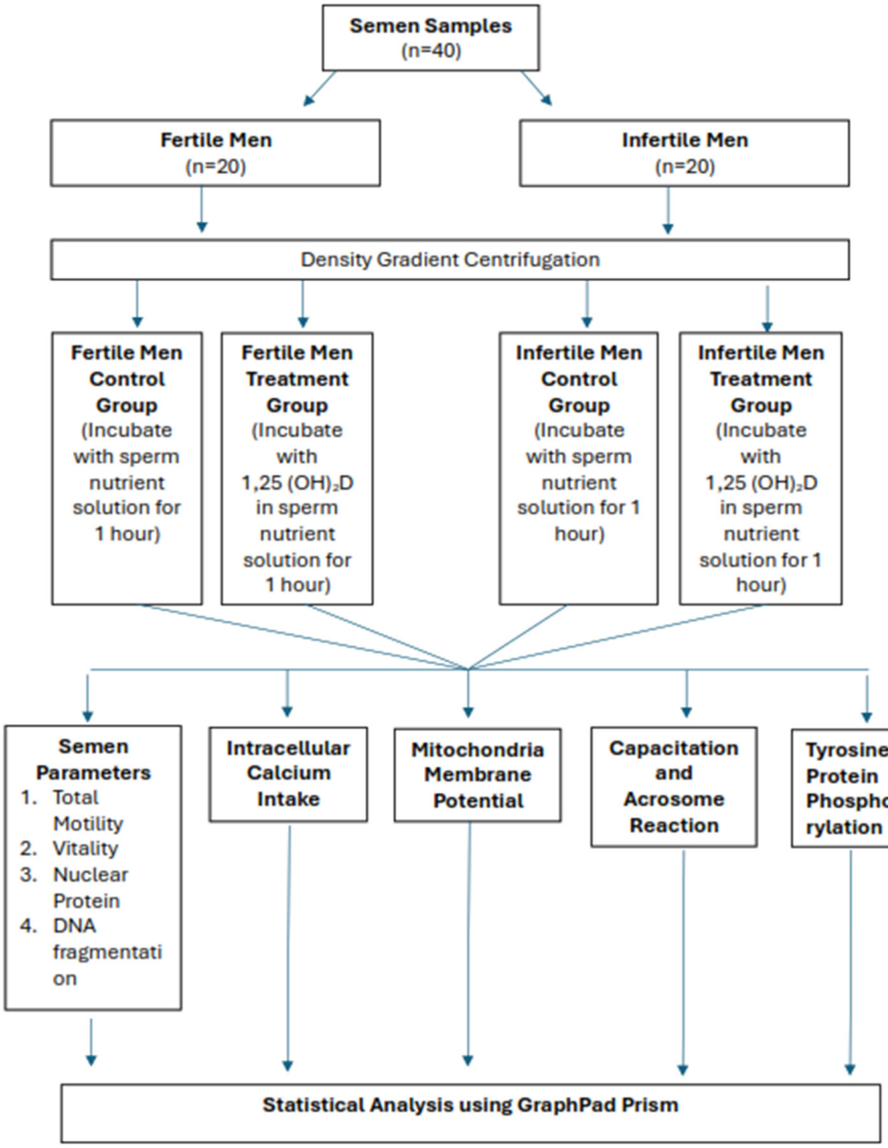

Figure 1: Flowchart of Research. Semen samples from patients were divided into fertile and infertile groups based on the results of basic semen parameter analyses conducted in the lab. Density gradient centrifugation was performed to separate motile sperm from immotile sperm. Subsequently, the samples were aliquoted into an *in vitro* vitamin D treatment group and a control group for fertile and infertile men. Following incubation, the samples were further aliquoted for semen analysis including total motility, vitality, nuclear protein, and DNA fragmentation; Fluo-4 AM staining to assess intracellular calcium intake, MitoPT JC-1 staining to assess mitochondrial membrane potential; Chlortetracycline (CTC) staining to assess capacitation and acrosome reaction; and immunofluorescence to assess tyrosine protein phosphorylation.

RESULTS

Sperm Motility

As shown in Figure 2A, after vitamin D treatment, significant improvements in total sperm motility (%) were observed in both the fertile (p=0.0012) and infertile (p=0.0135) samples. However, a higher average motility was recorded in fertile men, suggesting that vitamin D treatment may be particularly beneficial for improving motility in individuals with lower baseline levels of sperm motility. This variation in response could be attributed to the underlying differences in health and hormonal levels between the fertile and infertile groups, which may affect the bioavailability and efficacy of vitamin D on sperm function.

Sperm Vitality

Vitamin D treatment significantly enhanced sperm vitality, resulting in a higher proportion of live sperm in both fertile and infertile men. As demonstrated in Figure 2B, the eosin-nigrosin staining technique revealed distinct sperm characteristics: mature sperm heads were stained red, while immature sperm heads were stained blue. This differentiation allows for clear visualization of sperm health and maturity.

Furthermore, the quantitative analysis depicted in Figure 2C showed a substantial increase in the percentage of live sperm in both groups following vitamin D treatment. Fertile men exhibited a significant improvement (*p*<0.0001), as did infertile men (*p*<0.0001), indicating that vitamin D supplementation is effective in increasing sperm vitality, regardless of the baseline fertility status.

The marked enhancement in sperm vitality suggests that vitamin D may play a crucial role in improving overall sperm quality. The ability to increase live sperm percentages could be linked to the involvement of vitamin D in various biological processes, such as cellular proliferation, hormone regulation, and antioxidant defense mechanisms. Collectively, these factors contribute to improved sperm function and potentially improve reproductive outcomes.

Sperm Nuclear Protein

Figure 2D shows the aniline blue stain, which stains the mature sperm head red and immature sperm head purple. Vitamin D treatment did not significantly affect sperm chromatin packaging (Figure 2E). Additionally, no considerable variation was observed in the percentage of abnormal chromatin packaging between the control and treatment groups (Figure 2E) in either fertile (t(19)=0.8950, *p*=0.3820, mean difference 0.3000, 95% CI [-1.002, 0.4016]) or infertile men (t(19)=0.3251, *p*=0.7487, mean difference 0.1750, 95% CI [-1.302, 0.9518]). These unchanged parameters may be due to the inherent resilience of sperm chromatin structure against oxidative stress, which vitamin D treatment might not sufficiently alter, or it could indicate that the role of vitamin D in chromatin remodeling requires a longer duration or higher dosage to become apparent.

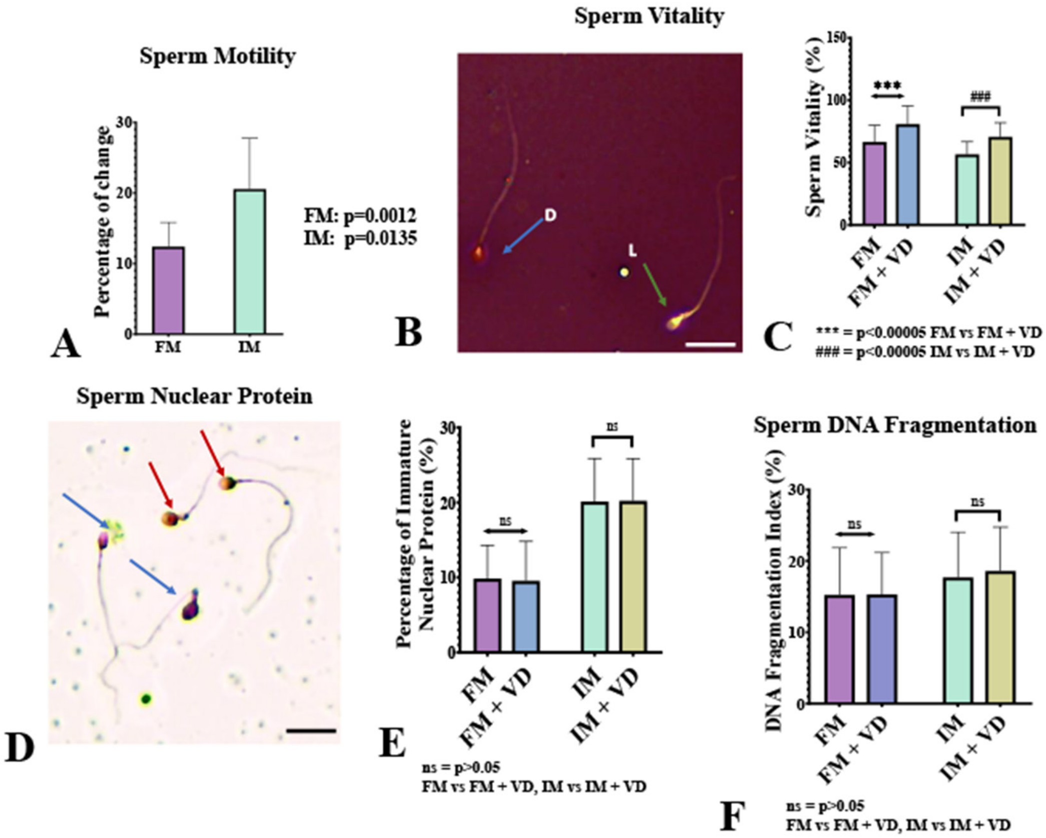

Figure 2: Basic Semen Analysis. A: Change in total sperm motility (%) in Fertile (FM) and Infertile Men (IM) after *in vitro* vitamin D treatment (*p*-values indicated). B: Eosin-Nigrosin staining showing sperm vitality; light blue arrows indicate dead sperm, and green arrows indicate live sperm. C: Percentage of live sperm in FM and IM with and without 1 nM vitamin D treatment (statistical significance from paired t-test). D: Aniline blue staining revealing nuclear protein; mature sperm heads are marked with red arrows, and immature heads with purple arrows. E: Percentage of immature nuclear protein for FM and IM with and without *in vitro* vitamin D treatment (paired t-test). F: DNA Fragmentation Index (DFI) percentages for FM and IM with and without *in vitro* vitamin D treatment (paired t-test). Values are mean±SEM, *n*=20. Magnification: 40X, Scale bar: 100 µm. Abbreviations: FM=Fertile men; FM + VD=Fertile men with *in vitro* vitamin D treatment; IM=Infertile men; IM + VD=Infertile men with *in vitro* vitamin D treatment.

Sperm DNA Fragmentation

Vitamin D treatment did not significantly affect DNA fragmentation in fertile or infertile men (Figure 2F). The DNA Fragmentation Index (DFI) remained unchanged post-treatment in fertile (t(19)=0.1092, *p*=0.9142, mean difference 0.075, 95% CI [-1.362, 1.512]) and infertile men (t(19)=1.634, *p*=0.1187, mean difference 0.9000, 95% CI [-0.2529, 2.053]). This lack of variation suggests that factors other than vitamin D, such as genetic predisposition or pre-existing oxidative stress levels, could influence DNA integrity. Vitamin D may not directly modulate DNA fragmentation under the conditions used in the present study.

Intracellular Calcium Intake

Sperm samples stained with Fluo-4 AM, as shown in Figure 3A, reveal significant differences in intracellular calcium levels following *in vitro* vitamin D treatment. The fluorescent staining technique allowed for the visualization of calcium mobilization, with vibrant green fluorescence indicating heightened intracellular calcium concentrations in the sperm.

The quantitative analysis presented in Figure 3B demonstrates a marked increase in intracellular calcium levels after treatment. Specifically, fertile men exhibited a substantial elevation in calcium levels (median difference=8131, *p*<0.0001), reflecting a significant enhancement in calcium mobilization due to vitamin D. Similarly, infertile men also experienced a notable increase in intracellular calcium levels (median difference=2807, *p*=0.0007), although this increase was comparatively lower than that observed in fertile men.

These results indicate that *in vitro* vitamin D treatment effectively enhances intracellular calcium intake in sperm, which is crucial for various sperm functions, including motility, capacitation, and the acrosome reaction. The differential responses between the fertile and infertile groups suggest that the underlying mechanisms of calcium signaling may vary based on baseline fertility status. Enhanced calcium influx into sperm could contribute to improved functional outcomes and reproductive potential, emphasizing the importance of vitamin D in male fertility.

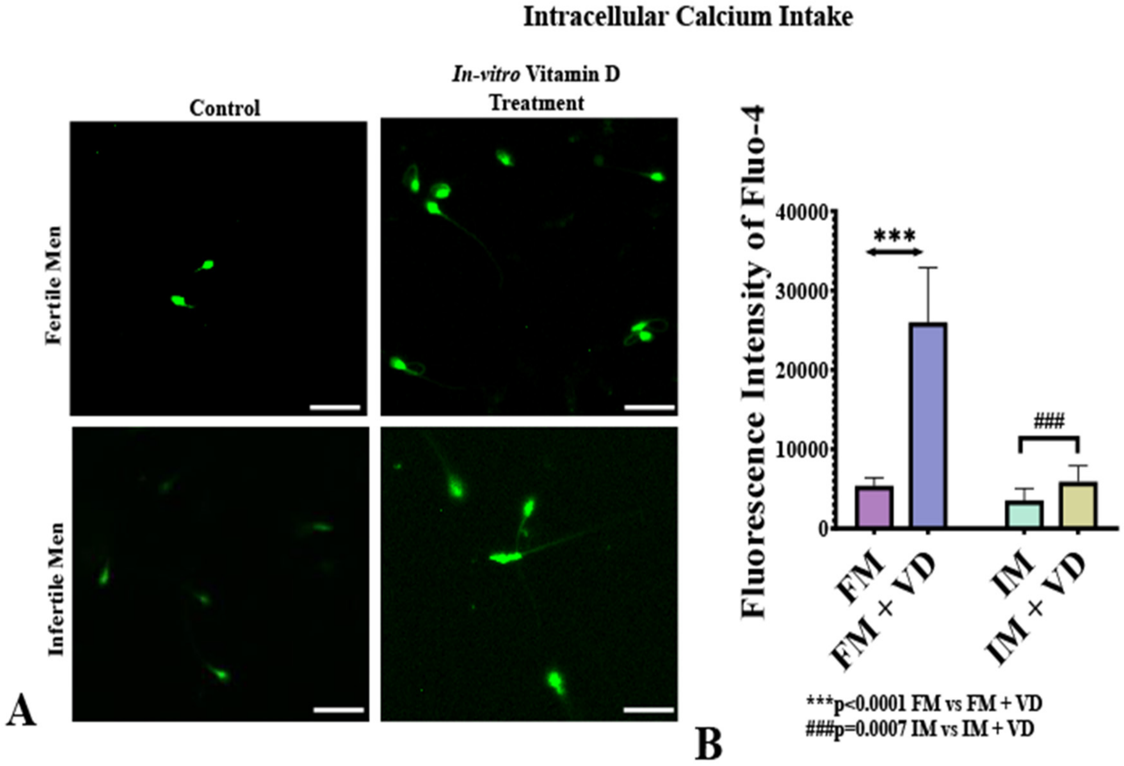

Figure 3: Calcium Mobilization. A: Fluo-4 AM staining of sperm from Fertile (FM) and infertile men (IM) with and without *in vitro* vitamin D treatment, indicating intracellular calcium mobilization (green fluorescence). Vitamin D treatment increased calcium uptake in both groups. B: Bar chart showing Corrected Total Cell Fluorescence (CTCF) of sperm from FM and IM with and without 1 nM vitamin D treatment (statistical significance assessed using paired t-test). Values are mean ±SD, *n*=20. Magnification: 40X, Scale bar: 100 µm. Abbreviations: FM=Fertile men; FM + VD=Fertile men with *in vitro* vitamin D treatment; IM=Infertile men; IM + VD=Infertile men with *in vitro* vitamin D treatment.

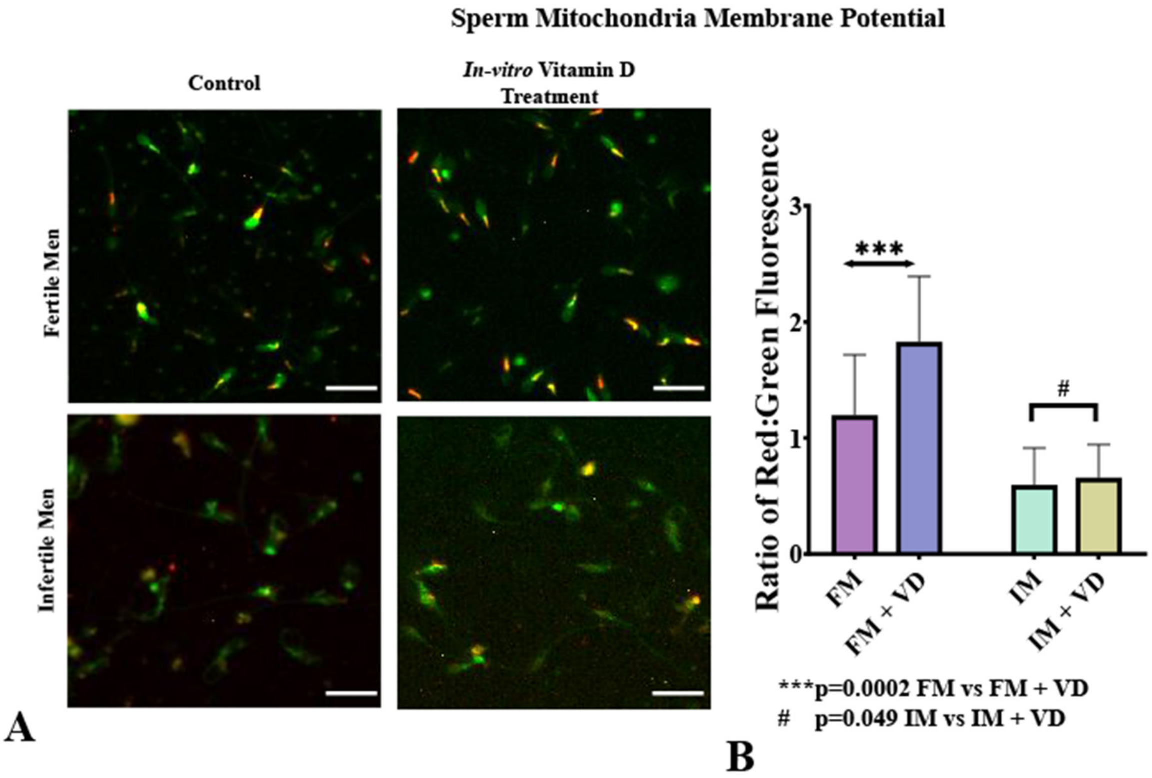

Figure 4: Mitochondrial Membrane Potential. A: Fluorescence microscopy using MitoPT JC-1 to assess sperm mitochondrial membrane potential in Fertile (FM) and Infertile Men (IM) with and without *in vitro* vitamin D treatment. Orange-red fluorescence indicates hyperpolarized cells, while green fluorescence indicates depolarized cells. B: Bar chart showing the ratio of red to green fluorescence for sperm from FM and IM with and without 1 nM vitamin D treatment (statistical significance assessed using paired t-test). Values are mean±SD, *n*=20. Magnification: 40X, Scale bar: 100 µm. Abbreviations: FM=Fertile men; FM + VD=Fertile men with *in vitro* vitamin D treatment; IM=Infertile men; IM + VD=Infertile men with *in vitro* vitamin D treatment.

Mitochondrial Membrane Potential

Figure 4A illustrates MitoPT JC-1 staining of sperm from fertile and infertile men, with comparisons made between control samples and those treated with vitamin D *in vitro*. JC-1 dye is vital for assessing Mitochondrial Membrane Potential (MMP), with healthy sperm exhibiting a higher ratio of red to green fluorescence.

Analysis of the results, as shown in Figure 4B, revealed significant improvements in MMP in both fertile and infertile sperms following vitamin D treatment. Fertile men demonstrated a notable increase in the red-to-green fluorescence ratio (t(19)=4.539, *p*=0.0002, mean difference=0.6323, 95% CI [0.3409, 0.9239]), indicating enhanced mitochondrial function and energy availability. This suggests that vitamin D positively influences the metabolic activity of sperms, potentially improving their overall viability and motility.

In infertile men, treatment also yielded a significant increase in MMP (t(19)=2.103, *p*=0.049, mean difference=0.06319, 95% CI [0.0003118, 0.1261]). Although the improvement was less pronounced compared to fertile men, it demonstrates that vitamin D can enhance mitochondrial function in sperm, which is critical for fertilization and subsequent embryonic development.

The observed increase in the ratio of red to green fluorescence across both groups suggests that vitamin D treatment effectively supports healthy mitochondrial dynamics. Improved MMP is essential because it is closely linked to sperm motility and the ability to undergo successful capacitation and acrosome reactions.

Capacitation and Acrosome Reaction

Figure 5A shows CTC staining used to assess the capacitation and Acrosome Reaction (AR) status of sperm samples. Following vitamin D treatment, there was a notable increase in the percentage of capacitated and acrosome-reacted sperm in both the fertile and infertile groups. Specifically, fertile men showed a significant increase (t(19)=4.661, *p*<0.0002, mean difference=7.475, 95% CI [4.119, 10.83]), indicating an enhanced readiness for fertilization. Similarly, infertile men exhibited a significant increase in capacitated sperm (t(19)=3.400, *p*<0.003, mean difference=5.075, 95% CI [1.951, 8.199]) following vitamin D treatment (Figure 5B). These findings suggest that vitamin D may facilitate the key

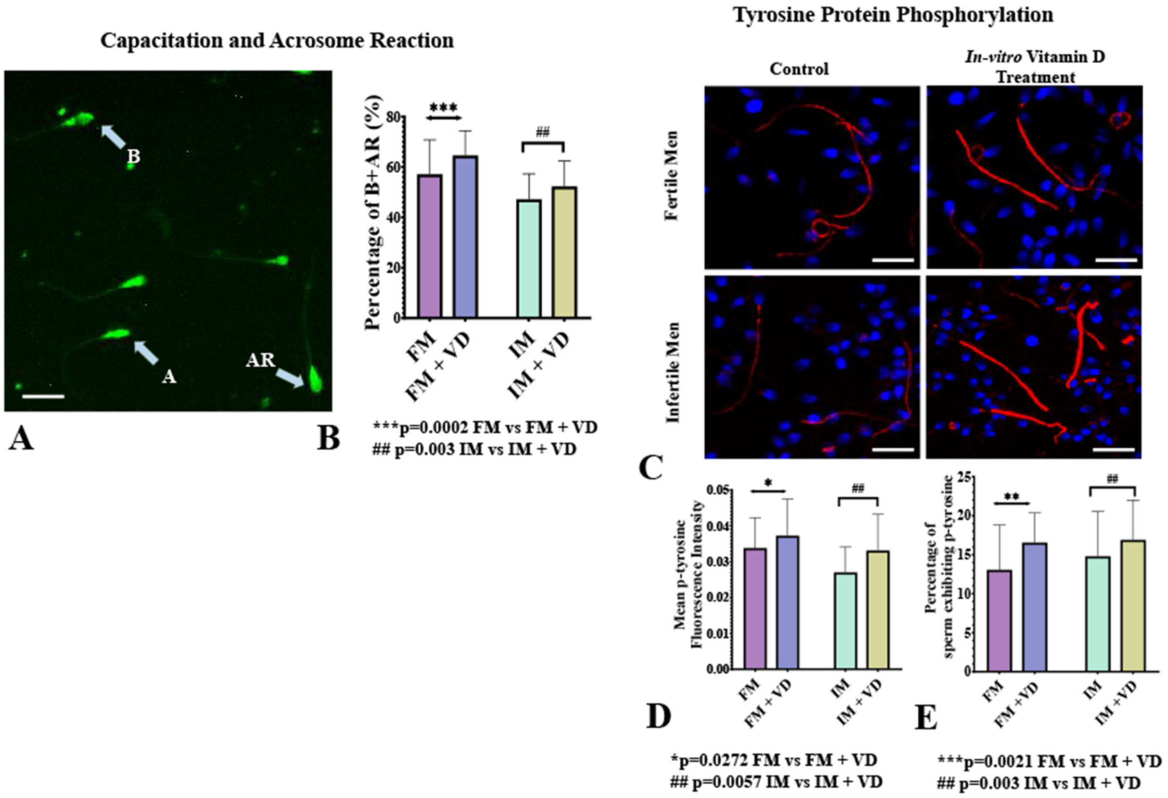

Figure 5: Sperm Capacitation. A: Assessment of sperm capacitation and acrosome reaction using Chlortetracycline (CTC) staining. Capacitated Acrosome-Reacted sperm (AR) exhibit dull fluorescence over the head and bright fluorescence in the midpiece, while intact sperm show uniform fluorescence. B: Bar chart showing the percentage of capacitated and acrosome-reacted sperm in Fertile (FM) and Infertile Men (IM) with and without 1 nM vitamin D treatment (paired t-test). C: Immunofluorescence of phospho-tyrosine in sperm; red indicates tyrosine phosphorylation, blue marks the nucleus. D: Bar chart showing Corrected Total Cell Fluorescence (CTCF) of phospho-tyrosine in sperm from FM and IM with and without *in vitro* vitamin D treatment (paired t-test). E: Bar chart showing the percentage of sperm exhibiting phospho-tyrosine in FM and IM with and without vitamin D treatment (paired t-test). Values are mean±SD, *n*=20. Magnification: 40X, Scale bar: 100 µm. Abbreviations: FM=Fertile men; FM + VD=Fertile men with *in vitro* vitamin D treatment; IM=Infertile men; IM + VD=Infertile men with *in vitro* vitamin D treatment.

processes essential for successful fertilization in both fertile and infertile men.

Tyrosine Protein Phosphorylation

Figure 5C illustrates the immunofluorescence results for tyrosine phosphorylation in sperm after vitamin D treatment. Corrected Total Cell Fluorescence (CTCF) values indicated a significant increase in phosphotyrosine protein levels in both groups. In fertile men, vitamin D treatment resulted in a notable increase in CTCF (t(19)=2.392, *p*=0.0272, mean difference=0.003470, 95% CI [0.0004340, 0.006506]), signifying an improvement in sperm capacitation. In infertile men, the increase was even more pronounced (t(19)=3.115, *p*=0.0057, mean difference=0.006107, 95% CI [0.002003, 0.01021]) (Figure 5D), further corroborating the positive effect of vitamin D on sperm functional capacity.

Moreover, a comparison of the percentage of sperms exhibiting phospho-tyrosine proteins revealed higher levels in fertile men treated with vitamin D than in the control group. The mean difference between these groups was 0.003470 with a 95% confidence interval ranging from 0.0004340 to 0.006506.

In the infertile group, a similar trend was noted, with a significant increase in the percentage of sperm demonstrating phosphotyrosine post-treatment. The mean difference in this case was 2.1, with a 95% Confidence Interval (CI) ranging from 0.7342 to 3.466.

DISCUSSION

The Global prevalence of infertility is increasing annually, and male factors contribute significantly to this burden. In recent years, vitamin D has attracted attention because of its effects on both male and female fertility (Cristina de Angelis *et al.*, 2017). However, their role in capacitation and sperm activity in males remains unclear. This study evaluated the effects of *in vitro* vitamin D treatment on sperm motility, capacitation, the acrosome reaction, and tyrosine protein phosphorylation in samples collected from both fertile and infertile men.

Previous studies have demonstrated a positive correlation between blood levels of Vitamin D (25-hydroxy-vitamin D3) and sperm parameters such as count, motility, and morphology (Rehman *et al.*, 2018; Yang *et al.*, 2012). However, few studies have reported no significant association between serum vitamin D levels and male fertility (Abbasihormozi *et al*., 2017). Another study reported that *in vitro* exposure to 1 nM vitamin D showed promising results in enhancing sperm motility (Blomberg Jensen *et al*., 2011), which is consistent with the findings of our study. However, there are contradictory reports on the effects of vitamin D supplementation on sperm quality. Multiple studies involving vitamin D supplementation have reported enhanced sperm motility in infertile males (Maghsoumi-Norouzabad *et al*., 2021), whereas other studies have not found any significant improvements in sperm quality (Wadhwa *et al*., 2020). Notably, many studies have suggested that semen vitamin D levels, rather than serum levels, are indicative of male infertility (Aquila *et al*., 2009; Jueraitetibaike *et al*., 2019).

Preliminary investigations are crucial for determining the optimal dosage and incubation time for vitamin D treatment. The optimal dosage of vitamin D was 1 nmol/L, with an incubation time of 1 h, which is similar to the dosage used in another study (Blomberg Jensen *et al*., 2011). Another study explored a range of concentrations (0.01 nM to 10 nM) and incubation durations (15 to 90 min), with the most favourable outcomes observed with 0.1 nM 1,25(OH)2D for 30 min (Aquila *et al*., 2009; Jueraitetibaike *et al*., 2019).

This study found that fertile men had higher average motility and vitality than infertile men before vitamin D treatment. This baseline difference was expected, as reduced sperm motility is one of the factors that contribute to male infertility, which is in agreement with the findings of another study (Anagnostis *et al*., 2013). Calcium signaling has emerged as a pivotal mechanism influenced by vitamin D treatment (Blomberg Jensen *et al*., 2011; Yahyavi *et al*., 2023). Calcium ions play multifaceted roles in sperm function, including motility, capacitation, and the acrosome response, all of which are essential for fertilization (Finkelstein *et al*., 2020). Vitamin D treatment increased the intracellular calcium levels in both groups, which is crucial for sperm motility. The greater relative improvement in the infertile group might be due to a lower baseline calcium level that had more room for enhancement, which is corroborated by previous findings demonstrating increased sperm motility and calcium influx following *in vitro* vitamin D treatment in both the fertile and infertile groups (Blomberg Jensen *et al*., 2016). Vitamin D treatment improved the mitochondrial membrane potential in both groups, which is essential for ATP production and sperm motility. The infertile group may have had a more compromised mitochondrial function initially, which accounts for the more noticeable improvement (Agnihotri *et al*., 2016; Azizi *et al*., 2018). This aligns with previous research showing a positive association between MMP and ATP levels, and sperm motility (Akbarinejad *et al*., 2020).

The Sperm Chromatin Dispersion (SCD) test showed that vitamin D treatment did not significantly affect DNA fragmentation in fertile and infertile men. Chromatin packaging is completed during spermatogenesis, which occurs over several weeks. Short-term *in vitro* treatments may not be sufficient to affect this process (Farkouh *et al*., 2022). In addition, The DNA Fragmentation Index (DFI) remained unchanged post-treatment in the fertile fish. Thus, the mechanisms causing DNA fragmentation (like oxidative stress or apoptosis) may not be directly influenced by vitamin D in mature sperm, and the protective effects of vitamin D against DNA damage might require longer exposure or *in vivo* supplementation (Jeremy *et al*., 2019).

Moreover, this study highlights the role of vitamin D therapy in boosting MMP in sperm cells, which is a crucial factor in ATP generation and other mitochondrial processes (Azizi *et al*., 2018; Zorova *et al*., 2018). The enhanced red-to-green fluorescence ratio observed in the Vitamin D therapy group suggests an improved mitochondrial membrane potential, which may lead to increased ATP synthesis. This enhancement in energy production could potentially improve sperm motility (Akbarinejad *et al*., 2020), capacitation, and the acrosome reaction (Ďuračka *et al*., 2023; Pinto *et al*., 2023).

Furthermore, energy production is vital for sperm motility and capacitation, and an increase in MMP can enhance sperm motility (Agnihotri *et al*., 2016; Barbagallo *et al*., 2020). Research has shown that MMP, ATP, and ROS levels are positively correlated with sperm motility and membrane integrity (Akbarinejad *et al*., 2020). Additionally, CatSper channels, which increase intracellular calcium during capacitation (Vicente-Carrillo *et al*., 2017), also enhance MMP, contributing to increased sperm motility, capacitation, and acrosome reaction (Ferreira *et al*., 2021).

This study found increased levels of CTCF and phospho-tyrosine proteins after vitamin D treatment, which is associated with improved capacitation and motility. The infertile group may have been more deficient in this process initially, leading to a more pronounced effect after vitamin D treatment (Brukman *et al*., 2019). Moreover, our findings suggest that the increase in sperm motility and mitochondrial membrane potential after vitamin D treatment is likely facilitated by protein phosphorylation, a process mediated by elevated intracellular calcium levels resulting from vitamin D binding to VDR in sperm (González-Mariscal *et al*., 2018). This enhancement in protein expression and phosphorylation underscores the potential of vitamin D to improve male fertility outcomes by promoting key molecular events that are essential for successful fertilization. However, further research is required to translate these findings into clinical application.

CONCLUSION

This study demonstrated the role of vitamin D in the modulation of sperm function and fertilization potential. The molecular mechanisms underlying vitamin D-mediated effects on sperm support the notion that *in vitro* vitamin D treatment can increase MMP and enhance sperm motility, which is consistent with previous research. Vitamin D has emerged as a promising avenue for understanding and addressing male infertility, offering potential improvements in fertility outcomes, and aiding couples in achieving successful pregnancies. However, it is essential to acknowledge the limitations of our study, including its *in vitro* nature, small sample size, and potential participant selection bias, which warrant consideration in future studies. Additional research evaluating the impact of vitamin D treatment on actual IVF outcomes is recommended as a next step. Further research focusing on IVF outcomes is essential to translate the findings of this study into clinical practice, and may potentially improve treatment strategies for infertile couples.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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ABBREVIATIONS

ATPase: Adenosine Triphosphatase; **CTC:** Chlortetracycline; **CTCF:** Corrected Total Cell; Fluorescence; **DAPI:** 4',6-diamidino-2-phenylindole; **DFI:** DNA Fragmentation Index; **ECL:** Enhanced Chemiluminescence; **IVF:** *In vitro* Fertilization; **JC-1:** 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; **MMP:** Mitochondrial Membrane Potential; **MREC:** Medical Research Ethics Committee; **PAWP:** Post-acrosomal WW domain-binding protein; **PBS:** Phosphate Buffered Saline; **PLCζ:** Phospholipase C zeta; **RIPA:** Radioimmunoprecipitation Assay; **SCD:** Sperm Chromatin Dispersion; **SERCA 2:** Sarcoplasmic/Endoplasmic Reticulum Ca2+-ATPases 2; **WHO:** World Health Organization; Z**P:** Zona Pellucida.

ETHICAL STATEMENT

This study was approved by the Medical Research Ethics Committee of the University Malaya Medical Centre (MREC ID NO: 2021629-10288. Written informed consent was obtained from all participants prior to sample collection.

AUTHORS' ROLES

AWY. performed the experiments, collected and analyzed the data, and wrote the original article. MBH, NG. designed, supervised, and funded the study and edited the manuscript.

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