Experimental
&
Clinical Article

**Reproductive Medicine;** *Endocrinology and Infertility*

# Semen Parameters Can be Used as a Credible Marker with Doppler Ultrasonography in the Diagnosis of Subclinical Varicocele Cases

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**ABSTRACT**

**OBJECTIVES:** The link between varicoceles and male infertility has been a problem of debate for more than half a century. A substantial amount of data about varicocelectomies' effects has been provided, but inadequate study designs and heterogeneity of current studies make these data rarely conclusive. This article investigates whether semen and sperm analysis in subclinical varicocele patients without any clinical signs diagnosed by Doppler Ultrasonography (USG) can be used as a diagnostic tool.

**STUDY DESIGN:** The current prospective cohort study included infertile male patients (n=44) enrolled in the Urology Clinic of Biruni University Hospital from January 2017 to January 2018. Patients were divided into two groups: 1st group as the control group (CG) (n=22): No varicocele as determined by Doppler USG, and 2nd group as the test group (TG) (n=22): Subclinical varicocele (SCV) as determined by Doppler USG. A semen analysis was done. Sperm were dyed with aniline blue and chromomycin A3 (CMA3), acridine orange (AO), propidium iodide (PI), and Rhodamine 123 (Rh123) for the determination of sperm maturation, sperm DNA fragmentation, apoptosis, and sperm mitochondrial membrane potential, respectively. Student t Test was used for statistical analysis. $p<0.05$ was considered significant.

**RESULTS:** The sperm concentration of the CG was higher than the TG ($p=0.98$, $p>0.05$). Forward sperm motility in the CG was higher than in the TG ($p<0.001$, $p<0.05$). Sperm with normal morphology in the TG was lower than in the CG ($p<0.001$, $p<0.05$). Sperm neck anomalies were higher in varicocele cases ($p<0.001$, $p<0.05$). Sperm maturation at the TG was lower than the CG ($p<0.001$, $p<0.05$). A high apoptotic sperm rate was found at the TG ($p<0.001$, $p<0.05$). Sperm mitochondria potential at the TG was lower than the CG ($p<0.001$, $p<0.05$). Sperm chromatin condensation at the TG was higher than the CG ($p<0.001$, $p<0.05$).

**CONCLUSIONS:** The analysis of sperm DNA and apoptosis in SCV cases can be used as a reliable diagnostic tool to confirm Doppler USG.

**Keywords:** DNA fragmentation; Sperm apoptosis; Sperm maturation; Subclinical varicocele; Ultrasonography

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## Introduction

A varicocele is a varicose vein in the scrotum resulting from an abnormal dilation of the internal spermatic vein and pampiniform venous plexus within the spermatic cord. The causes of varicocele largely remain unknown, but increased testis temperature, hormonal imbalances, increased venous pressure, oxidative stress, hypoxia, and reflux of toxic metabolites from adrenals or renal origin are thought to contribute to varicocele-related testicular failure (1).

Varicocele is the most common treatable cause of male infertility, affecting approximately 40% of men with primary infertility and 80% of men with secondary infertility. Varicocele is detected in 25% of men with abnormal semen parameters,

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compared with 12% of men with normal semen parameters, providing data that it is associated with impaired testis function and male infertility. Only 20% of men have fertility problems among adults with documented varicocele, so it may be concluded that no treatment may be required for the remaining 80% of men suffering from varicocele (1). Subclinical varicocele (SCV) is an early phase in the progression of its clinical counterpart. Because of few relevant studies about the treatment strategies, no conclusive answer can be found regarding how to manage SCV (2). The aim of treating varicocele in adults is to regain fertility and improve current fertility status. In contrast, in most cases, the treatment purpose for adolescent varicocele is to prevent testis injury and maintain testis function i.e. normal spermatogenesis and hormone production for future fertility.

Physical examination is the main diagnostic tool for the diagnosis of varicocele The examination should be performed in a warm room and the patient must be examined in both supine and standing positions. The scrotum should be examined visually and by palpation, and a Valsalva maneuver should be performed (1). Examination grades for both adult and adolescent varicoceles can be described as follows: Grade I, palpable impulse in the spermatic cord veins during Valsalva maneuver without enlargement of the veins at rest; Grade II, palpable engorged veins with the patients standing without Valsalva maneuver, but not visible; Grade III, veins easily visible through the scrotal skin while the patient is standing (1).

The size of each testis should be determined using an orchidometer and/or ultrasonography. Ultrasonography offers greater accuracy than using an orchidometer for testicular volume measurement (3). Nevertheless, in clinical practice, an orchidometer is still a reliable tool to measure testicular volume since there is a close relationship between ultrasonography-derived and orchidometer-derived testicular volume (4). In either case, it is critical to determine whether there is a discrepancy in volume between the left and right testicles, as such a discrepancy could guide intervention.

Varicoceles impair semen parameters and sperm DNA, so they are linked with lower pregnancy and live birth rates. Abnormal sperm parameters are detected in men with varicoceles compared with healthy men (5). A frequently observed, definite relationship exists between varicocele and hypospermia (6). The presence of varicocele negatively affects sperm density, motility, and morphology (7).

Patients suffering from clinical varicocele had a higher DNA fragmentation index (DFI) [DFI with Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL), Comet, and Sperm Chromatin Structure Assay (SCSA) than healthy men as controls, indicating that varicocele can damage sperm DNA, resulting in decreased fertility potential of patients (8). A decrease in mitochondrial membrane potential (MMP) and ATP content in patients with varicocele, in addition to sperm parameter quality loss and increased sperm DNA fragmentation, implicates severe sperm mitochondrial dysfunction in men with varicocele.

The beneficial effects of varicocelectomy on semen quality and pregnancy outcomes were observed in couples with confirmed infertility if the male partner possessed a clinically palpable varicocele and affected semen parameters (9,10).

SCV, i.e., an early phase in the progression of varicocele, is nonpalpable on physical examination of the scrotum, even with Valsalva, but can be confirmed via scrotal ultrasound, spermatic venography, and thermography (11-13). Because there are not enough relevant studies on varicocele treatment strategies, no conclusive answer can be made regarding how SCV should be managed. Evidence-based guidelines do not generally show a definite way for the management of surgical treatments in SCV patients (14). Therefore, semen parameters become important for the detection and determination of treatment modalities for SCV (8). This article investigates whether semen and sperm analysis in SCV patients without any clinical signs but diagnosed with Doppler Ultrasonography (Doppler USG) can be used as a diagnostic tool.

## Material and method

Patients profile: Prospective cohort study was conducted on patients who applied to Biruni University Hospital Department of Urology for semen analysis between August 2019 and September 2019. This study was conducted according to the principles of the Declaration of Helsinki, and all patients signed written informed consent before enrollment in the study. The study was reviewed and approved by the ethics committee of Biruni University (Ethics approval reference number: 2019/31, Date: 24.07.2019).

Inclusion criteria can be listed as 1) Diagnosis of varicocele and healthy condition using USG and Valsalva maneuver, 2) The patients must be aged between 20 and 45, 3) The patients must not show endocrinological and metabolic disorders, 4) A period of sexual abstinence must be applied for at least three and at most seven days.

There were two groups: 1) The control group (n=22) and 2) The patient group (n=22). Both groups consisted of Turkish citizens.

Spermiogram: Sperm concentration and motility were assessed microscopically using a Neubauer chamber under 200 × magnification. For morphology, sperm with abnormal shapes were divided into 7 categories: proximal cytoplasmic droplets (PD), mid-piece abnormalities (MP), abnormal tails and detached heads (T/H), pyriform heads (Py), knobbed acrosome (KA), swollen acrosome (SA), and vacuoles/teratoid (V/T). They were also counted in percentages. Smears and staining procedures were conducted according to Yimer et al.

(2011) (15). Trans-scrotal ultrasonography and breeding soundness evaluation of bulls in a herd of dairy and beef cattle with poor reproductive performance by mixing a drop of net semen with 3 drops of eosin–nigrosin stain for 30 s. Then, 5 µL of the mixture was used to prepare a smear. After air drying, the smear was examined under a light microscope at 1000× magnification to count a total of 200 spermatozoa and determine the percentages of abnormalities found in each category (15).

Step-by-step instructions of the World Health Organization (WHO) Laboratory Manual 2010 for the examination and processing of human semen were followed (16). According to these criteria, if the sperm concentration range is in the range of 15-259 million/ml, spermatozoa morphology is in the range of 4-48%, and progressively motile sperm is in the range of 32-75%, the person is considered healthy with a normal semen analysis. These healthy people also formed the control group.

The Aniline Blue Staining (ABS): The aniline blue staining technique is a standard test for determining whether sperm DNA has chromatin condensation, i.e., sperm DNA maturation. To perform this staining, 20 µl fresh sperm smear from each case was air dried and then fixed in 2% buffered glutaraldehyde in 0.2M phosphate buffer (pH 7.2) for 30 min at room temperature (17). Each smear was treated with 5% aqueous AB stain (5 g powder in 100 ml distilled water in 2% acetic acid (pH 3.5)) for 5 min. At least 200 spermatozoa were counted on each slide by light microscopy. Unstained or pale blue-stained cells were considered normal (mature) sperm, while dark blue cells were considered abnormal sperm. At least 200 spermatozoa were evaluated on each slide, and the percentage of abnormal spermatozoa was reported (17).

The Acridine Orange Staining (AOS): The AO assay measures the ability of sperm nuclear DNA to denature by acid, forming a metachromatic shift of AO fluorescence from green (native DNA) to red (denatured DNA). The fluorochrome AO intercalates in double-stranded DNA as a monomer, which binds to single-stranded DNA. The monomeric AO-bound to native DNA fluoresces green, whereas the aggregated on AO denatured DNA fluoresces red (18,19).

The AO assay may be used for fluorescence microscopy. To perform this assay for fluorescent microscopy, 20 µL of thick semen layers were fixed in Carnoy's fixative (Methanol: Acetic acid 3:1) for 5 mins at +4°C. After fixation, the slides were stained with AO dye (1% AO, 10 ml 0.1M citric acid, 300 µl 0.3 M Na2HPO4) for 5 minutes in the dark (18,19) The slides were washed with distilled water, covered with a coverslip, and examined under a fluorescent microscope. An average of 200 sperm cells was evaluated on each slide by the same examiner. Spermatozoa, which show green fluorescence, were considered to have standard DNA content, whereas sperms displaying a yellow-orange to red fluorescence were considered to have damaged DNA. The ratio between (yellow-to-red)/(green+yellow-to-red) fluorescence was considered as DFI percentages, and the percentage of the sample showing a ratio <1 was calculated in the group (18,19).

The chromomycin A3 (CMA3) staining: Protamine deficiency is associated with diminished sperm quality, but the threshold at which protamine deficiency occurs best correlates with reduced sperm quality. Indirect methods for sperm protamine assessment based on chromomycin A3 (CMA3) staining have recently been described (20). The CMA3 assay may be used for fluorescence microscopy (19). To perform this assay for fluorescent microscopy, 20 µL of thick semen layers were fixed in Carnoy's' fixative (Methanol: Acetic acid 3:1) for 5 minutes. After fixation, the slides were stained with CMA3 dye [0.25 mg of CMA3 dissolved in 1 ml of Mcilvaine buffer solution (200 mM Na2HPO4.7H2O, 100 mM citric acid, 10 mM MgCl2; pH 7.0) for 20 minutes in the dark. The slides were washed with distilled water, covered with a coverslip, and examined under a fluorescent microscope (20). The percentage value is calculated by counting an average of 200 sperm by the same examiner. Green-stained sperm are considered to contain protamine, and yellow-stained sperm are considered to have decondensed DNA.

Rhodamine 123 (Rh123)/Propidium Iodide (PI) Dual Staining: The percentage of live sperm cells with functional mitochondria was assessed using a combination of fluorescent stains Rhodamine123 (Rh123) and propidium iodide (PI) as described previously (21). Sperm at a concentration of 5x106 were placed in 1 mL of Dulbecco's Modified Eagle Medium (DMEM) to prepare a sperm suspension. 5 µl Rh123 (5 mg Rh123 in 5 ml PBS) was added to sperm suspension for the incubation process for about 15 minutes. Then, 1 µL of PI dye (2 µL PI in 1 ml DMEM) was added to the same solution. The solution was incubated in the dark for about 5 minutes (21). 20 µL of the stained suspension was spread on a slide. The slides were left to dry. Slides were examined under a fluorescent microscope. Green-stained sperm were considered to have high mitochondrial membrane potential, and red-stained sperm were determined to be apoptotic. The percentage value is determined by counting an average of 200 sperm (21).

***Statistical Analysis***

Statistical analysis was done using the SPSS program (IBM Corp., Armonk, NY, USA). The Shapiro-Wilk test was used to detect normal distribution. The results were determined by using the Student t-test. Results are shown in mean±standard deviation (SD). A p-value <0.05 was considered statistically significant.

## Results

Results are shown in Table I. The test results of patients were performed on Turkish citizens aged between 20-45.

***Table I:*** *The results of sperm tests*

| | Healthy group (n=22) | Varicocele group (n=22) | p |
|---|---|---|---|
| Sperm concentration (million/mL) | 56.09±37.88 | 40.50±20.71 | **0.098** |
| Sperm motility (%) | 50.09±8.73 | 24.40±5.7 | **<0.001*** |
| Morphology (%) | 6.18±1.25 | 3.31±1.46 | **<0.001*** |
| Sperm maturation (AB +; %) | 20.27±5.31 | 73.27±7.66 | **<0.001*** |
| DNA fragmentation index (AO +; %) | 18.63±5.21 | 76.81±8.21 | **<0.001*** |
| Sperm chromatin condensation (CMA3 +; %) | 23.45±8.03 | 71.59±14.55 | **<0.001*** |
| Sperm mitochondria membrane potential (Rh123 +; %) | 79.90±7.25 | 13.86±16.93 | **<0.001*** |
| Apoptotic Sperm Index (%) (PI +; %) | 20.09±7.25 | 89.18±4.08 | **<0.001*** |

*Results are shown in mean±standard deviation (SD). *p value<0.05 was considered statistically significant. AB: Aniline blue, AO: Acridine orange, CMA3: Chromomycin A3, Rh123: Rhodamine 123, PI: Propidium Iodide.*

Sperm concentration (p>0.05), sperm motility (p<0.05), sperm morphology (p<0.05), sperm maturation (p<0.05; Fig. 1A), and sperm mitochondrial membrane potential (p<0.05) were decreased in the varicocele group in comparison to the control group (Table I). The increase in DNA fragmentation index (p<0.05; Fig. 1B), sperm chromatin condensation (p<0.05; Fig. 1C), and apoptotic sperm index (p<0.05; Fig. 1D) were detected in the varicocele group in comparison to the control group (Table I). The morphology section of Table I shows that sperm neck anomalies are higher in varicocele patients(20.68±6.95 vs. 28.27±6.35, p<0.05).

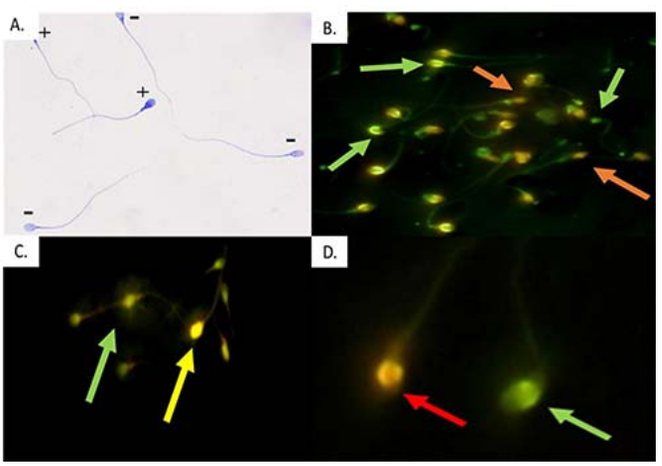


***Figure 1A:*** *AB staining photos. Dark blue stained sperm are accepted as positive (+) sperm, which show a DNA maturation defect. Unstained sperm are accepted as unfavorable (-) sperm with normal chromosome condensation (Light microscopy magnification 100x);* ***B:*** *AO staining photos. Colors in green and orange represent normal sperm and defective sperm with DNA fragmentation (Fluorescent microscopy magnification 100x);* ***C:*** *CMA3 staining photos. Colors in green and orange represent normal and defective sperm with decondensed sperm chromatin (Fluorescent microscopy magnification 100x);* ***D:*** *RH123/PI staining photos. Colors in green and red represent normal live sperm and apoptotic sperm (Fluorescent microscopy magnification 100x)*

## Discussion

In this study, it was found that sperm concentration, motility, morphology, maturation, and mitochondrial membrane potential were decreased in the varicocele group; however, DNA fragmentation index, sperm chromatin condensation, and apoptotic sperm index were increased.

Panidis et al. (1990) studied sperm morphology defects in semen specimens from 107 men with varicocele. They found that morphological abnormalities of the neck are significantly more frequent in spermatozoa with an abnormal head than in those with a normal head. In addition, they showed that tail morphological abnormalities and cytoplasmic droplets are more frequent in spermatozoa with an abnormal head (22). Morini et al. (2021) investigated the progression of semen parameters after surgical varicocele repair (23). They determined that sperm in normal morphology were increased. However, abnormal sperm with defects of the head (e.g., microcephaly, absent) and the tail (e.g., absent tail) decreased after surgical interventions. In addition, other semen parameters, such as sperm number and motility, were also improved (23).

Elahi et al. (2023) showed the concentration, progressive motility, normal morphology, mitochondrial membrane potential (MMP), and ATP contents of sperm in varicocele cases decreased significantly in comparison to the healthy group (24). In addition, the sperm DFI was significantly higher in the varicocele group than in the healthy group in a cross-sectional study including 50 normozoospermic men (standard) and 50 varicocele patients without varicocelectomy (24).

Chromatin condensation and decondensation in the sperm nucleus depend on protamine 1 (P1) and protamine 2 (P2), which are coded by PRM1 and PRM2 genes located on chromosome 16.1 (25). Nayeri et al. (2020) conducted a case-control study with 128 varicocele patients, with the polymorphisms of sperm protamine genes as PRM1 and PRM2 genes. They also used CMA3 staining (25). They showed that PRM1

and PRM2 variations in varicocele patients were closely associated with increased spermatozoa numbers with protamine deficiency (25). They concluded that protamine deficiency is significantly related to abnormal sperm penetration, sperm DNA fragmentation, and embryo quality (25).

A cross-sectional study conducted by Dieamant et al. (2017) used semen samples from 239 men with varicocele (26). They showed that the percentages of sperm with DNA fragmentation, abnormal chromatin packaging, and abnormal MMP were significantly higher in varicocele patients than in healthy men. In addition to their results, they also showed that early apoptosis and abnormal MMP had a significant correlation with high sperm DNA fragmentation. They concluded that conventional semen parameters were markedly worse in varicocele patients in comparison to healthy men (26).

Generally, varicocele develops during puberty and occurs more often on the left side due to anatomic reasons. However, its diagnosis cannot be easily made and is often delayed, especially in asymptomatic cases, until the man contacts an andrologist for the couple's infertility (23). The physical examination allows us to classify varicocele using a three-graded scale. After that, varicocele diagnosis is confirmed using USG evaluation for definitive classification (23). Cauni et al. (2022) worked with a group of patients with secondary infertility and asymptomatic varicocele, which would not have been diagnosed in the absence of Doppler USG investigations (27). They emphasized the importance of Doppler USG for patients younger than 36 years of age in the diagnosis of infertility (27).

Although the role of Doppler USG is well-established in varicocele diagnosis, the need for imaging itself is debated. In Europe, USG is recommended to confirm clinically suspected varicoceles, while in the USA and Asia, routine use of USG imaging is not recommended (28). There is no agreement on how to perform the USG examination. Different classifications are used based on different sonographic parameters, even within the same country, depending on the practice experience of the individual sonologist and the referring clinician (29). In addition, several international guidelines recommend surgical treatment for varicocele when it is palpable and/or if abnormal semen parameters are detected (23). Keene et al. (2016) evaluated the trends in sperm parameters among adolescent varicocele patients aged 12 to 17 years with symmetrical testicular volume, idiopathic varicocele diagnosis, and no varicocelectomy. They found that sperm concentration and forward motility are not correlated with age in adolescents with idiopathic varicocele and symmetrical testicular volume (30).

When you evaluate the information above, you can understand that although several publications and/or clinical studies have been conducted, there are still dilemmas regarding the use of diagnostic and prognostic techniques for varicocele. However, it's understandable that the combination of physical examination, USG, and semen parameters provide accurate information for varicocele diagnosis, leading to the selection of the appropriate management strategy, such as surgery.

Morphology, genome damage, and quantity are recognized in clinical practice as significant parameters in evaluating sperm quality (18,22,25,26,31). The AOS technique for detecting denatured DNA, the ABS technique for detecting sperm DNA maturation, the Rh123/PI staining technique for detecting live sperm cells with functional mitochondria, and the CMA3 staining technique for assessing sperm protamine were involved in our current study. These staining techniques for evaluating semen/sperm parameters have been applied for decades. They are associated with successful in vitro fertilization (IVF) (18,32-34). In our study, results from different techniques confirmed each other, indicating that decreased sperm concentration, motility, morphology, maturation, and MMP were positively correlated with increased sperm DNA fragmentation and apoptosis in varicocele patients. In addition, the results of our study have a significant positive correlation with Doppler USG results. Additionally, the worse semen parameters detected in our study are consistent with those found in previous studies (18,22,24-26,31,34).

In conclusion, although there are still unresolved questions on the compatibility and interpretation of current methods in varicocele diagnostics, especially for SCV, and the results of various techniques are not always in mutual concordance, our results showed that cases that are asymptomatic and/or undetected/neglected by Doppler USG examination, semen parameters with conventional staining techniques can help to put diagnosis and monitor the prognosis.

*Declarations*

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*Availability of data/materials: The data supporting this study is available through the corresponding author upon reasonable request.*

*Author contributions: TI participated in conception and design, experiments, supervision, and writing. BS assisted in experiments, data collection, and analysis; ME conducted data collection and analysis, interpretation, commentary, and revision writing; ES participated in experiments, data collection and analysis, and supervision. All authors read and approved the final manuscript.*

*Conflict of interest: The authors declare that they have no competing interests.*

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