Exp Biomed Res 2024; 7(1): 1-8 https://doi.org/10.30714/j-ebr.2024.202

Experimental Biomedical Research Original article

Investigation of sperm chromatin condensation by aniline blue staining in infertile men with normal and abnormal semen parameters

Neslihan Hekim*[1], Zeynep Dolu[1], Sezgin Gunes[1], Ramazan Asci[2]

[1]*Department of Medical Biology, Faculty of Medicine, Ondokuz Mayis University, Samsun, Türkiye*

[2]*Department of Urology, Faculty of Medicine, Ondokuz Mayis University, Samsun, Türkiye*

ABSTRACT

Aim: To evaluate the relationship between sperm chromatin condensation assessed by aniline blue staining and semen parameters in infertile men.

Methods: Infertile men applied to our urology clinics and diagnosed with normozoospermia (n=50), asthenozoospermia (n=17), oligozoospermia (n=3), teratozoospermia (n=2) oligoasthenoteratozoospermia (OAT) (n=10) according to their semen analysis were included in the study. Semen samples were evaluated for sperm chromatin condensation by aniline blue staining.

Results: The percentage of aniline-positive spermatozoa in the OAT and teratozoospermia group was found to be higher than in the normozoospermic and asthenozoospermic infertile groups (p=0.002, p=0.044 with normozoospermia group and p=0.026, p=0.007 with asthenozoospermia group, respectively). Sperm chromatin condensation was negatively correlated with sperm concentration (p=0.003, r=-0.322), total sperm count (p=0.004, r=-0.313), total progressive motile sperm count (p=0.005, r=-0.307), and normal morphology (p<0.0001, r=-0.554); and positively correlated with the percentage of immotile sperm (p=0.037, r=0.230).

Conclusion: Sperm chromatin condensation was found to be different in infertile men differently diagnosed based on their semen analysis. The results of the study suggest that chromatin condensation, together with routine sperm parameters, may constitute a valuable parameter in the evaluation of male fertility.

Keywords: Aniline blue, oligoasthenoteratozoospermia, semen analysis, sperm chromatin.

**Neslihan Hekim, Department of Medical Biology, Faculty of Medicine, Ondokuz Mayis University, Samsun, Türkiye*

E-mail: neslihan.taskurt@omu.edu.tr

Received: 2023-09-14

Accepted: 2023-11-20 / Published: 2024-01-01

Introduction

Infertility is a health problem in which couples cannot achieve pregnancy despite at least one year of unprotected and regular sexual intercourse [1]. According to global infertility prevalence estimates by World Health Organization (WHO) for 2022, infertility affects almost one in six people worldwide at some point in their life [2]. Approximately in the half of the couples with struggling infertility, the problems are caused by male partners [3]. Routine semen analysis, together with the medical history and physical examination, are essential elements in the evaluation of male infertility. Semen analysis provides information about sperm count, sperm motility and viability, sperm morphology, and functional status of accessory sex glands. However, semen analysis may be limited in evaluating male fertility chances [4].

The reorganization of sperm chromatin is a sperm-specific epigenetic rearrangement and

allows paternal DNA to be fitted into the sperm nucleus [5]. This process takes place at the stage of spermiogenesis and is called protamination. After protamination, approximately 85% of histones in the structure of sperm chromatin are replaced by protamines [6]. This structural alterations of sperm chromatin is necessary for sperm motility and fertilization. Protamination also protects to the paternal genome from oxidation, nucleases and mutagenic molecules in the female reproductive system [5]. The first stages of sperm chromatin rearrangement are hyperacetylation of histones and the incorporation of testis-specific histone variants into the chromatin structure [6]. Thus, topoisomerases form strand breaks in DNA, opening the super helix, loosening the nucleosome structure and making it easier for histones to separate from the chromatin. The histone variants are then replaced by transition proteins (TP1 and TP2), which are then entirely replaced by protamine 1 and protamine 2 (P1 and P2) [5,7]. Smaller than histones, protamines bind strongly to phosphate groups in sperm DNA with their arginine-rich domains. Paternal DNA then transforms into a toroid, an O-shaped structure by placing protamines. The toroidal structure is stabilized by phosphorylation, disulfide bonds and zinc bridges in the epididymis. Repackaging of sperm chromatin is completed during the epididymal transition of spermatozoa. However, endogenous breaks in sperm DNA afterward may indicate an error in chromatin packaging in spermiogenesis, thus an incomplete maturation stage [8].

Aniline blue is an acidic dye and binds strongly to lysine-rich histones. Thus, it is used to detect immature spermatozoa characterized by errors in histone-protamine exchange [9]. Histone-rich immature sperm are distinguished under the microscope as dark blue compared to arginine-rich protamine-packed mature sperm [10]. The study aimed to evaluate the relationship between semen parameters and sperm chromatin condensation as assessed by aniline blue staining in infertile men. For this purpose, semen samples of infertile patients with oligozoospermia, asthenozoospermia, teratozoospermia, oligoasthenoteratozoospermia (OAT) and normozoospermia were compared in terms of the percentage of aniline blue positive spermatozoa.

Materials and methods

Patients and semen analysis

The participants included in the study were patients who had semen analysis from infertile couples who applied to the Urology Clinics of OMU Faculty of Medicine between August 2022 and June 2023. Ethical approval for the study was given by the Ondokuz Mayıs University (OMU) Clinical Research Ethics Committee with the decision number OMU KAEK 2022/237. According to the results of routine semen analysis, infertile patients aged between 18-50 years who were diagnosed with oligozoospermia, asthenozoospermia, teratozoospermia, OAT and normozoospermia were included in the study. Semen samples were obtained by masturbation after 2-5 days of sexual abstinence, and routine semen analysis was evaluated according to the 6th edition of WHO's laboratory manual for examination and processing of human semen [11].

In this context, all semen parameters in normozoospermia were above the lower fifth percentile, azoospermia was defined as no sperm in the ejaculate, oligozoospermia as total sperm count was lower than $16x10^6$ spermatozoa/ml, asthenozoospermia as the percentage of progressive motile sperm was below 30%. Teratozoospermia was defined when the

percentage of morphologically normal spermatozoa was lower than <4%. OAT, on the other hand, was defined as the simultaneous observation of oligozoospermia, asthenozoospermia and teratozoospermia [11]. Patients diagnosed with obstructive or non-obstructive azoospermia, in which the female factor plays a role in infertility and/or karyotype anomalies, Y-chromosome microdeletions, cystic fibrosis transmembrane regulator gene (*CFTR*) mutations were not included in the study.

Analysis of sperm chromatin condensation with aniline blue

After the semen analysis, the samples were brought to OMU Medical Biology Department. Fresh semen samples were centrifuged twice with 1x phosphate salt solution (phosphate buffer saline; PBS) for 10 minutes at 2000 rpm after separating the seminal plasma. Semen pellets obtained after centrifugation were spread on clean slides. Slides were air dried and fixed with 3% glutaraldehyde. The samples were then soaked in a 5% aniline blue 4% glacial acetic acid solution. The slides prepared at the end of the staining process were analyzed under a light microscope (Olympus CX-31) at 1000x magnification in OMU Medical Biology Laboratory. Analysis was performed by counting at least 200 sperm from each slide. Histone-rich spermatozoa were then calculated as percentages.

Statistical analysis

Whether the percentage of histone-rich spermatozoa and the semen parameters of the participants showed a normal distribution were evaluated with the Shapiro-Wilk test. Data were given as mean ± standard deviation or mean difference ± standard error. Comparison of the data between groups was performed with one-way ANOVA followed by post-hoc Tukey HSD if they followed a normal distribution and with Kruskal Wallis and post-hoc Tamhane test if not. The strength and direction of the relationship between the two variables analyzed by Spearman's rank correlation. All tests were evaluated as two-tailed and a p value of <0.05 was considered statistically significant. SPSS 22.0 program (IBM, Quebec, Canada) was used for all statistical analyses.

Results

A total of 82 infertile men with oligozoospermia (n=3), asthenozoospermia (n=17), teratozoospermia (n=2), OAT (n=10) and normozoospermia (n=50) were included in the study. For sperm chromatin condensation, sperm stained dark blue as a result of aniline blue staining were evaluated as histone-rich, while sperm stained pale blue or not stained were considered protamine-rich (Figure 1).

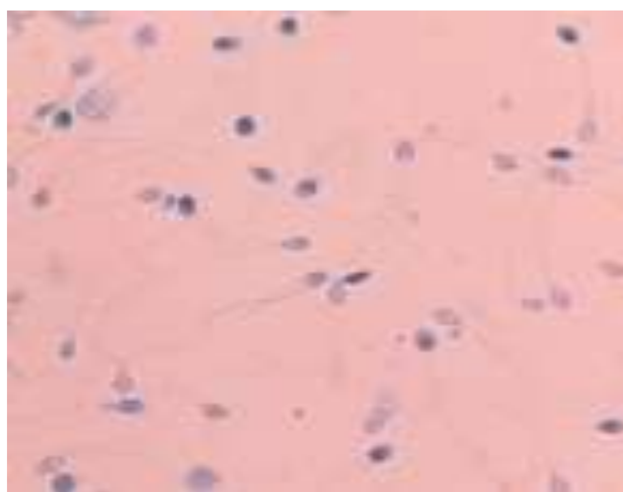

Figure 1. Dark stained histone-rich and unstained protamine-rich sperms as a result of aniline blue staining (Olympus CX-31, 1000x magnification)

The percentages of histone-rich spermatozoa analyzed by aniline blue staining, semen analysis, and age among the infertile groups are given in Table 1. As expected between groups, semen volume (p=0.008), sperm concentration (p<0.0001), total sperm count (p<0.0001), total progressive motile sperm count (p<0.0001), motility (p<0.0001) and normal morphological spermatozoa (p<0.0001) were different.

Additionally, infertile groups were found to differ from each other in terms of histone-rich spermatozoa (p=0.001).

Table 2 presents multiple comparisons of parameters found to be different between infertile groups. Accordingly, among the groups, the percentage of histone-rich spermatozoa in the OAT group was higher than in men with asthenozoospermia (p=0.026) and normozoospermia (p=0.002).

Results of aniline blue analysis of men with teratozoospermia similarly revealed higher histone-rich spermatozoa in this group compared to asthenozoospermia and normozoospermia groups (p=0.007 and p=0.044, respectively). On the other hand, there was no difference in sperm chromatin condensation between oligozoospermia men and asthenozoospermia, OAT, normozoospermia, and teratozoospermia groups (p>0.05). In addition, no difference was found between the normozoospermia group and the asthenozoospermia group in terms of thepercentage of histone-rich spermatozoa (p>0.05).

Table 1. Results of histone-rich spermatozoa, semen analysis, and age among the infertile groups.

Parameters	Diagnosis					
	Oligozoospermia	Astenozoosspermi	OAT	Normozoospermia	Teratozoospermia	*p*
Age	30.00±3.61	31.94±4.63	34.20±4.96	31.58±5.31	33.00±4.24	**.001**[a]
Histone-rich spermatozoa (%)	34.59±15.37	31.91±16.50	50.84±12.45	28.19±14.21	55.26±3.46	.603[b]
Volume (ml)	3.00±1.00	3.36±0.97	3.30±1.32	2.53±0.65	3.50±0.71	**.008**[a]
Leucocyte (10^3/ml)	266.67±57.74	300.00±35.36	350.00±108.01	326.00±77.75	300.00±0.00	.294[a]
pH	7.93±0.12	8.01±0.11	8.11±0.23	8.09±0.20	8.00±0.00	.294[a]
Sperm Concentration (10^6/ml)	8.33±4.73	21.41±6.63	5.78±6.91	31.00±12.94	23.00±0.00	**<.0001**[a]
Total Sperm Count (10^6/ejaculate)	22.00±9.17	71.06±26.42	14.99±14.68	76.28±32.36	80.50±16.26	**<.0001**[b]
TPMSC	10.33±3.79	6.46±6.55	3.43±4.22	36.28±18.14	32.00±5.66	**<.0001**[a]
Progressive Motility (A) (%)	49.67±4.51	9.47±7.29	20.10±9.96	46.86±7.66	41.00±1.41	**<.0001**[a]
Non-Progressive Motility (B) (%)	5.00±0.00	4.59±1.42	5.10±0.32	5.20±1.00	6.50±2.12	.381[a]
Immotility (%)	45.33±4.51	85.94±8.17	74.80±9.91	47.94±7.34	52.50±3.54	**<.0001**[a]
Motility (A+B) (%)	54.67±4.51	14.06±8.17	25.20±9.91	52.06±7.34	47.50±3.54	**<.0001**[a]
Normal Morphology (%)	6.00±1.73	4.88±0.78	3.10±1.29	6.00±1.53	1.50±0.71	**<.0001**[a]

Values are mean±SD. a. Kruskal Wallis Test, b. One-Way ANOVA, OAT=Oligoasthenoteratozoospermia, TPMSC=Total progressive motile sperm count. Statistically significant results are given in bold.

Table 2. Multiple comparisons of data of infertile groups according to semen analysis results.

Dependent Variable			Mean Difference	Std. Error	*P*	95% Confidence Interval	
						Lower Bound	Upper Bound
Histone-rich spermatozoa[a]	OAT	Astenozoospermia	18.93	5.61	**.026**	1.58	36.29
		Normozoospermia	22.65	4.42	**.002**	8.02	37.28
	Teratozoospermia	Astenozoospermia	23.35	4.69	**.007**	6.28	40.43
		Normozoospermia	27.07	3.17	**.044**	1.22	52.92
Volume[a]	Astenozoospermia	Normozoospermia	0.83	0.25	**.032**	0.05	1.62
Sperm Concentration[a]	OAT	Astenozoospermia	-15.63	2.71	**<.0001**	-24.25	-7.01
		Normozoospermia	-25.22	2.85	**<.0001**	-34.00	-16.44
		Teratozoospermia	-17.22	2.18	**<.0001**	-25.25	-9.19
	Normozoospermia	Oligozoospermia	22.67	3.29	**.020**	4.99	40.34
		Astenozoospermia	9.59	2.44	**.002**	2.48	16.70
		OAT	25.22	2.85	**<.0001**	16.44	34.00
		Teratozoospermia	8.00	1.83	**.001**	2.63	13.37
Total Sperm Count[b]	Oligozoospermia	Normozoospermia	-54.28	17.25	**.019**	-102.47	-6.09
	Astenozoospermia	OAT	56.07	11.57	**<.0001**	23.76	88.38
	OAT	Astenozoospermia	-56.07	11.57	**<.0001**	-88.38	-23.76
		Normozoospermia	-61.29	10.05	**<.0001**	-89.38	-33.20
		Teratozoospermia	-65.51	22.48	**.037**	-128.31	-2.71
	Normozoospermia	Oligozoospermia	54.28	17.25	**.019**	6.09	102.47
		OAT	61.29	10.05	**<.0001**	33.20	89.38
	Teratozoospermia	OAT	65.51	22.48	**.037**	2.71	128.31
TPMSC[a]	Normozoospermia	Oligozoospermia	25.95	3.37	**<.0001**	14.07	37.82
		Astenozoospermia	29.82	3.02	**<.0001**	21.08	38.57
		OAT	32.85	2.89	**<.0001**	24.42	41.27
Progressive Motility[a]	Astenozoospermia	Oligozoospermia	-40.20	3.15	**.002**	-57.16	-23.23
		Normozoospermia	-37.39	2.07	**<.0001**	-43.67	-31.11
		Teratozoospermia	-31.53	2.03	**<.0001**	-38.67	-24.38
	OAT	Oligozoospermia	-29.57	4.09	**.001**	-45.02	-14.12
		Normozoospermia	-26.76	3.33	**<.0001**	-38.31	-15.21
		Teratozoospermia	-20.90	3.30	**.001**	-32.69	-9.11
Immotility[a]	Astenozoospermia	Oligozoospermia	40.61	3.27	**.001**	24.65	56.57
		Normozoospermia	38.00	2.24	**<.0001**	31.14	44.86
		Teratozoospermia	33.44	3.19	**.034**	4.83	62.05
	OAT	Oligozoospermia	29.47	4.07	**.001**	14.04	44.90
		Normozoospermia	26.86	3.30	**<.0001**	15.38	38.34
		Teratozoospermia	22.30	4.01	**.023**	3.68	40.92
Motility[a]	Astenozoospermia	Oligozoospermia	-40.61	3.27	**.001**	-56.57	-24.65
		Normozoospermia	-38.00	2.24	**<.0001**	-44.86	-31.14
		Teratozoospermia	-33.44	3.19	**.034**	-62.05	-4.83
	OAT	Oligozoospermia	-29.47	4.07	**.001**	-44.90	-14.04
		Normozoospermia	-26.86	3.30	**<.0001**	-38.34	-15.38
		Teratozoospermia	-22.30	4.01	**.023**	-40.92	-3.68
Normal Morphology[a]	Astenozoospermia	OAT	1.78	0.45	**.016**	0.27	3.29
		Normozoospermia	-1.12	0.29	**.003**	-1.96	-0.28
	OAT	Astenozoospermia	-1.78	0.45	**.016**	-3.29	-0.27
		Normozoospermia	-2.90	0.46	**<.0001**	-4.42	-1.38

a.Tukey HSD, b. Tamhane, OAT=Oligoasthenoteratozoospermia, TPMSC=Total progressive motile sperm count.

Sperm chromatin condensation by aniline blue staining was found to be negatively correlated with sperm concentration (*p*=0.003, r=-0.322), total sperm count (*p*=0.004, r=-0.313), total progressive motile sperm count (*p*=0.005, r=-0.307), and normal morphology (*p*<0, 0001, r=-0.554); and positively correlated with the percentage of immotile sperm (*p*=0.037, r=0.230) (Table 3).

Table 3. Correlation between sperm chromatin condensation and semen parameters.

Spearman's Rank Correlation		Sperm Concentration (10^6/ml)	Total Sperm Count (10^6/ejaculate)	TPMSC	Progressive Motility (A) (%)	Non-Progressive Motility (B) (%)	Immotility (%)	Motility (A+B) (%)	Normal Morphology (%)
Histone-rich sperm (%)	r	**-.322****	**-.313****	**-.307****	**-.227***	.074	**.230***	**-.230***	**-.554****
	P	**.003**	**.004**	**.005**	**.040**	.512	**.037**	**.037**	**<.0001**
	N	82	82	82	82	81	82	82	82

***. Correlation is significant at the 0.01 level (2-tailed) *. Correlation is significant at the 0.05 level (2-tailed) r= Correlation Coefficient. TPMSC=Total progressive motile sperm count. Statistically significant results are given in bold.*

Discussion

In this study, the percentage of histone-rich spermatozoa was revealed to be different in infertile patients with different semen analysis. Replacement of protamines with histones occurs during the elongated stage of spermiogenesis after meiosis is completed. These spermatids undergo processes that affect the motility and fertility ability of mature sperm during protamination [12]. Incomplete chromatin packing is known to be a primary cause of sperm DNA damage and eventually, this may lead to reduce fertility [13]. As a sperm chromatin integrity analysis, aniline blue staining method is based on an increased affinity of the dye to histones of the sperm nucleus [13]. This fast and relatively easy-to-apply method can be used with a light microscope. Pourmasumi et al. (2019) evaluated semen samples of 1044 infertile men diagnosed with normozoospermia, oligoasthenozoospermia, asthenozoospermia, oligozoospermia and idiopathic, in terms of sperm chromatin condensation. Researchers reported that histone-rich aniline blue-positive sperm were more common in the infertile group compared to the normozoospermic group [14]. However, when infertile subgroups were analyzed separately, aniline blue positive spermatozoa was found to be higher in oligoasthenozoospermia, asthenozoospermia, and oligozoospermia groups compared to idiopathic infertile groups, similarly with the present study. We also found that histone-rich immature spermatozoa, especially in the OAT group, were determined to be higher than the other infertile subgroups. In addition, accordingly with our results, negative correlations were shown between the aniline blue positive spermatozoa and sperm count and spermatozoa with normal morphology [14]. Similarly, Al-Sultani et al (2015) determined that there was a difference in sperm chromatin condensation between fertile and infertile men with aniline blue staining. Also, spermatozoa with abnormal aniline blue staining were negatively correlated with sperm morphology, concentration, and progressive motility [15]. However, in this study, it was found that there

was a difference in sperm chromatin integrity between normozoospermic and fertile individuals [15]. This difference may suggest that sperm chromatin condensation differs not only in those with abnormal semen parameters but also in normozoospermic infertile men compared to fertile men. Nuclear condensation, which occurs with protamination in paternal DNA, may be considered as a determinant of the fertilization capacity of sperm. As a matter of fact, studies show that errors in histone protamine transition may be associated with male infertility and early miscarriages [9,14,16,17]. After all, the chromatin integrity of the sperm of men who have not been able to have children with assisted reproductive techniques was shown to be affected compared to the controls [18]. In addition, some studies have shown the difference between sperm samples from infertile patients and healthy individuals in terms of staining with aniline blue but could not find a relationship between chromatin condensation and semen parameters [19]. Sellami et al. (2013) found a significant association between aniline blue staining and sperm chromatin condensation in infertile men and the mean number of sperm head abnormalities but did not find any association with sperm motility, viability, and number [20]. Studies conducted in our laboratory, sperm chromatin condensation has been also shown to be negatively correlated with sperm motility and concentration [15,16]. The limitation of our study is mainly due to the small number of volunteers constituting some of our patient groups. However, addition of a fertile control group in future studies may also increase the study power.

Conclusions

In conclusion, our study, together with other studies, reveals that sperm chromatin condensation analyzed by aniline blue staining is correlated with routine semen parameters and may differ in men with different semen analysis results. However, together with semen analysis, it constitutes a valuable parameter evaluating of male fertility.

Funding: *The present study was partially supported by the project budget of the OYP program*

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

Ethical statement: *Ethical approval for the study was given by the Ondokuz Mayıs University (OMU) Clinical Research Ethics Committee with the decision number OMU KAEK 2022/237.*

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