Review Article

Sperm DNA fragmentation and reproductive outcomes

Bindu Bajaj[1], Garima Kapoor[2]

[1]Department of Obstetrics and Gynaecology, Safdarjung Hospital and VMMC, New Delhi, India
[2]Paschim Vihar, New Delhi, India

Abstract

In the last few years, male infertility is increasingly catching the attention of researchers. The impact of spermatozoal factors on the fertilization and early embryo development is being deciphered. An association of sperm aneuploidy status in unexplained recurrent pregnancy loss has been observed. Sperm DNA damage or the sperm DNA fragmentation (sDF) is now measurable as DNA fragmentation index. The methods of evaluating sDF are many and they differ in their technique and paraphernalia needed to perform them. The most common methods are SCSA, TUNEL, SCD, COMET. TUNEL utilizes tagging of DNA in places where there is a break and then quantifying them. SCD is based on the principal of susceptibility of fragmented DNA to acid denaturation. COMET assay involves variable movement of intact and fragmented DNA under the influence of electric field. There are different methods to perform each of the previous mentioned tests though the principle remains the same. Each laboratory performing these tests needs to standardize them and should have their own cutoffs, which should relate to the procedure [intrauterine insemination/*in vitro* fertilization (IVF)/intracytoplasmic sperm injection] being performed. The role of sDF is slowly getting defined in recurrent implantation failures, unexplained infertility, varicocele, and IVF failures. Yet the jury is still out.

Keywords: Assisted reproductive technology, DNA fragmentation index, intracytoplasmic sperm injection, intrauterine insemination, *in vitro* fertilization, sperm DNA

Address for correspondence: Bindu Bajaj, Department of Obstetrics and Gynaecology, Safdarjung Hospital and VMMC, New Delhi, India.
E-mail: bindubajaj15@gmail.com
Submission: 05–12–2021, **Revised:** 24–02–2022, **Accepted:** 31–03–2022, **Published:** 29–June–2022

It is well established now that for a couple with infertility, male factor is contributory in 50%. Male factor infertility refers to the inability of the male to cause pregnancy in a clinically healthy female. Semen analysis is routinely employed to evaluate the male factor.[1] However, semen analysis shows intra- and interassay variability apart from intraindividual variability. It is thus imperative to look for methods that are more objective and also are more predictive of fertility potential of the sperms. One of the key determinants of embryo quality is the quality of the oocyte and sperm that have created the embryo. Several studies have explored the effect of oocyte parameters on embryo quality, but the effects of sperm quality on the embryo have not been comprehensively evaluated. Male fertility can be affected by several factors that may be congenital, endocrinal, immunologic, oncologic, infectious, or lifestyle related.[2,3] It can be classified as nonidiopathic, that is, where cause of male infertility is known as in cryptorchidism, varicocele, hormonal imbalances, and chemotherapy or idiopathic, that is, cause not known. Idiopathic male infertility when further probed unravels chromosomal microdeletions to be responsible. All these genetic defects may interfere

Access this article online

Quick Response Code:

Website: www.fertilityscienceresearch.org

DOI: 10.4103/fsr.fsr_49_21

How to cite this article: Bajaj B, Kapoor G. Sperm DNA fragmentation and reproductive outcomes Sperm DNA fragmentation and reproductive outcomes. Fertil Sci Res 2022;9:10-5.

with the development of the male reproductive system and urogenital tract, arrest germ-cell production and maturation, and lead to the production of nonfunctional spermatozoa. In recent times, a gray area of sperm biology includes that of sperm DNA damage and epigenetic or methylation anomalies which are believed to be potential candidates responsible for infertility. The sperm contributes only its nuclear material and centrosome to the zygote as most of its cytoplasm and other organelles are lost during spermiogenesis, maturation, and fertilization. Oocyte cytoplasm provides the first signal to control gene expression from male genome. Studies on association of the paternal genome to embryonic development show significantly lower cleavage rates and blastocyst formation rates when frozen or morphologically abnormal sperm were used to fertilize oocytes in *in vitro* fertilization (IVF) and lower blastocyst formation rates after intracytoplasmic sperm injection (ICSI), impact of spermatozoa factors on early embryo development is only still being deciphered.

At present, several sperm selection techniques such as density gradient centrifugation and swim-up are routinely used in most ART clinics with the aim of selecting the best sperm for fertilization. Unfortunately, not many of these techniques target the assessment of important sperm characteristics such as apoptosis, DNA integrity, membrane maturation, and ultrastructure, all of which contribute significantly toward improved embryo quality. Improving ART outcome by isolating mature, structurally intact, and nonapoptotic spermatozoa with high DNA integrity remains an ongoing challenge with several methods based on surface charge (electrophoresis and zeta potential), apoptosis (magnetic cell sorting and glass wool), ultramorphology (high magnification), or membrane maturity (hyaluronic acid binding) being investigated.

Abnormalities in the sperm nucleus can be broadly classified into sperm chromosomal abnormalities (aneuploidies) and sperm DNA abnormalities such as abnormal packing, DNA integrity, or DNA fragmentation.

As the semen consists of a concentrated suspension of spermatozoa, stored in the epididymis, and, at the time of ejaculation, diluted with the secretions of the accessory glands of the genital tract (mostly prostate and seminal vesicles), some parameters can reflect the sperm capacity of the testicle, the patency of the ejaculatory ducts (the total number of spermatozoa), and the secretory capacity of the accessory glands (the total fluid volume). The testicular sperm DNA damage is much less than that in the epididymis and in the ejaculated sample.[4] Integrity of sperm DNA is increasingly being recognized as a parameter of semen quality and an important fertility predictor. Sperm DNA damage is defined as any chemical change in the normal structure of DNA. Among the damage, it is single strand- or double-strand break in the genetic material which is the most common sperm DNA fragmentation (sDF). This may occur during spermatogenesis or later due to pathologic and environmental conditions. sDF does not affect the fertilization capacity of the sperm but it affects the embryo development, implantation, and pregnancy in both natural and assisted reproduction.[5,6]

SPERM DNA TESTING[7-9]

(1) Neat semen sample should be used for sDF testing.
(2) A fixed ejaculatory abstinence before collection of semen sample should be applied.
(3) A standardized protocol with stringent quality control is essential for a reliable sDF testing result.
(4) sDF threshold reflects the probability on reproductive outcome.

INDICATIONS FOR SDF TESTING[7-10]

(1) Varicocele
(2) Unexplained infertility
(3) Recurrent intrauterine insemination (IUI) failure
(4) Recurrent pregnancy loss
(5) IVF and ICSI failure

1. **Varicocele:** sDF testing is recommended in patients with grade 2/3 varicocele with normal conventional semen parameters.

2. **Unexplained infertility:** High sDF is found in men with normal semen parameters in couple with unexplained infertility.

3. **Recurrent IUI failure:** High sDF is associated with lower IUI pregnancy rates.

4. **Recurrent pregnancy loss:** sDF testing should be offered to infertile couples with RPL.

5. **IVF and ICSI failure:** sDF modestly affects IVF pregnancy rates; sDF does not affect ICSI pregnancy rates.

The use of testicular sperm rather than ejaculated sperm may be beneficial in men with oligozoospermia, high sDF, and recurrent IVF failure.

6. **Life style risk factors:** sDF testing should be offered to patients who have a modifiable lifestyle risk factor of male infertility.

Assays that can be used to evaluate sDF vary greatly in both the method and the type of damage they are detecting. Terminal deoxynucleotidyl transferase (dUTP) nick end labeling (TUNEL) and single-cell gel electrophoresis (COMET) assays directly assess the presence of single- and/or double-strand breaks in the DNA, whereas acridine orange flow cytometry and sperm chromatin dispersion (SCD) test assays detect the susceptibility of chromatin to treatment by acid which indirectly reflects sperm DNA damage.

sDF test comes under the extended examination category in WHO manual 2021. The diagnostic thresholds (also called cutoff values) of the methods used to calculate sDF is specific to each assay and method by which it is performed. For clinical use, the appropriate thresholds should be determined and validated by the performing laboratory.

1. TUNEL

The principle of TUNEL is to label the breaks present in DNA with deoxynucleotides (usually deoxyuridine triphosphate, dUTP). The dUTP is directly conjugated to a fluorescent dye or tagged by biotin, the method is capable of directly assessing both single and double strand breaks, thus the more DNA strand break sites present, the more label is incorporated within a cell. After labeling, the percentage of fluorescent spermatozoa can be determined by fluorescent microscopy or a flow cytometer. In the case of biotin-tagged probes, streptavidin-horseradish peroxidase (HRP) and a chromogenic HRP substrate are necessary to reveal marked spermatozoa. TUNEL can be performed in multiple ways. Depending on the protocol used, different laboratories have reported differing limits that have been used to discriminate between a healthy semen sample and those correlated with male infertility. Therefore, every laboratory should establish its own reference range using appropriate controls, based on positive and negative predictive values, and be clear what the predictive value relates to (e.g., conception, miscarriage, or other phenomena).

2. SCD TEST OR HALO TEST

The SCD test is a light microscopy method to evaluate the susceptibility of sperm DNA to acid denaturation. SCD is based on the principle that intact DNA loops expand following denaturation and extraction of nuclear proteins, whereas when DNA is fragmented, dispersion does not develop, or is minimal. This method relies on the capacity of the intact sperm chromatin to form dispersion halos, after being exposed to acid and a lysing solution; the halos correspond to relaxed DNA loops attached to the residual nuclear structure, which are released after the removal of nuclear proteins. The DNA breaks, as they are susceptible to denaturation, prevent this dispersion. The halos of the spermatozoa in the samples can be classified according to the criteria of Fernández *et al.*[11]

(a) Large: Halo width is similar to or larger than the minor diameter of the core.
(b) Medium: Halo size is between those with large and with small halo.
(c) Small: Halo width is similar to or smaller than one third of the minor diameter of the core.
(d) Without halo
(e) Without Halo Degraded: Those that show no halo and present a core irregularly or weakly stained. This Halo Degraded category is associated with severe damage affecting both DNA and protein compound. The results should be represented as a percentage of each category. The percentage of spermatozoa with fragmented DNA is the sum of those with small halo, without halo and without halo degraded.

Sperm without fragmented DNA = Sperms with large halo + Sperms with medium halo

$$\text{sDF\%} = \frac{100 \times \text{Number of sperm with fragmented DNA}}{\text{Number of sperm counted}}$$

Many commercial kits using SCD technology are available at present. They are easy to use and only need routine andrology laboratory microscope.

3. COMET ASSAY

The COMET assay is a method to evaluate sDF in individual sperm based on the differential migration of broken DNA strands under the influence of an electric field depending on the charge and size of the strands. The name of the test is related to the "COMET" appearance under fluorescence microscope of the stained unwound DNA fragments that are detached from the sperm head

after electrophoretic movement. The intact DNA constitutes the COMET's head, whereas the fragmented strains of DNA constitute the COMET's tail. COMETs with no heads should be considered as sperm containing 100% DNA damage COMET assay involves multiple methodologic steps, demands a high level of expertise for interpretation of the results, and has an important level of interlaboratory variation, its use may not be appropriate for some laboratories.

The physiologic temperature for sperm in the female reproductive tract is supposedly around 36°C, so if sDF is examined during and after a period of incubation at this temperature, one is likely to detect individuals who are more prone to DNA damage than others. In fact, under incubation conditions that mimic the temperature in the female reproductive tract, it may be possible to show a rapid increase in SDF within the first 2 hours of incubation. In some individuals, this increase is estimated to occur as high as 8% per hour, and in some cases, it may exceed 80% after 24 hours of incubation.[2] From a biologic standpoint, this indicates that if the semen sample is used for IVF or ICSI, the level of DNA fragmentation of sperm inoculated or coincubated with the oocyte may be greater at the time of fertilization than that observed in the initial assessment. In routine IVF, oocytes are exposed to sperm overnight with a maximum of 20 hours, and in some cases, this extended period of coincubation has been shown to cause problems in the normal embryo development; in fact, some studies have recommended only short periods of coincubation to achieve better rates of fertilization.[12] It is possible that these problems in embryonic development are related to an increased incidence of sperm DNA damage that occurs during coincubation.

Any sDF associated with iatrogenic-induced damage should be minimized through a reduction in poor or prolonged sperm handing *in vitro*, and this can be achieved by minimizing the sperm storage time postejaculation so as to ensure that the semen sample is processed and used for fertilization, soon after liquefaction or cryopreservation. Iatrogenic sDF can also be reduced by avoiding incubation of the neat ejaculate at 37°C; instead, sperm either be maintained at room or chilled at 15°C, as the methodology used in other mammalian species, to reduce the rate of sDF. Secondly, serial ejaculations once every 24 hours for 4 days before sperm recruitment will diminish the level of sDF after sperm selection and provide semen sample that is more representative of the patient.[13] Finally, the creation of a personal sperm mini-cryobank for each patient to allow for the selection of ejaculates with the lowest levels of sDF for fertilization can be a useful protocol. All these procedures will help to standardize and optimize sperm quality so that the real relationship between sDF and pregnancy can be established.

The stability of the sperm chromatin structure is of fundamental importance for embryo development and quality. Disturbance of the stability of the sperm chromatin is associated with lower fertilization in assisted reproduction. sDF is inversely related to sperm motility and concentration. Increasing levels of DNA fragmentation are correlated with both low sperm concentration and total motile sperm count.[14]

To date, a higher percentage of sDF (>30%) was found in infertile subjects compared with fertile subjects (approximately 5–15% sDF). In particular, sDF levels between 30% and 40% are negatively associated with sperm quality and sDF levels of >26% seem implicated in recurrent miscarriage.[2] Based on the observations that relate to the integrity of sperm DNA and the outcomes of pregnancy, the sDF evaluation in the diagnostic process of an infertile couple is becoming increasingly important. Infections affecting the genitourinary tract are often diagnosed too late, after they have already spread to one or more accessory sex glands, thereby becoming chronic and more difficult to eradicate. Follow-up on the alterations found in semen analysis, that is, infection suggested by pus cells, and evidence of DNA fragmentation can help drive eventual treatments and characterize undiagnosed and unexplained infertility.[15-17]

Routine sDF estimation will direct ART specialists to circumvent DNA fragmentation by using new advanced sperm selection techniques.

Recent, new types of sperm analyzers have come up which can apart from routine parameters of motility, concentration, morphology, and vitality can provide a count of DNA-fragmented sperms and also acrosome intact and acrosome-reacted spermatozoa.

There is extreme heterogeneity in the studies with respect to method of finding DNA fragmentation index (DFI). Also DFI cutoffs vary from <15% to >25%. There is no uniformity in cutoff of what should be considered low DFI and otherwise. Study by Katherine *et al*.[15] demonstrates that sDF is inversely related to sperm motility and concentration. The effect

of sDF on fertilization, blastulation, and aneuploidy rates is negatively impacted if fertilization is conventional and it is neutralized if fertilization is performed via ICSI. It has been postulated in this study that the ICSI treatment group may have provided a higher chance of repairing DNA damage through better quality oocytes. DNA repair can occur during and after fertilization in the oocyte and the developing zygote. The exact mechanism by which the oocyte repairs sperm DNA damage remains unknown. Human oocytes were found to express DNA repair genes and the repair process is believed to be related mainly to maternal mRNA. The effect of the fertilized oocyte with damaged sperm DNA depends on the extent and type (double-stranded DNA breaks worse) of DNA damage and the capacity of the oocyte to repair this damage.

It is to be appreciated that none of the four sDF assays can measure the DNA damage of the actual sperm used to fertilize the oocyte.

A systematic review was performed by Osman *et al*. on the effect of high sDF on IVF-ICSI outcome. A total of 103 manuscripts were retrieved and evaluated in detail. A total of 97 articles did not meet the inclusion criteria. Only six articles fulfilled the inclusion criteria and the risk ratio in most studies were in favor of association of low DNA fragmentation and live birth rate [Table 1].

The major limitation of this analysis is the heterogeneity among the assay of sDF, which were used, also the threshold of DNA fragmentation was different in each study.

Another Systematic Review on 67 articles of which 41 were included in the meta-analysis by Simon *et al*.[17] These included 56 IVF and/or ICSI performed in 8068 treatment cycles, 16 IVF studies performed on 3734 treatment cycles, 24 ICSI studies performed on 2282 treatment cycles, and 16 mixed IVF + ICSI studies on 2052 treatment cycles. Of the total 8068 cycles, 34.9% SCSA was used to measure sperm DNA damage, in 29.2% SCD, 26% by TUNEL, and COMET in 9.9% cycles. The study-by-study comparisons were synthesized by a standard meta-analytical approach applied to the odds ratios (ORs) of two-by-two tables. Statistically fixed or the random effect models for meta-analysis were used to calculate an overall ORs [Table 2].

Table 2 does not show pregnancy, as the OR of clinical pregnancy has been calculated separately for each study, considering it is a large data and only OR compared here.

Table 1: Systematic review by Osman *et al*[16] depicting relationship of DNA fragmentation to live birth rate

Study	Type ofstudy	patient population	semen	assay	DFI thresh hold	No. of patients	RR (Risk Ratio) LBR
Bungum etal 2004	Prospective	IVFICSI	RAW	SCSA	27%	10966	1.110.81
Check et al 2005	Prospective	ICSI	RAW	SCSA	30%	106	1.11
Ozmen et al 2007	Prospective	ICSI	Swim Up	TUNEL	10%	41	1.21
Frydman et al 2008	Prospective	IVF	RAW	TUNEL	35%	117	1.79
Speyer et al 2010	Prospective	IVFICSI	RAW	SCSA	30%	12496	1.191.06
Simon et al 2013	Prospective	IVFICSI	RAW	COMET	50%	203136	1.191.14

RR, risk ratio; LBR, live birth rate; IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection; DFI, DNA fragmentation index; ICSI, intracytoplasmic sperm injection.

Table 2: Relationship between sperm DNA damage assay and clinical pregnancy by type of ART using fixed and random-effects models

Assays	Types	No. of studies	Fixed effects model (OR)	P value	Random effects model (OR)	Pvalue	%age of variation across studies (I^2)	Test of hetero-geneity (Q^2)
SCSA	IVF	6	1.32	0.1471	1.43	0.1670	35.9	0.1678
SCSA	ICSI	12	0.96	0.7800	0.96	0.7800	0.0	0.5811
SCSA	Mixed	5	1.69	0.0234	1.93	0.2147	70.5	0.0089
TUNEL	IVF	6	1.81	0.0007	1.78	0.0039	20.1	0.2822
TUNEL	ICSI	7	2.11	0.0005	2.38	0.0042	42.4	0.1078
TUNEL	Mixed	5	2.92	0.0000	3.17	0.0038	61.5	0.0344
COMET	IVF	3	5.86	0.0000	8.39	0.0021	67.8	0.0448
COMET	ICSI	2	1.84	0.0859	1.84	0.00859	0.0	0.6692
COMET	Mixed	2	3.36	0.0000	2.27	0.3150	81.9	0.0187
SCD	IVF	1	1.12	0.6405	1.12	0.6405	NA	NA
SCD	ICSI	3	1.42	0.0896	2.65	0.1770	85.9	0.0008
SCD	Mixed	4	2.07	0.0007	2.14	0.0272	60.9	0.0534

OR, odds ratio; IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection; NA, not applicable.

CONCLUSION

In conclusion, there is a modest but statistically significant detrimental effect of sperm DNA damage on clinical pregnancy rate after IVF and/or ICSI. However, this effect is observed to vary according to type of assay used to measure sperm DNA damage. Standardized protocols are lacking in showing reproducible results across a range of laboratories. The threshold for each essay for sperm DNA fragmentation is not validated.

Any technique to analyze sDF in clinical andrology or ART laboratories should be simple, reproducible, and preferably without the need for new, complex, or expensive instrumentation.[16] The new improved SCD tests are simple, fast, accurate, and highly reproducible method for the analysis of sDF without the need for complex instrumentation. They can be used with automation. There is a very good correlation between the results from the SCD test and SCSA. Finally, laboratory technicians can easily, quickly, and reliably assess the test end points. Therefore, the improved SCD test could allow for the routine screening of sDF in the andrology laboratories.

There is role of performing sDF in patients of recurrent pregnancy loss, IVF failure, unexplained infertility, and recurrent IUI failure. The evidence is limited for its role in varicocele with or without altered semen parameters. ICSI overcomes the male factor and thus sDF for patients undergoing ICSI seems not useful, but with limited evidence.

Despite these generalities, there are still cases wherein patients with sDF values of over 30% are in fact fertile and males with an SCD value below 15% are clinically infertile. It is therefore important that sDF be considered not as a seminal characteristic in isolation but as an additional parameter to the complete semen analysis and with reference to specific type of fertility treatment utilized (e.g., IUI, IVF, and ICSI).

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

REFERENCES

1. WHO. WHO Laboratory Manual for the Examination and Processing of Human Semen. 6th ed. 2021. p. 86–104.
2. Gosálvez J, Corteś-Gutierrez EI, López-Fernández C, *et al.* Sperm deoxyribonucleic acid fragmentation dynamics in fertile donors. Fertil Steril 2009;92:170–3.
3. Sánchez-Martín P, Sánchez-Martín F, González-Martínez M, Gosálvez J. Increased pregnancy after reduced male abstinence. Syst Biol Reprod Med 2013;59:256–60.
4. Esteves SC, Sánchez-Martín F, Sánchez-Martín P, *et al.* Comparison of reproductive outcome in oligozoospermic men with high sperm DNA fragmentation undergoing intracytoplasmic sperm injection with ejaculated and testicular sperm. Fertil Steril 2015;104:1398–405.
5. Colaco S, Sakkas D. Paternal factors contributing to embryo quality. J Assist Reprod Genet 2018;35:1953–68.
6. Simon L, Emery BR, Carrell DT. Review: impact of sperm DNA damage in assisted reproduction. Best Pract Res Clin Obstet Gynaecol 2017;44:38–56.
7. Agarwal A, Cho CL, Majzoub A, Esteves SC. Clinical practice guidelines for sperm DNA fragmentation testing in male infertility. Transl Androl Urol 2017;6:S720–33.
8. Tan J, Taskin O, Albert A, Bedaiwy MA. Association between sperm DNA fragmentation and idiopathic recurrent pregnancy loss: a systematic review and meta-analysis. Reprod Biomed Online 2019;38:951–60.
9. Zini A, Dohle G. Are varicoceles associated with increased deoxyribonucleic acid fragmentation? Fertil Steril 2011;96:1283–7.
10. Esquerré-Lamare C, Walschaerts M, Chansel Debordeaux L, *et al.* Sperm aneuploidy and DNA fragmentation in unexplained recurrent pregnancy loss: a multicenter case-control study. Basic Clin Androl 2018;28:4.
11. Fernández JL, Muriel L, Goyanes V, *et al.* Simple determination of human sperm DNA fragmentation with an improved sperm chromatin dispersion test. Fertil Steril 2005;84:833–42.
12. Bungum M, Bungum L, Humaidan P. A prospective study, using sibling oocytes, examining the effect of 30 seconds versus 90 minutes gamete co-incubation in IVF. Hum Reprod 2006;21: 518–23.
13. Antinori M, Licata E, Dani G, *et al.* Intracytoplasmic morphologically selected sperm injection: a prospective randomized trial. Reprod Biomed Online 2008;16:835–41.
14. Agarwal A, Majzoub A, Esteves SC, *et al.* Clinical utility of sperm DNA fragmentation testing: practice recommendations based on clinical scenarios. Transl Androl Urol 2016;5:935–50.
15. Green KA, Patounakis G, Dougherty MP, *et al.* Sperm DNA fragmentation on the day of fertilization is not associated with embryologic or clinical outcomes after IVF/ICSI. J Assist Reprod Genet 2020;37:71–6.
16. Osman A, Alsomait H, Seshadri S, El-Toukhy T, Khalaf Y. The effect of sperm DNA fragmentation on live birth rate after IVF or ICSI: a systematic review and meta-analysis. Reprod Biomed Online 2015;30:120–7.
17. 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;19:80–90.