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A Review on Salient Methods of Sperm DNA Fragmentation

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Abstract

Based on a study of semen samples, the typical seminal profile has traditionally been used to diagnose male infertility. Generally, fragmentation of sperm DNA associated to infertility in males. It reveals that it should be considered when making male infertility evaluations. The utilisation of sperm DNA fragmentation (SDF) as a technique for assessing male potency is well recognised. The purpose of SDF test is to measure the cell's proportion with damage to the DNA, but it has not been determined which ones are most effective at predicting infertility. There aren't many articles that compare these techniques in-depth for the same demographic. The objective of this review is to analyse the various testing methods of acridine orange (AO) test, aniline blue (AB) staining, tunnel assay (TA), comet assay (CA), sperm chromatin structure assay (SCSA), sperm chromatin dispersion test (SCD), chromomycin in A3 (CMA3). The present study has reviewed various literatures from the year 1980 to 2019. The study uses survey methodology to analyse various testing methods. In order to choose candidates for varicocelectomy, SDF analysis is suggested for patients affected with the clinical varicocele and borderline to control semen values. The study concludes that continued advancements in DNA damage assessment techniques will contribute to improved understanding and applications in genotoxicology, environmental toxicology, and cancer research will contribute to improved understanding, diagnosis, as well as treatment of male infertility.
Keywords: sperm, testing methods, DNA damage techniques, diagnosis, infertility evaluation

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Обзор основных методов фрагментации ДНК спермы

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Резюме

Типичный профиль семенной жидкости, основанный на исследовании образцов спермы, традиционно используется для диагностики мужского бесплодия. Как правило, фрагментация ДНК сперматозоидов связана с бесплодием у мужчин. Использование фрагментации ДНК сперматозоидов в качестве метода оценки мужской потенции хорошо известно. Целью теста SDF является измерение доли клеток с повреждением ДНК, однако не установлено, какие из них наиболее эффективны для прогнозирования бесплодия. Существует не так много статей, в которых эти методы подробно сравниваются для одной и той же демографической группы. Целью этого обзора является анализ различных методов тестирования: тест акридинового оранжевого (AO), окрашивание анилиновым синим (AB), туннельный анализ (TA), кометный анализ (CA), анализ структуры хроматина сперматозоидов (SCSA), дисперсия хроматина сперматозоидов, тест (SCD) и хромомицин в A3 (CMA3). В настоящем исследовании были рассмотрены различные источники литературы за период с 1980 по 2019 г., а также используется методология опроса для анализа различных методов тестирования. В исследовании делается вывод о том, что дальнейшее развитие методов оценки повреждений ДНК будет способствовать улучшению понимания проблемы, а их применение в генотоксикологии, экологической токсикологии и исследованиях рака будет способствовать улучшению понимания, диагностики и лечения мужского бесплодия.
Ключевые слова: сперма, методы тестирования, методы повреждения ДНК, диагностика, оценка бесплодия

■ INTRODUCTION

DNA is made up of two long chains that are looped around one another and are built of sugars and phosphates on either end. DNA is the genetic material in people and other living things. The information in DNA is encoded by four different types of molecules known as bases, which are connected to the DNA strands like rungs on a ladder. Despite the fact that all living things share the same basic components, the information needed to create and maintain an organism depends on how those components are arranged. Sperm

DNA fragmentation happens when a nucleotide mutation or a physical break occurs in one or both of the chromosomes present in the sperm. Numerous techniques have been deployed for assessing the integrity of DNA in sperms (NJCA). Semen analysis forms the basic, noninvasive investigation for male infertility (NJCA). Conventional semen analysis has a restricted ability to identify male fertility potential as well as the success of assisted reproductive technology (ART) [12]. New indicators are required, and it is becoming clearer how crucial sperm DNA integrity is to human reproduction. Tests for sperm DNA fragmentation (SDF) provide a chance to look into the significant genetic material that is handed down to next generations (Evenson, Darzynkiewicz and Melamed). Semen analysis findings shows the sperm quality in a DNA package carrier. SDF tests are carried out additionally which is similar to the traditional semen characteristics. The findings of SDF tests partially reflect sperm quality [15].

Alternatively, the ability of sperm with substantial DNA fragmentation to have regular motility as well as morphology raises the possibility that the evaluation has extra predictive value. It has been demonstrated conclusively that male infertility is related with greater levels of DNA strand breaks and other DNA abnormalities [28]. About half of all occurrences of infertility are caused by male causes [26]. A traditional semen analysis (SA), which is used for the male patient's initial evaluation, can fall short of fully illuminating his reproductive potential. It is challenging for utilising SA to make management choices because of variability in sperm quantity and quality [4, 5]. Sperm function tests can be used to determine if sperm are capable of carrying out complicated tasks such as transport of sperm via the reproductive system of female, acrosome response, and penetration in zona pellucida [20]. Men associated with aberrant semen parameters [13] as well as normozoospermic partners of infertile couples [24] are also shown to have greater levels of SDF. Recent research has established the importance of SDF. Additionally, it reveals SDF as a self-regulating evaluation of semen quality comparing to the traditional analysis of semen in the evaluation of male infertility [9].

Sperm DNA integrity has been linked negatively to fertility in both in vitro and in vivo investigations [18]. Fertility may be impacted by increased SDF, which may impede fertilisation, earlier development of embryo, implantation, and pregnancy [16]. While SDF is more often present in urologists' toolkits utilised for the assessment of impotent men, its precise clinical implications are still not well known. Although the significance of DNA fragmentation in spermatozoa has been recognised in the most recent guidelines of American Urological Association (AUA) and European Association of Urology (EAU) in the field of male infertility, there does not appear to be enough evidence for the general utilisation of SDF in male factor analysis at this time [8]. Studies establishing particular indications for DNA testing are increasingly appearing [25], despite the fact that a specific knowledge about the efficacy of test in various therapeutic contexts is still missing.

The present study reviews on various testing methods of acridine orange (AO) test, aniline blue (AB) staining, tunnel assay (TA), comet assay (CA), sperm chromatin structure assay (SCSA), sperm chromatin dispersion test (SCD), chromomycin in A3 (CMA3). The purpose of the review paper is to deliver an overview of the principles, advantages, limitations, as well as applications of the CMA3 method in DNA research.

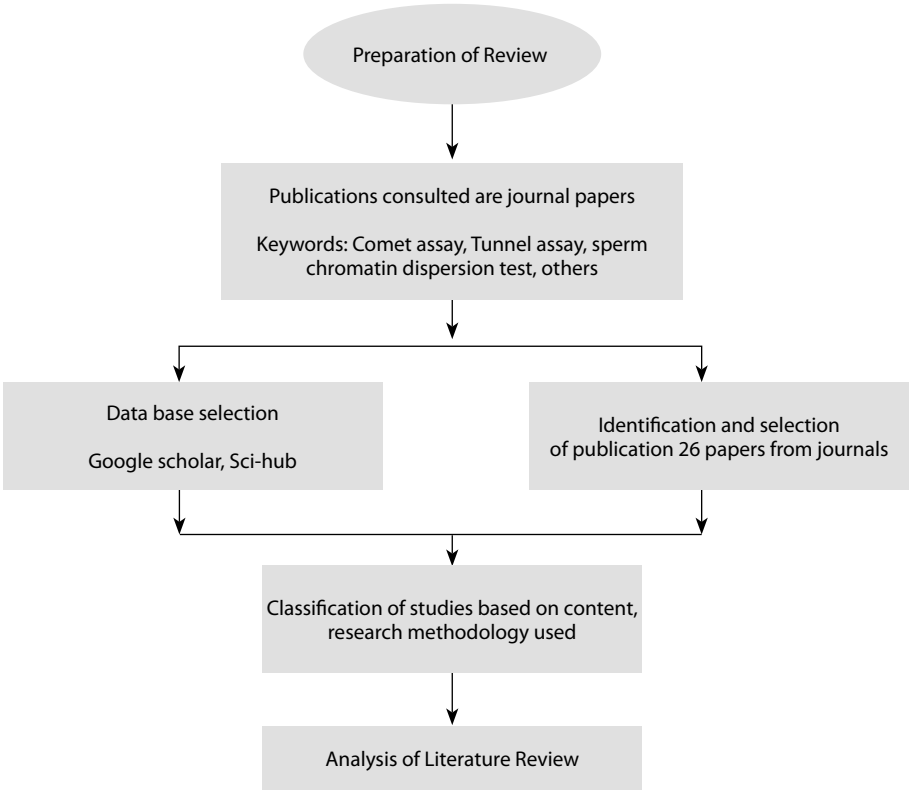
This review paper will help shed light into different testing techniques that will help identify the best possible choice for SDF testing. The upcoming sections present a comparative analysis of different testing methods.

■ SURVEY METHODOLOGY

The use of Google Scholar generated the survey methodology. The terms applied for searching keywords include "Sperm DNA Fragmentation", "acridine orange (AO) test", "aniline blue (AB) staining", "tunnel assay", "comet assay", "sperm chromatin dispersion test", "sperm chromatin structure assay", and "chromomycin in A3". The suitable and related publications were selected from the papers published between 1980 and 2019. Initially, the title-related papers were selected. Further, abstract screening was performed, and while suitable, the overall text was also considered. Additionally, citations of the paper were evaluated and comprised as required, with total refined studies of 26 corresponding to the concept. Figure denotes the survey methodology used for the study.

■ ACRIDINE ORANGE (AO) TEST AND ANILINE BLUE (AB) STAINING METHODS

AO test and the AB method are two widely employed techniques for determining cellular viability. This review provides an in-depth analysis of these methods, focusing on their principles, advantages, and limitations.



Survey methodology

Acridine Orange (AO) Test

AO test is associated with the principle of nucleic acid staining, where AO permeates the cell membrane and stains both living and dead cells. The viable cells exhibit green fluorescence due to the intercalation of AO with DNA, while the non-viable cells appear orange or red due to the exclusion of AO by compromised membranes. The AO test offers several advantages, including simplicity, speed, and compatibility with various cell types. Moreover, its ability to distinguish live, apoptotic, and necrotic cells makes it a versatile tool in cell biology and toxicology studies. However, the AO test has limitations, such as the requirement for fluorescence microscopy or flow cytometry equipment and the potential interference from extracellular AO staining [11].

Aniline Blue (AB) Method

The AB method is based on the principle of selective staining of dead or damaged cells. AB, a cationic dye, preferentially binds to the negatively charged components of dead or injured cells. The staining results in blue or purple coloration of non-viable cells, while viable cells remain unstained or appear pale. This method offers simplicity, cost-effectiveness, and compatibility with various sample types, including tissue sections and plant cells. However, the Aniline Blue method may lack specificity in certain cases and requires careful interpretation of staining results [2].

Comparative Analysis

In a comparative analysis, both the AO test and the AB method demonstrate efficacy in determining cellular viability. However, the choice between these methods depends on specific experimental requirements, sample types, and available resources. The AO test provides detailed information about different cell populations, including live, apoptotic, and necrotic cells, while the AB method primarily distinguishes viable from non-viable cells. Furthermore, the AO test requires fluorescence microscopy or flow cytometry equipment, whereas the AB method can be assessed using standard light microscopy. Researchers must consider these factors when selecting an appropriate technique for their studies. The main disadvantage in the AO and AB methods are Variations between laboratories and a lack of reliability [23].

Table 1
Acridine Orange (AO) Test

Parameters	Results
Diagnostic Significance	Permit a rapid, accurate diagnosis of T. vaginalis infection.
Clinical Correlation	Sensitive to pH levels, do not differentiate between single-stranded DNA and RNA, using the stain alone.
Wavelength	502 nm to 525 nm

Table 2
Aniline Blue (AB) Method

Parameters	Results
Diagnostic Significance	Tracing pathological and autolytic changes in lysosomes
Clinical Correlation	Negative correlation with sperm count, sperm motility and vitality
Wavelength	500 nm to 506 nm

■ TUNNEL ASSAY (TA) AND COMET ASSAY (CA) METHOD

DNA damage is a critical event that can lead to genomic instability, cell death, and the development of various diseases. The TA and CA are widely employed methods for evaluating DNA damage.

Tunnel Assay (TA)

TA is associated with the detection of breaks in DNA strand using terminal deoxynucleotidyl transferase (TdT) enzyme. It catalyses the addition of labelled nucleotides to the 3′ ends of DNA fragments. The assay's main steps are cell cultivation and harvesting, cell fixing and permeabilization to allow reaction reagents of TUNEL to enter the nucleus, association of labelled dUTPs to the -OH moieties of broken DNA using TdT, and visualisation of the labelled dUTPs. The visualisation will be either fluorescent or enzymatic depending on the label. The incorporated labelled nucleotides can be visualized using fluorescence microscopy or by means of flow cytometry. The TA offers advantages such as high sensitivity, ability to detect apoptotic DNA fragmentation, and compatibility with various sample types. However, it has limitations, including the requirement for specialized equipment, potential interference from endogenous DNA damage repair processes, and limited quantification capabilities [3, 19].

Comet Assay (CA)

The CA detects damage in DNA by analysing the migration of fragmented DNA present in an electrophoresis gel. The steps in the comet assay process are 1) cell fixation on a microscope slide, 2) cell lysis as well as 3) agarose gel electrophoresis under direct electric current. The DNA is then dyed and shown in step 4). If there are breaks in the DNA strand, the comet will be visible on the gel. Cells embedded in agarose are subjected to electrophoresis, causing the fragmented DNA to migrate and form a comet-like structure. The degree of DNA migration reflects the level of DNA impairment. The CA provides advantages such as simplicity, cost-effectiveness, and the ability to assess individual cell damage. Moreover, it allows for quantitative analysis of DNA damage by measuring parameters such as tail length and tail intensity. However, the CA has limitations, including potential variability in comet measurements and challenges in assessing specific types of DNA damage [3, 10].

Table 3
Tunnel Assay (TA)

Parameters	Results
Diagnostic Significance	Detects DNA breakage by labeling the free 3'-hydroxyl termini
Clinical Correlation	Proliferating cells with increased rates of DNA repair may exhibit false TUNEL positivity
Wavelength	493 nm to 593 nm

Table 4
Comet Assay (CA)

Parameters	Results
Diagnostic Significance	A valuable tool in measuring the extent of DNA damage
Clinical Correlation	Not predictive of irinotecan toxicities and thus do not have the potential to personalize the dose administered.
Wavelength	450 nm – 490 nm

Comparative Analysis

Both the TA and CA methods are effective for assessing DNA damage, but their choice depends on specific experimental requirements and objectives. The TA is particularly useful for detecting apoptotic DNA fragmentation and is compatible with a wide range of sample types. On the other hand, the CA provides quantitative information about DNA damage and enables the assessment of individual cell damage. Researchers must consider the nature of DNA damage they intend to study, the available resources, and the desired level of quantitative analysis when selecting an appropriate technique.

■ SPERM CHROMATIN DISPERSION TEST (SCD) AND SPERM CHROMATIN STRUCTURE ASSAY (SCSA)

Assessment of sperm DNA integrity is crucial in understanding male fertility potential and diagnosing infertility. The SCD test and SCSA are widely used techniques for assessing sperm DNA integrity.

SCD

The SCD test assesses the vulnerability of sperm DNA in the process of acid-induced denaturation. In this method, sperm cells are treated with acid, leading to DNA denaturation in damaged sperm. The undamaged DNA remains protected and forms a halo-like dispersion around the nucleus when stained with a DNA-specific dye. The SCD test offers advantages such as simplicity, speed, and compatibility with a variety of sample types. It provides qualitative information about sperm DNA integrity, distinguishing between intact and damaged DNA (Evenson, Larson and Jost).

SCSA

SCSA evaluates the vulnerability of sperm DNA to acid-induced denaturation and subsequent staining with a DNA dye, such as acridine orange. In this technique, sperm cells are treated with acid, followed by discolouration with acridine orange. The damaged DNA exhibits greater susceptibility to acid-induced denaturation and fluoresces red, beside the intact DNA fluoresces green. The red to green fluorescence ratio provides a quantitative measure of sperm DNA fragmentation. The SCSA offers advantages such as quantitative assessment, sensitivity to subtle DNA damage, and compatibility with various sample types. However, it requires specialized equipment for flow cytometry analysis and may be influenced by inter-laboratory variability [14].

Comparative Analysis

Both the SCD and SCSA are valuable approaches for evaluating integrity of sperm DNA. The SCD test provides a simple and qualitative assessment of DNA damage, distinguishing

Table 5
SCD test

Parameters	Results
Diagnostic Significance	Critical function of sperm DNA integrity for healthy embryonic development and successful reproductive outcome
Clinical Correlation	Sperm with fragmented DNA fail to produce the characteristic halo of dispersed DNA loop
Wavelength	488 nm – 532 nm

Table 6
SCSA

Parameters	Results
Diagnostic Significance	Distinguishing normal sperm from those with fragmentation in their DNA
Clinical Correlation	Highly correlated with the heterospermic performance of semen
Wavelength	450 nm -490 nm

between intact and damaged DNA. Alternatively, the SCSA offers a quantitative measure of DNA fragmentation, allowing for more precise evaluation of sperm DNA integrity. Researchers should consider their specific requirements, available resources, and the desired level of quantitative analysis when selecting an appropriate method.

■ CHROMOMYCIN IN A3

The Chromomycin A3 (CMA3) method is a widely used technique for evaluating DNA structure and composition, particularly the detection of GC-rich regions and chromatin condensation. The CMA3 method relies on the selective binding of Chromomycin A3, a fluorescent dye, to regions rich in guanine and cytosine (GC-rich regions) within DNA molecules. Upon binding, CMA3 undergoes a spectral shift, emitting fluorescence signals that can be visualized as well as quantified using fluorescence microscopy or flow cytometry. This method provides valuable information about the distribution, organization, and condensation of GC-rich regions within DNA.

There are various benefits in CMA3 method and it is significant to specifically target and stain GC-rich regions, which are related with important functional elements like gene promoters and telomeres. By selectively labelling these regions, researchers can gain insights into DNA structure, chromatin organization, and gene expression regulation. Additionally, the CMA3 method is relatively simple to perform and can be easily integrated with other techniques for comprehensive DNA analysis. However, the CMA3 method does have some limitations. It primarily targets GC-rich regions, which may limit its applicability in studying DNA regions with lower GC content. Furthermore, the interpretation of CMA3 staining patterns requires careful consideration as fluorescence signals can be influenced by factors such as DNA conformation, chromatin compaction, and DNA-protein interactions. Therefore, the CMA3 method should be utilised in aggregation with complementary systems to achieve a broad understanding of DNA structure and function [1].

■ DISCUSSION

The present study does a comparative analysis of various DNA integrity assessment techniques highlights the diverse range of methods available for studying DNA structure

Table 7
Chromomycin in A3

Parameters	Results
Diagnostic Significance	An antibiotic that binds to the GC-rich sequence of DNA in the presence of divalent cations, inhibiting DNA replication and transcription
Clinical Correlation	Potently inhibit DNA binding, inhibit apoptosis
Wavelength	445 nm – 575 nm

and composition. Each technique offers unique advantages and limitations, making them suitable for different experimental contexts and research objectives. Male infertility is mostly caused by damage to sperm DNA, which has an adverse impact on how well couples' pregnancies perform. The possible utilisation of SDF analysis in some clinical circumstances is suggested by recent clinical practise recommendations. This would increase the sperm DNA fragmentation assay's potential to be used internationally as a predictive and an analytical tool in a range of situations including male infertility and their management [8].

According to WHO recommendations, the main approach for determining men's fertility is semen analysis by checking traditional criteria. It is evident that regular semen analysis only sometimes explains the root of male infertility and can only offer a limited prognosis of a man's reproductive potential. In reality, conventional studies of semen quality frequently miss sperm DNA abnormalities. This circumstance is a foremost cause of male impotency. The association among semen parameters as well as the sperm DNA fragmentation index (DFI) is still debatable and uncertain.

While the study [17] found a strong link among DFI as well as human sperm parameters, other research were unable to do so [2]. Even though, there are numerous methods for doing that test at the moment, the SCD analysis appears to be a common and accessible test. SCD testing requires minimal equipment and is not just easy and affordable. Additionally, it has been claimed that the SCD test has a strong connection with other tests like TA, AO, or SCSA [27]. Except for the SCD test, expensive equipment is needed for the majority of the fragment DNA sperm testing. Using traditional microscopes, the distinction between fragmented and non-fragmented sperm was made. SCD test is referred to as the method where fragmented sperm are unable to generate the distinctive halo of scattered DNA loops which are observed in sperm with non-fragmented DNA. It is a fairly straightforward, efficient, and precise method of determining SDF [6].

While the AO test provides detailed information on live, apoptotic, and necrotic cell populations, the Aniline Blue method primarily distinguishes viable from non-viable cells. The TA and CA provide valuable tools for assessing DNA damage. While the TA is suitable for detecting apoptotic DNA fragmentation and offers compatibility with various sample types, the CA allows for quantitative analysis and assessment of individual cell damage. Additionally, the TA method was less sensitive to the examination of fragmented sperm DNA than the SCD test method [27]. It requires specialized equipment and may be affected by endogenous repair processes, limiting its quantitative capabilities. The CMA3 method targets GC-rich regions within DNA, providing insights into DNA structure and chromatin condensation. It has wide-ranging applications in studying chromatin organization, gene expression regulation, and DNA integrity. However, its applicability is limited to GC-rich regions, and interpretation requires careful consideration of various factors.

■ CONCLUSION

Continued advancements in DNA damage assessment techniques will contribute to improved understanding and applications in genotoxicology, environmental toxicology, and cancer research will contribute to improved understanding, diagnosis, as well as treatment of male infertility. Researchers should carefully evaluate the specific requirements of their experiments and choose the most suitable technique accordingly. Continued advancements in viability assessment techniques will undoubtedly contribute

to improved understanding and applications in cell biology, toxicology, and medical diagnostics. Even though each method has its pros and cons and it depends on specific use-case that the choice on method depends on, looking at the overall picture of various techniques discussed here, the SCD method seems to be the most advantageous due to various reasons of ease of use, cost and other aspects discussed as part of this review paper. Therefore, the comparative study of the SDF techniques have been discussed here. The study limits in analysing the side effects of the methods, which has an effect on birth defect, miscarriage and so on.

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