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DNA Fragmentation and Vitamin K2 Levels in Sperm Maturation and Apoptosis

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Abstract. Background: There is mounting evidence that vitamin K2 (menaquinones) plays a role as a hormone and transcriptional regulator, which has raised interest in its possible impact on male reproductive health. The vitamin K series as a whole has a long history of therapeutic use around the world. Because of its essential function in removing damaged germ cells and preserving the quality of spermatozoa, vitamin K may have an antioxidant impact that is advantageous to spermatogenesis, a process regulated by apoptosis. Aim of the study: Association of DNA fragmentation as an apoptotic marker and Vit K2 levels with sperm maturation. Patients, materials and methods: Sixty-seven infertile men were included in this Cross-sectional study. All the patients underwent Vit K assessment by ELISA, Seminal fluid analysis according to WHO 2021 and DNA fragmentation test using aniline blue staining. Results: The study found highly significant inverse correlation between vit K2 level and DNA fragment proportion of the study sample of semen but there is inverse insignificant correlation of fragmentation with sperm motility while other semen parameters showed insignificant positive correlation including concentration, vitality and normal morphology. Progressive motile sperms (AB) and immotile sperms (D) were found with high significant difference between sperm DNA fragmentation and non-sperm DNA fragmentation. Also, there were no significant differences between infertile men with and without sperm DNA fragmentation in age, smoking, alcohol conception, varicocele and type of infertility. Conclusions: The findings revealed a highly significant negative correlation between vitamin K2 levels and sperm with DNA fragmentation, as well as a highly significant positive correlation between vitamin K2 levels and sperm without DNA fragmentation. The findings indicate that vitamin K2 may play a protective function in protecting sperm DNA integrity.

Highlights:

1. Vitamin K2 Protects Sperm DNA – Higher K2 levels correlate with lower DNA fragmentation.
2. Minimal Impact on Motility – Fragmentation shows weak links to sperm motility and other parameters.
3. No Link to Lifestyle – Factors like smoking and varicocele show no significant effect.

Keywords: Vit K, Apoptosis, DNA Fragmentation, Biomarker, ROS

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Introduction

Male infertility is a multifactorial condition that affects a significant proportion of couples worldwide, with sperm abnormalities accounting for approximately 50% of cases. [1] Infertile men, who have lower odds of conceiving naturally, exhibit high levels of sperm DNA fragmentation (SDF). The mechanisms driving SDF include abnormal spermatogenesis, oxidative stress damage, and abnormal sperm apoptosis [2]. Apoptosis, or programmed cell death, is a fundamental process that regulates cellular homeostasis and tissue remodeling in multicellular organisms. In the context of spermatogenesis, since it was demonstrated that spermatogenesis can be controlled by apoptosis, as a result of its crucial role in eliminating defective germ cells and maintaining the quality of spermatozoa, many reports analyzed the association of DNA fragmentation, a hallmark of apoptosis, with semen quality. [3] On the other hand, as abnormal sperm apoptosis can induce SDF and subsequent radical changes leading to male infertility.[2] Because increased levels of DNA fragmentation have been associated with impaired sperm motility, reduced fertilization rates, and higher rates of pregnancy loss.[4] Moreover, although, the conventional semen analysis remains the cornerstone for assessing male fertility potential, however, it often fails to provide comprehensive insights into the underlying mechanisms of sperm dysfunction. So, DNA fragmentation, has emerged as a potential biomarker for assessing sperm quality and fertility potential.[5] Therefore, there is a growing interest in exploring novel biomarkers and molecular pathways associated with sperm maturation and function. Vitamin K series has long been recognized as a therapeutic agent globally, particularly vitamin K2 (menaquinones), and evidence suggests its role as a transcriptional regulator and a hormone.[6] An even broader impact of vitamin K (menaquinone) which is a fat-soluble vitamin on human health has been revealed by the identification of non-hemostatic vitamin –K-dependent (VKD) proteins (Matrix Gla Protein (MGP), osteocalcin, Gla Rich Protein (GRP) and four transmembrane proteins (TMG s) that have been confirmed to undergo an unusual post-translational modification, which is the conversion of specific Glu residues to carboxylated Glu (Gla). Gla generation is required for the activation of VKD proteins, and occurs in the endoplasmic reticulum during their secretion to either the cell surface or from the cell.[7] Recently, it has garnered attention for potential role of vitamin k in male

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reproductive health. Other study has reported that, as in other cell type the MGP has also been found in human spermatozoa. Moreover, it offers a plausible protection against DNA damage and apoptotic changes to the germ line.[8]

As well as beyond its well-established role in blood coagulation and bone metabolism, emerging evidence suggests that vitamin K2 may exert protective effects on spermatozoa by modulating oxidative stress and apoptosis. However, the relationship between vitamin K2 levels and sperm maturation remains poorly understood, warranting further investigation.[9] Based on mentioned evidence, we hypothesize that increased DNA fragmentation levels may be inversely correlated with sperm maturation, while higher vitamin K2 levels may be associated with improved sperm quality and maturation. So, the aim of this study is to explore the associate\ion between DNA fragmentation as an apoptotic marker and vitamin K2 levels with sperm maturation. [10]

Materials and Method

1. Design of the Study

Cross- sectional study

2. Sample and Setting of the Study

The present study includes 67 infertile men who attended the male infertility clinic at the High Institute for Infertility Diagnosis and Assisted Reproductive Technologies /Al-Nahrain University between June and August 2022. The study was approved by the ethical committee of the High Institute for Infertility Diagnosis and Assisted Reproductive Technologies / Al-Nahrain University. History details were obtained regarding age, infertility duration, type of infertility (primary or secondary infertility), residency, varicocele and smoking. Semen samples and blood samples were taken from all the patients.

3. Data Collection Method and Instrument

a. DNA Fragmentation Test

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Semen underwent seminal fluid analysis according to WHO 2021 and assessment for DNA integrity by use of acidic aniline blue stain. The Aniline blue stain is used to assess sperm chromatin conformation, revealing differences in nuclear protein composition of human spermatozoa. Histone-rich nuclei absorb the blue stain, while protamine-rich nuclei lack it, indicating differences in the basic nuclear protein composition of immature and mature spermatozoa. [11-13] Using Aniline blue powder (5g) dissolved in distilled water (100 ml) containing 4% acetic acid (pH 3.5) at the final concentration of 5% according to WHO 2021. Sperm samples were air-dried, preserving in 4% formaldehyde and aqueous AB stain for 30 minutes. At least 200 spermatozoa were counted using a high-resolution lens and light microscopy. Dark blue and unstained cells were considered abnormal, while pale blue stained cells were normal. The percentage of sperm chromatin was calculated, and an ejaculate with less than 20% blue-stained nucleus sperm was considered normal.

b. Vit K Measurement

The human vitamin K2 (VK2) is assayed using a kit using the enzyme-linked immune sorbent assay (ELISA), which is based on the Biotin double antibody sandwich technology. First, add Vitamin K2 (VK2) to the wells that have been pre-coated with Vitamin K2 (VK2) monoclonal antibody. Anti-VK2 antibodies labeled with biotin to unite with streptavidin-HRP, which forms immune complex. After incubation and washing the unbound enzymes were removed. Then substrate A and B were added. The solution turned blue and then turned yellow under the influence of acid. The concentration of Human Vitamin K2 (VK2) and the hues of the solution correspond positively.

4. Statistical Analysis

The results were analyzed using the Chi-square test, paired t-test, frequency, and percentage using the statistical software IBM SPSS (Statistical package for social sciences) program (Version-25).

5. Limitations of the Study

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While this study gives valuable details, numerous limitations should be noted. The sample size may have compromised the ability to find subtler associations between vitamin K2 levels and sperm parameters.

Results

Correlation Between Vitamin K2 Levels, Semen Characteristics, and Sperm DNA Fragmentation Index in the Study Sample.

There is high significant inverse correlation between level of vitamin K2 and DNA fragment proportion of the study sample of semen (r= -0.459. P = 0.0001) as showed in Table 1 and Figure 1. There is inverse insignificant correlation of fragmentation with sperm motility (r= -0.04, p= 0.798) while other semen parameters showed insignificant positive correlation including body mass index, concentration vitality and normal morphology (p>0.05).

Table 1. Correlations Between Vitamin K2 and Some Semen Characteristics with Sperm DNA Fragmentation Index in Study Sample

Sperm factors	% DNA fragmentation	
	R	**p value***
Vitamin K2	-0.459	0.0001
BMI	0.188	0.183
Concentration	0.027	0.758
Sperm motility	-0.041	0.798
Vital sperms	0.028	0.880
Frequency of normal morphology	-0.008	0.970

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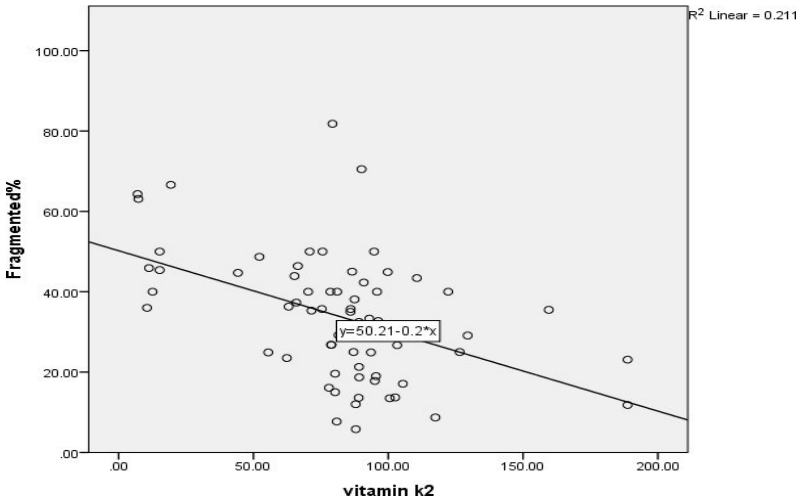

Figure 1. Regression Relationship Between Vitamin K2 Level and DNA Fragment Proportion in Sperms of Study Sample

Figure 2 illustrates the mean serum vitamin K2 levels in relation to sperm DNA fragmentation percentages. The bar chart shows that individuals with sperm DNA fragmentation below 20% had a higher mean serum vitamin K2 level (98.60) compared to those with 20% or more DNA fragmentation (77.24). The error bars indicate variability in the data. This finding supports the study's conclusion that higher vitamin K2 levels are associated with lower sperm DNA fragmentation, suggesting a potential protective role of vitamin K2 in maintaining sperm DNA integrity.

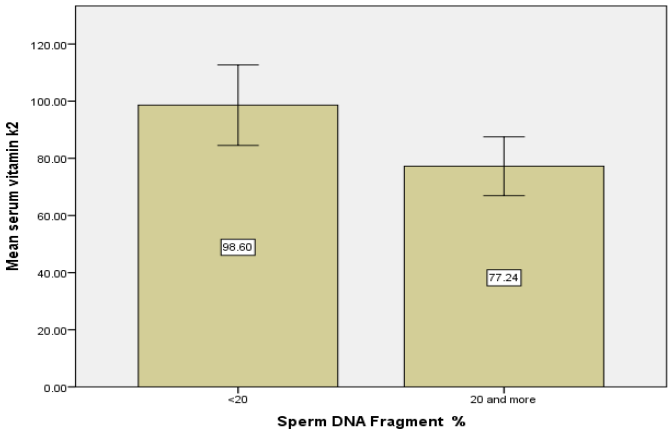

Figure 2. Mean Vitamin K2 Serum Level by Sperm DNA Fragments in Study Sample

Figure 3 illustrates the relationship between age (years) and the percentage of fragmented sperm samples. The regression equation y=28.82+0.14xy = 28.82 +

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0.14xy=28.82+0.14x and the low R^2 value (0.005) indicate a weak and statistically insignificant correlation between age and sperm DNA fragmentation. This finding suggests that age has little influence on the extent of sperm DNA fragmentation in the study population, aligning with the article's results that no significant differences were found between infertile men with and without sperm DNA fragmentation based on age.

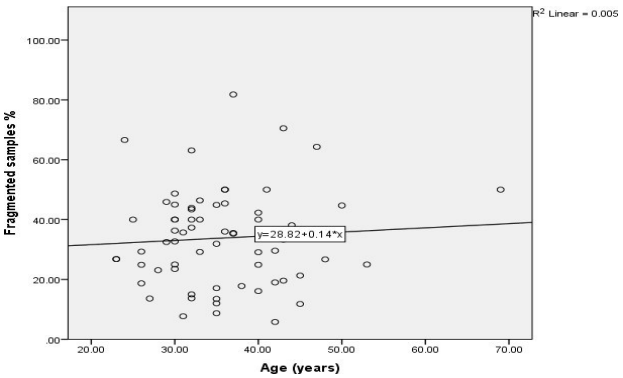

Figure 3. Regression Relationship Between DNA Sperm Fragment Proportion and Patient's Age of Study Sample (r=0.071. p=0.566)

Figure 4 illustrates the relationship between body mass index (BMI) and the percentage of fragmented sperm samples. The regression equation y=51.45−0.59xy = 51.45 - 0.59xy=51.45−0.59x and the low R2R^2R2 value (0.035) indicate a weak negative correlation between BMI and sperm DNA fragmentation. This suggests that higher BMI may be slightly associated with lower sperm DNA fragmentation, but the correlation is not statistically significant. This finding aligns with the article's results, which reported an insignificant relationship between BMI and sperm DNA fragmentation.

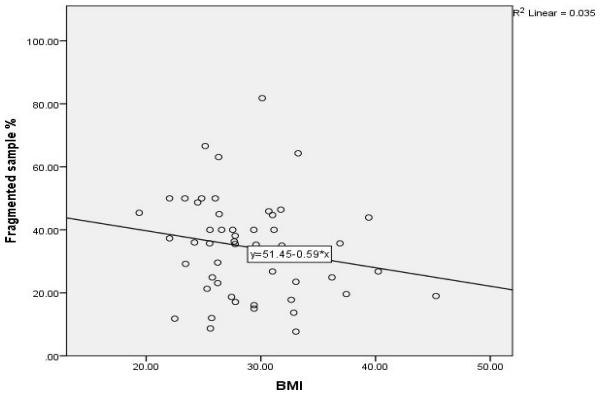

Figure 4. Regression Relationship Between DNA Sperm Fragment Proportion and Patient's BMI of Study Sample (r=1.88, p= 1.83)

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Analysis of Semen Parameters in Relation to Sperm DNA Fragmentation

Progressive motile sperms (AB) and immotile sperms (D) were found with high significant between sperm DNA fragmentation and non- sperm DNA fragmentation (P Value =0.014 and 0.028) respectively as shown in table 2.

Table 2. Comparison of Semen Parameters According to Sperm DNA Fragmentation

Motility		**N**	**Mean**	**Std. Deviation**	**Std. Error**	**95% Confidence Interval for Mean**		**P Value**
						Lower Bound	**Upper Bound**	
AB %	< 20	33	35.42	17.432	3.035	29.24	41.61	0.014
	≥ 20	55	26.64	14.815	1.998	22.63	30.64	
	Total	88	29.93	16.319	1.740	26.47	33.39	
C %	< 20	33	15.94	7.163	1.247	13.40	18.48	0.801
	≥ 20	55	15.51	8.071	1.088	13.33	17.69	
	Total	88	15.67	7.704	.821	14.04	17.30	
D%	< 20	33	48.64	19.819	3.450	41.61	55.66	0.028
	≥ 20	55	57.85	18.094	2.440	52.96	62.75	
	Total	88	54.40	19.179	2.044	50.33	58.46	

Sociodemographic Comparison Between Infertile Men With and Without Sperm DNA Fragmentation (N=67)

Table 3 shows there were no significant differences between infertile men with and without sperm DNA fragmentation in age, smoking, alcohol conception, varicocele and type of infertility P value 0.597, 0.251, 0.776**,** 0.258 and 0.243 respectively.

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Table 3. Comparison Between Infertile Men With Sperm DNA Fragmentation and Non-Sperm DNA Fragmentation in Sociodemographic Characteristics (N=67).

Patient Characteristics	Fragmented Samples		Total	P Value
	< 20%	**≥ 20%**		
Age Group (Years)				
< 40	10 (66.7%)	35 (67.3%)	45 (67.2%)	0.597
≥ 40	5 (33.3%)	17 (32.7%)	22 (32.8%)	
Smoking				
No	4 (26.7%)	23 (44.2%)	27 (40.3%)	0.251
Yes	11 (73.3%)	29 (55.8%)	40 (59.7%)	
Alcohol				
No	15 (100.0%)	51 (98.1%)	66 (98.5%)	0.776
Yes	0 (0.0%)	1 (1.9%)	1 (1.5%)	
Varicocele				
No	11 (73.3%)	44 (84.6%)	55 (82.1%)	0.258
Yes	4 (26.7%)	8 (15.4%)	12 (17.9%)	
Type of Infertility				
Primary	8 (53.3%)	35 (67.3%)	43 (64.2%)	0.243
Secondary	7 (46.7%)	17 (32.7%)	24 (35.8%)	
Total	15 (100.0%)	52 (100.0%)	67 (100.0%)	

Discussion

The present study aimed to explore the correlation between vitamin K2 levels and various sperm parameters, with a particular focus on DNA fragmentation. The significant negative correlation between vitamin K2 levels and sperm DNA fragmentation, showed in Figure 1, suggests that higher levels of vitamin K2 may be associated with

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lower rates of DNA damage in sperms. This is consistent with the increasing number of research showing the significance of vitamin K2 in cellular health and its possible antioxidant capabilities.[14] Previous research has indicated that oxidative stress is one of the key causes of DNA damage in sperm, and vitamin K2's role in lowering oxidative stress may explain the protective effect reported in this study. [15, 16] The positive correlation between vitamin K2 and sperm with intact DNA, in the current research, further supports this hypothesis. Interestingly, in Table 1 the study discovered an inverse but not statistically significant correlation between sperm motility and DNA fragmentation ($r = -0.04$, $p = 0.798$). While this reveals a weak tendency in which sperm with less DNA fragmentation have slightly higher motility, the absence of statistical significance suggests that the association may not be strong in this population. Sperm motility is frequently influenced by a variety of variables, including oxidative stress, but the current findings suggest that DNA fragmentation and motility are not closely associated in this dataset. [17] Table 3 showed the correlations between DNA fragmentation and other semen indicators, such as body mass index, sperm concentration, vitality, and normal morphology, were likewise statistically not significant ($p > 0.05$). This shows that, while these parameters are important for overall semen quality, they may not directly affect or be influenced by sperm DNA fragmentation in the context of this study. However, it is vital to consider the possibility of unremarkable interactions that were missed due to sample size or other study constraints. Previous researches have shown inconsistent conclusions on the association between DNA fragmentation and sperm morphology, concentration, and motility. [18, 19] According to some research, defective sperm morphology or poor concentration can be connected to increased DNA fragmentation, others have found no significant associations. [20, 21] The lack of a significant association in this study shows that vitamin K2's preventive activity against DNA fragmentation may function independently of these standard semen parameters. The processes by which vitamin K2 may protect against DNA fragmentation are not entirely known. One proposed explanation is that it controls oxidative stress and promotes calcium homeostasis. Oxidative stress is a well-known contributor in sperm DNA damage, and vitamin K2 may help alleviate this by acting as an antioxidant or increasing the activity of antioxidant enzymes. [22, 23] Additionally, calcium is essential for sperm

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motility and function, and vitamin K2's role in calcium management may indirectly enhance sperm health. [24] Also, VitK1 and Menatetrenone MK-4 prevent oxidative cell death by inhibiting the activation of 12-LOX and ROS production. [25]

Clinical Implications

The study's findings could have a substantial impact on male fertility treatments. Vitamin K2 supplementation may be a non-invasive way to increase sperm quality, especially for men who have significant levels of DNA fragmentation. Additional study, including randomized controlled trials, is required to confirm these findings and investigate the possible therapeutic effect of vitamin K2 in male infertility.

Limitations

Due to the cross-sectional design, causation cannot be proven; additional longitudinal studies are needed to assess whether raising vitamin K2 levels can directly reduce DNA fragmentation over time.

Conclusions

The findings revealed a highly significant negative correlation between vitamin K2 levels and sperm with DNA fragmentation, as well as a highly significant positive correlation between vitamin K2 levels and sperm without DNA fragmentation. The findings indicate that vitamin K2 may play a protective function in protecting sperm DNA integrity. By elucidating the relationship between DNA fragmentation, vitamin K2 levels, and sperm maturation, our findings have the potential to enhance our understanding of male infertility mechanisms and inform the development of targeted therapeutic strategies for couples struggling with fertility issues.

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