CMMB
https://doi.org/10.31689/rmm.2025.32.2.161

ORIGINAL PAPERS

Impact of Vitamin D_3 and Reactive Oxygen Species on Sperm Functional Parameters in Patients with Grade III Varicocele

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Abstract

This study aimed to investigate the impact of reactive oxygen species (ROS) and vitamin D_3 on sperm parameters in patients diagnosed with grade III varicocele.

This case-control study included 70 patients diagnosed with varicocele grade III and 70 volunteers as the control group. These subjects were matched in age, BMI, geographical location, and socioeconomic background to minimize variances related to healthcare access, nutrition, and environmental factors, but not in those with varicocele. Semen parameters were assessed using an automatic CASA sperm analyzer. Semen ROS levels were measured using a fluorescence assay of 2',7'-dichlorofluorescein diacetate assay (DCFH-DA), and vitamin D_3 was quantified using an automatic biochemistry analyzer. Multiple linear regression analysis was used to assess the effect of vitamin D_3 and ROS on sperm parameters. Findings showed significant alteration of semen parameters in patients with grade III varicocele, reduced mean concentration of vitamin D_3 (14.61 ng/ml) compared to control (25.13 ng/ml) ($P < 0.001$), and increased mean ROS in semen (4791.29 RFU) compared to control (1607 RFU) ($P < 0.001$). Multiple linear regression analysis showed that vitamin D_3 positively affected sperm concentration ($\beta = 0.219$, $P = 0.006$) and total sperm motility ($\beta = 0.178$, $P = 0.016$). ROS negatively affected sperm concentration ($\beta = -0.418$, $P < 0.001$), total motility ($\beta = -0.534$, $P < 0.001$), progressive motility ($\beta = -0.518$, $P < 0.001$), and sperm morphology ($\beta = -0.318$, $P < 0.001$). Lower vitamin D_3 and higher semen ROS levels have a significant negative impact on sperm parameters in grade III varicocele patients. Treatment with antioxidant supplementation, specifically vitamin D_3, could improve semen quality in these patients.

Keywords: Varicocele; Reactive oxygen species; Vitamin D_3; Sperm parameters.

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INTRODUCTION

Male infertility significantly affects the lives of millions globally [1]. One-third of these cases can be attributed to varicocele [2]. Varicocele is characterized by enlargement of the venous plexus of the scrotum owing to dysfunctional venous valves and elevated venous pressure in the testes, causing signs of scrotal swelling, discomfort, and pain [3]. Although the link between varicocele and male infertility remains largely unknown, several factors were proposed to impact male infertility among this group of individuals. These include tissue hypoxia, waste accumulation, limitation in the hormonal supply, and hyperthermia[4]. Collectively, these factors can trigger oxidative stress, which is resulted from the imbalance between the production of reactive oxygen species (ROS) and antioxidant capacity of the tissue [5]. Higher levels of ROS were associated with significant alteration in normal sperm motility, viability, and fertilizing ability of sperm. Several studies correlated higher ROS levels to lower pregnancy rates following natural conception and infertility treatment [6]. High ROS levels were reported in semen of patients with varicocele along with significant impairment in testicular function, abnormal sperm parameters, and infertility [7,8].

Varicocelectomy is generally considered as the best clinical approach in treating varicocele. Studies reported reduction in the clinical signs associated with varicocele and improvement in the normal markers of male infertility [9]. Additional approaches included the use of antioxidant metabolites for their properties in fighting damage caused by oxidative stress and improving semen quality [5]. Vitamin D_3 is well known for its potent antioxidant and anti-inflammatory properties that significantly minimize the impact of oxidative stress on the testes and protect the cellular components of the sperm, including lipids, proteins, and DNA, from free radical accumulation [10,11]. In contrast, low levels of vitamin D_3 are associated with a higher prevalence of infertility, lower sperm motility, and higher ROS build-up [12,13]. Patients with advanced varicocele grades (grade III) display vitamin D_3 levels below the physiological range [14]. Providing patients with vitamin D_3 supplementation can significantly lower levels of oxidative stress, enhance sperm DNA integrity, and achieve higher success rates with infertility treatment [15]. Moreover, a recent investigation by Ağtürk *et al.* showed that vitamin D_3 supplementation in a rat model of varicocele led to lower expression of oxidative stress markers in the testes and a significant improvement in the structures of seminiferous tubules compared to the control group [16]. These studies suggest an intricate and direct association between oxidative stress and vitamin D_3 in men with varicoceles. However, only a limited number of clinical and experimental studies have been conducted to validate this association. Therefore, this study aimed to examine the association between ROS and vitamin D_3 levels and the sperm parameters of patients with varicocele. The findings of this study should offer insight into the impact of oxidative stress and vitamin D_3 on sperm parameters in patients with varicocele and the potential applications of supplementation with vitamins and antioxidants, specifically vitamin D_3, as an adjunct treatment for pre- and post-surgical intervention.

METHODS

This case-control study recruited 70 patients with bilateral varicocele grade III at the time of referral to the outpatient clinic of urology, Teaching Hospital of Hilla City, Iraq, for routine medical investigation and fertility assessment between November 2022 and December 2023. Patients with bilateral varicocele grade III were diagnosed by specialized doctors in urology according to the clinical signs of enlarged visible testicular veins, swollen scrotum, and scrotal pain [17]. Exclusion criteria included patients with a history of varicocelectomy, acute or chronic medical conditions that could affect sperm quality; sexually transmitted diseases; FSH, LH, and testosterone abnormalities; autoimmune diseases; tobacco smoking and alcohol drinking; and patients on antioxidants or vitamin D_3 supplementation.

The study included a control group consisting of 70 male volunteers who were referred to the same outpatient urology clinic for non-fertility-related medical conditions, including benign prostatic hyperplasia, mild urinary tract infections, kidney stones, and hydrocele. These subjects were selected on the basis of matching the varicocele group with respect in age, BMI, residential area, and socioeconomic background to minimize variances related to healthcare access, nutrition, and environmental factors, but not in those with varicocele. The exclusion criteria for the control group included diagnoses of varicocele at any grade of the disease, history of varicocelectomy, acute or chronic medical conditions that could impact sperm production and quality, such as bacterial and viral infection of the testes and genital tract, hormonal abnormalities, tobacco smoking and alcohol drinking, and medication or vitamin supplementations for the last six months prior to the study.

The study was approved by the Research Ethics Committee of the College of Medicine, University of Al-Qadisiyah, Iraq, on June 6th, 2022 (reference no. 31). Written informed consent was obtained from all participants, and the collection of subjects' information and samples was carried out in accordance with the Helsinki Declaration.

For semen collection and analysis, participants were asked to abstain for three days before collection with masturbation. Semen samples were then incubated at 37 °C for 30 min. before analysis using an automatic CASA sperm analyzer (ASCEN Technology, China).

To measure ROS levels in semen, samples were aliquoted in a 96-well microplate, and a 2',7'-dichlorofluorescein diacetate assay (DCFH-DA / Sigma, Germany) was performed according to Benedetti *et al.* [18]. Semen ROS levels were then quantified by measuring the fluorescence signal on a microplate reader (Bio-Rad, USA) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

Total vitamin D_3 levels were quantified in serum samples using the electrochemiluminescence binding assay on a Roche Cobas-e-411 automatic biochemistry analyzer (Roche Ltd., Germany).

Data collection and analysis were performed using the Statistical Package for the Social Sciences (SPSS), version 28 (IBM-USA). The sample sizes of the study and control groups were determined using power analysis (effect size = 0.15, P = 0.05, power = 0.8). Normality of variables was tested using the Kolmogorov-Smirnov test, and normally distributed variables were expressed as mean ± standard deviation (S.D.). To assess differences between study and control groups, an independent sample t-test was applied, and $P < 0.05$ was considered a significant difference. Multiple linear regression analysis was applied to predict the impact of vitamin D_3 and ROS on sperm parameters, and the coefficient of determination (R^2), standardized coefficients (β), and 95% confidence interval (95% CI) were determined. GraphPad Prism software version 9 was used to generate graphs.

RESULTS

The collected variables from all included subjects and a comparative analysis between the study and control groups are presented in Table 1. Statistical analysis showed no significant differences between the groups terms of age, BMI, and semen volume ($P > 0.05$). In contrast, semen pH, sperm concentration, total motility, progressive motility, non-progressive motility, immotile sperm, and sperm normal morphology were significantly different between the control and varicocele patients ($P < 0.05$). In the varicocele group, semen pH, sperm concentration, sperm total motility, progressive motility, sperm non-progressive motility, and sperm normal morphology were significantly lower than those in the control group and were below the reference values outlined in the fertility guidelines by WHO (2021). In contrast, the percentage of immotile sperm was significantly higher in the varicocele group than in the control group ($P < 0.001$) and higher than that of the WHO reference value (2021). The observed deviation of these parameters from WHO reference values suggests a strong association between varicocele and male infertility.

Quantifying vitamin D_3 in the serum showed a lower mean concentration of vitamin D_3 among varicocele patients (14.61 ng/ml) compared to control (25.13 ng/ml) ($P < 0.05$). Varicocele patients were also found to have significantly higher mean ROS in the semen (4791.29 RFU) than in the control (1607 RFU) ($P < 0.05$) (Table 1). To evaluate the effects of individual variations on the levels of vitamin D_3 and semen ROS, correlation analysis was performed to explore the association of age and BMI with vitamin D_3 and semen ROS. There was no correlation between age and vitamin D_3 ($P = 0.882$) or semen ROS ($P = 0.628$). BMI also showed no significant relationship with vitamin D_3 ($P = 0.893$) or ROS ($P = 0.857$) (Figures 1 and 2). These findings indicate that the observed differences in levels of vitamin D_3 and semen ROS between the control and study groups are related primarily to varicocele and not to any individual variations.

The impact of vitamin D_3 and semen levels of ROS on sperm parameters was assessed using multiple linear regression analysis (Table 2). The findings showed that vitamin D_3 was significantly associated with sperm concentration (β = 0.219, $P = 0.006$) and total sperm motility (β = 0.178, $P = 0.016$). Progressive sperm motility and morphology were not significantly associated with vitamin D_3 levels ($P > 0.05$). In contrast, semen ROS levels appeared to have more substantial and negative effects on semen quality than vitamin D_3 through its significant impact on sperm concentration

(β = -0.418, P < 0.001), total motility (β = -0.534, P < 0.001), progressive motility (β = -0.518, P < 0.001), and sperm morphology (β = -0.318, P < 0.001). Comparing the β regression coefficients undoubtedly indicate a higher influence of semen ROS on sperm parameters in contrast to vitamin D_3. Moreover, the findings also showed a significant negative correlation between serum vitamin D_3 and semen ROS levels (r = -0.42; P < 0.001), (Figure 3). This finding suggests that lower levels of vitamin D_3 could not counteract the prevalent damage caused by elevated ROS levels in the semen of patients with varicocele.

Table 1. Summarizes patients' variables and comparative analysis between control and varicocele groups.

Variables	Control group (n=70) Mean ± S. D.	Varicocele group (n=70) Mean ± S. D.	P value
Age (years)	37.51 ± 6.84	38.60 ± 7.28	0.356
BMI (kg/m^2)	22.99 ± 4.39	22.34 ± 4.61	0.396
Semen pH	7.61 ± 0.59	7.39 ± 0.54	0.023*
Semen volume (ml)	3.09 ± 1.01	2.92 ± 1.22	0.392
Sperm concentration (million/ejaculate)	61.66 ± 23.31	26.55 ± 16.34	<0.001***
Sperm total motility (%)	70.47 ± 19.49	31.68 ± 15.35	<0.001***
Sperm progressive motility (%)	33.16 ± 14.51	9.18 ± 5.58	<0.001***
Sperm non-progressive motility (%)	37.30 ± 16.68	22.51 ± 11.03	<0.001***
Immotile sperm (%)	29.53 ± 19.49	68.32 ± 15.35	<0.001***
Sperm normal morphology (%)	8.43 ± 5.17	3.42 ± 2.25	<0.001***
Vitamin D_3 (ng/ml)	25.13 ± 11.05	14.61 ± 7.66	<0.001***
ROS (RFU)	1607 ± 625.28	4791.29 ± 2058.42	<0.001***

**Significant at P values <0.05; ***Significant at P values <0.001; RFU: Relative fluorescence unit.*

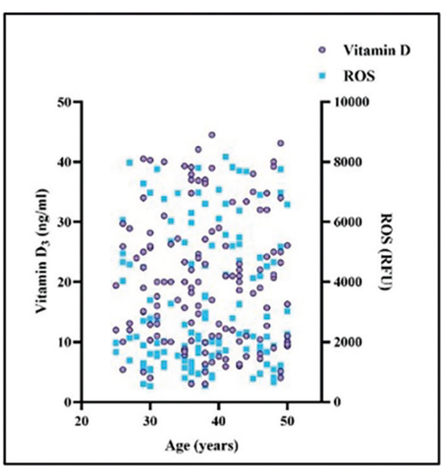

Figure 1. A two-sided scatterplot shows the relationship between age with vitamin D_3 (○, plotted on the left side of the y-axis), and semen ROS (□, plotted on the right side of the y-axis) for all subjects of the study. No significant correlations were found between age and vitamin D_3 (P = 0.882), and age and semen ROS (P =0.628).

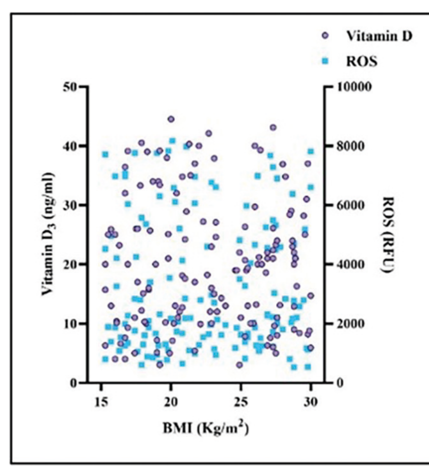

Figure 2. A two-sided scatterplot shows the relationship between BMI with vitamin D_3 (○, plotted on the left side of the y-axis), and semen ROS (□, plotted on the right side of the y-axis) for all subjects of the study. No significant correlations were found between BMI and vitamin D_3 (P = 0.893), and BMI and semen ROS (P = 0.857).

Table 2. Multiple linear regression analysis for predicting impact of vitamin D_3 and semen ROS on sperm parameters.

Parameters	R^2	Vitamin D_3			ROS		
		β	(95% CI)	*P* value	β	95% CI	*P* value
Semen pH	0.049	0.128	-0.030 – 0.178	0.146	- 0.134	-0.181 – 0.027	0.145
Semen volume (ml)	0.016	-0.113	-0.298 – 0.071	0.226	-0.123	-0.307 – 0.061	0.189
Sperm concentration (million/ml)	0.299	0.219	0.063 – 0.374	0.006 **	-0.418	-0.574 - -0.262	<0.001 ***
Sperm total motility (%)	0.397	0.178	0.034 – 0.323	0.016 *	-0.534	-0.678 - -0.390	<0.001***
Sperm progressive motility (%)	0.343	-0.132	- 0.018 – 0.283	0.085	-0.518	-0.669 - -0.367	<0.001 ***
Sperm non-progressive motility (%)	0.168	0.148	-0.022 - 0.317	0.097	0.326	-0.495 - -0.156	<0.001 ***
Immotile sperm (%)	0.397	- 0.178	-0.323 - -0.034	0.019 *	0.534	0.390 – 0.678	<0.001 ***
Sperm normal morphology (%)	0.166	0.155	-0.015 – 0.325	0.074	-0.318	-0.488 - -0.148	<0.001 ***
Vitamin D_3	0.175	-	-	-	-0.418	-0.571 - -0.265	<0.001 ***
ROS	0.175	-0.418	-0.571 - -0.265	<0.001 ***	-	-	-

**Significant at P values <0.05; **Significant at P values <0.01; ***Significant at P values <0.001.*

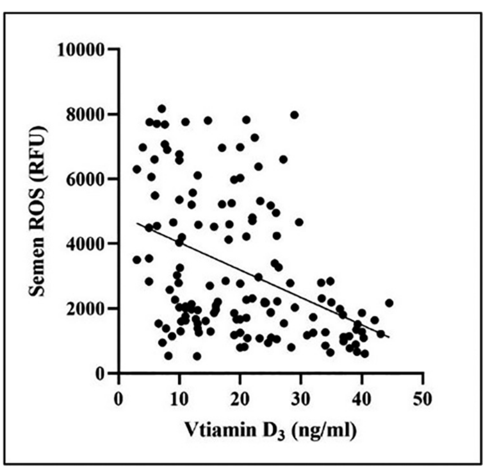

Figure 3. Scatterplot shows significant negative correlation between serum vitamin D_3 concentration and semen ROS levels ($P < 0.001$).

DISCUSSION

The findings from the current analysis provide important insights into the impact of varicocele (grade III) on normal sperm parameters and male infertility. The analysis showed that the semen characteristics of patients with varicocele were significantly altered compared to those of the control group. For instance, there were significant decreases in the mean values of sperm concentration, sperm total motility, sperm progressive motility, non-progressive motility, and sperm normal morphology in the varicocele group. The analysis also showed that the mean value of immotile spermatozoa in the varicocele group was higher than that in the control group. In fact, those patients showed mean values of semen parameters that were found to be abnormally deviated from the reference values of the WHO fertility guidelines [19]. The current findings are in line with previous research on the impact of varicocele on testicular function, sperm functional characteristics, and lower rates of spontaneous conception among this population [8,20]. Patients with advanced varicocele usually present with a significant reduction in sperm concentration and total sperm count per ejaculate compared to patients with mild to moderate grades of varicocele [21]. Higher severity of varicocele was also reported to have higher rates of abnormal sperm morphology, an increase in the percentage of immotile sperm, and higher sperm DNA fragmentation [22].

The impact of varicocele on semen characteristics is believed to be related to elevated levels of ROS [8]. ROS have been found to negatively impact sperm formation and maturation, sperm motility, morphology, and fertilizing ability of sperm [23]. ROS also adversely affects testosterone production from Leydig cells, causing impaired spermatogenesis, abnormal sperm maturation in the epididymis, and male infertility [2]. Studies have also shown that ROS have a direct effect on the integrity of the sperm plasma membrane and mitochondrial function, resulting in lower sperm motility and higher incidence of sperm DNA fragmentation [5,24]. In our study, ROS levels were found to be higher in the semen of the varicocele group and were significantly linked to reduced semen quality. In addition, regression analysis revealed a significant negative effect of ROS on sperm concentration, total motility, progressive motility, and morphology. These results are in line with several previous reports, which collectively highlight a significant correlation between semen ROS, abnormal sperm parameters, and male infertility [5,7]. Elevated levels of ROS were identified by the current analysis as a risk factor and predictor of abnormal sperm characteristics among varicocele patients. However, other studies have reported a less notable impact of varicocele on normal sperm parameters and male infertility [8,25]. Variation across studies on the impact of varicocele on male infertility could be related to differences in study size, demographic factors, varicocele grade, and other methodological approaches adopted by different studies.

Moreover, patients with varicocele exhibit a lower mean concentration of vitamin D_3 which is associated with altered sperm parameters. These findings are in line with multiple previous studies highlighting that vitamin D_3 deficiency can impose severe changes on sperm functional parameters that can ultimately lead to male infertility [14,26]. Vitamin D_3 is essential for health and reproduction due to its role as an antioxidant, anti-inflammatory, calcium ion regulator, and its widely-documented positive impact on sperm quality and male sex hormones [13,27,28]. In contrast, vitamin D_3 insufficiency has been reported to negatively affect the normal levels of testosterone, spermatogenesis, and semen quality [29]. A study by Derakhshan and others identified vitamin D_3 deficiency as a potential risk factor for male infertility [30]. In addition, infertility outcomes with IVF treatments can significantly increases with higher levels of vitamin D_3 [31]. Although there are a limited number of studies on vitamin D_3 and varicocele, studies by Cannarella *et al.* and Ağtürk *et al.* demonstrated a significant association between varicocele and lower vitamin D_3 levels [14,16]. In line with these studies, the reported significant association between vitamin D_3 and varicocele indicates that vitamin D_3 deficiency can be considered a risk factor and a predictor for changes in semen characteristics among varicocele patients, and that it can be employed as a biomarker for fertility evaluation, at least within the context of varicocele.

Furthermore, the observed negative association between vitamin D_3 and ROS suggests a potential protective role of vitamin D_3 for sperm parameters against elevated oxidative stress in the semen of varicocele patients. This was witnessed in the current study first through the positive association between vitamin D_3 and sperm concentration and sperm total motility, (Table 1), and second by examining the significantly higher β regression coefficients for ROS in comparison to the β regression coefficients for vitamin D_3, (Table 2). Decreased vitamin D_3 levels below physiological levels could contribute to the generation of higher oxidative stress in the semen, (Figure 3). This was evident by Shahid *et al.* who reported a similar negative correlation between vitamin D_3, ROS, and sperm parameters [32]. However, this is the first study to report this correlation among patients with varicocele.

There are several limitations to the current investigation. First, it included 70 patients with varicocele from a single center of urology, which can be considered a relatively localized and small study size. A larger and more diverse multi-centered patients' collection should provide a better evaluation of the impact of varicocele, vitamin D_3, and ROS levels on sperm parameters of varicocele patients. Second, the study lacked patients over time follow-up examination, which can be considered necessary for determining the long-term effects of varicocele on semen parameters, vitamin D_3, and ROS, in addition to the potential improvements that can be acquired from surgical treatment or vitamin D_3 supplementation to enhance semen quality and reduce oxidative stress. Despite these limitations, this study provided important insights into the relationship between grade III varicocele, oxidative stress, vitamin D_3, and semen quality.

CONCLUSION

Patients with grade III varicocele showed significant alterations in semen and sperm functional parameters, including low sperm count, low motility, and abnormal morphology. These changes were significantly associated with elevated levels of ROS in semen and a reduction in the levels of vitamin D_3 in the serum of patients with varicocele. The negative correlation between vitamin D_3 and ROS levels suggest that vitamin D_3 deficiency could exacerbate the impact of oxidative stress on sperm parameters, further damaging sperm quality. Further studies are required to determine the potential effectiveness of varicocelectomy or vitamin D_3 supplementation in reducing semen ROS and improving semen quality in patients with varicocele.

Ethics Statement and Conflict of Interest Disclosures

Financial support and sponsorship: All authors have declared that no financial support was received from any organization for the submitted work.

Ethics Consideration: The authors declare that all the procedures and experiments of this study respect the ethical standards in the Helsinki Declaration of 1975, as revised in 2008(5), as well as the national laws. Written informed consent was provided by all participants in this study.

Conflict of interest: No known conflict of interest correlated with this publication.

Availability of data and materials: The data used and/or analyzed throughout this study are available from the corresponding authors upon reasonable request.

Competing interest: The authors declared that they have no competing interests.

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