ANDROLOGY
Original Article

The effect of varicocele on sperm morphology and DNA maturity: does acridine orange staining facilitate diagnosis?

Ali Ersin Zümrütbaş[1], Ömer Gülpınar[2], Murat Mermerkaya[2], Evren Süer[2], Önder Yaman[2]

ABSTRACT

Objective: The aim of this study was to determine whether Acridine Orange (AO) can be used as a routine procedure in a physician's office to detect sperm deoxyribonucleic acid (DNA) fragmentation in patients with varicoceles.

Material and methods: Forty-five patients with a mean age of 30.4 years, who had been diagnosed with varicoceles by physical examination and 30 healthy males with a mean age of 28.3 years and without any signs of varicoceles were included in this study. Doppler Ultrasonography was performed to each individual in addition to physical examination. Semen analyses were performed by the same biologist. Sperm morphology was evaluated according to the World Health Organization (WHO) criteria and Kruger's strict criteria. After routine semen analysis, the samples were fixed separately for further examination with AO staining.

Results: In routine semen analyses, total sperm count, total motile sperm count, and fast direct forward motile and direct forward motile sperm counts were significantly lower in patients with varicoceles, and the immotile sperm count was significantly higher in patients with varicoceles compared to the control group. Kruger's examination revealed a lower sperm count with normal morphology in patients with varicoceles. After AO staining, the ratio of spermatozoa demonstrating red and green coloration were 6.5±11.0% and 93.5±11.0%, respectively in the varicocele group and 1.0±1.0% and 99.0±1.0%, respectively in the control group ($p<0.001$).

Conclusion: Semen analysis using AO staining can be performed under a clinician's office conditions with a fluorescent microscope without any additional equipment. Further studies are needed to validate the AO staining with more extensively used and well-known methods. Therefore, AO staining can be used as a simple and reliable method that can be performed daily in a physician's office in infertility and andrology clinics.

Key words: DNA; infertility; sperm maturity; sperm morphology; varicocele.

Introduction

Varicocele is the most common cause of male infertility and can be detected in 30-40% of these patients, but how fertility is affected by varicoceles is not clear. [1] Different theories regarding the disease pathophysiology include retrograde flow, increased scrotal temperature and damage caused by reactive oxygen species (ROS) due to blood stasis.[2-4] The relationship between varicocele and altered semen parameters and sperm functions has been well described.[5,6]

The nucleus of the sperm becomes condensed during the final stages of spermatogenesis, and histones are replaced by cysteine-rich protamines. Sperm deoxyribonucleic acid (DNA) becomes more resistant to damage mediated by heat stress and other factors, especially ROS. Varicocele may affect the final stages of spermatogenesis and lead to changes at sperm parameters and functions.[7-9] Unexplained infertility or low fertility potential that cannot be determined by conventional semen analysis may be explained with this issue.[10] Several techniques were described to assess the DNA integrity of sperm, and these techniques include TdT (terminal deoxyribonucleotidyl transferase)-mediated dUTP nick-end labeling (TUNEL) assay, aniline blue straining, chromomycin A3 and acridine orange (AO) staining.[11-13] The maturity of sperm can be detected using acridine orange dye. Double stranded nucleic acids fluoresce green with acridine orange staining, while single stranded nucleic acids fluoresce red.[12,14] Significantly higher red fluorescence than green fluorescence suggests that the percentage of immature spermatozoa is increased and the fertilizing capacity is decreased.[15] The

[1]Department of Urology, Faculty of Medicine, Pamukkale University, Denizli, Turkey

[2]Department of Urology, Faculty of Medicine, Ankara University, Ankara, Turkey

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Correspondence: Ali Ersin Zümrütbaş Department of Urology, Faculty of Medicine, Pamukkale University, 20070 Denizli, Turkey Phone: +90 532 789 89 61 E-mail: alizumrut@yahoo.com

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aim of this study was to evaluate the effect of varicocele on sperm DNA integrity by acridine orange staining.

Material and methods

Ankara University School of Medicine ethics committee approved the study, and written consent was obtained from the participants. Forty-five patients who have clinically diagnosed varicoceles and 30 healthy men without varicoceles were included in the study. Patients in the varicocele group admitted to our infertility clinic with infertility for one or more years and without any female factors for infertility. The healthy group consisted of 30 men without any known health problems. Scrotal Doppler ultrasonography was performed for the entire varicocele group, and varicoceles were confirmed. Varicoceles were diagnosed by palpation in a standing position. The mean age of patients with varicocele was 30.4 years, and the mean age of control group patients was 28.3 years. A semen sample was taken from all patients after a period of sexual abstinence for 3-5 days with masturbation. Semen analysis was performed by the same biologist according to the World Health Organization (WHO) and Kruger's criteria.

After semen analysis acridine orange staining was performed. Sperm from each specimen was smeared onto glass slides and air dried for one night. The smears were fixed for 20 minutes with Carnoy's solution (methanol and glacial acetic acid, 3:1). Acridine orange solution was prepared with 10 mL of stock solution, 40 mL of 0.1 M citric acid and 2.5 mL of 0.3 M disodium hyperoxide. The stock solution is prepared using 1000 mL distilled water and 1 g acridine orange hemi-zinc chloride (Sigma-Aldrich) and was stored at 4°C in the dark. The solution was buffered to pH 2.5, and staining was performed by dropping 2-3 mL of solution onto the smears, incubating for 15 minutes, and then washing with distilled water. After washing, lamels were closed on the glasses and fixed.

The nuclei of 100 spermatozoa from each specimen were examined under a fluorescence microscope with 490 nm excitation, 530 nm barrier filter (Axioplan Zeiss). Nuclei were evaluated as fluorescing green (Figure 1), red, orange and yellow (Figure 2). Red, orange and yellow fluorescing spermatozoa were counted as red because it was thought that DNA damage had started. Green stained spermatozoa were considered normal.

Statistical analysis

Statistical analysis of the study data was performed with Statistical Package for Social Sciences Program (SPSS Inc., Chicago, IL, USA). Differences between groups were evaluated with Mann-Whitney U test, and P values under 0.05 were considered significant.

Results

Mean ages of the varicocele and control groups were 30.4±6.2 years (range 19-47) and 28.3±3.2 years (range 24-35), respectively. Doppler USG revealed the median diameter of the left testicular vein as 3.1 in the varicocele group. Semen analysis revealed that the total sperm count, total motile sperm count and progressive motile sperm count were significantly lower in varicocele group than the control group ($p<0.001$). The immotile sperm count was significantly higher in the varicocele group. According to Kruger's criteria, sperm with normal morphology were significantly higher in the control group, whereas sperm head anomalies were significantly higher in the varicocele group (Table 1).

When the samples were evaluated with acridine orange staining, the percentage of red fluorescing spermatozoa was 6.5±11.0 and 1.0±1.0 for the varicocele and control groups, respectively. The percentage of green fluorescing spermatozoa was 93.5±11.0 and 99.0±1.0 for the varicocele and control groups, respectively (Table 2). The acridine orange staining percentage was significantly higher in the control group compared to the varicocele group ($p<0.001$).

Discussion

The management of male infertility is still a controversial subject that incites continuing debate. Approximately half of infertile men are known to lack knowledge of male infertility management or experience any benefit from the current medical and surgical treatment options.[16] Most infertile couples therefore are directed to assisted reproductive techniques.

The negative effect of varicocele on semen parameters and infertility is well known; however, several questions remain regarding the exact pathophysiology of this condition. Moreover, the abso-

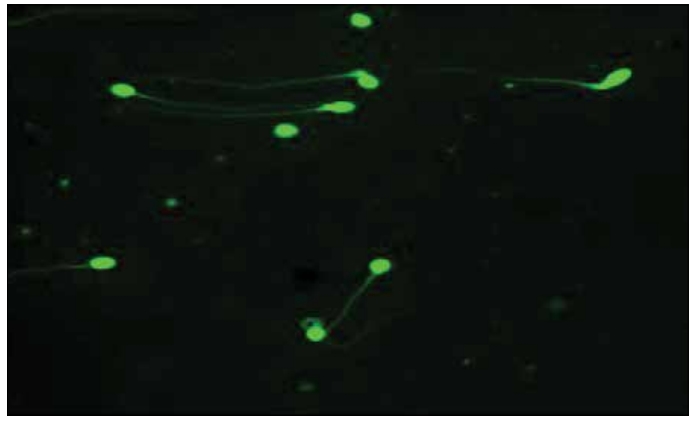

Figure 1. Sperm nuclei fluorescing green with acridine orange

lute indications for varicocelectomy become debatable because of the uncertainty about the patients who will benefit from this treatment. The morphology of sperm in varicocele patients display what is known as the "stress pattern", which is the elongation and thinning of the head and amorphous cells. The results of this study also showed that the number of spermatozoa with normal morphology decreased, and subgroup analysis revealed that sperm-head anomalies and cytoplasmic excesses were significantly higher in the varicocele group. Additionally, the total and motile sperm counts were significantly lower in that group, which is consistent with previously reported results.

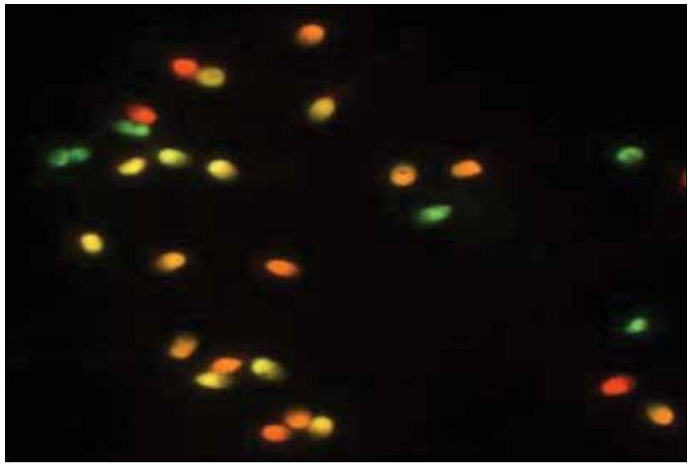

Figure 2. Sperm nuclei fluorescing red, yellow or orange with acridine orange

Several factors have demonstrated the relationship between varicocele and infertility, and recent studies have particularly highlighted sperm DNA damage.[17] The final steps of spermatogenesis have a major role in sperm viability and fertility.[7,8] Disorders affecting these steps, such as varicocele, may lead to abortive apoptosis or altered sperm fertility potential.[8,10] Total and motile sperm counts may decrease or fertility may be affected, independently of these parameters, and sperm DNA damage, which cannot be determined by routine semen analysis, may be the reason for these problematic issues.[10] For this reason, routine semen analysis may not be sufficient to detect the etiology of infertility/subfertility. Additional tests are required to determine sperm DNA damage. Several tests such as TdT (terminal deoxyribonucleotidyl transferase)-mediated dUTP nick-end labeling (TUNEL) assay, aniline blue straining, chromomycin A3 and acridine orange (AO) staining have been described to determine the extent of DNA damage.[11-13]

Acridine orange stains normal double stranded DNA green and single stranded DNA red.[18] Increased red fluorescence indicates increased denaturation. Spermatozoa that are sensitive to denaturation were shown to have fragmented DNA.[19] Aniline blue stains lysine rich nucleoproteins, which is another sign of deterioration of chromatin condensation and was shown to correlate with AO staining.[20] In this study, the increased ratio of red stained cells in the varicocele group represents the increased sperm DNA denaturation in patients with varicocele.

One of the initial studies using AO staining was conducted by Tejada et al.[21] who attempted to simplify the SCSA technique

Table 1. Semen analysis results of the varicocele group and control group

	Varicocele Group			Control Group			p**
	Mean	**SD**	**Range**	**Mean**	**SD**	**Range**	**Value**
Total Sperm Count*	151.3	146.5	1.6-513	478.6	460.9	11-1575	<0.001
Motile Sperm Count*	73.8	94.4	0-346	264.3	271.9	1-910	<0.001
a (%)	4.2	9.5	0-42	13.5	16.9	0-58	<0.001
b (%)	35.6	22.5	0-84	39.8	21.7	1-74	0.396
a+b (%)	39.9	23.6	0-84	53.3	20.5	1-76	0.021
c (%)	9.9	9.7	0-47	8.4	5.2	4-22	0.776
d (%)	50.1	25.2	8-100	38.3	21.5	17-94	0.032
Normal Morphology (%)	3.6	4.9	0-14	8.9	5.5	3-25	<0.001
Head anomaly (%)	60.9	8.2	44-76	56.0	9.5	34-70	0.037
Neck anomaly (%)	21.5	7.3	5-34	20.7	5.4	11-33	0.544
Tail anomaly (%)	11.3	6.5	2-35	8.8	4.6	1-17	0.141

* Million, **Significant ($p < 0.05$) values are written bold

a: progressive motility, b: non-linear motility, c: non-progressive motility, d: immotile sperm

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Table 2. Acridine orange staining percentages of varicocele and control groups

	Varicocele Group			Control Group			p
	Mean	SD	Range	Mean	SD	Range	Value
Red	6.5	11.0	(0-58)	1.0	1.0	(0-3)	0.001
Green	93.5	11.0	(42-100)	99.0	1.0	(97-100)	0.001

using a light microscope; however, due to technical challenges, this method was not popularized. Subsequently, Gopalkrishnan et al.[10] who used a different filter system and a fluorescence microscope demonstrated that sperm analysis using AO staining can be performed with only a fluorescent microscope in a physician's office. They also showed that the ratios of green stained cells were 44% and 67% in varicocele and control groups, respectively.

Other studies showed increased sperm DNA damage in patients with varicocele using different techniques. Saleh et al[9] used the sperm DNA fragmentation index (DFI) and found that the DFI ratio was significantly higher in the varicocele group compared to the control group (25% vs. 15%). If the results of AO staining can be confirmed with the other tests such as TUNEL, aniline blue or chromomycin A3, this technique may be widely used for the assessment of varicocele patients in a physician's office, as it is an inexpensive, reliable and simple test for the evaluation of infertile patients with or without varicocele.

Because this study is epidemiologically designed for patients with varicocele, the effects of varicocelectomy were not investigated. However, a study with computer assisted semen analysis (CASA) revealed that progressive sperm motility was increased after varicocelectomy.[22] In a recent study, sperm DNA integrity and chromatin compaction were improved significantly after microsurgical varicocelectomy.[23] However, other studies showed that the increase in sperm motility was only demonstrable in the patient subgroups who had impregnated their partners after varicocelectomy.[24] A recent meta-analysis showed that patients with varicoceles had significantly higher sperm DNA damage than controls with a mean difference of 9.8%. Furthermore, varicocelectomy was also shown to improve sperm DNA integrity.[17]

A limitation of our study is that the acridine orange staining results were not confirmed using other established DNA integration tests such as SCSA (sperm chromatin structure assay), TUNEL or DFI, which would strengthen the value of the test. Furthermore, a larger sample size would provide additional information about the test.

In conclusion, varicoceles still have a major role in infertility and affect semen parameters and sperm DNA integrity. In our group of patients, routine semen analysis is not enough to determine the cause of infertility. Additionally, acridine orange may provide further information about sperm DNA that was shown to effect fertility. This technique may also be used to assess the effects of surgical treatment, and its simplicity, usefulness and inexpensiveness suggest that it may be included in routine investigations of patients with infertility problems and varicoceles.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Ankara University School of Medicine.

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Peer-review: Externally peer-reviewed.

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