Review Article

# Cytochemical tests to investigate sperm DNA damage: Assessment and review

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## Abstract

Male factor infertility has been diagnosed as the cause of infertility in about 20% of infertile couples. Sperm analysis is the most common method for diagnosing infertility in a laboratory. However, approximately 15% of infertile men have a normal sperm analysis. Therefore, the result of a routine sperm analysis often cannot be a definitive diagnosis for male factor infertility. Also, approximately 8% of infertile men with normal sperm parameters have high levels of abnormal sperm DNA. This indicates the role of the integrity of sperm DNA in male infertility. Here, we review the current tests available to evaluate the sperm DNA integrity along with their benefits and limitations.

**Keywords:** Aniline blue, chromatin, chromomycin A3, sperm, toluidine blue, TUNEL

## Introduction

Today, it has been found that sperms with normal morphology and motility can have chromatin or DNA abnormalities.[1,2] Approximately, 15% of infertile men have a normal sperm analysis.[3] Therefore, definite diagnosis of male infertility is difficult with a standard routine semen analysis.[4] In the past decade, some studies were done in the assessing of the sperm DNA role in male infertility.[5,6] According to available evidence, the normality of sperm DNA is an important marker of normal sperm function and it was a good predictor to prognosis in male reproductive potential.[7] Approximately, 8% of infertile men with normal sperm parameters have high levels of abnormal sperm DNA,[3] and selection of these sperm in Assisted Reproduction Techniques (ART), can reduced the success rate of fertilization significantly.[8] Therefore, in andrology laboratory, it was necessary in addition to checking the routine sperm parameters, the health of sperm chromatin and DNA should be ensured. Several methods and techniques are invented to investigate the quality of chromatin and the possible occurrence of sperm DNA fragmentation. Although each of them has a special characteristic to demonstrate defect in structure of chromatin and incompetency in chromatin condensation, but in general, all of them can show sperm chromatin integrity.[9]

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## Methods of Investigation of Sperm DNA Damage

There are two different methods to measure DNA damage in the sperm: a) direct methods: methods that examine the DNA fracture in both single and double strand, such as terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and Comet assay[10]; b) indirect methods: methods that assesses the health of sperm chromatin by measuring the sensitivity of chromatin, and in particular sperm DNA, to denaturation under specific condition, such as the sperm chromatin structure assay (SCSA), sperm chromatin dispersion (SCD), and staining of nuclear proteins including aniline blue (AB), toluidine blue,



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acridine orange (AO), and chromomycin A3 (CMA3).[11] Several factors can affect chromatin staining, such as range of chromatin density, secondary structure, and total DNA binding proteins.[12] Also, molecular size, electrical load, concentration, PH, and staining methods are important.[13] The staining mechanism can be performed in both intercalary and external ways, in which, in the intercalary type, pigment molecules placed between the alkali regions of the DNA and do not enter in the DNA polymerization process. In the external mechanism, the pigment molecules are bound to DNA phosphate groups and can enter in the DNA polymerization process.[14]

## Direct methods

### *TUNEL assay*

This technique was introduced for the first time by Gorczyca *et al.* in 1993 and directly shows the level of fracture in one or two strands of DNA in mammalian sperm.[15] Of course, the tunnel technique was initially used for somatic cells and then used for sperm cells.[16] In this technique, labeled nucleotides can labeled fracture nucleotides in the end-of DNA strands. Through this process Deoxyuridine triphosphate (dUTP) modified by a terminal deoxynucleotidyl transferase called TDT, combined with biotin or dicoxygenin at the OH 3 'end of broken string. Then the modified nucleotides identify with a fluorescent antibody. To detect damaged sperm, can be used an optical microscope, fluorescence or flow cytometry.[17] In the fluorescent microscope method, sperms with normal DNA do not have fluorescent colorant, while sperm with a fragmented DNA have a bright fluorescent colorant. The increase in fluorescence observed indicates a fracture in the DNA strand and show more damage in DNA.[18] But in the enzymatic tunnel method, streptavidin-horseradish peroxidase (streptovidin-labeled) bonds to biotin nucleotides. This complex is characterized by a hydrogen peroxide substrate (H2O2) and a chromogen stain called diaminobenzidine, consequently sperm with fragmented DNA marked by dark brown color in the sperm head and can be detected by optical microscopy.[19] The TUNEL technique was first used to diagnose the population of apoptosis sperm in ejaculatory samples and also detects spermatozoa that have entered the apoptosis.[20] Studies had showed a significant negative correlation between TUNEL+ sperm cells and sperm parameters, fertilization rate, and embryo development.[21,22]

### *Comet assay*

The comet assay is widely used as one of the most sensitive tests for DNA damages.[23] This method was introduced to determine the DNA fragmentation of sperm cells in 1984 by Kalderon *et al.*[24] Comte assay depending on the type of DNA damage can be performed under neutral conditions (pH = 7.5) or alkaline (pH > 10).

Neutral comet assay is used to determine damage in double strand DNA (dsDNA) and is more sensitive to identify infertility-related DNA damage, while the alkaline comet method is used to detect each defect in single-strand DNA (ssDNA).[25] This method is useful because it recognizes different types of DNA fragmentation, such as necrosis and apoptosis.[26] For performing this test, after sperm cell lysis, uses detergents such as sodium dodecyl sulfate (SDS) or mercaptoethanol to move and detach DNA proteins, and then they are placed on agarose in an electric playing field.[27] During electrophoresis, fragmented DNA that are characterized by fluorescence colors are separated from the chromatin and move in the direction of the anode. Sperms with fragmented DNA have a longer tail in a comet form and look like a tear drop that is due to the migration and stretching of short DNA fragments. The staining intensity of the sperm tail indicates the fracture value in the sperm DNA that moves from the negative end to the positive end, and this amount of movement indicates different degrees of DNA fragmentation.[28] In 2015, Garolla *et al.* determined that the comet test was helpful in predicting the embryo quality after *In vitro* fertilization (IVF), especially in couples with unexplained infertility.[29] Increasing in DNA damage leads to failure in embryo development after intracytoplasmic sperm injection (ICSI).[30] In addition, Nasr-Esfahani *et al.* evaluated the level of fragmentation by the comet assay and showed DNA damage occurs in sperms that contain low protamine content.[31]

## Indirect methods

### *The SCSA*

This technique is a very sensitive method for assessing the degree of DNA damage that was first introduced by Evenson.[32] SCSA measures the sensitivity of DNA to denaturation factors, such as acid or heat, by applying a flow cytometry method along with staining of AO staining.[33] AO is a metachrome color that turns red when attached to a denatured DNA, but in the state of binding to the DNA with its natural structure, it turns green. Acidic pH or heat can cause DNA to be denatured. Then the chromatin of sperms that have been damaged is characterized by AO color and finally counted by flow cytometry. In this method, 5 to 10,000 spermatozoa are counted every second, and the percentage of errors is very low.[34] The SCSA method examines several parameters: The DNA fragmentation index (DFI), which expresses the rate of fracture detected in the DNA structure, and it is the most important parameter of SCSA. Another parameter is histone deacetylases (HDS), which indicates fractures in two strands and shows the distortion of histone displacement with protamine.[32] Collins *et al.* Reported that pregnancy can be predicted using the SCSA test so, when the DFI is above 30–28%, the pregnancy rate decreases.[35] In 2002, Evenson *et al.*, on the basis of fertility potential, categorized people in terms of the degree of DNA damage to four groups: High DFI with less than 15%, A good group with DFI is 24–15%, The average DFI range is 30–25% and the weak group, with DFI more than 30%. Also, when the HDS index is more than 15%, fertility potential in men is classified in one group only. They also showed that if DFI is more than 30%, apart from the sperm number, motility and morphology, male fertility will be disturbed.[36] Spanò *et al.* showed that if the DFI level is more than 20%, the pregnancy rate is severely affected, and if it

reaches 40%, fertility will reach zero.[37] Aravindan *et al.* showed that there is a significant relationship between SCSA, comet, and TUNEL tests for DNA damage.[38]

## SCD test

One of the easy, cost-effective and sensitive methods recently used to test the sperm DNA damage is SCD testing.[39] Considering the mechanism of more chromatin sperm aggravation than somatic cells, this test can be used to evaluate different levels of DNA fragmentation using a lysis buffer that breaks down the disulfide bands and exits proteins.[40] In this case, sperm with nonfragmented DNA, their DNA loops are released and a large halo around the core structure of the nucleus is formed. But spermatozoa with fragmented DNA have a very small halo or no halo. The size of the halo can be checked by light or fluorescent microscope based on the type of color (wright color or fluorescent).[40] The SCD test for predicting the amount of DNA fragmentation has a predictive value similar to those of TUNEL and SCSA.[22] Therefore, the SCD technique as a relatively simpler method can be a good alternative to the SCSA method. The results of a recent study show that there is a significant relationship between the amount of sperm DNA-fragmented determined by SCD, with the fertility rate and quality of the fetus. In this test, they proposed a threshold of 18% for the amount of sperm DNA fragmentation and as a predictive value for fertility.[41]

## AB staining

This technique was founded by Terquem and Dadoune. They showed that AB staining was acidic and specifically showed the existence of additional histones in the structure of sperm chromatin.[42] The histone protein contains a large number of lysine amino acids, which produce alkaline properties in this protein, while protamine is rich in amino acids of arginine and cytine.[43] Acidic AB reacts with lysine amino acids and under the light microscope, the sperm nucleus with high levels of histone is seen in blue. Sperms, whose chromatin becomes blue with this color, are defected during the transposition of histone by protamine in the spermyogenesis stage and, as a result, the nucleus structure will have additional histones. In other words, AB staining indirectly shows the presence of low levels of protamine in the sperm nucleus. These sperms are immature sperm with low chromatin density.[7] Recently, it has been shown that adding Eosin color as a contrast color to AB staining can increase the chance of detecting immature sperm with an additional histone and thus increase the sperm chromatin density estimation.[44] In 1988, Dadoune *et al.* showed that 20% of the normal spermatozoa in the fertile men are stained with AB.[45] The results of this test show a clear correlation between sperm chromatin abnormality and infertility then it can be a good predictor of IVF results. Although this test cannot be a good determinant of the fertility potential, cleavage and pregnancy rate after ICSI but is useful to detect the sperm chromatin defects in cases who are candidate for ICSI.[47] It has also been shown that AB staining has a positive correlation with the AO test.[48]

## Toluidine blue (TB) staining

TB is a basic nucleus color for metachromatic and orthochromatic staining for chromatin. Anomalous and low density chromatin, due to the availability of DNA phosphate groups, has a strong tendency to TB, and this color is linked to the DNA through an external mechanism. The more chromatin abnormalities, the more phosphate groups are available and the amount of DNA staining is increased. Therefore, a spectrum of bright blue (sperm with normal chromatin), dark blue (sperm with some abnormal chromatin), violet (sperm with abnormal chromatin), and finally purple (sperm with highly abnormal chromatin) can be detected.[49] TB staining is very simple, useful, and inexpensive to evaluate the quality of chromatin and DNA abnormalities. Long-term storage of lames and their examination by light microscopy is another advantage over AO staining. On the contrary, since sperm morphology can also be studied with TB color, this staining is preferable to colors such as AB, which is used only for the chromatin density assay.[50] Tsarev *et al.* showed that the threshold for TB testing to predict male fertility is 45%.[51] It has been reported that SCSA and TB are effective at measuring the maturity of sperm chromatin in fertile men, and their results are related. Erenpreiss *et al.* also observed a relationship between the level of sperm $TB^+$ and $TUNEL^+$.[52]

# Acridine Orange Test (AOT)

The AOT was first introduced by Tejada *et al.* in 1984.[53] In summary, it can be said that AO is a fluorescent metachloromatic color that is used to differentiate normal double-stranded DNA from denatured single-stranded DNA.[54] If the AO reacts with a double-stranded DNA molecule, it is seen in green under a fluorescent microscope with a wavelength of 460 nm and is visible in red or orange, if it reacts with single-stranded DNA. The moderate form is also seen in a bright yellow color.[55] If, in AO staining, the percentage of spermatozoa that turned fluorescent red is more than 56% (threshold), it indicates an abnormal chromatin state in the patient.[56] In another study, it was reported that the presence of more than 50% of the fluorescent red sperm could have a negative effect on fertility after the IVF cycle.[57] DNA denaturation can be induced by acidic environments or by heat. Intact DNA and optimal chromatin concentration in sperm will resistant to acid and heat and will not be denatured. The presence of di-sulfide bands in sperm chromatin prevents the degradation of this molecule by denaturing agents (such as acid and heat).[58] However, AO has a wide application, but for some reasons its use is limited in very precise cases:

1. The AO fluorescence decreases steadily and is reduced to a very small level after 48 hours.
2. The different regions of a slide are stained heterogeneously.
3. This defect, along with visual errors, has resulted in large amounts of false-positive and false-negative sperms.
4. Because, AO is absorbed by the lam glasses, and on the

other hand lames and plates are not completely flat, Small microscopic ponds are made up of color, called Microlake, in which the color concentration varies with other areas. This problem leads to the creation of artifacts in this test.[25]

In general, although, the AO test is not very efficient in very precise cases, it has still been used in many experiments, and it can be used to measure the DNA anomalies and sperm chromatin density.[59] Also, AO test is negatively correlated with the rate of sperm motility.[54]

## Chromomycin A3 (CMA3)

CMA3 is an antitumor agent that is derived from a bacterium called *Streptomyces cerevisiae*.[60] The CMA3 color contains two monomers, which are joined by magnesium ion ($Mg2^+$). The bonding of this color to the rich areas of guanine and cytosine breaks down hydrogen bonds between these two organic base and leads to wider and deeper the small grooves and squeezing the large gaps in DNA. CMA3 competes with protamine, for binding to small grooves DNA and guanine and cytosine-rich regions. And therefore indirectly shows the amount of protamine deficiency in the chromatin structure.[61] If an abnormality occurs during the histone–protamine displacement, or generally in the percentage of P1 or P2, this color can be used.[62] These abnormal spermatozoa are called $CMA3^+$ and they can be seen in bright yellow under the fluorescent microscope.[63] CMA3 can be a predictor of success in the IVF process.[64] Several studies have shown that there is a significant relationship between sperm protein protamine deficiency ($CMA3^+$) and fertilization rate after ICSI and IVF.[65] It has been shown that samples containing more than 30% sperm $CMA3^+$ have a direct relation to the low fertility rate in the ICSI programs.[66] It has also been reported that patients who fail to achieve a natural pregnancy show a high percentage of sperm $CMA3^+$.[67] Therefore, this technique can be a good tool for evaluating in male infertility and also a good predictor of fertility and pregnancy rates.

### Sperm chromatin decondensation assay

Sperm chromatin decondensation assay is performed by SDS. The formation of disulfide bonds between and within protamine molecules is performed to produce chromatin stability during sperm transport from epididymis.[68] SDS, as a detergent, can enter the nucleus after the destruction of the membrane of the sperm nucleus and, due to the presence of a polyanionic property, will compete with protamine to bind DNA, leading to protamine separation from DNA and its replacement. It also revitalizes the disulfide bands, and leads to the formation of free groups of thiol in chromatin, and the swelling of sperm nucleus. The rate of sperm nucleus swelling depends on the amount of S-S bands and noncovalent interactions between zinc sulfhydryl groups (HS. Zn.SH). However, as these bands and chromatin stability are lower, more bands are broken down by the SDS and the sperm heads produce more swelling. For this reason, SDS is also called nuclear chromatin decondensation testing. Depending on the amount of chromatin excretion, spermatozoa can be ranked.[69]

Talebi *et al.* developed a modified method for determining the stability of sperm chromatin. They used a combination of TB and SDS and then evaluated changes in both sperm heads and chromatin dye. Accordingly, TB is a metachromatic color that, after the reduction of sulfide bands in the chromatin by SDS and the availability of DNA, is linked to its phosphate group. Obviously, the lower the chromatin density, DNA staining increases by TB. Finally, by TB staining, a spectrum of colors, including bright blue, violet, and purple, is obtained. But since observing the swelling of sperm's head and determining its ranking is difficult, usage of TB staining along with it is far more accurate than measuring the sperm's head alone. They ranked the swelling of the sperm nucleus after staining TB in four different groups. Accordingly, sperm without swelling in the head and bright blue under the name S0 (sperm with natural chromatin), sperm with relatively swollen head and dark blue under the name S1 (sperm with relatively abnormal chromatin), sperm with swollen head and violet color, under the name S2 (sperm with abnormal chromatin), Finally, sperm with a very swollen head and a purple head called S3 (sperm with a very abnormal chromatin). The final scoring was done according to the following formula: Total score = $[S_0 \times 0] + [S_1 \times 1] + [S_2 \times 2] + [S_3 \times 3]$

It has been shown that, if in a sample of semen, in the presence of SDS with Ethylenediaminetetraacetic acid (EDTA), more than 30% of the sperm are healthy (don't have chromatin de-condensation) this sample has a high level of sperm, with a stable chromatin.[13]

## Conclusion

Finally, as it is known, sperm chromatin complementary tests were useful to examine any defect in sperm chromatin and can provide comprehensive information on the sperm DNA integrity that cannot be evaluated with standard and routine sperm analysis in the andrology laboratories Therefore, it is suggested that these tests should be used in the infertility laboratories to evaluate the quality of sperm chromatin.

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### Conflicts of interest

There are no conflicts of interest.

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