Chapter 9
Cytochemical Tests of Sperm Chromatin Maturity

Juris Erenpreiss and Ksenija Zubkova

9.1 Introduction

Infertility affects approximately 15% of couples trying to conceive, and a male cause is believed to be a sole or contributing factor in approximately half of these cases [1]. In clinical practice, the traditional, manual-visual light microscopic methods for evaluating semen quality maintain their central role in assessment of male fertility potential. However, often a definitive diagnosis of male fertility cannot be made as a result of basic semen analysis due to the overlap of sperm concentration, motility, and morphology between fertile and infertile men [2].

It has been demonstrated that abnormalities in the male genome, characterized by disturbed chromatin packaging and damaged sperm DNA may be a cause for male infertility regardless of routine semen parameters [3, 4]. Focus on the chromatin maturity and integrity of the male gamete has been intensified by the growing concern about transmission of damaged DNA through assisted reproductive techniques (ARTs), especially by means of intracytoplasmic sperm injection (ICSI) that bypasses processes of natural selection during sperm-oocyte interaction, which are still present in conventional in vitro fertilization (IVF). There are concerns relating to potential chromosomal abnormalities, congenital malformations, and developmental abnormalities in ICSI-born progeny [5–8]. Accumulated evidence suggests a negative relationship between abnormal sperm chromatin structure and the fertility potential of spermatozoa both in vivo and in vitro [9–13].

Abnormalities in the sperm chromatin organization may be indicative of male infertility regardless of normal semen parameters [3, 4]. Evaluation of sperm chromatin structure is an independent measure of sperm quality that provides good prognostic and diagnostic capabilities. Therefore, it may be considered a reliable predictor of a couple's inability to conceive.

J. Erenpreiss (✉) • K. Zubkova
Riga Stradins University, Riga, Latvia
e-mail: jerenpreiss@gmail.com

A. Zini, A. Agarwal (eds.), *A Clinician's Guide to Sperm DNA and Chromatin Damage*, https://doi.org/10.1007/978-3-319-71815-6_9

Many techniques have been described for evaluation of the chromatin status and maturity. There is a group of methods based on the ability of some stains to test the conformation of sperm chromatin, which in turn depends on sperm DNA breaks and DNA interaction with proteins. These assays, often referred to as "cytochemical," include acidic aniline blue (AAB), toluidine blue (TB), and chromomycin A3 tests.

9.2 Cytochemical Properties of Human Sperm Chromatin

In many mammals, spermatogenesis leads to the production of highly homogenous spermatozoa. For example, mouse sperm nuclei contain more than 95% protamines in their nucleoprotein component [14]. This allows the mature sperm nuclei to adopt a volume 40 times less than that of normal somatic nuclei [15]. The final, very compact packaging of the primary sperm DNA filament is produced by DNA-protamine complexes, which contrary to nucleosomal organization in somatic cells provided by histones approach the physical limits of molecular compaction [16]. Human sperm nuclei, however, contain considerably fewer protamines (around 85%) than sperm nuclei of bull, stallion, hamster, and mouse [17, 18]. Human sperm chromatin, therefore, is less regularly compacted and frequently contains DNA strand breaks [19, 20].

To achieve this uniquely condensed state, sperm DNA must be organized in a specific manner, which differs substantially from that of somatic cells [15]. The fundamental packaging unit of mammalian sperm chromatin is a toroid containing 50–60 kb of DNA. Individual toroids represent the DNA loop domains highly condensed by protamines and fixed at the nuclear matrix; toroids are cross-linked by disulfide bonds, formed by oxidation of sulfhydryl groups of cysteine present in the protamines [16, 21]. Thus, each chromosome represents a garland of toroids, while all 23 chromosomes are clustered by centromeres into a compact chromocenter positioned well inside the nucleus with telomere ends united into dimers exposed to the nuclear periphery [22, 23]. This condensed, insoluble, and highly organized nature of sperm chromatin acts to protect genetic integrity during transport of the paternal genome through the male and female reproductive tracts. It also ensures that the paternal DNA is delivered in the form that sterically allows the proper fusion of two gametic genomes, their centromeric rings, and enables the developing embryo to correctly express the genetic information [23–25].

In comparison with other species [26], human sperm chromatin packaging is exceptionally variable. This variability has been mostly attributed to its basic protein component. The retention of 15% histones, which are less basic than protamines, leads to the formation of less compact chromatin structure [16]. Moreover, human spermatozoa contain two types of protamines, P1 and P2, with a second type deficient in cysteine residues [27]. This results in diminished disulfide cross-linking responsible for more stable packaging as compared to species containing P1 alone [28].

Chromatin structural probes using aromatic cationic dyes allow to analyze chromatin structure in terms of protein packaging correctness and disulfide cross-linking density. These probes are both sensitive and simple to use and therefore attractive for clinical use. However, their cytochemical background is rather complex. Several factors influence the staining of the chromatin by planar ionic dyes: (1) secondary structure of DNA, (2) regularity and density of chromatin packaging, and (3) binding of DNA to chromatin proteins.

9.2.1 DNA Secondary Structure and Conformation

Fragmented DNA is easily denatured [29]. However, even a single DNA strand break causes conformational transition of the DNA loop domain from a supercoiled state to a relaxed state. Supercoiled DNA avidly takes up intercalating dyes (like acridine orange) because this reduces the free energy of torsion stress. In contrast, the affinity for intercalation is low in relaxed DNA and is lost in fragmented DNA. In this case, an external mechanism of dye binding to DNA phosphate residues and dye polymerization (metachromasy) is favored [30, 31]. Nevertheless, fragmentation of DNA is not the only factor affecting the determination between metachromatic and orthochromatic staining. Chromatin packaging density also influences this balance.

9.2.2 Chromatin Packaging Density

If the chromatin is regularly arranged and sufficiently densely packed, dye coplanar polymerization providing metachromatic shift (change of color) is favored [32, 33]. However, if the chromatin is packaged even more densely (as in normal sperm), the polymerization of the dye is hindered [34] and may even prevent dye binding, especially by large, bulky dyes at an unfavorable pH. The latter case is seen with aniline blue at low pH where it stains basic proteins loosely associated with DNA and is unable to bind to the chromatin of normal sperm, which is very densely packaged and low charged. Binding of protamine molecules to DNA facilitates DNA condensation and toroid formation [35]. Substitution of histones for more basic protamines occurring during spermiogenesis neutralizes the DNA negative charge and decreases the accessibility of DNA-specific cationic dyes. Thus, the fluorescence staining intensity of a haploid sperm is much lower than the fluorescence intensity of a haploid round spermatid. However, after removal of nuclear proteins (e.g., by acid extraction), increase in sperm DNA stainability can vary depending on the chemical structure of the dye and the binding type the dye forms with the DNA substrate [36].

9.2.3 Chromatin Proteins

Chromatin proteins affect the binding of DNA dyes in the way that they themselves bind differently to relaxed (fragmented) or supercoiled DNA. DNA supercoiling requires covalent binding of some nuclear matrix proteins and tighter ionic interactions between DNA and chromatin proteins to support negative supercoils [37]. Relaxed and fragmented DNA has looser ionic interactions with chromatin proteins, which can be more easily displaced from the DNA, thus favoring external metachromatic binding of the dye to DNA phosphate groups. Both mechanisms of dye binding, external and intercalating, compete within each constraint loop domain (toroid) depending on its conformational state.

9.3 Sperm Chromatin Structural Probes

Chromatin proteins in sperm nuclei with the impaired DNA appear to be more accessible to binding with the acidic dye, as found by the AAB test [38, 39]. An increase in the ability to stain sperm by AAB indicates a looser chromatin packaging and increased accessibility of the basic groups of the nucleoprotein. This is due to the presence of residual histones [40]. Chromomycin A3 (CMA3) is another staining technique, which has been used as a measure of sperm chromatin condensation anomalies. CMA3 is a fluorochrome specific for GC-rich sequences and is believed to compete with protamines for association with DNA. The extent of staining is therefore related to the degree of protamination of mature spermatozoa [41, 42]. In turn, phosphate residues of sperm DNA in nuclei with loosely packed chromatin and/or impaired DNA will be more liable to binding with basic dyes. Such conclusions were also deduced from the results of staining with basic dyes, such as TB, methyl green, and Giemsa stain [43, 44].

9.3.1 Acidic Aniline Blue

The AAB stain discriminates between lysine-rich histones and arginine/cysteine-rich protamines. This technique provides a specific positive reaction for lysine and reveals differences in the basic nuclear protein composition of human spermatozoa. Histone-rich nuclei of immature spermatozoa are rich in lysine and will consequently take up the blue stain. On the contrary, protamine-rich nuclei of mature spermatozoa are rich in arginine and cysteine and contain relatively low levels of lysine, which means they will not take up the stain [45].

Technique: slides are prepared by making a smear of 5 μl of either raw or washed semen sample. The slides are air-dried and fixed for 30 min in 3% glutaraldehyde in phosphate-buffered saline (PBS). The smear is dried and stained for 5 min in 5%

aqueous AB solution (pH 3.5). Sperm heads containing immature nuclear chromatin stain blue and those with mature nuclei do not. The percentage of spermatozoa stained with AB is determined by counting 200 spermatozoa per slide under bright-field microscopy [46].

A strong association between the results of AAB staining and male infertility has been shown [47]. It has been reported by some studies that chromatin condensation as visualized by the AAB staining is predictive for IVF outcome [48], but it was not a case for ICSI. Other studies, in turn, have reported an association also with the ICSI outcomes: fertilization and cleavage rate [49, 50]. Also, an association of poor chromatin condensation as detected by the AAB test with the abortion rates following the IUI cycles has been reported [51].

9.3.2 Toluidine Blue Stain Assay

TB is a basic planar nuclear dye used for metachromatic and orthochromatic staining of the chromatin. The phosphate residues of sperm DNA in nuclei with loosely packed chromatin and/or impaired DNA become more liable to binding with TB, providing a metachromatic shift due to coplanar dimerization of the dye molecules from light-blue to purple-violet color [38, 52]. This stain is a sensitive structural probe for DNA structure and packaging.

Technique: thin smears were prepared on pre-cleaned defatted slides and then air-dried for 30 min. Dried smears are fixed with freshly made 96% ethanol-acetone (1:1) at 40 °C for 30 min to 12 h and air-dried. Hydrolysis is performed with 0.1 N HCl at 40C for 5 min followed by three changes of distilled water, 2 min each. Toluidine blue (0.05% in 50% Mcllvaine's citrate phosphate buffer at pH 3.5) is applied for 5 min. The slides are rinsed briefly in distilled water, dehydrated in tertiary butanol and xylene (both two times for 3 min) at room temperature, and mounted with DPX.

The results of the TB test are estimated using oil-immersion light microscopy. Sperm heads with good chromatin integrity stain light blue, and those with diminished integrity stain violet (purple) [53]. The proportion of cells with violet heads (high optical density) is calculated based on 200 sperm cells examined per sample. Based on the different optical densities of sperm cells stained by the TB, the image analysis cytometry test had been elaborated [54].

TB staining may be considered a fairly reliable method for assessing sperm chromatin. Abnormal nuclei (purple-violet sperm heads) have been shown to correlate with counts of red-orange sperm heads as revealed by the acridine orange test [38]. Also, correlations between the results of the TB test, sperm chromatin structure assay (SCSA), and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) tests have been demonstrated [53]. A threshold for proportion of sperm cells with abnormal sperm chromatin structure (violet staining) was set at 45%, providing 92% specificity and 42% sensitivity for infertility detection [55]. The

association between the TB test and the outcome of IVF/ICSI cycles has not been investigated.

TB staining is simple and inexpensive and has advantage of providing permanent preparations for use with an ordinary microscope. However, these methods may have the inherent limits of repeatability dictated by a limited number of cells, which can be reasonably scored.

It has been shown that sperm chromatin maturity as demonstrated both by AAB and TB tests is associated by zygote development following ICSI [56].

9.3.3 Chromomycin A3 Assay

Chromomycin A3 is a fluorochrome that specifically binds to guanine-cytosine DNS sequences. It reveals chromatin that is poorly packed in spermatozoa by visualization of protamine-deficient DNA. Chromomycin A3 and protamines compete for the same binding sites in the DNA. Therefore, high CMA3 fluorescence is an indicator of the low protamination state of the chromatin of spermatozoa [41].

Technique: for CMA3 staining, semen smears are first fixed in methanol-glacial acetic acid (3:1) at 40C for 20 min and are then allowed to air-dry at room temperature for 20 min. The slides are treated for 20 min with 100 μl of CMA3 solution that consists of 0.25 mg/mL CMA3 in Mcllvaine's buffer (pH 7.0) supplemented with 10 mmol/L $MgCl_2$. The slides are rinsed in buffer and mounted with 1:1 v/v PBS-glycerol. The slides are then kept at 40C overnight. Fluorescence is evaluated using a fluorescence microscopy. A total of 200 spermatozoa are randomly evaluated on each slide. CMA3 staining is evaluated by distinguishing spermatozoa that stain bright yellow (CMA3 positive) from those that stain dull yellow (CMA3 negative) [41].

CMA3 staining has demonstrated a sensitivity of 73% and specificity of 75% for the IVF success (>50% oocytes fertilized) [57]. It appears that semen samples with high CMA3 positivity (>30%) may have significantly lower fertilization rates if used for ICSI, and poor chromatin packaging can contribute to a failure in the decondensation process and reduced fertility [58, 59].

The CMA3 assay yields reliable results as it is strongly correlated with other assays used in the evaluation of sperm chromatin. The sensitivity and specificity of the CMA3 stain (75% and 82%, respectively) are comparable with those of the AAB stain (60% and 91%, respectively) if used to evaluate the chromatin status in infertile men.

It has been shown that the results of the AAB and TB tests (both detecting chromatin condensation and conformation) are well correlated [60, 61]. However, the results of the CMA3 test differ from these two in some specific biological conditions, for example, after induced sperm capacitation [61], providing another evidence not only for the complexity of the cytochemical background behind these assays as described above but also for the complexity of sperm chromatin biology. It also shows the complexity of sperm chromatin remodeling during sperm

functioning processes like sperm capacitation when the change of sperm chromatin conformation (or condensation) can be detected by assays like AAB and TB, but assays like CMA3 (competing with protamines) or sperm chromatin structure assay (SCSA, targeting the susceptibility of abnormal sperm chromatin for in situ denaturation) do not detect any change in sperm chromatin [61].

A relationship between poor sperm chromatin maturity and integrity is detected by means of all three methods (AAB, TB, and CMA3), and recurrent spontaneous pregnancy loss has been shown [62, 63]. Regarding the utility of these methods in context of the ART, there is still inconsistency regarding the published data whether sperm chromatin maturity and integrity as tested by these cytochemical methods are related to the outcome of the IVF and ICSI. For example, some studies have shown an association between CMA3 test and fertilization rates following ICSI, failing to demonstrate the same associations for the AAB test [59], while other studies are showing such a relationship between the AAB test and ICSI outcome [64]. Some studies do not find any association between the results of all three tests (AAB, TB, and CMA3) and the outcome of ICSI [65]. Therefore, unlike the good predictive power of these tests for in vivo fertility capacity of men, their utility in IVF/ICSI cycles is still debatable.

9.4 Conclusion

Cytochemical sperm chromatin assays described here (AAB, TB, and CMA3 assays) are simple, inexpensive to perform, and sensitive tests for the evaluation of sperm chromatin structure, although their cytochemical backgrounds and targeting tools in sperm chromatin are different. They are reliable methods for the more refined diagnosis of male in vivo fertility and are also shown to be predictive of in vitro fertilization processes, although the accumulation of more evidence for the relationship between the results of these methods and in vitro fertilization is needed. A very robust reproducibility of these assays might be their weak point because of the assessment of the limited numbers of sperm cells (usually 200–300) under the bright-field or fluorescence microscopy. On the other hand, the acquisition of the permanent preparations by the AAB and TB assays must be mentioned as the strength of these tests.

References

1. Oehninger S. Strategies for the infertile man. Semin Reprod Med. 2001;19:231–7.
2. Guzick DS, Overstreet JW, Factor-Litvak P, National Cooperative Reproductive Medicine Network, et al. Sperm morphology, motility, and concentration in fertile and infertile men. N Engl J Med. 2001;345:1388–93.
3. Lopes S, Jurisicova A, Sun JG, Casper RF. Reactive oxygen species: potential cause for DNA fragmentation in human spermatozoa. Hum Reprod. 1998;13:896–900.

4. Sakkas D, Tomlinson M. Assessment of sperm competence. Semin Reprod Med. 2000;18:133–9.
5. Hansen M, Kurinczuk JJ, Bower C, Webb S. The risk of major birth defects after intracytoplasmic sperm injection and in vitro fertilization. N Engl J Med. 2002;346:725–30.
6. Schieve LA, Meikle SF, Ferre C, Peterson HB, Jeng G, Wilcox LS. Low and very low birth weight in infants conceived with use of assisted reproductive technology. N Engl J Med. 2002;346:731–7.
7. Moll AC, Imhof SM, Cruysberg JR, Schouten-van Meeteren AY, Boers M, van Leeuwen FE. Incidence of retinoblastoma in children born after in-vitro fertilisation. Lancet. 2003;361:309–10.
8. Orstavik KH, Eiklid K, van der Hagen CB, Spetalen S, Kierulf K, Skjeldal O, Buiting K. Another case of imprinting defect in a girl with Angelman syndrome who was conceived by intracytoplasmic semen injection. Am J Hum Genet. 2003;72:218–9.
9. Aitken RJ, de Iuliis GN. Origins and consequences of DNA damage in male germ cells. Reprod BioMed Online. 2007;14:727–33.
10. Zini A, Sigman M. Are tests of sperm DNA damage clinically useful? Pros and cons. J Androl. 2009;30:219–29.
11. Zini A, Jamal W, Cowan L, Al-Hathal N. Is sperm DNA damage associated with IVF embryo quality? A systematic review. J Assist Reprod Genet. 2011;28:391–7.
12. Zini A. Are sperm chromatin and DNA defects relevant in the clinic? Syst Biol Reprod Med. 2011;57:78–85.
13. Simon L, Zini A, Dyachenko A, Ciampi A, Carrell DT. A systematic review and meta-analysis to determine the effect of sperm DNA damage on in vitro fertilization and intracytoplasmic sperm injection outcome. Asian J Androl. 2017;19(1):80–90.
14. Bellve AR, McKay DJ, Renaux BS, Dixon GH. Purification and characterization of mouse protamines P1 and P2. Amino acid sequence of P2. Biochemistry. 1988;27:2890–7.
15. Ward WS, Coffey DS. DNA packaging and organization in mammalian spermatozoa: comparison with somatic cells. Biol Reprod. 1991;44:569–74.
16. Fuentes-Mascorro G, Serrano H, Rosado A. Sperm chromatin. Arch Androl. 2000;45:215–25.
17. Gatewood JM, Cook GR, Balhorn R, Bradbury EM, Schmid CW. Sequence-specific packaging of DNA in human sperm chromatin. Science. 1987;236:962–4.
18. Bench GS, Friz AM, Corzett MH, Morse DH, Balhorn R. DNA and total protamine masses in individual sperm from fertile mammalian subjects. Cytometry. 1996;23:263–71.
19. Sakkas D, Mariethoz E, Manicardi G, Bizzaro D, Bianchi PG, Bianchi U. Origin of DNA damage in ejaculated human spermatozoa. Rev Reprod. 1999;4:31–7.
20. Irvine DS, Twigg JP, Gordon EL, Fulton N, Milne PA, Aitken RJ. DNA integrity in human spermatozoa: relationships with semen quality. J Androl. 2000;21:33–44.
21. Ward WS. Deoxyribonucleic acid loop domain tertiary structure in mammalian spermatozoa. Biol Reprod. 1993;48:1193–201.
22. Zalensky AO, Allen MJ, Kobayashi A, Zalenskaya IA, Balhorn R, Bradbury EM. Chromosoma. 1995;103:577–90.
23. Solov'eva L, Svetlova M, Bodinski D, Zalensky AO. Nature of telomere dimers and chromosome looping in human spermatozoa. Chromosom Res. 2004;12:817–23.
24. Ward WS, Zalensky AO. The unique, complex organization of the transcriptionally silent sperm chromatin. Crit Rev Eukaryot Gene Expr. 1996;6:139–47.
25. De Jonge CJ. Paternal contributions to embryogenesis. Reprod Med Rev. 2000;8:203–14.
26. Lewis JD, Song Y, de Jong ME, Bagha SM, Ausio J. A walk through vertebrate and invertebrate protamines. Chromosoma. 1999;111:473–82.
27. Corzett M, Mazrimas J, Balhorn R. Protamine 1 : protamine 2 stoichiometry in the sperm of eutherian mammals. Mol Reprod Dev. 2002;61:519–27.
28. Jager S. Sperm nuclear stability and male infertility. Arch Androl. 1990;25:253–9.
29. Darzynkiewicz Z. Acid-induced denaturation of DNA in situ as a probe of chromatin structure. Methods Cell Biol. 1994;41:527–41.

30. Erenpreisa EA, Zirne RA, Zaleskaia ND, S'iakste TG. Effect of single-stranded breaks on the ultrastructural organization and cytochemistry of the chromatin in tumor cells. Biull Eksp Biol Med. 1988;106:591–3. [Article in Russian].
31. Erenpreisa EA, Sondore OI, Zirne RA. Conformational changes in the chromatin of tumor cells and the phenomenon of nuclear achromasia. Eksp Onkol. 1988;10:54–7. [Article in Russian].
32. Sculthore HH. Metachromasia. Med Lab Sci. 1978;35:365–70.
33. Erenpreisa J, Zaleskaya N. Effect of triton X-100 on cytochemical and ultrastructural pattern of chromatin. Acta Morphol Hung. 1983;31:387–93.
34. Erenpreisa J, Freivalds T, Selivanova G. Influence of chromatin condensation on the absorption spectra of nuclei stained with toluidine blue. Acta Morphol Hung. 1992;40:3–10.
35. Brewer LR, Corzett M, Balhorn R. Protamine-induced condensation and decondensation of the same DNA molecule. Science. 1999;286:120–3.
36. Evenson D, Darzynkiewicz Z, Jost L, Janca F, Ballachey B. Changes in accessibility of DNA to various fluorochromes during spermatogenesis. Cytometry. 1986;7:45–53.
37. Benyajati C, Worcel A. Isolation, characterization, and structure of the folded interphase genome of Drosophila melanogaster. Cell. 1976;9:393–407.
38. Erenpreiss J, Bars J, Lipatnikova V, Erenpreisa J, Zalkalns J. Comparative study of cytochemical tests for sperm chromatin integrity. J Androl. 2001;22:45–53.
39. Auger J, Mesbah M, Huber C, Dadoune JP. Aniline blue staining as a marker of sperm chromatin defects associated with different semen characteristics discriminates between proven fertile and suspected infertile men. Int J Androl. 1990;13:452–62.
40. Terquem T, Dadoune JP. Aniline blue staining of human spermatozoa chromatin. Evaluation of nuclear maturation. In: Adre J, editor. The sperm cell. The Hague: Martinus Nijhoff Publishers; 1983. p. 249–52.
41. Manicardi GC, Bianchi PG, Pantano S, Azzoni P, Bizzaro D, Bianchi U, Sakkas D. Presence of endogenous nicks in DNA of ejaculated human spermatozoa and its relationship to chromomycin A3 accessibility. Biol Reprod. 1995;52:864–7.
42. Bianchi PG, Manicardi GC, Bizzaro D, Bianchi U, Sakkas D. Effect of deoxyribonucleic acid protamination on fluorochrome staining and in situ nick-translation of murine and human mature spermatozoa. Biol Reprod. 1993;49:1083–8.
43. Mello ML. Induced metachromasia in bull spermatozoa. Histochemistry. 1982;74:387–92.
44. Andreetta AM, Stockert JC, Barrera C. A simple method to detect sperm chromatin abnormalities: cytochemical mechanism and possible value in predicting semen quality in assisted reproductive procedures. Int J Androl. 1995;18(1):23–8.
45. Hammadeh ME, Zeginiadov T, Rosenbaum P, et al. Predictive value of sperm chromatin condensation (aniline blue staining) in the assessment of male fertility. Arch Androl. 2001;46:99–104.
46. Baker H, Liu D. Assessment of nuclear maturity. In: Acosta A, Kruger T, editors. Human spermatozoa in assisted reproduction. London: CRC Press; 1996. p. 193–203.
47. Foresta C, Zorzi M, Rossato M, Varotto A. Sperm nuclear instability and staining with aniline blue: abnormal persistence of histones in spermatozoa in infertile men. Int J Androl. 1992;15:330–7.
48. Hammadeh ME, Stieber M, Haidl G, Schmidt W. Association between sperm cell chromatin condensation, morphology based on strict criteria, and fertilization, cleavage and pregnancy rates in an IVF program. Andrologia. 1998;30:29–35.
49. Irez T, Sahmay S, Ocal P, Goymen A, Senol H, Erol N, Kaleli S, Guralp O. Investigation of the association between the outcomes of sperm chromatin condensation and decondensation tests, and assisted reproduction techniques. Andrologia. 2015;47:438–47.
50. Asmarinah, Syauqy A, Umar LA, Lestari SW, Mansyur E, Hestiantoro A, Paradowszka-Dogan A. Sperm chromatin maturity and integrity correlated to zygote development in ICSI program. Syst Biol Reprod Med. 2016;62:309–16.

51. Mohamed EE, Mohamed MA. Effect of sperm chromatin condensation on the outcome of intrauterine insemination in patients with male factor infertility. J Reprod Med. 2012;57:421–6.
52. Sato S, Matsumoto S, Freivalds T, Erenpreisa J. Consideration on the metachromatic spectra of toluidine blue dimers formed on DNA oligomers. Bull Chem Soc Jpn. 2010;83(10):1216–22.
53. Erenpreiss J, Jepson K, Giwercman A, Tsarev I, Erenpreisa J, Spano M. Toluidine blue cytometry test for sperm DNA conformation: comparison with the flow cytometric sperm chromatin structure and TUNEL assays. Hum Reprod. 2004;19:2277–82.
54. Erenpreisa J, Erenpreiss J, Freivalds T, Slaidina M, Krampe R, Butikova J, Ivanov A, Pjanova D. Toluidine blue test for sperm DNA integrity and elaboration of image cytometry algorithm. Cytometry. 2003;52:19–27.
55. Tsarev I, Bungum M, Giwercman A, Erenpreisa J, Ebessen T, Ernst E, Erenpreiss J. Evaluation of male fertility potential by toluidine blue test for sperm chromatin structure assessment. Hum Reprod. 2009;24:1569–74.
56. Esterhuizen AD, Franken DR, Lourens JG, Prinsloo E, van Rooyen LH. Sperm chromatin packaging as an indicator of in-vitro fertilization rates. Hum Reprod. 2000;15:657–61.
57. Sakkas D, Urner F, Bianchi PG, Bizzaro D, Wagner I, Jaquenoud N, Manicardi G, Campana A. Sperm chromatin anomalies can influence decondensation after intracytoplasmic sperm injection. Hum Reprod. 1996;11:837–43.
58. Sakkas D, Urner F, Bizzaro D, Manicardi G, Bianchi PG, Shoukir Y, Campana A. Sperm nuclear DNA damage and altered chromatin structure: effect on fertilization and embryo development. Hum Reprod. 1998;13(4):11–9.
59. Iranpour FG. Impact of sperm chromatin evaluation on fertilization rate in intracytoplasmic sperm injection. Adv Biomed Res. 2014;3:229.
60. Kim HS, Kang MJ, Kim SA, Oh SK, Kim H, Ku SY, Kim SH, Moon SY, Choi YM. The utility of sperm DNA damage assay toluidine blue and aniline blue staining in routine semen analysis. Clin Exp Reprod Med. 2013;40:23–8.
61. De Lamirande E, San Gabriel MC, Zini A. Human sperm chromatin undergoes physiological remodeling during in vitro capacitation and acrosome reaction. J Androl. 2012;33:1025–35.
62. Talebi AR, Vahidi S, Aflatoonian A, Ghasemi N, Ghasemzadeh J, Firoozabadi RD, Moein MR. Cytochemical evaluation of sperm chromatin and DNA integrity in couples with unexplained recurrent spontaneous abortions. Romanian J Morphol Embryol. 2011;52:645–51.
63. Kazerooni T, Asadi N, Jadid L, Kazerooni M, Ghanadi A, Ghaffarpasand F, Kazerooni Y, Zolghadr J. Evaluation of sperm's chromatin quality with acridine orange test, chromomycin A3 and aniline blue staining in couples with unexplained recurrent abortion. J Assist Reprod Genet. 2009;26:591–6.
64. Simon L, Liu L, Murphy K, Ge S, Hotaling J, Aston KI, Emery B, Carrell DT. Comparative analysis of three sperm DNA damage assays and sperm nuclear protein content in couples undergoing assisted reproduction treatment. Hum Reprod. 2014;29:904–17.
65. Sadeghi MR, Lakpour N, Heidari-Vala H, Hodjat M, Amirjannati N, Hossaini Jadda H, Binaafar S, Akhondi MM. Relationship between sperm chromatin status and ICSI outcome in men with obstructive azoospermia and unexplained infertile normozoospermia. Andrologia. 2012;44(1):462–70.