doi: 10.53446/actamednicomedia.1342822

Acta Medica Nicomedia

Cilt: 6 Sayı: 3 Ekim 2023 / Vol: 6 Issue: 3 October 2023
https://dergipark.org.tr/tr/pub/actamednicomedia

Research Article | Araştırma Makalesi

CYTOCHEMICAL EVALUATION OF SPERM DNA INTEGRITY AND RELATION TO CONVENTIONAL SEMEN PARAMETERS IN IDIOPATHIC INFERTILE MEN

SPERM DNA BÜTÜNLÜĞÜNÜN SİTOKİMYASAL DEĞERLENDİRİLMESİ VE İDİOPATİK İNFERTİL ERKEKLERDE KONVANSİYONEL SEMEN PARAMETRELERİ İLE İLİŞKİSİ

Sevilay Erimsah[1*], Yusufhan Yazir[2]

[1]Istanbul Nisantasi University, Medical Faculty, Histology and Embryology Department, Istanbul, Türkiye. [2]Kocaeli University, Faculty of Medicine, Histology and Embryology Department, Kocaeli, Türkiye.

ABSTRACT

Objective: The aim of this study is to investigate the relationship between sperm DNA integrity and conventional semen parameters in idiopathic infertile men using cytochemical methods.

Methods: Sperm DNA integrity was evaluated by using acidic aniline blue staining specifies nuclear chromatin condensation and TUNEL method identifies sperm DNA breaks in 40 idiopathic infertile men. Stained sperm heads on smears were counted as TUNEL+ and aniline blue+, showing sperm DNA damage. The findings were used to determine the correlation between rutin semen parameters and sperm DNA integrity tests results.

Results: There was a significant negative correlation between the percentage of aniline blue+ staining and sperm morphology and progressive motility. And a negative correlation was observed between the percentage of TUNEL+ staining and normal morphology and progressive motility ($p<0.01$), where no correlation was found regarding sperm concentration and sperm total motility.

Conclusion: Methods such as acidic aniline blue and TUNEL can show structural defects of sperm independent of conventional semen parameters. Although these methods are associated with some semen parameters, their using especially for the idiopathic infertile patient group may contribute positively to the success of assisted reproductive techniques.

Keywords: Sperm, chromatin condensation, DNA fragmentation, TUNEL, aniline Blue

ÖZ

Amaç: Bu çalışmanın amacı idiyopatik infertil erkeklerde sperm DNA bütünlüğü ile konvansiyonel semen parametreleri arasındaki ilişkinin sitokimyasal yöntemlerle araştırılmasıdır**.**

Yöntem: 40 idiopatik infertilitesi olan bireyde, nükleer kromatin yoğunlaşmasını belirten asidik anilin mavisi boyama yöntemi ve sperm DNA kırıklarını belirten TUNEL yöntemi kullanılarak sperm DNA bütünlüğü değerlendirildi. Yayma preperatlarda, sperm DNA hasarı gösteren TUNEL+ ve anilin blue+ boyalı sperm başları sayıldı. Bulgular, rutin semen parametreleri ile sperm DNA bütünlük testleri sonuçları arasındaki ilişkiyi belirlemek için kullanıldı.

Bulgular: Anilin mavisi+ boyanma oranı ile sperm morfolojisi ve progresif hareketliliği arasında anlamlı bir negatif korelasyon izlendi. TUNEL+ boyanma oranı ile normal morfoloji ve ilerleyici hareketlilik arasında negatif bir korelasyon gözlendi ($p<0,01$), ancak sperm konsantrasyonu ve sperm toplam hareketliliği ile ilgili herhangi bir korelasyon bulunamadı.

Sonuç: Asidik anilin mavisi ve TUNEL gibi yöntemler, geleneksel semen parametrelerinden bağımsız olarak spermin yapısal kusurlarını gösterebilmektedir. Bu yöntemler bazı semen parametreleri ile ilişkili olsa da özellikle idiyopatik infertil hasta grubu için kullanılması yardımcı üreme tekniklerinde başarıya olumlu katkı sağlayabilir.

Anahtar Kelimeler: Sperm, kromatin yoğunlaşması, DNA fragmantasyonu, TUNEL, anilin mavisi

***Corresponding author/İletişim kurulacak yazar:** Sevilay Erimsah; Istanbul Nisantasi University, Medical Faculty, Histology and Embryology Department, Istanbul, Türkiye.
Phone/Telefon: +90 (505) 629 85 60 **e-mail/e-posta:** sevilayerimsah@yahoo.com
Submitted/Başvuru: 14.08.2023 • **Accepted/Kabul:** 18.09.2023 • **Published Online/Online Yayın:** 21.10.2023

Introduction

Worldwide, moreover 70 million couples experience infertility and need assisted reproductive technology (ART).[1] Current data suggests that the male factor is one-third of these infertility disorders.[2] Conventional semen analysis including seminal volume, pH, sperm count, sperm motility, and morphology is still the most essential initial step in male factor infertility.[3] However, these parameters provide information on the penetration ability of the sperm and embryo development but have limited capacity to determine the underlying changes to molecular and cellular processes that play a crucial role in reproductive functions.[4,5] Some determinant sperm parameters such as apoptosis and chromatin condensation are overlooked in routine semen analysis.[6,7]

According to the results of some studies, sperm DNA damage is observed in men clasified as idiopathic infertility with normal semen parameters.[8] Therefore, routine semen analysis in male infertility is not sufficient to make a definitive diagnosis.[9,10] For the patient group with idiopathic infertility, comprehensive sperm evaluation is a priority for successful treatment. Sperm chromatin condensation is decisive for the fertilization ability of sperm. In addition, increased sperm DNA fragmentation (SDF) reduces fertilization rates.[11] Acidic aniline blue is a widely used method in studies to determine sperm chromatin condensation.[12,13] Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) is also used as a method based on detecting the breaks in sperm DNA structure.[14] In some studies, the relationship between sperm chromatin condensation and sperm parameters has been investigated. [15,16,17]

The aim of this study is to evaluate sperm DNA integrity with cytochemical methods and investigate the relation to semen parameters in semen samples of idiopathic infertile men.

Methods

Subjects and Evaluation of Standard Sperm Parameters

This study was performed on semen samples of men with idiopathic infertility (n=40, aged 29-55 years) attending Bolu Abant Izzet Baysal University Hospital for semen analysis. After giving detailed information about the study, informed consent forms were signed. The study was approved by the Ethics Committee of Clinical Research (2019/13) of Bolu Abant Izzet Baysal University, Medical Faculty, Bolu, Turkey. The semen samples were obtained by masturbation, after 2-7 days of sexual abstinence and allowed to liquefy for 30 minutes at room temperature. Each liquefied semen sample was divided into three fractions, for analyzing standard sperm parameters and assessment of sperm chromatin condensation and DNA fragmentation.

Standard sperm parameters were evaluated according to the World Health Organization (WHO) Criteria including sperm concentration, motility, and morphology.[18] Morphology assessments were done according to strict criteria.[19] Semen smears were made on slides for Papanicolaou staining and considered morphologically with immersion objective.

Assessment of Chromatin Condensation by Aniline Blue

Aniline blue staining was used to evaluate sperm chromatin condensation on semen samples as described by Hammadeh et al.[20] Raw semen samples were washed with PBS followed by centrifugation at 300 g for 10 min. Then, the washed samples were smeared and dried. Then, each smear was fixed in %3 glutaraldehyde in PBS for 30 minutes and stained with 5% aqueous acidic aniline blue (pH 3.5) solution for 5 min. After the smears were washed with distilled water, mounted slides were evaluated for sperm chromatin condensation, and a total of 200 spermatozoa were counted with immersion objective (100x magnification) and scored. The spermatozoa unstained or stained light blue scored as AB-negative (normal chromatin) and stained blue scored as AB-positive (abnormal chromatin). The percentage of AB-positive staining at 200 spermatozoa was calculated for each smear.

Assessment of Sperm DNA Fragmentation by TUNEL Assay

In this study, sperm DNA fragmentation (SDF) was evaluated by TUNEL assay using the In Situ Cell Death Detection Kit (Merck Millipore, Darmstadt, Germany). Raw semen samples were washed with Pbs followed by centrifugation at 300 g for 10 min. Then, the washed samples were smeared and dried. Then each smear was fixed with methanol for 30 minutes following the TUNEL (Millipore) staining protocol. The mounted slides were evaluated for sperm chromatin condensation, and a total of 200 spermatozoa were counted with immersion objectives and scored. The spermatozoa stained brown scored as TUNEL-positive and, unstained as TUNEL-negative. The percentage of TUNEL-positive sperm was calculated by counting 200 spermatozoa for each smear. For each smear, the percentage of TUNEL-positive staining at 200 spermatozoa was calculated.

Statistical Analysis

The data of sperm DNA integrity and parameters were evaluated with the Kolgomorov-Smirnov test in terms of homogeneity and normal distribution, and the results were expressed as mean ± standard deviation, median, minimum, and maximum values. The Pearson correlation between semen parameters and DNA integrity was analyzed. Statistical analysis was performed by using the SPSS 21 version (IBM, Armonk, New York, United States), and a $p<0.05$ was considered statistically significant.

Results

The findings of conventional semen parameters, acidic aniline blue+, and TUNEL+ staining, in which the DNA structure of idiopathic infertile patients is evaluated, are

shown in Table 1. TUNEL test (46±23.91%) detected the rates of sperm with DNA breaks and sperm with defects in chromatin condensation (47±24.76%) with acidic aniline blue staining (Figure 1). When the relationship between sperm DNA integrity and conventional sperm parameters was investigated; a significant negative correlation between aniline blue staining and sperm morphology and progressive motility was observed ($p<0.01$). Similarly, a negative correlation was observed between TUNEL+ staining and sperm morphology and progressive motility ($p<0.01$). However, there was a nonsignificant correlation between sperm DNA integrity, concentration, and total motility. In addition, a significant positive correlation was observed between the results of AB+ and TUNEL+ staining ($p<0.01$).

Table 1. Descriptive statistical values of semen parameters and sperm DNA integrity tests

Parameters	Idiopathic infertile men (n=40) Mean±SD	Median (min–max)
Age	37.48±6.05	35 (29-55)
Sperm Concentration (x10^{6}ml)	49.67±14.39	45 (39-90)
Normal Morphology (%)	14±6.9	12 (4-27)
Total Motility (%)	67.27±16	60 (40-95)
Progressive motility (%)	42±58	39 (29-71)
Asidic aniline blue + (%)	47±24.76	42,5 (7-98)
TUNEL+ (%)	46±23.91	37 (13-95)

SD: Standart Deviation, Min: Minimum, Max: Maximum

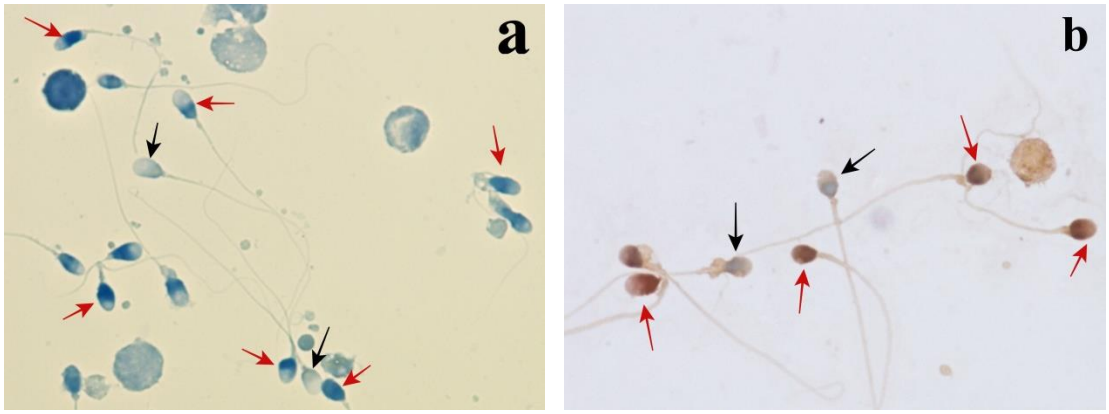

Figure 1. Sperm cells stained with acidic aniline blue and TUNEL method**; a.** AB-negative spermatozoa (condense chromatin) marked with black arrow and AB-positive spermatozoa marked with red arrow, **b.** TUNEL-negative spermatozoa stained brown marked with black arrow and TUNEL-positive spermatozoa marked with red arrow.

Discussion

Idiopathic male infertility is a condition that can not be determined exactly. Some studies in recent years have suggested that there are significant defects in sperm DNA integrity in men with idiopathic male infertility.[21] This suggests that one of the potential underlying causes of male infertility may be damage to sperm DNA integrity. Sperm DNA integrity refers to whether the genetic material of the sperm cell is preserved and any damage that occurs in the genetic material.

In our study, we detected sperm DNA integrity in patients with normal semen parameters with male infertility according to the results of routine spermiogram analysis. Although semen parameters such as concentration, motility, and morphology were normal values, a high percentage of sperm with abnormal chromatin condensation was observed. In many studies, male infertility can be seen with chromatin condensation over 30%.[6,22] A higher percentage of the smears from idiopathic infertile men was stained by aniline blue (47±24,76%). It is very difficult to obtain sufficient information about sperm structure with standard sperm parameters. Acidic aniline blue staining is a method that provides information about sperm chromatin condensation during the development of sperm. It is widely used in studies due to easy and quick access to information. [10,12,13] Insufficient packaging of the paternal genome during the maturation process of the sperm also reduces the fertilization ability of the sperm and also embryo development and health in the future.[23] The high rate of aniline blue+ staining in this patient group actually reports the scarcity of sperm with fertilization ability. This information allows us to make the selection of sperm in the clinic by considering this information.

In addition, the TUNEL+ sperm percentage was high in the semen samples. This information informs us that there are high rates of DNA breaks in patients with idiopathic infertility. With these methods, which are frequently used to determine sperm DNA integrity in studies, we were able to determine the proportion of sperm with normal standard semen parameters but with impaired DNA structure (Table 1). On the other hand, our results showed a negative correlation between normal morphology and TUNEL+ staining and AB+ staining, similar to some studies.[12,24] However, unlike some studies, a high negative correlation was observed between the rate of progressively motile sperm and positive staining rates in our study.[24] We found that the concentration and total motile sperm ratio used in standard semen analysis were not related to DNA integrity. Similarly, some studies show a correlation between morphology and sperm chromatin structure.[8,17] In this study, we evaluated sperm chromatin condensation and DNA fragmentation, considered as an independent measure of sperm quality, which can provide new approaches to diagnosis and treatment better than conventional sperm parameters (concentration, morphology, and motility). A negative correlation has been found between some semen parameters and sperm DNA integrity as well as, there was no relationship between other semen parameters. These relationships contributed to new information about the source of DNA damage.

Finally, our study shows that standard semen parameters alone are not sufficient for the idiopathic infertile patient group, and detailed analyzes including sperm DNA structure are needed to determine a successful treatment in the clinical approach.

Compliance with Ethical Standards

Bolu Abant Izzet Baysal University Faculty of Medicine Ethics Committee approved this study (decision number 2019/13; date: February 12, 2019). Informed consent was obtained from all participants.

Conflict of Interest

The authors declare no conflicts of interest.

Author Contribution

Authors contributed equally to this work.

Financial Disclosure

Financial disclosure none.

References

1. Ombelet W. Global access to infertility care in developing countries: a case of human rights, equity and social justice. *Facts Views Vis Obgyn*. 2011;3(4):257-266.
2. Kumar N, Singh AK. Trends of male factor infertility, an important cause of infertility: A review of literature. *J Hum Reprod Sci*. 2015;8(4):191-196. doi:10.4103/0974-1208.170370
3. Cooper TG, Noonan E, von Eckardstein S, et al. World Health Organization reference values for human semen characteristics. *Hum Reprod Update*. 2010;16(3):231-245. doi:10.1093/humupd/dmp048
4. Wang C, Swerdloff RS. Limitations of semen analysis as a test of male fertility and anticipated needs from newer tests. *Fertil Steril*. 2014;102(6):1502-1507. doi:10.1016/j.fertnstert.2014.10.021
5. Oehninger S, Ombelet W. Limits of current male fertility testing. *Fertil Steril*. 2019;111(5):835-841. doi:10.1016/j.fertnstert.2019.03.005
6. Hammadeh ME, Zeginiadov T, Rosenbaum P, Georg T, Schmidt W, Strehler E. Predictive value of sperm chromatin condensation (aniline blue staining) in the assessment of male fertility. *Arch Androl*. 2001;46(2):99-104.
7. Khalili MA, Nazari S, Dehghani-Firouzabadi R, et al. Comparing the roles of sperm chromatin integrity and apoptosis in intrauterine insemination outcomes of couples with mild male and female factor infertility. *J Reprod Infertil*. 2014;15(1):35-40.
8. Saleh RA, Agarwal A, Nelson DR, et al. Increased sperm nuclear DNA damage in normozoospermic infertile men: a prospective study. *Fertil Steril*. 2002;78(2):313-318. doi:10.1016/s0015-0282(02)03219-3
9. Agarwal A, Allamaneni SS. Sperm DNA damage assessment: a test whose time has come. *Fertil Steril*. 2005;84(4):850-853. doi:10.1016/j.fertnstert.2005.03.080
10. Softness KA, Trussler JT, Carrasquillo RJ. Advanced sperm testing. *Curr Opin Urol*. 2020;30(3):290-295. doi:10.1097/MOU.0000000000000761
11. Aitken RJ, De Iuliis GN. On the possible origins of DNA damage in human spermatozoa. *Mol Hum Reprod*. 2010;16(1):3-13. doi:10.1093/molehr/gap059
12. Franken DR, Franken CJ, de la Guerre H, de Villiers A. Normal sperm morphology and chromatin packaging: comparison between aniline blue and chromomycin A3 staining. *Andrologia*. 1999;31(6):361-366. doi:10.1046/j.1439-0272.1999.00290.x
13. Irez T, Sahmay S, Ocal P, et al. Investigation of the association between the outcomes of sperm chromatin condensation and decondensation tests, and assisted reproduction techniques. *Andrologia*. 2015;47(4):438-447. doi:10.1111/and.12286
14. Ribas-Maynou J, García-Peiró A, Fernández-Encinas A, et al. Comprehensive analysis of sperm DNA fragmentation by five different assays: TUNEL assay, SCSA, SCD test and alkaline and neutral Comet assay. *Andrology*. 2013;1(5):715-722. doi:10.1111/j.2047-2927.2013.00111.x
15. Atshan M, Kakavand K, Hosseini SH, Sadighi Gilani MA, Mohseni Meybodi A, Sabbaghian M. Evaluation of sperm DNA fragmentation and chromatin structure in infertile men with immotile short-tail sperm defect. *Andrologia*. 2020;52(1). doi:10.1111/and.13445
16. Hologlu D, Gunes S, Asci R, Henkel R, Guvenc T. Association among sperm chromatin condensation, sperm DNA fragmentation and 8-OHdG in seminal plasma and semen parameters in infertile men with oligoasthenoteratozoospermia. *Andrologia*. 2022;54(1): e14268. doi:10.1111/and.14268
17. Salsabili N, Mehrsai A, Jalalizadeh B, Pourmand G, Jalaie S. Correlation of sperm nuclear chromatin condensation staining method with semen parameters and sperm functional tests in patients with spinal cord injury,

varicocele, and idiopathic infertility. *Urol J*. 2006;3(1):32-37.
18. Sánchez-Álvarez J, Cano-Corres R, Fuentes-Arderiu X. A Complement for the WHO Laboratory Manual for the Examination and Processing of Human Semen (First Edition, 2010). EJIFCC. 2012;23(3):103-106.
19. Menkveld R, Stander FS, Kotze TJ, Kruger TF, van Zyl JA. The evaluation of morphological characteristics of human spermatozoa according to stricter criteria. *Hum Reprod*. 1990;5(5):586-592. doi:10.1093/oxfordjournals.humrep.a137150
20. Hammadeh ME, al-Hasani S, Stieber M, et al. The effect of chromatin condensation (aniline blue staining) and morphology (strict criteria) of human spermatozoa on fertilization, cleavage and pregnancy rates in an intracytoplasmic sperm injection programme. *Hum Reprod*. 1996;11(11):2468-2471. doi:10.1093/oxfordjournals.humrep.a019139
21. Aitken RJ, Baker MA, Sawyer D. Oxidative stress in the male germ line and its role in the aetiology of male infertility and genetic disease. *Reprod Biomed Online*. 2003;7(1):65-70. doi:10.1016/s1472-6483(10)61730-0
22. Agarwal A, Said TM. Role of sperm chromatin abnormalities and DNA damage in male infertility. *Hum Reprod Update*. 2003;9(4):331-345. doi:10.1093/humupd/dmg027
23. Muriel L, Garrido N, Fernández JL, et al. Value of the sperm deoxyribonucleic acid fragmentation level, as measured by the sperm chromatin dispersion test, in the outcome of in vitro fertilization and intracytoplasmic sperm injection. *Fertil Steril*. 2006;85(2):371-383. doi:10.1016/j.fertnstert.2005.07.1327
24. Sadek A, Almohamdy AS, Zaki A, Aref M, Ibrahim SM, Mostafa T. Sperm chromatin condensation in infertile men with varicocele before and after surgical repair. *Fertil Steril*. 2011;95(5):1705-1708. doi:10.1016/j.fertnstert.2011.01.008