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In vitro effect of vitamin D on sperm motility and oxidative stress in spermatozoa from infertile men

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Abstract

Calcium influx plays a role in optimizing sperm motility and capacitation that correlates with favorable pregnancy outcomes in both natural and assisted reproductive technologies. Vitamin D was found to promote calcium homeostasis in the male reproductive system by genomic and nongenomic pathways. Our study aimed to estimate in vitro effects of vitamin D on sperm motility and antioxidant capacity by adding vitamin D in purified spermatozoa. Forty-one samples with normal sperm motility according to the World Health Organization 2010 criteria were recruited. Density gradient centrifugation was used to isolate sperm in two fractions, pellet and ring layers, divided to measure effects of vitamin D on sperm motility and kinematics compared with untreated samples using computer-assisted semen analysis. Afterwards, samples underwent the intracellular reactive oxygen species test measured by Dichlorofluorescein diacetate (DCFH-DA). No significant changes were observed in the pellet layer but the ring layer revealed improved sperm motility (+ 4.2% versus − 5.4%) and kinematic parameters. However, DCFH-DA measurement showed no significant difference in both groups. In this study, increasing sperm motility and kinematic parameters were shown in ring layer sperm after adding vitamin D.

Introduction

Male factor infertility is found in up to 15% of infertile couple, but the exact underlying causes are rarely identified.(1) Optimal spermatogenesis and sperm transport are crucial to achieve fertilization. Unfortunately, only 0.01% of ejaculated spermatozoa are identified in the oviduct, so adequate amounts of high motility spermatozoa per ejaculation are needed. Semen parameters were investigated showing correlations with fertility outcomes; concentration, motility and morphology. Total motile sperm count (TMSC) is used to predict successful infertility treatment and determine choice of infertility treatment.(2) With TMSC less than 10 million per ejaculation, in vitro fertilization (IVF) is preferred to IUI because of its higher cost-effectiveness.(3)

Capacitation is a crucial step that activates sperm hypermotility and enhances contact to chemotactic factors that direct them to oocytes.(4) It has been reported that intracellular calcium plays an important role in tyrosine phosphorylation providing sperm hypermotility during capacitation occurs.(5) Furthermore, reactive oxygen species (6) such as hydrogen peroxide (H_2O_2) and peroxynitrite (ONOO) modulate sperm capacitation as well.(7)

Vitamin D is a major substance in calcium homeostasis by genomic and nongenomic action.(8) In mammal studies, vitamin D receptor (VDR) knockout mice exhibited lower sperm concentration and motility(9) leading to studies to elicit vitamin D pathways in the reproductive system. Later, VDR and metabolizing enzyme were identified in the male reproductive tract including spermatozoa.(10) It was also reported that vitamin D insufficient men had lower sperm motility compared with those with normal vitamin D status.(11) Vitamin D supplementation to improve sperm motility was investigated in clinical

trials but revealed insignificant differences in sperm motility after 150 months of treatment.[12] Nonetheless, in vitro studies have used 1α, 25 $(OH)_2$ vitamin D shown to improve sperm motility. Furthermore, vitamin D is found to support mitochondrial function resulting in less oxidative stress. Therefore, it has been used among patients as an antioxidant.[13] DCFH-DA could measure intracellular H_2O_2 that is a major ROS of spermatozoa. Also, it has been reported to associated with sperm capacitation[5] and objectively measured using a more simplified process. Moreover, DCFH-DA protocol for testing in spermatozoa was validated and correlation to infertility treatment outcomes has been reported, such as pronuclear formation rate. Nonetheless, association with vitamin D status is currently lacking.[14]

Samples size of related experimental studies was small and lacked of appropriate control groups; thus, effects of vitamin D on sperm motility could not be concluded. Using a larger sample size, stratification of sperm by quality and adequate control group using untreated and solvent-added samples would be more informative and applicable. Our study endeavored to estimate effects of 1α, 25 $(OH)_2$ vitamin D-added to sperm, divided into 2 fractions by motility, to improve sperm motility compared with untreated and dimethyl sulfoxide (DMSO)-added samples.

Results

Semen samples were allocated from 41 patients attending the Infertility Clinic, Ramathibodi Hospital from October 2019 to March 2020. All were screened for any chronic medical illness. Semen analysis was performed by two embryologists to justify whether they were compatible with the inclusion criteria.

Pretreatment characteristics

Demographic patient's characteristics including semen analysis are shown in Table 1. Moreover, baseline semen analysis, such as sperm motility and sperm morphology using strict Kruger criteria were also reported. After sperm preparation using density gradient centrifugation and dividing in six parts, three from pellet layers and others from ring layers, CASA was performed and the results are revealed in Table 2. The median concentration of pellet was higher than ring layer (30.7 versus 16.5 million ml^{-1}). Motility was significantly higher in pellet layer, 88.8% versus 61.4%. Also, sperm kinematic parameters revealed mean VAP, VSL and VCL significantly differed between the two layers. On the contrary, mean BCF was not different.

Table 1
Baseline patient characteristics

Characteristic	Mean ± SD
Male age (years)	35.0 ± 5.34
BMI (kg m^{-2})	23.7 ± 1.74
Semen analysis	75 (57, 127)
• Concentration (M ml^{-1})†	59.5 ± 12.76
• Motility (%)	51.5 ± 17.51
• Progressive motility (%)	1.4 ± 0.81
• Morphology (%)	112 (75.2, 202.3)
• TMSC (M) †	
† Data are showed as median (IQR)	

Table 2
Pre-treatment computer-assisted semen analysis

Characteristic	Pellet layer	Ring layer	*p*-value
Semen analysis (CASA)	**30.7 (17.40, 53.83)**	**16.5 (11.24, 44.35)**	**0.013**
• Concentration (M ml^{-1}) †	**88.8 ± 9.36**	**61.4 ± 16.94**	**< 0.001**
• Motility (%)	**54.8 ± 17.12**	**32.9 ± 15.37**	**< 0.001**
• Progressive motility (%)	**27.9 (14.58, 48.93)**	**9.6 (5.82, 33.75)**	**< 0.001**
• TMSC (M) †	**16.70 (7.55, 24.44)**	**5.38 (2.68, 16.22)**	**< 0.001**
• Progressive TMSC (M) †			
Sperm kinematics	**66.3 ± 11.33**	**49.6 ± 7.63**	**< 0.001**
• VAP	**53.1 ± 10.44**	**39.7 ± 7.09**	**< 0.001**
• VSL	**111.0 ± 19.99**	**83.9 ± 14.76**	**< 0.001**
• VCL	**4.5 ± 0.82**	**3.9 ± 0.63**	**< 0.001**
• ALH	**25.2 ± 4.04**	**25.2 ± 3.42**	**0.954**
• BCF			
Motility grade distribution	85.5 ± 11.78	52.9 ± 17.86	< 0.001
• Rapid (grade a, %)	3.3 ± 3.22	8.2 ± 3.19	< 0.001
• Medium (grade b, %)	7.9 ± 7.89	22.4 ± 11.35	< 0.001
• Slow (grade c, %)	3.2 ± 3.44	16.5 ± 14.96	< 0.001
• Static (grade d, %)			

† Data are showed as median (IQR)

Comparisons between pellet and ring layer used the Wilcoxon signed-rank test for paired within-subject data (same specimens); p = 0.014 for concentration remained significant, consistent with the unpaired result reported previously.

Posttreatment sperm analysis

The mean difference between vitamin D-added versus untreated and DMSO-added versus untreated samples are shown in Table 3. In the pellet layer mean motility was lower in both vitamin D-added and DMSO-added samples (-1.6% versus − 2.3%) including progressive motility (-0.5% versus − 4.4%) resulting in lowered TMSC. However, coefficient analysis showed vitamin D-added samples tended to have better sperm motility compared with DMSO-added samples but without significance. Nonetheless, in the ring layer, vitamin D-added samples resulted in significantly higher motility (+ 4.2% versus − 5.4%)

and progressive motility (+ 3.7% versus − 5.2%) with coefficients of + 9.6% and + 8.9% respectively (p = 0.001 and 0.005).

Table 3
Post-treatment computer-assisted semen analysis and kinematics

Characteristic	Vitamin D	DMSO	Coefficient (95% CI)	*p*-value
Pellet layer	-1.6 ± 7.12	-2.3 ± 7.83	+ 0.6 (-2.06, + 3.35)	0.218
• Motility (%)	-0.5 ± 16.20	-4.4 ± 14.42	+ 3.9 (-1.36, + 9.14)	0.051
• Progressive motility (%)	-1.0 ± 16.20	-2.0 ± 7.65	+ 0.4 (-2.35, + 3.06)	0.429
• TMSC (%)	-4.1 ± 7.03	-4.5 ± 7.48	+ 0.3 (-2.08, + 2.69)	0.778
• VAP	-2.5 ± 9.47	-3.8 ± 8.95	+ 1.4 (-1.22, + 3.95)	0.302
• VSL	-3.8 ± 8.47	-5.2 ± 11.48	+ 1.4 (-1.23, + 3.98)	0.221
• VCL	-4.7 ± 10.63	-4.6 ± 10.43	-0.1 (-3.64, + 3.40)	0.766
• Rapid (grade a, %)	+ 2.3 ± 10.80	+ 3.4 ± 9.76	-1.1 (-4.59, + 2.35)	0.343
• Elongation (%)				
Ring layer	+ 4.2 ± 18.73	-5.4 ± 13.61	+ 9.6 (+ 4.26, + 15.04)	0.001
• Motility (%)	+ 3.7 ± 22.13	-5.2 ± 15.99	+ 8.9 (+ 2.30, + 15.55)	0.005
• Progressive motility (%)	+ 4.0 ± 22.13	-5.0 ± 13.54	+ 9.8 (+ 4.40, + 15.20)	0.001
• TMSC (%)	+ 1.1 ± 11.59	-4.2 ± 12.96	+ 5.4 (+ 1.23, + 9.48)	0.025
• VAP	-0.2 ± 14.09	-3.4 ± 15.17	+ 3.2 (-1.41, + 7.82)	0.270
• VSL	+ 2.2 ± 15.68	-3.7 ± 12.61	+ 5.9 (+ 1.44, + 10.34)	0.012
• VCL	-3.0 ± 30.74	-11.7 ± 27.07	+ 8.8 (+ 0.25, + 17.27)	0.013
• Rapid (grade a, %)	+ 4.9 ± 13.36	+ 1.1 ± 12.78	+ 3.8 (+ 0.51, + 7.11)	0.022
• Elongation (%)				

Coefficient = paired mean difference (vitamin D-added minus DMSO-added, both expressed relative to each subject's own untreated baseline); p-values from the Wilcoxon signed-rank test for paired within-subject comparisons. After Benjamini-Hochberg FDR correction for multiple comparisons within each layer, motility, progressive motility, TMSC, VAP, VCL, rapid motility and elongation remained significant in the ring layer (adjusted $p < 0.05$); only VSL was not significant in the ring layer, and no comparison reached significance in the pellet layer.

Changes of sperm kinematics compared with untreated samples are also shown in Table 3. VAP, VSL and VCL did not improve in both vitamin D-added and DMSO-added groups in the pellet layer, but slightly increased VAP and VCL were shown in vitamin D-added ring layer samples (p = 0.025 and 0.012). Other parameters, ALH and BCF, were not statistically different after adding of 1α, 25 $(OH)_2$ Vitamin D.

Changes of intracellular ROS measured by DCFH-DA

Remaining samples were transferred to perform the DCFH-DA test. Changes of DCFH-DA were recorded every 5 minutes up to 30 minutes. Compared with untreated samples, DCFH-DA changed insignificantly in both DMSO and vitamin D-added sperm, as shown in Figs. 2 and 3.

Discussion

Related published literature revealed evidence of vitamin D metabolism in the male reproductive tract and association with fertility outcomes. Also, experimental studies have affirmed significant positive effects on motility after vitamin D was added to purified spermatozoa. In this study, sperm motility was unimproved after adding 1 nmol l^{-1} of 1α, 25 $(OH)_2$ vitamin D in the pellet layer compared with untreated samples, but increased motility was found in the ring layer with mean motility change of + 4.2% versus − 5.4%. It could be postulated that high quality spermatozoa in the pellet layer expressed adequate VDR able to provide an optimal response, but not in poor quality spermatozoa.[15] Supplementation of higher than physiologic dose of 1α, 25 $(OH)_2$ vitamin D might potentiate those mid-level spermatozoa expressing lower VDR levels, such as infertile males with low sperm motility (Asthenozoospermia). Also, it was supported by positive trend of vitamin D status on sperm motility that has revealed in observational data with experiments that revealed a rapid increase in intracellular calcium after addition of vitamin D.[11] Another related study showed lower magnitude of sperm motility gain with mean difference of + 2.9% after incubating of pre-preparation semen with 1 nmol l^{-1} of 1α, 25 $(OH)_2$ vitamin D[16], because it contained mixed population of spermatozoa and was confounded by substances from seminal fluid.

Sperm kinematic changes were also significantly improved in the ring layer but not the pellet layer. Compared with another in vitro study, a slight improvement in sperm kinematic parameters, VSL, VCL and VAP was revealed at 30 minutes after adding 0.1 and 1 nmol l^{-1} of 1α, 25 $(OH)_2$ vitamin D.[17] Even the cut-point of sperm kinematic parameters was lacking, it has been reported that sperm kinematic parameters, especially VCL and VAP positively correlates with fertility outcome in observational study.[18] In addition, the proportion of rapidly motile (grade a) spermatozoa increased and head elongation improved in the ring layer after vitamin D exposure, and both changes remained significant after correction for multiple comparisons. Because grade-a motility and head elongation are recognized morphokinetic correlates of sperm maturity and fertilizing competence, this pattern is consistent with vitamin D selectively upgrading a subset of the lower-quality, ring-layer population rather than uniformly affecting all spermatozoa. Adding vitamin D was showed to increase not only quantity but also sperm quality on motility parameters that could be beneficial to achieve fertility.

DCFH-DA constitutes a ROS testing kit that has been used in spermatozoa samples and has shown correlation with intrinsic H_2O_2 that correlated with sperm viability and DNA fragmentation. However, in this study, no significant ROS change was observed after incubating for 30 minutes. Possibly, 1α, 25

$(OH)_2$ vitamin D might not play a major role in intracellular H_2O_2 production and available direct data of vitamin D on ROS were limited. Nicotinamide adenine dinucleotide (NADH) concentration, one of several coenzymes on mitochondrial surface in antioxidative pathways, has been reported slightly positive response after adding 1α, 25 $(OH)_2$ vitamin D in small experiment, but it was technically complicated due to cell lysate and long incubation period were needed.[17]

This study has several strengths. This experimental study was designed to compare differences among each individual sample. Thus, other confounding factors from baseline characteristics of each participant were eradicated. Adding DMSO was unavoidable and related studies have showed correlations, both positive and negative, between DMSO and sperm quality.[19] However, confounding effects from DMSO were also adjusted by assignment of another control group as mentioned.

Moreover, this was the first experimental study intending to stratify spermatozoa by quality using the density gradient method to provide clear effects of intervention. These results could be applied to investigate further among patients with poor semen analysis, such as asthenozoospermia.

Limitations of this study include the lack of vitamin D status among participants, especially in seminal fluid. But available report revealed very low level of 25(OH) vitamin D detected in seminal fluid and correlation between vitamin D status and response to vitamin D-treated spermatozoa was unknown.[17] Thus, serum 25(OH) vitamin D testing was not performed. Furthermore, other sperm functions that are essential on fertilization, such as acrosome reaction and zona binding, were not evaluated. In addition, this study did not include a formal a priori sample-size or power calculation, and intracellular calcium — the proposed mechanistic link between vitamin D and sperm motility — was not measured directly; direct measurement in future work would help confirm the underlying pathway. These within-subject comparisons were re-analyzed using the paired Wilcoxon signed-rank test with Benjamini-Hochberg FDR correction for multiple comparisons within each layer; the improvements in motility, progressive motility, TMSC, VAP, VCL, rapid motility and elongation in the ring layer remained significant after this correction (adjusted $p < 0.05$), supporting the robustness of the primary findings.

Looking forward, these findings carry a direct implication for sperm-preparation protocols in assisted reproductive technology. Density gradient centrifugation routinely separates pellet from ring-layer spermatozoa, and clinical practice generally prioritizes the higher-quality pellet fraction while the ring layer is discarded or used only when the pellet yield is insufficient. Because vitamin D supplementation selectively improved motility, kinematics, rapid-grade motility and elongation in the ring layer, without a corresponding benefit in the pellet layer, it may offer a simple, low-cost adjunct specifically for samples in which the ring layer cannot be discarded, such as severe oligoasthenozoospermia, cryptozoospermia, or post-thaw and post-surgical sperm retrieval, where the total motile sperm count is often the binding constraint on treatment choice between intrauterine insemination, in vitro fertilization and intracytoplasmic sperm injection. In such settings, even a modest improvement of the magnitude observed here could shift a borderline sample above the threshold conventionally used for a less invasive treatment, or increase the number of usable spermatozoa available for micromanipulation.

Experimental studies focusing on fair quality spermatozoa, such as those from asthenozoospermic men, could test this directly and clarify whether the effect translates into a clinically meaningful gain in usable, progressively motile sperm. However, adequate sperm preparation to obtain enough spermatozoa for both density gradient separation and subsequent functional testing would remain a practical challenge in this population, and any adoption into clinical sperm-processing protocols would first require confirmation in fresh clinical samples, dose-ranging studies, and assessment of downstream outcomes such as fertilization and pregnancy rates rather than motility parameters alone.

In conclusion, no benefit of sperm motility was shown after adding 1 nmol l^{-1} of 1α, 25 $(OH)_2$ vitamin D in good quality capacitating spermatozoa, but not in midlevel cohorts, concordant with sperm kinematic parameters. On the other hand, intracellular H_2O_2 remained unchanged in all samples.

Methods

Population and sample size

Forty-one volunteer male patients, 20 to 45 years old attending the Infertility Clinic were requested to use their leftover semen for research and provided informed consent.

Inclusion criteria

The inclusion criteria are listed below.

1. Semen from infertile volunteers age 20–45 years at the Infertility Clinic, Department of Obstetrics and Gynecology, Ramathibodi Hospital, Thailand
2. Duration of infertility more than 1 year
3. Abstinence period within 2 to 7 days
4. Semen volume of at least 1.5 ml per ejaculation
5. Sperm concentration of at least 15 million ml^{-1}
6. Sperm motility of at least 40%

Exclusion criteria

The exclusion criteria are listed below.

1. Unable to ejaculate by masturbation
2. History of previous testicular/penile surgery or vasectomy
3. Positive serologic screening test for HIV, syphilis or hepatitis B infection
4. Poor prognostic female factors, such as bilateral tubal obstruction, age of 40 years or older
5. Chronic medical illness such as liver disease, kidney disease, diabetes mellitus and thyroid disease
6. Current alcohol consumption or smoking

The study was approved by the Ethics Clearance Committee on Human Rights Related to Research Involving Human Subjects, Faculty of Medicine, Ramathibodi Hospital, Mahidol University (approval number: COA/MURA 2020/341). All experiments were performed in accordance with relevant guidelines and regulations, and in accordance with the Declaration of Helsinki. Informed consent was obtained from all patients enrolled between 1st October 2019 and 31th March 2020. Through suggestions from nurses and physicians, 41 patients were recruited.

Semen collection and assessment

Participants produced semen samples by masturbation and provided information of the duration of abstinence to technicians as routine. Volume and pH were measured then samples were left to liquify to evaluate sperm motility using a Makler counting chamber under a microscope at 40X magnification by two technicians. Grading of sperm motility according to WHO was performed. Finally, smears were prepared and eosin/methylene blue staining (Dip Quick Stain, S.E. Synergist, Thailand) was performed to evaluate sperm morphology using a 100X oil-immersion bright-field objective lens based on Kruger strict criteria.

Samples for in vitro studies

The dose-response relationship experiment was performed using 5 semen samples. Sperm preparation was performed by density gradient centrifugation technique with 1250 rpm using density gradient media (Sil-Select Plus Upper/Lower Layer 45%/90%; Fertipro™, Belgium) following manufacturer instructions to collect the ring layer located between different concentration of density gradient media and the pellet layer separately. All samples were evaluated sperm motility using computer-assisted semen analysis (CASA). Afterwards, each sample was diluted in sperm washing media and divided into 5 tubes: untreated, DMSO-added, 0.1, 1 and 10 nmol l^{-1} of 1α, 25 $(OH)_2$ vitamin D-added (Insolution™, Calbiochem® 50uL/112 ml, 1 umol l^{-1}), according to related literature revealing 1α, 25 $(OH)_2$ vitamin D at concentrations of 0.1 and 1 nmol l^{-1} provided optimal effect on sperm motility.[14, 15] All ten samples were incubated for 30 minutes in 37^{O}C with 5% CO_2 and evaluate changes of motility using CASA. Among 5 samples, 1 nmol l^{-1} of 1α, 25 $(OH)_2$ vitamin D provided the optimal response on sperm motility and was used in eligible samples. Each was extracted spermatozoa in 2 cohorts, pellet and ring layers, then divided in 3 parts: untreated, DMSO-added and vitamin D-added.

Computer-assisted semen analysis of post-preparation spermatozoa

After sample separation, all test tubes were evaluated using computer assisted semen analysis (CASA, Hamilton Throne Research, Version 12 IVOS; Beverly, MA, USA) to ensure equal distribution using a 10 uL drop of sample in a Makler counting chamber (SeffiMedical Instruments, Haifa, Israel). Baseline sperm analysis was performed using a 10X lens on five random fields with standard settings provided by the manufacturer. Motility and progressive motility were measured together with kinematic parameters:

average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF). Spermatozoa were also classified by motility grade, using the CASA system's velocity thresholds to categorize each cell as rapid (grade a), medium (grade b), slow (grade c) or static (grade d), and by head elongation (%), a CASA-derived shape descriptor giving the proportion of spermatozoa with a normal, elongated head form. Afterwards, 1 nmol l^{-1} of 1α, 25 $(OH)_2$ vitamin D or DMSO were added in each test tube and another was left untreated, as shown in Fig. 1, then all specimens were incubated for 30 minutes at 37^{o}C and 5% CO_2 and CASA was repeated. Finally, specimens were sent to perform the Dichlorofluorescein test to measure intracellular ROS.

Determination of ROS by Dichlorofluorescein test

The chemical, 2', 7'-dichlorofluorescin diacetate (DCFH-DA; Sigma Chemical Co., Hamburg, Germany) is a specific probe for H_2O_2 with cell-permeable staining property without the need of cell lysate. When oxidized into DCF, it could bind to DNA and express green light in fluorescence. It has been used to evaluate sperm quality following the induction of ROS and show correlation with sperm viability.[16] The sample was diluted by DMSO added in to achieve 0.02 mmol/l then 70 uL was transferred to a microplate reader (TECAN Infinite→ M200 PRO, Männedorf, Switzerland). After 20 minutes of incubation in the microplate reader at 37^{o}C, the green fluorescent signal was evaluated from 480 to 540 nm. Data was reported as percentage of fluorescent spermatozoa.

Statistical analysis

Statistical analyses were performed using STATA Statistics for Windows, Version 14.0 (Stata Corp LLC, Texas, USA). Baseline demographics, clinical and laboratory characteristics, used mean ± standard deviation (SD) and median (IQR) for continuous data and percentage for categorical data. Baseline group-level characteristics were compared using the Mann-Whitney U test. Because the pellet-versus-ring layer comparisons and the vitamin D-added-versus-DMSO-added comparisons were obtained from the same specimens, these within-subject comparisons were analyzed using the paired Wilcoxon signed-rank test (confirmed with the paired t-test); for the post-treatment analysis, Benjamini-Hochberg false discovery rate (FDR) correction was applied across the outcomes compared within each layer. The level of statistical significance was set at $p < 0.05$.

Declarations

Competing interests

The authors declare no competing interests.

Author Contribution

P.M., A.T. and C.S. conceived and designed the study.P.M., S.C. and A.T. performed the experiments and acquired the data.P.M., A.T. and C.S. analyzed and interpreted the data.P.M. drafted the manuscript.A.T. and C.S. supervised the study and revised the manuscript critically.All authors reviewed and approved the final manuscript.

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Data Availability

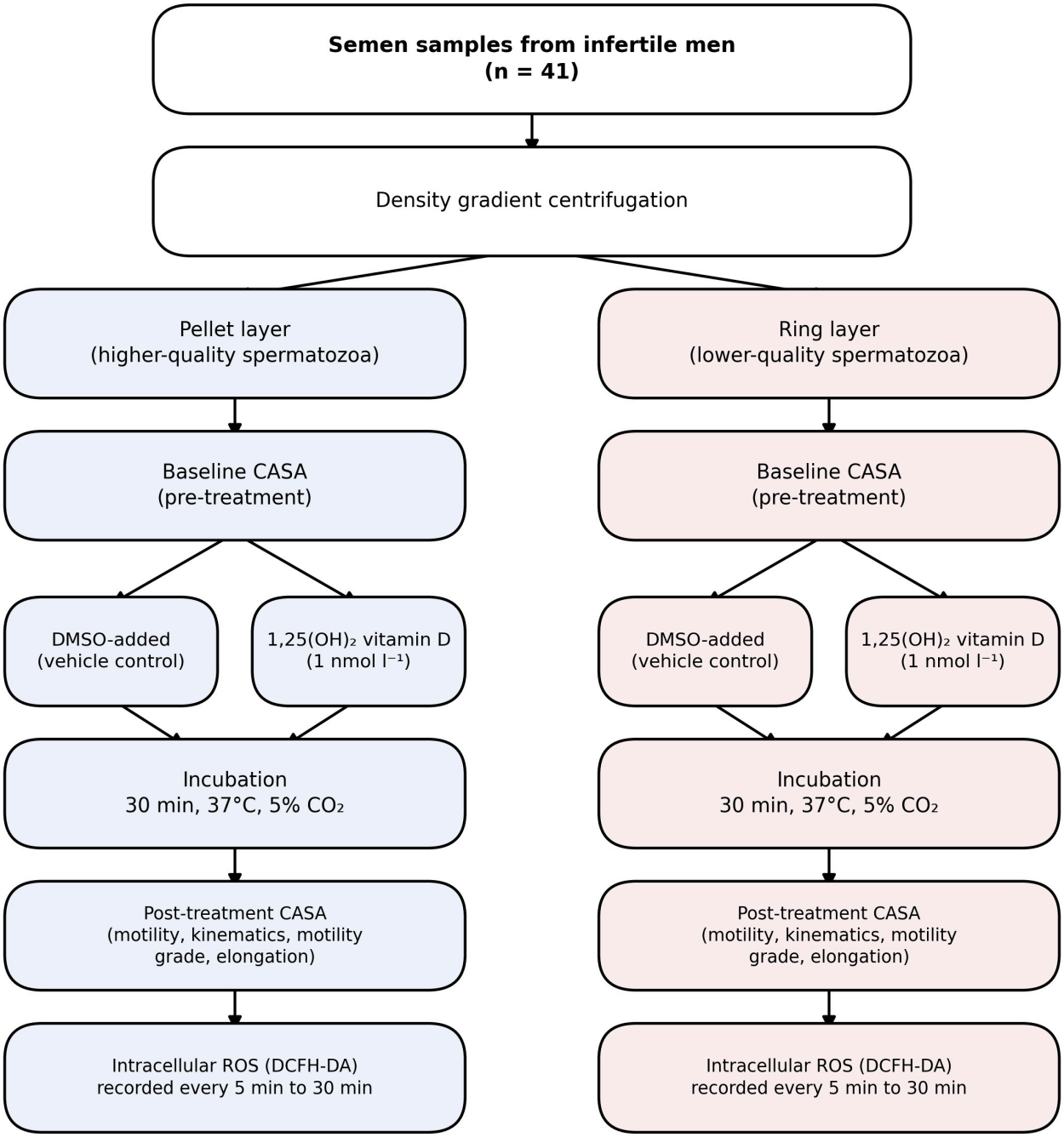
The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures



Pellet-layer and ring-layer specimens from the same subject were compared using paired statistics (Wilcoxon signed-rank test); DMSO-added and vitamin D-added conditions within each layer were likewise paired within subject.

Figure 1

Overview of the experimental workflow. Semen samples from 41 infertile men were processed by density gradient centrifugation, which separates spermatozoa into two fractions of differing quality: a pellet layer enriched for higher-quality, more motile spermatozoa, and a ring layer containing a lower-quality subpopulation. Baseline computer-assisted sperm analysis (CASA) was performed on each fraction before any treatment. Each fraction was then split into two paired aliquots from the same specimen: a

DMSO-added vehicle-control aliquot and a 1,25(OH)2 vitamin D-added aliquot (1 nmol l-1), added in parallel so that any effect of the DMSO solvent itself could be distinguished from the effect of vitamin D. Both aliquots were incubated for 30 minutes at 37°C in 5% CO2. After incubation, CASA was repeated to obtain post-treatment motility, kinematic (VAP, VSL, VCL, ALH, BCF), motility-grade (rapid/grade a, medium/grade b, slow/grade c, static/grade d) and head-elongation measurements, and intracellular reactive oxygen species were measured using the DCFH-DA fluorescence assay every 5 minutes for 30 minutes. Because the pellet and ring fractions, and the DMSO- and vitamin D-added aliquots, were all derived from the same individual specimen, every comparison in this study is paired (within-subject) rather than between independent groups, and was analyzed accordingly using the Wilcoxon signed-rank test.

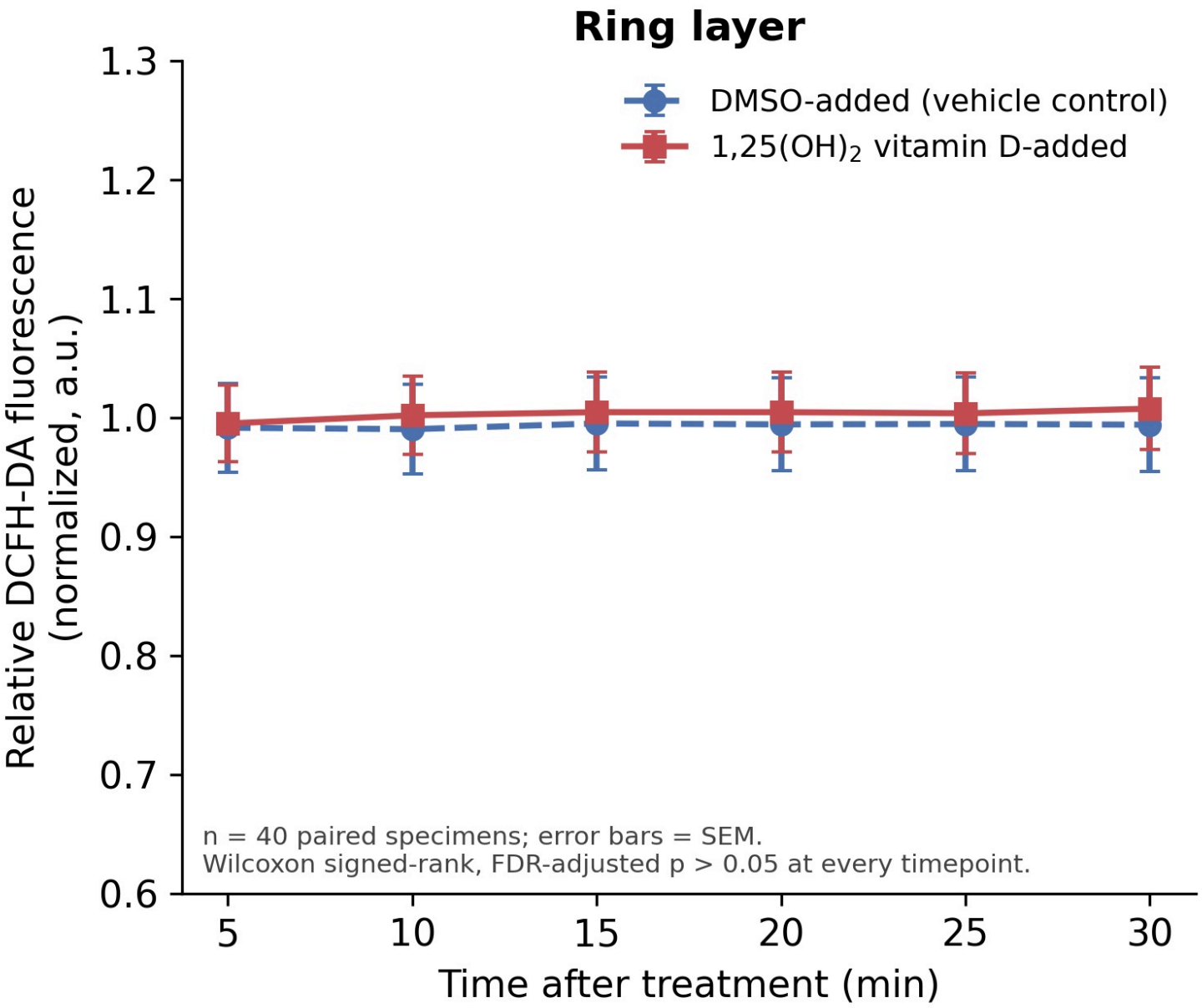


Figure 2

Intracellular reactive oxygen species (6) in the pellet layer after vitamin D or DMSO addition. Pellet-layer spermatozoa were split into paired DMSO-added (vehicle control) and 1,25(OH)2 vitamin D-added (1 nmol l-1) aliquots from the same specimen, and intracellular ROS was measured using the DCFH-DA fluorescence assay every 5 minutes for 30 minutes. Values are expressed as DCFH-DA fluorescence normalized within each specimen (relative units). Points show the mean and error bars show the standard error of the mean; n = 40 paired specimens at every timepoint (one of the 41 enrolled subjects had missing ROS measurements and was excluded from this analysis). Vitamin D-added and DMSO-added aliquots did not differ significantly at any timepoint (paired Wilcoxon signed-rank test; Benjamini-Hochberg FDR-adjusted p > 0.05 throughout).

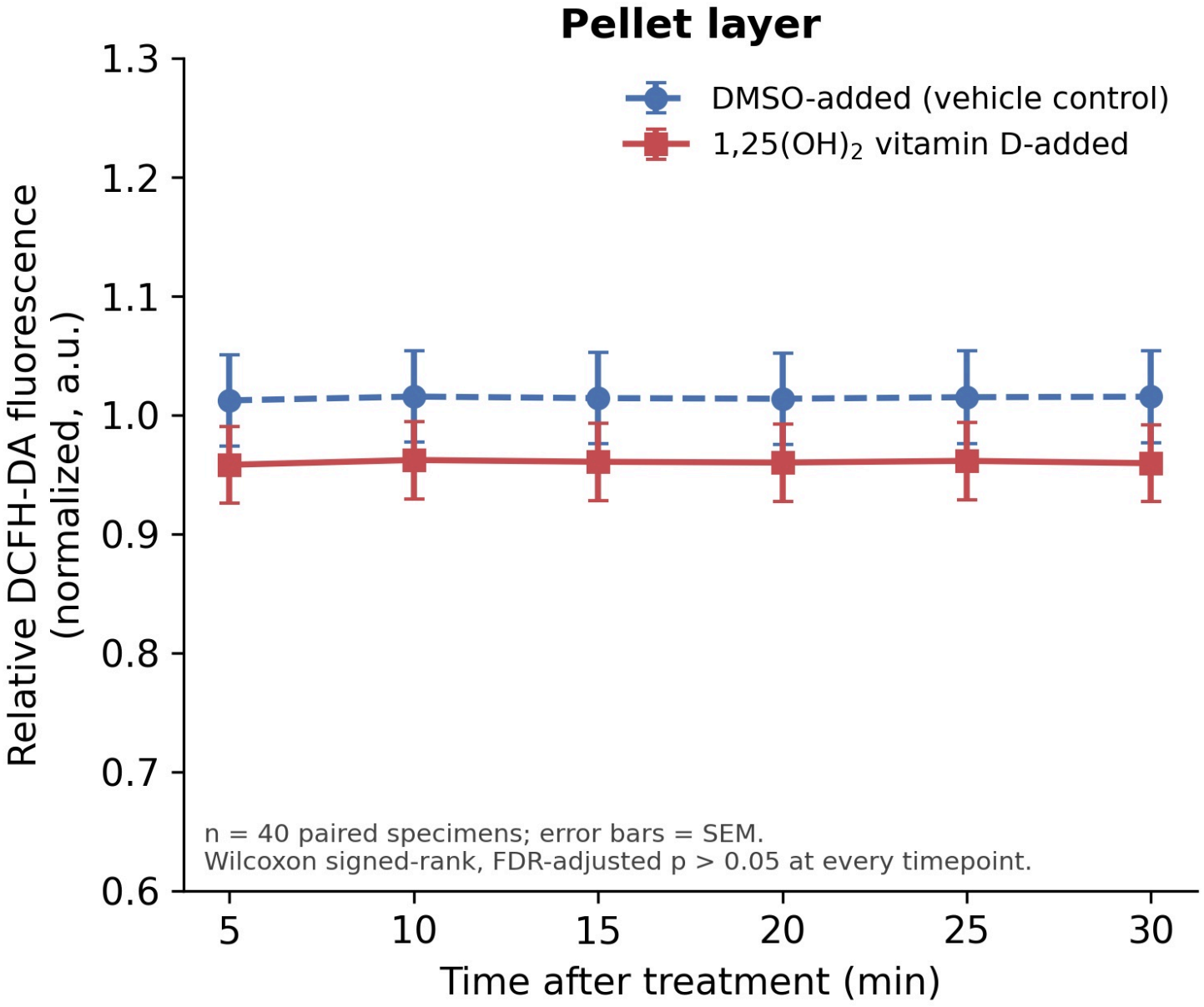


Figure 3

Intracellular reactive oxygen species (6) in the ring layer after vitamin D or DMSO addition. Ring-layer spermatozoa were split into paired DMSO-added (vehicle control) and 1,25(OH)2 vitamin D-added (1 nmol l-1) aliquots from the same specimen, and intracellular ROS was measured using the DCFH-DA fluorescence assay every 5 minutes for 30 minutes. Values are expressed as DCFH-DA fluorescence normalized within each specimen (relative units). Points show the mean and error bars show the standard error of the mean; n = 40 paired specimens at every timepoint (one of the 41 enrolled subjects had missing ROS measurements and was excluded from this analysis). Vitamin D-added and DMSO-added aliquots did not differ significantly at any timepoint (paired Wilcoxon signed-rank test; Benjamini-Hochberg FDR-adjusted $p > 0.05$ throughout).