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Differentiating between oligospermic men and fertile men using aniline blue staining, a marker for sperm chromatin defects

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ABSTRACT

The present study aimed to investigate the relationship between sperm nuclear chromatin condensation and semen parameters in men diagnosed with oligospermia. The study cohort included 40 oligospermia patients with idiopathic infertility and 21 fertile individuals as controls. Sperm chromatin condensation was evaluated using the aniline blue staining technique. According to the staining intensity, mature (condensed) and immature (less condensed) chromatin were distinguished. The percentage of aniline-positive (AB-positive) spermatozoa in the patient group was statistically higher than in the control group ($P<0.001$). This finding suggests impaired chromatin condensation may be associated with oligospermia and idiopathic infertility. Our study shows significant correlations between sperm parameters, including morphology, concentration, and motility. In oligospermia men, we observed impaired sperm structural integrity and increased sperm head and tail abnormalities. Collectively, these findings indicate that sperm chromatin condensation plays a crucial role in assessing male fertility potential. In addition to semen analysis for the comprehensive evaluation of male fertility, it is crucial to include chromatin condensation testing as a reliable and practical method in diagnostic protocols, especially in idiopathic cases.

Keywords: Aniline blue staining, oligospermia, infertility.

1. INTRODUCTION

Spermatogenesis is the process by which sperm - the male reproductive cell - is formed and, is a complex biological process that consists of several stages (Hermann et al., 2018). The process begins when spermatogonia cells multiply through mitosis and form primary spermatocytes. Subsequently, these cells undergo meiosis. During meiosis, the cell nucleus divides twice, and four daughter cells (round spermatids) are formed. These round spermatids develop into mature sperm cells (spermatozoa) over time (Sharma and Agarwal, 2011). Spermiogenesis is the process by which round spermatids develop into mature sperm cells (spermatozoa). This process produces essential changes in the cell.

For example, the sperm cell develops structures such as the flagella, which enables movement, and the acrosome, which facilitates piercing the egg cell. In addition, the cell reorganizes its organelles and condenses its nucleus into a smaller, tighter structure (Teves and Roldan, 2022). Nuclear condensation replaces 85-95% of histone proteins in nucleosomes with essential non-histone transition proteins (TNP1 and TNP2). Protamines replace transition proteins and form tight, packed nuclear genome protamine-DNA toroids (Gunes and Esteves, 2021). Phosphorylation of serine amino acids, formation of cysteine disulfide bonds, and the presence of zinc bridges in the protamine structure stabilize the toroidal structure (Carrell, 2012).

Protamine contains high levels of arginine and cysteine. The cysteine molecules form disulfide bonds which provide mechanical and chemical stability and help to neutralize the positive charge of protamine and the negative charge of DNA. The strong intermolecular interaction between the DNA skeleton and protamine facilitates DNA binding to protamine with essential characteristics and causes chromatin condensation (Kasinsky et al., 2011). This process enables the paternal genome to diminish in size to approximately one-twentieth of the nucleus's volume. The specialized structure of sperm chromatin allows sperm DNA to be transported and stored during the passage of spermatozoa in the epididymis (Talibova et al., 2022; Zhang et al., 2022).

Protamines tightly pack most of the chromatin structure of spermatozoa, whereas histones pack 5-15% more loosely. Infertile men have higher histone/protamine ratios than fertile men (more than 15%). This condition is termed "abnormal chromatin packaging" and leads to increased sensitivity of spermatozoa to external stress (Güneş and Kulaç, 2013). Abnormal chromatin packaging affects the compaction of nuclear chromosomes Ribas-Maynou et al., (2015), which can damage sperm DNA (Talebi et al., 2018). Research has shown that this damage can adversely affect sperm function and male fertility (Eskandari et al., 2018). Additionally, scientists have observed that protamine deficiencies cause the formation of nuclear vacuoles (NV) in the sperm head region.

These vacuoles indicate sperm DNA damage and reflect a morphological abnormality (Franco et al., 2012). Aniline blue (AB) staining is one of the methods used to assess the integrity of sperm chromatin. The basis of this methodology is the high affinity of the dye for histones found within the sperm nucleus (Hammadeh et al., 2001). The nuclei of immature sperm cells are rich in histones and contain large amounts of lysine. Therefore, they give a blue color under the microscope when stained with aniline blue. In contrast, the nuclei of mature sperm cells are rich in protamine and contain high levels of arginine and cysteine but low levels of lysine.

Therefore, they do not stain well with aniline blue (Zhang et al., 2016). This study aims to investigate the relationship between semen parameters, sperm morphology, and chromatin condensation in fertile and infertile men. The researchers analyzed semen samples from oligospermic (low sperm count) infertile men and normozoospermic (normal sperm count) fertile men to determine the percentage of spermatozoa staining positively with aniline blue.

2. MATERIALS AND METHODS

Subject Selection

The present study comprised a case-control study. The study population included 40 patients diagnosed with oligospermia and 21 fertile men with a proven fertile history and typical semen analysis results. We analyzed 40 selected patients with oligozoospermia from Dicle University Medical Faculty Hospital in Turkey. We also included fertile men from the same region as a control group. Oligospermia was diagnosed according to the World Health Organisation criteria (Mazzilli et al., 2024).

The diagnosis of oligospermia was confirmed by two semen analyses performed by the World Health Organization criteria, specifically a sperm concentration of 15 million/ml, normal motility, and normal sperm morphology. Patients diagnosed with oligospermia were required to meet the following criteria for inclusion in this study: primary infertility, a sperm concentration of 5-10 million/ml in the ejaculate, a typical male genital tract, and age between 20 and 45 years.

Sperm chromatin condensation analysis with aniline blue

After analyzing the ejaculate samples, we transferred them to the Department of Medical Biology at Dicle University. We separated the seminal plasma and subjected approximately 1 ml of fresh semen samples to two rounds of centrifugation at 2000 rpm for 10 minutes using 1x phosphate buffer saline (PBS). After centrifugation, we spread the semen pellets onto clean slides, air-dried them, and fixed them with 3% glutaraldehyde.

For staining, we immersed the samples in a solution of 5% aniline blue and 4% glacial acetic acid, followed by two minutes of submersion in pure water. We repeated this staining process twice. We examined the slides under a light microscope (Olympus CX-31) at 1000x magnification. For each slide, we enumerated and examined a minimum of 200 spermatozoa and determined the percentage of histone-rich spermatozoa. We excluded patients with karyotype abnormalities, Y chromosome microdeletions, or patients with mutations linked to cystic fibrosis from the study.

3. RESULTS

Study participants' demographic profile

Our study's patient group consisted of 40 idiopathic infertile individuals diagnosed with oligospermia. The control group included 21 fertile men. The mean age of the patient group was 31.65 ± 0.80 years, and the mean age of the control group was 33.43 ± 1.11 years. There was no statistically significant difference between the two groups regarding age (P = 0.811). Table 1 shows the semen parameters of the patient and control groups.

Table 1 Comparison of Semen Parameters of Patient and Control Group

		OLIGOSPERMIA			CONTROL		p
	n	Mean+SD	Median (Min-Max)	n	Mean+SD	Median (Min-Max)	
Sperm concentration	40	9.72±0.45	9.7 (2.17-14.6)	21	122.30±13.46	124.10 (20.2-296.2)	0.000
Nonprogressive Motile Spermatozoa %	40	60.43±3.48	67 (10-99)	21	37.30±4.4	29 (16-76)	0.000
Total motile sperm	40	13.82±1.76	13.75 (0,2-53,8)	21	93.7±17.5	71 (10.11-362.6)	0.000
Total progressive motile sperm	40	8.92±1.32	6.85 (0-27,14)	21	81.14±15.48	67 (7.9-331.2)	0,000
Normal sperm morphology	40	3.28±0.59	2.9 (0-21,30)	21	47.49±5.93	44.7 (1.1-89)	0.000
Aniline blue (+)	40	33.05±0.71	32 (25,46)	21	26.62±0.69	26 (18-34)	0.122
Head Anomaly	40	9.6±0.23	10 (7-12)	21	3.81±0,.6	10 (7-12)	0.171
Tail Anomaly	40	7.78±0.21	8 (6-11)	21	3.67±0.28	4 (2-6)	0.741

We used the aniline blue staining technique to assess the integrity of sperm chromatin. Histone-rich spermatozoa displayed a dark blue staining reaction when treated with aniline blue, while protamine-rich spermatozoa appeared pale blue or remained unstained (Figure 1 for visual representation).

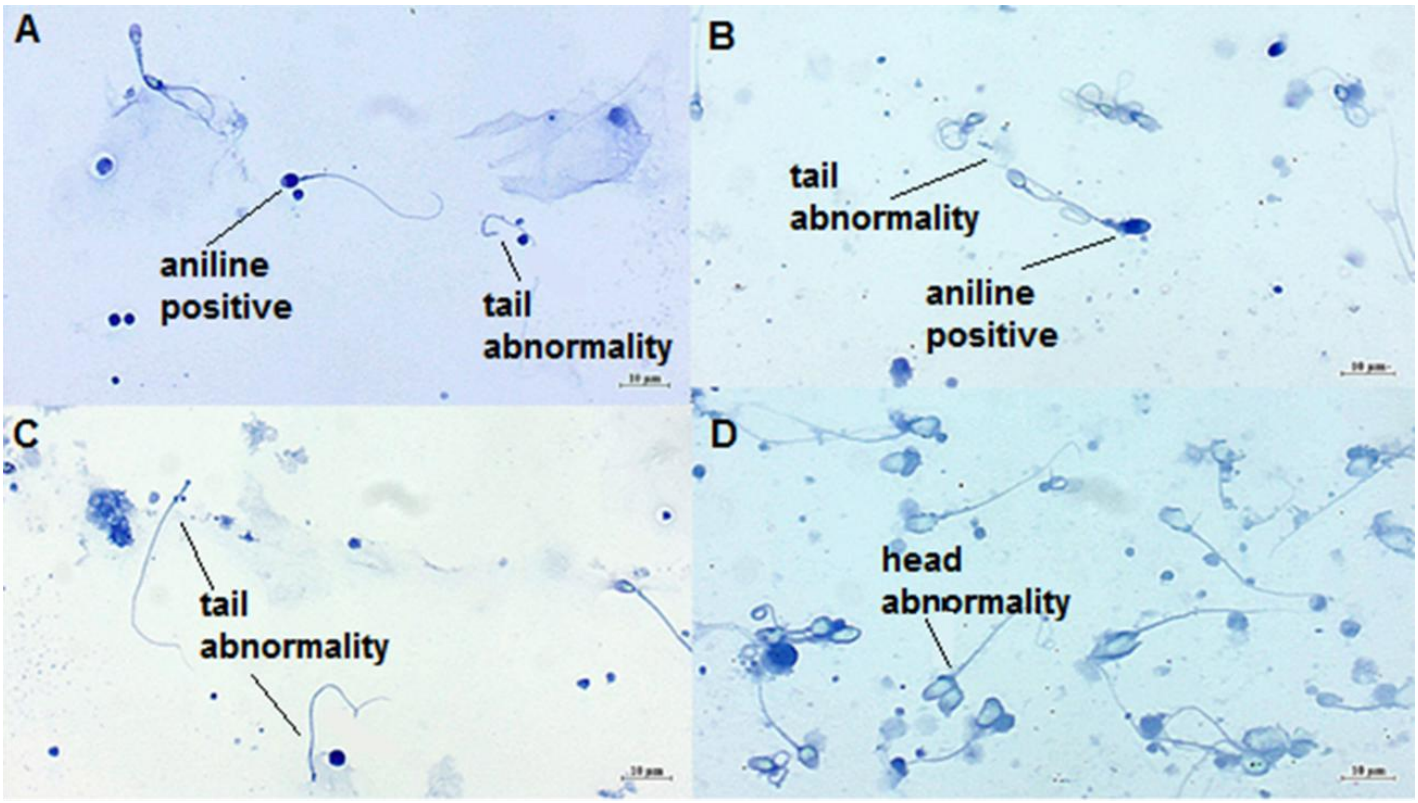

Figure 1 The findings of this study, employing the aniline blue staining technique, demonstrate that samples A and B exhibited positive staining with aniline blue and tail anomaly. Moreover, sample C displayed positive staining with aniline blue and tail anomaly. Notably, sample D exhibited sperm head anomaly.

We calculated the discriminative power of the percentage of aniline-positive spermatozoa between patients and control group individuals, yielding an AUC of 0.908 ($P<0.001$). We estimated the cut-off value for the proportion of aniline-positive spermatozoa at 28.5%, which demonstrated a sensitivity of 92.5% and a specificity of 81% (Figure 2).

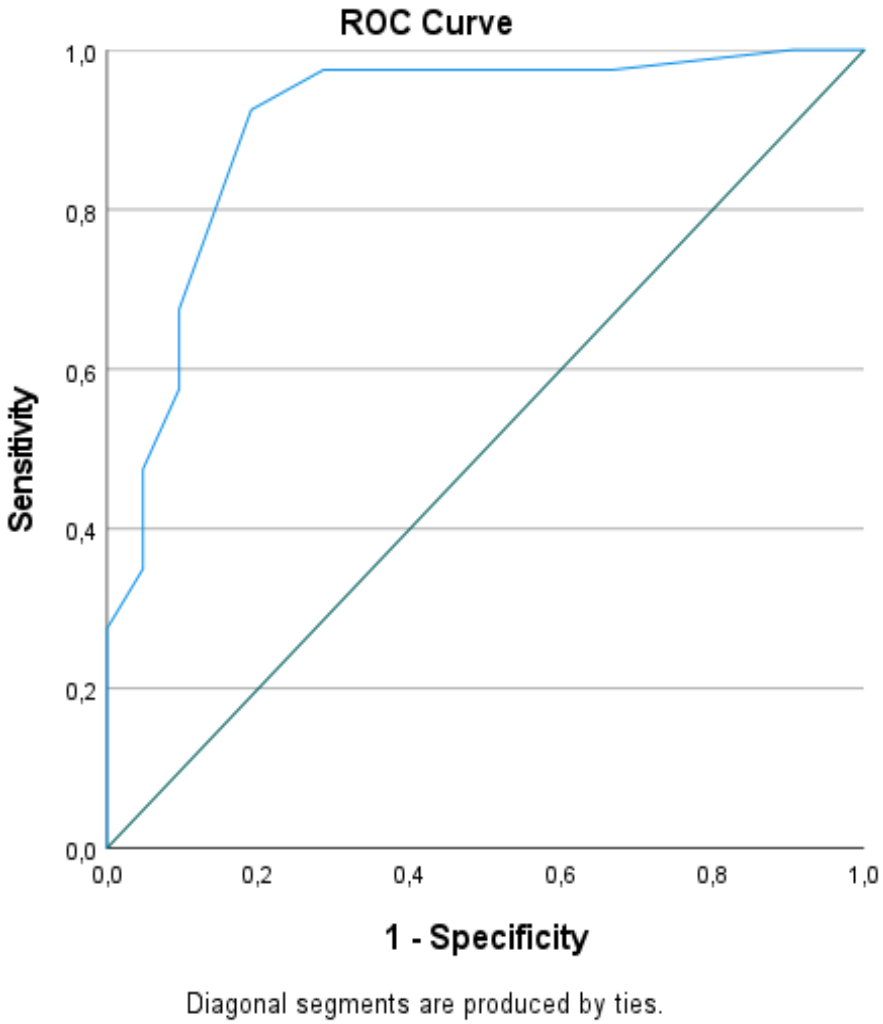

Figure 2 ROC curve for the percentage of aniline-positive spermatozoa.

This study revealed that the rate of aniline blue-positive (AB-positive) spermatozoa in the oligospermia group was significantly higher than in the control group (P<0.001), as shown in (Table 2).

Table 2 Comparison of percentage of aniline-positive spermatozoa between groups

Aniline Positive (+)	Oligospermia	Control	p
n	40	21	0.000
Minimum value	31.61	2.18	
Maximum value	34.49	28.06	
Median	32	26	
Skewness	1.159±0.374	-0.113±0.501	
Kurtosis	1.38±0.733	2.899±0.972	

We observed a negative correlation between the percentage of aniline blue-positive spermatozoa and age (r = -0.145, P = 0.265), sperm concentration (r = -0.628, P < 0.001), total motile spermatozoa (r = -0.578, P < 0.001), total number of progressive motile spermatozoa (r = -0.595, P < 0.001), and normal morphology (r = -0.625, P < 0.001). However, we found a positive correlation between

the percentage of aniline blue-positive spermatozoa and immobility (r = 0.495, P < 0.001), head abnormality (r = 0.542, P < 0.001), tail abnormality (r = 0.543, P < 0.001), and normal morphology (r = 0.543, P < 0.001), as shown in (Table 3).

Table 3 The effect of aniline-positive sperm percentage on semen parameters

Anilin - positive spermatozoa		*Age*	*Sperm Concentration*	*Immobility %*	**Total motile sperm**	**Total progressive motile sperm**	**Normal sperm morphology**	**Head Anomaly**	**Tail Anomaly**
	r	-0.145	-0.628	0.495	-0.578	-0.595	-0.625	0.542	0.543
	P	0.265	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	N	61	61	61	61	61	61	61	61

4. DISCUSSION

The current diagnostic criteria for assessing male fertility are based on traditional semen parameters defined by the World Health Organisation (WHO). Standard semen analysis provides valuable information about sperm concentration, motility, and viability. It also includes information on morphological characteristics, the functionality of the reproductive glands, and the ejaculation process. The hypotheses formulated in our study indicated statistically significant differences between the control and patient groups in parameters such as the total number of progressively motile spermatozoa, the proportion of progressively motile spermatozoa, the proportion of motile spermatozoa, the number of fixed spermatozoa and normal sperm morphology.

We detected higher levels of abnormalities in sperm parameters in the patient group compared to the control group (P < 0.001). Agarwal et al., (2014) demonstrated that fertile males exhibited normal semen parameters. In contrast, oligospermic infertile males demonstrated abnormal semen parameters, a finding that is also consistent with the results of our study. In this study, we used aniline blue staining to assess sperm chromatin integrity. The study revealed that the percentage of aniline blue-positive (AB-positive) spermatozoa in the patient group was significantly higher than in the control group (P < 0.001).

We found a negative correlation between the percentage of AB-positive spermatozoa and semen parameters, including sperm morphology, sperm concentration, and progressive motility. In contrast, we observed a positive correlation between sperm head anomalies and sperm tail anomalies (P < 0.001). In a study encompassing 100 infertile and 20 fertile men, Al-Sultani et al., (2014) identified a significant negative correlation between the percentage of AB-positive sperm and sperm morphology, sperm concentration, and progressive motility parameters, aligning with the findings of our study.

Similarly, Pourmasumi et al., (2019) examined semen samples from 1044 infertile and 342 fertile men for sperm chromatin integrity, while Hekim et al., (2024) examined semen samples from 50 fertile and 32 infertile men for sperm chromatin integrity and reported that the percentage of AB-positive sperm was higher in the infertile group in both studies. Many studies have suggested that aniline blue staining may be a valuable tool in assessing male fertility along with standard sperm parameters (Pourmasumi et al., 2019; Hekim et al., 2024).

In the present study, we observed a statistically significant correlation between the percentage of AB-positive spermatozoa and sperm head abnormalities (r = 0.542, P < 0.001) as well as tail abnormalities (r = 0.543, P < 0.001). These findings are consistent with those reported in the literature and demonstrate a significant correlation between sperm morphology and the percentage of AB-positive sperm (Lazaros et al., 2011; Sellami et al., 2013). Kazerooni et al., (2009) showed that individuals diagnosed with teratozoospermia exhibited a significantly higher prevalence of AB-positive spermatozoa compared with the normozoospermic group.

In addition, researchers established a significant association between sperm head abnormalities and abnormalities in nuclear condensation, suggesting that sperm dysmorphisms may be partly due to inadequate nuclear compaction in sperm (Zini et al., 2009). Sellami et al., (2013) also found that the mean sperm head abnormalities increased as the percentage of AB-positive sperm increased in infertile men. This finding emphasizes the potential effects of sperm morphological abnormalities on fertility. The study suggests that structural abnormalities in the sperm head may adversely affect the functional ability of sperm and thus contribute to male infertility.

5. CONCLUSIONS

Semen analysis alone is insufficient to assess the functional capacity of sperm. It is necessary to complement traditional semen analysis with advanced assessments, including tests for sperm chromatin integrity. This provides more information about the integrity of sperm DNA and a more comprehensive evaluation of a man's fertility potential.

Author's Contribution

GGE and MB (Mahmut Balkan) were responsible for the analysis and interpretation of the data and drafting of the manuscript. Mi, OD, and ZÇ were responsible for patient recruitment and semen collection. RG was responsible for adjusting the aniline blue dye ratios. İY (İsmail Yıldız) performed the statistical analysis.

GGE, MB, and İY (İlyas YÜCEL), DO, ST, were responsible for the design, analysis, interpretation, discussion, drafting, and editing of the manuscript.

Ethical approval

Dicle University Medical Faculty Ethics Committee (Ethical approval code: 179 Date: 14/06/2023).

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Informed consent

Written & Oral informed consent was obtained from individual participants included in the study.

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Conflict of interest

The authors declare that there is no conflict of interests.

Data and materials availability

All data sets collected during this study are available upon reasonable request from the corresponding author.

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