

Clinical utility of sperm DNA fragmentation testing: practice recommendations based on clinical scenarios

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Abstract: Sperm DNA fragmentation (SDF) has been generally acknowledged as a valuable tool for male fertility evaluation. While its detrimental implications on sperm function were extensively investigated, little is known about the actual indications for performing SDF analysis. This review delivers practice based recommendations on commonly encountered scenarios in the clinic. An illustrative description of the different SDF measurement techniques is presented. SDF testing is recommended in patients with clinical varicocele and borderline to normal semen parameters as it can better select varicocelectomy candidates. High SDF is also linked with recurrent spontaneous abortion (RSA) and can influence outcomes of different assisted reproductive techniques. Several studies have shown some benefit in using testicular sperm rather than ejaculated sperm in men with high SDF, oligozoospermia or recurrent in vitro fertilization (IVF) failure. Infertile men with evidence of exposure to pollutants can benefit from sperm DNA testing as it can help reinforce the importance of lifestyle modification (e.g., cessation of cigarette smoking, antioxidant therapy), predict fertility and monitor the patient's response to intervention.

Keywords: Sperm DNA fragmentation (SDF); male infertility; varicocele; unexplained infertility; assisted reproductive technology (ART)

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Introduction

Male factors are responsible for approximately half of all infertility cases (1). The initial assessment of the male patient involves a conventional semen analysis (SA), which may fail to provide a complete understanding of fertility potential. Due to variations in sperm quantity and quality (2), it is difficult to use SA to make management decisions. Sperm function tests can help assess whether they are likely to complete complex actions such as sperm transport through the female reproductive tract, acrosome reaction and penetration of the zona pellucida (3). However, with the advent of intracytoplasmic sperm injection (ICSI), sperm function

tests have fallen out of favor in most andrology laboratories.

Within the last decade, infertility researchers have turned their attention to sperm molecular architecture for good reason—mammalian fertilization and subsequent embryo development depend in part on the inherent integrity of sperm DNA (4). Sperm DNA is bound to protamine and is naturally present in a compact state, protecting it from damage during transport (5). Some damage can occur, which can be repaired in the cytoplasm of the oocyte. When the damage exceeds the cytoplasm's repair threshold, however, infertility can ensue (6).

Both *in vitro* and *in vivo* studies have shown that sperm DNA integrity is negatively associated with fertility (7-10). Elevated

Sperm DNA fragmentation (SDF) may affect fertility by hindering fertilization, early embryo development, implantation, and pregnancy (11).

The etiology of SDF is multifactorial. A number of cellular events contribute to impaired fertility and sperm DNA damage including abnormal chromatin packaging and/or remodeling during spermatogenesis (12,13), excessive reactive oxygen species (ROS) production (14,15) and/or decreased seminal antioxidants (16), and apoptotic events during sperm maturation within the epididymis (17). Exposure to environmental toxins and pollutants, drugs, chemo-radiation, cigarette smoking, febrile illness, varicocele and advanced age have also been proposed as factors that can increase SDF (18-20).

While SDF is increasingly being available in the urologists' armamentarium for the evaluation of infertile men, its accurate clinical implication remains poorly understood. Currently, there seems to be insufficient evidence to support the routine use of SDF in male factor evaluation (21) nevertheless the importance of DNA fragmentation in spermatozoa has been acknowledged in the latest American Urological Association (AUA) and European Association of Urology (EAU) guidelines on male infertility (21,22). Although a precise understanding of the specific utility of such test in different clinical scenarios is still lacking, studies defining specific indications for DNA testing are now emerging (23-25).

This review will help explain the current indications of sperm DNA testing as well as the management of increased SDF. Using clinical scenarios, it is intended to be a useful reference for assisting practicing urologists and reproductive specialists outside the expertise of genetics in identifying the circumstances in which SDF testing should be of greatest clinical value.

Evidence acquisition

A comprehensive search was performed through PubMed up until June 2016. Original and review articles investigating the significance of SDF testing were included. A panel comprised of five urologists (Ahmad Majzoub, Sandro C. Esteves, Edmund Ko, Ranjith Ramasamy, Armand Zini) and one andrologist (Ashok Agarwal) with expertise in male infertility were selected to provide evidence-based recommendations. These colleagues have been considered opinion leaders according to the following criteria: clinical experience with the use of SDF testing in male infertility scenarios and/or assisted reproductive

technology, demonstrated by peer-reviewed publications and presentation at major international meetings.

For the first part, the group of experts prepared an illustrative review about the tests clinically available for SDF testing. In the second part, clinical scenarios commonly found in the urologic office of participants were described, followed by an evidence-based analysis of the clinical utility of SDF under that particular case and recommendations by consensus.

Evidence synthesis

SDF tests

There are two types of assays that have been developed to measure SDF: those that can directly measure the extent of DNA fragmentation through the use of probes and dyes and those that measure the susceptibility of DNA to denaturation, which occurs more commonly in fragmented DNA. The eight described methods to assess SDF are briefly presented below and summarized in *Table 1*. The most commonly used tests are terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), the sperm chromatin dispersion test (SCD), and the sperm chromatin structure assay (SCSA) (26).


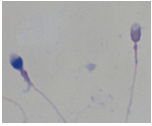
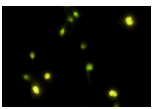
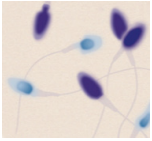
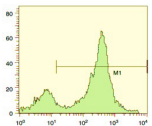
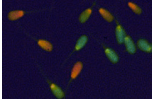
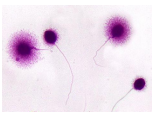
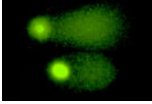
Acridine orange (AO) test

AO is a nucleic acid-selective cationic fluorescent dye that interacts with double strand (ds)DNA or single strand (ss)DNA by intercalation or electrostatic attraction, respectively. When bound to dsDNA, it mimics fluorescein, having an excitation maximum at 502 nm and an emission maximum at 525 nm (green). However, when it associates with ssDNA, the excitation maximum shifts to 460 nm (blue) and the emission maximum shifts to 650 nm (red). It is this metachromatic shift that allows the extent of sperm DNA damage to be determined.

Following mild acid denaturation of sperm DNA, AO binds to ds (i.e., non-denatured) DNA producing green fluorescence or to ss (i.e., denatured) DNA producing red fluorescence. The number of cells with red fluorescence can be measured, which approximates the quantity of sperm with DNA damage in the sample.

There are a number of advantages to this technique—it is fast, simple and inexpensive. Nonetheless, the presence of heterogeneous slide staining with multiple intermediate colors and the considerable inter-laboratory variations and lack of test reproducibility make AO a less

Table 1 Sperm DNA fragmentation (SDF) testing methods

Test	Principle	Advantage	Disadvantage
 [1]	AO test Metachromatic shift in fluorescence of AO when bound to single strand (ss)DNA. Uses fluorescent microscopy	Rapid, simple and inexpensive	Inter-laboratory variations and lack of reproducibility
 [2]	AB staining Increased affinity of AB dye to loose chromatin of sperm nucleus. Uses optical microscopy	Rapid, simple and inexpensive	Inter-laboratory variations and lack of reproducibility
 [3]	CMA3 staining CMA3 competitively binds to DNA indirectly visualizing protamine deficient DNA. Uses fluorescent microscopy	Yields reliable results as it is strongly correlated with other assays	Inter-observer variability
 [4]	TB staining Increased affinity of TB to sperm DNA phosphate residues. Uses optical microscopy	Rapid, simple and inexpensive	Inter-observer variability
 [5]	TUNEL Quantifies the enzymatic incorporation of dUTP into DNA breaks. Can be done using both optical microscopy and fluorescent microscopy. Uses optical microscopy, fluorescent microscopy and flow cytometry	Sensitive, reliable with minimal inter-observer variability. Can be performed on few sperm	Requires standardization between laboratories
 [6]	SCSA Measures the susceptibility of sperm DNA to denaturation. The cytometric version of AO test. Uses flow cytometry	Reliable estimate of the percentage of DNA-damaged sperm	Requires the presence of expensive instrumentation (flow cytometer) and highly skilled technicians
 [7]	SCD or Halo test Assess dispersion of DNA fragments after denaturation. Uses optical or fluorescent microscopy	Simple test	Inter-observer variability
 [8]	SCGE or comet assay Electrophoretic assessment of DNA fragments of lysed DNA. Uses fluorescent microscopy	Can be done in very low sperm count. It is sensitive and reproducible	Requires an experienced observer. Inter-observer variability

[1] Acridine orange (AO) stains normal DNA fluoresces green; whereas denatured DNA fluoresces orange-red. [2] Aniline blue (AB) staining showing sperm with fragmented DNA and normal sperm. [3] Chromomycin A3 (CMA3) staining: protamine deficient spermatozoa appear bright yellow; spermatozoa with normal protamine appear yellowish green. [4] Toulidine blue (TB) staining: normal sperm appear light blue and sperm with DNA fragmentation appear violet. [5] Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay fluorescent activated cell sorting histogram showing percentage of SDF. [6] Sperm chromatin structure assay (SCSA): flow cytometric version of AO staining. [7] Sperm chromatin dispersion (SCD) test: spermatozoa with different patterns of DNA dispersion; large-sized halo; medium-sized halo [2]; very small- sized halo. [8] Comet images showing various levels of DNA damage.

reliable test of SDF (27,28).

Aniline blue (AB) staining

AB staining is another technique that depends on the use of dyes. AB is an acidic dye that has a great affinity for lysine-rich histones in the nucleus of immature sperm, which stain blue (29). On the other hand, the protamine-rich nuclei of mature spermatozoa with abundant arginine and cysteine react negatively and remain unstained. Increased AB staining of sperm indicates loose chromatin packing.

This is a simple and inexpensive technique requiring a simple bright field microscope for analysis. While the results of AB staining correlate well with those of the AO test (30), heterogenous slide staining remains a prominent drawback of this technique.

Toluidine blue

Toluidine blue is a basic thiazine metachromatic dye with a high affinity for sperm DNA phosphate residues. It becomes heavily incorporated in damaged chromatin where it produces a violet-blue intense coloration. The sample can be analyzed using an ordinary microscope. However, intermediate coloration increases the inter-observer variability. Toluidine blue staining generally correlates well with other methods of sperm DNA testing (31).

Chromomycin A3 (CMA3) staining

CMA3 is a guanine-cytosine-specific fluorochrome that competes with protamines for the same binding sites in the DNA. Therefore, when the test is highly positive, it reflects a low DNA protamination state associated with poorly packaged sperm chromatin (32). When compared with AB staining, the CMA3 assay provided equivalent results during sperm chromatin evaluation (32,33).

SCSA (6)

The SCSA measures the susceptibility of sperm DNA to denaturation when it is exposed to heat or acids. It is a flow cytometry-based assay that can evaluate large numbers of cells (10,000 cells) rapidly and robustly (11). It is the flow cytometric version of AO staining in which the extent of DNA denaturation is determined by measuring the metachromatic shift from green fluorescence to red fluorescence (34). An advantage of SCSA is that it has a standardized protocol for all users, minimizing inter-laboratory variation. The clinical threshold is an SDF index of 30% meaning that samples can contain up to 30% of

DNA damaged cells and still be considered normal. Its disadvantage is that it requires a flow cytometer, making it less attractive to clinical andrology laboratories due to equipment costs. Without this equipment, specimens must be sent to a central laboratory, which lengthens the turnaround time.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

As its name suggests, this assay detects “nicks” or free ends of DNA by utilizing fluorescent nucleotides (35). The samples are evaluated with flow cytometry or a standard fluorescence microscope; the assay quantifies the incorporation of dUTP into ss- or dsDNA breaks through an enzymatic reaction creating a signal, which increases with the number of DNA breaks. While TUNEL is believed by many to be the gold standard for SDF testing (36), it lacks strict standardization, which makes comparison between laboratories more difficult and explains why many clinical thresholds exist (37).

Mitchell and colleagues recently modified the TUNEL assay to reduce inter-laboratory discrepancies. They attempted to relax the entire chromatin structure with dithiothreitol (DTT) before fixation to allow access to all “nicks” (38). Recently, a modified TUNEL protocol using bench top flow cytometer was shown to measure SDF accurately and in a large number of samples simultaneously (39).

Single cell gel electrophoresis assay (comet)

The comet assay (40) quantifies the amount of DNA damage per spermatozoon. The name of the assay comes from the mass of DNA fragments that stream out of the sperm head, resembling a ‘comet’ tail. The staining intensity and length of the comet tail represents the amount of migrated DNA, indicating different degrees of SDF (40). One major advantage of this assay is that it can be used in patients with severe oligozoospermia as only about 5,000 sperm are required (11). In addition to its ability to detect single and double strand breaks, the comet assay can identify altered bases. While the comet assay is informative because it is possible to analyze the different types of DNA damage in a single cell using neutral and alkaline electrophoresis, the method is not suited for rapid diagnosis and requires highly specialized personnel to analyze the results.

SCD

The SCD, also known as the Halo test (41), is based on the concept that sperm with fragmented DNA do not produce

the characteristic halo of dispersed DNA loops that are observed in sperm with non-fragmented DNA following acid denaturation and removal of nuclear proteins.

Agarose-embedded sperm are subjected to a denaturing solution to remove nuclear proteins and expose the damaged DNA (ssDNA, fragmented DNA). After lysis, spermatozoa with intact DNA exhibit characteristic loops around the sperm nucleus (creating a halo effect) (42) whereas spermatozoa with DNA damage do not.

Halos can be observed via bright field microscope if the staining is done with an eosin and azure B solution. If DNA-directed fluorochromes are used, the analysis requires a fluorescence microscope. The technique is simple and does not require complex instrumentation. There may be some inter-observer subjectivity, however, when categorizing the halos, which otherwise is a competent assay for SDF quantification.

Generally, SDF measurement provides a more accurate representation of a male's fertility status principally because it has a lower biologic variability than conventional semen studies (43-45). Despite that, a few obstacles still hinder its wide availability among andrology laboratories or prevent it from being routinely used for male fertility evaluation. We still do not understand the true nature of sperm DNA damage and exactly what it is that each test measures. The prognostic accuracy of SDF tests depends on the precision of their technique of implementation. Considerable inter-laboratory variability exists, influencing the reliability of test results. Furthermore, thresholds (or cutoffs) for many of these tests have not been clearly described. Finally, SDF results can be greatly affected by laboratory or clinical conditions such as the degree of sperm nuclear decondensation or the ejaculation abstinence period (46,47).

Indications for SDF testing

Clinical varicocele

Clinical scenario #1: a 28-year-old man presents with primary infertility of 2 years' duration. SA shows mild oligozoospermia [based on the 2010 WHO reference ranges (48)]. His wife is 28 years old with a normal fertility evaluation. During physical examination, a small varicocele is detected during Valsalva maneuver [grade 1 based on Dubin & Amelar classification (49)]. He is otherwise healthy with no significant reproductive or medical/surgical history.

Varicocele is a clinical condition associated with considerable debate. While its detrimental effects on

semen parameters and overall pregnancy rate are well documented (50), and it is prevalent in up to 20% of the adult male population (51), a substantial number of affected men are able to conceive without difficulties.

Because surgery is the mainstay treatment for varicocele, efforts were made to search for adjunct laboratory tests that would help improve patient selection and identify those who would benefit most after surgery. Interest in SDF testing began after a significantly positive association with varicocele was detected in early reports (7).

The occurrence of SDF can be explained by a brief understanding of varicocele pathophysiology. While several theories have been proposed to explain the deleterious effects of varicocele on testicular function, testicular hyperthermia is perhaps the most commonly accepted one. It has long been observed that minor changes in testicular temperature can affect spermatogenesis (52) as many of the enzymes responsible for DNA synthesis in the testis are temperature dependent (53), mainly favoring temperatures lower than 98.2 ± 0.72 °F (normal body temperature). The anatomic position of the testis in the scrotal sac together with the countercurrent cooling mechanism provided by the pampiniform plexus of veins are responsible regulating testicular temperature (54). The blood stasis that occurs with a varicocele disrupts the countercurrent cooling effect, causing the testicular temperature to rise, which subsequently results in abnormal DNA synthesis and defective spermatogenesis (55).

Intratesticular blood stasis is another theory that may help explain the occurrence of DNA damage in men with varicocele. The abnormal dilatation of the pampiniform plexus of veins reduces testicular blood inflow resulting in hypoxia and oxidative stress. It is generally accepted that oxidative stress is the most important intermediary state in the development of testicular dysfunction (56,57). A small amount of oxidative stress is required for normal sperm functions including sperm capacitation, hyperactivation, and sperm-oocyte fusion along with other critical cellular processes. However, negative consequences occur when levels exceed antioxidant capacity (58). Spermatozoa are extremely sensitive to oxidative stress as they lack the necessary enzyme repair systems (59). As a result, free radicals negatively affect spermatozoa in three main ways: membrane lipid peroxidation, DNA damage, and induction of apoptosis (60,61). DNA damage occurs because free radicals directly attack the purine and pyrimidine bases destabilizing the DNA molecule and causing anomalies such as point mutations, polymorphisms, deletions,

translocations, and double-stranded breaks (62).

SDF levels in varicocele patients

Many studies have explored the prevalence of SDF in varicocele patients. In their literature review, Zini and Dohle (63) identified 16 case-control studies that investigated the association between varicocele and SDF. In nine studies, infertile men with varicocele were compared to infertile men without varicocele. A significant association between infertile men with varicocele and sperm DNA damage was demonstrated in 4 of the 9 studies. In the remaining 7 studies involving fertile men, there was also an association between varicocele and SDF (63). In a recent multicenter study involving 593 men, Esteves *et al.* (23) evaluated SDF in various etiologic conditions, including 98 men with varicocele and 80 fertile controls. The highest SDF rates were observed in the men with varicocele (35.7%±18.3%) and in those with leukocytospermia (41.7%±17.6%). Rates of SDF in testicular cancer and repeated *in vitro* fertilization (IVF)/ICSI failure (P<0.05) were high in these two groups as well.

Interestingly, a specific subpopulation with massive nuclear SDF, so-called degraded sperm, was distinguished from the whole population of fragmented sperm. This class was not exclusive of varicocele patients but was overrepresented in this group (P<0.001). Using receiver operating characteristics (ROC) analysis, DDSi—defined as the proportion of degraded sperm in the whole population of spermatozoa with fragmented DNA—identified patients with varicocele with 94% accuracy (23).

Influence of varicocele surgery on SDF

The association between varicocele and increased SDF was further validated by several investigators who examined the effect of varicocelectomy on sperm DNA damage. Zini and Dohle reviewed 511 patients belonging to 9 prospective and 3 retrospective studies (63) comparing men with clinical varicocele with a control group. A reduction of SDF (measured with 8-hydroxy-2-deoxyguanosine, COMET, TUNEL, SCSA and AB staining) was reported by all studies after varicocelectomy (63).

More recent studies have reported similar results but further assessed the impact of this reduction on pregnancy rates. Smit *et al.* examined 49 patients who had a 1-year history of infertility and underwent varicocelectomy (64). Postoperatively, SDF assessed by SCSA significantly decreased from 35.2% to 30.2% (P=0.019). Natural pregnancy was reported by 37% of patients who had

a significantly lower SDF than patients who did not conceive naturally or who conceived with assisted reproduction (64).

Ni *et al.* compared 42 subfertile patients with left clinical varicocele with 10 normozoospermic healthy donors with proven fertility (65). Patients were evaluated with polymerase chain reaction and SCSA to analyze the sperm protamine-1/2 mRNA ratio and DNA fragmentation index before and after surgery (65). The female partners of 10 of the patients naturally conceived 6 months after surgery (23.81%), and these men had a statistically significant reduction in their protamine-1/2 mRNA ratio and DNA fragmentation index after surgery (65). It has also been shown in a meta-analysis of seven studies that varicocelectomy decreases SDF with a mean difference of -3.37% (95% CI, -4.09 to -2.65; P<0.00001) compared to no treatment (66).

Low grade varicocele and SDF

The patient in this clinical scenario had a low-grade varicocele, which further complicates the case. Little is known about the impact of varicocele grade on SDF. Almost all studies exploring the association between DNA damage and varicocele failed to examine this association among different grades. Sadek *et al.* reported similar preoperative measurements in clinical grades 2 and 3 while evaluating the influence of varicocelectomy on SDF measured with AB staining (67). In the aforementioned study, only grade 3 varicocele patients had a statistically significant reduction in SDF after surgery (67). Ni *et al.* reported a significant reduction in the protamine-1/2 mRNA ratio in grade 3 varicocele and a significant reduction in DNA fragmentation in grades 2 and 3 disease after surgery (65).

Clearly, there is insufficient evidence to highlight the clinical utility of DNA fragmentation testing in low-grade varicocele. As such, clinical decisions are based on the available literature, which has consistently shown a significant association between low-grade varicocele and subfertility. A recent study evaluated 482 infertile patients with varicocele who underwent surgical ligation (68). There was a statistically significant improvement in semen parameters after surgery in all three grades of varicocele. More importantly, lower grade varicocele patients had postoperative natural pregnancies that were similar to those of grade 3 varicocele patients (68). The authors recommend SDF testing for patients with low-grade varicocele and borderline SA because it would aid in surgical decision

Table 2 Indications for sperm DNA testing, rationale and evidence

Indications	References
Varicocele	
Significant association between SDF and varicocele has been detected	Zini and Dohle (63); Esteves <i>et al.</i> (23)
Varicocelectomy improves percentage of SDF resulting in improved pregnancy rates	Zini and Dohle (63); Smit <i>et al.</i> (64); Ni <i>et al.</i> (65)
Little is known about the effect of low grade varicocele on SDF. High SDF has been reported in clinical varicocele, particularly grades 2 and 3; improvement of SDF in all grades of varicocele have been reported after varicocelectomy	Sadek <i>et al.</i> (67); Ni <i>et al.</i> (65); Krishna Reddy <i>et al.</i> (68)
Unexplained Infertility	
High SDF is found in men with normal semen analysis	Saleh <i>et al.</i> (7); Oleszczuk <i>et al.</i> (69)
SDF is an independent predictor of male fertility status	Bungum <i>et al.</i> (70); Oleszczuk <i>et al.</i> (69)
SDF levels can predict the likelihood of natural pregnancy	Evenson <i>et al.</i> (6)
Recurrent pregnancy loss	
High SDF is associated with greater incidence of abortion	Ford <i>et al.</i> (71); Khadem <i>et al.</i> (72); Absalan <i>et al.</i> (73)
Recurrent IUI failure	
High SDF is associated with lower IUI pregnancy rates	Duran <i>et al.</i> (74); Bungum <i>et al.</i> (70)
IVF and ICSI failures	
SDF modestly affect IVF pregnancy rates	Zini and Sigman (75); Osman <i>et al.</i> (76); Jin <i>et al.</i> (77)
SDF does not affect ICSI pregnancy rates	Zini and Sigman (75); Zhao <i>et al.</i> (78)
High SDF is associated with greater incidence of abortion in both IVF and ICSI	Zini and Sigman (75); Zini <i>et al.</i> (79); Simon <i>et al.</i> (80); Lin <i>et al.</i> (81); Robinson <i>et al.</i> (82)
Testicular sperm have lower SDF than ejaculated sperm	Moskovtsev <i>et al.</i> (83); Greco <i>et al.</i> (84); Esteves <i>et al.</i> (24)
Higher IVF/ICSI success rates with testicular sperm	Esteves <i>et al.</i> (24); Greco <i>et al.</i> (84); Pabuccu <i>et al.</i> (85)
Lifestyle risk factors	
Age, obesity, smoking and environmental/occupational exposures have detrimental effects on SDF	Shi <i>et al.</i> (86); Bosch <i>et al.</i> (87); Slotter <i>et al.</i> (88); Yang <i>et al.</i> (89); Elshal <i>et al.</i> (90); Tunc <i>et al.</i> (91); Rybar <i>et al.</i> (92); Kort <i>et al.</i> (93); Wijesekara <i>et al.</i> (94); Sanchez-Pena <i>et al.</i> (95); Rahman <i>et al.</i> (96)

making. Furthermore, surgery recommendations based solely on conventional SA results, especially in this subset of patients, could miss those with already compromised sperm function and otherwise “normal” conventional parameters.

Recommendation

While further studies are required, current evidence

suggests that DNA fragmentation testing may allow clinicians to better select varicocelectomy candidates among those men with clinical varicocele and borderline to normal semen parameters (*Table 2*). SDF is recommended in patients with grade 2/3 varicocele with normal conventional semen parameters and in patients with grade 1 varicocele with borderline/abnormal conventional semen parameter results (*Table 3*, grade C recommendation).

Table 3 Grades of recommendations according to quality of evidence*

Grade A
Based on clinical studies of good quality and consistency with at least one randomized trial
Grade B
Based on well-designed studies (prospective, cohort) but without good randomized clinical trials
Grade C
Based on poorer quality studies (retrospective, case series, expert opinion)

*, modified from Oxford Centre for Evidence-Based Medicine (<http://www.cebm.net/oxford-centre-evidence-based-medicine-levels-evidence-march-2009/>).

Unexplained infertility/recurrent pregnancy loss/intrauterine insemination (IUI) failure

Clinical scenario #2: a 29-year-old man presents with secondary infertility of 3 years' duration. He was evaluated with several conventional semen analyses, the results of which were within reference limits (48). His wife is 24 years old with a normal fertility evaluation. The couple has a history of three miscarriages, all of which occurred before the 12th week of gestation. Subsequently, they underwent 2 IUI cycles (total motile sperm inseminated >5 million) with no clinical pregnancy. The man is otherwise healthy with no significant reproductive or medical/surgical history.

Unexplained infertility is a term given when the results of a fertility evaluation are normal. These include SA, ovulation assessment and a hysterosalpingogram. While estimates of the prevalence rate of unexplained infertility vary, it is thought to occur in about 10–30% of couples seeking evaluation (97,98) and is perhaps an obvious demonstration of the limitations of conventional semen testing. Furthermore, it has changed our understanding of the pathophysiology of infertility and initiated the search for new diagnostic tools that can further expand our knowledge (99).

SDF in unexplained infertility

Over the last few years, several studies have investigated the correlation between SDF and conventional sperm parameters (100). Some studies reported an inverse association between DNA fragmentation rates and sperm quality overall, as assessed by sperm concentration, motility and morphology (101–104). Several other studies failed to find such a significant association (105,106). Thus, men with unexplained infertility may indeed have a high SDF index, suggesting that impairment of sperm

DNA integrity can arise in men with otherwise normal semen parameters (70). Oleszczuk *et al.* compared 119 men with unexplained infertility to 95 men with proven fertility; the SDF index was above 30% in 17.7% of men with unexplained infertility and in 10.5% of the proven fertile men ($P=0.005$) (69). In another study, Saleh *et al.* observed that the SDF index, assessed by the SCSA assay, was higher in infertile men with normal SA (23%; interquartile range, 15–32%) than in fertile controls (15%; interquartile range, 11–20%) (8).

SDF and natural pregnancy

SDF has been found to be a valuable prognostic tool in assessing the chances of natural pregnancy in couples. The chances of natural pregnancy are reduced when the SDF index, measured by SCSA, is between 20–30% and is virtually nonexistent when the SDF index is higher than 30% (6). A meta-analysis involving three studies and 616 couples demonstrated that a high SDF, determined by the SCSA test, was associated with failure to achieve natural pregnancy with an odds ratio (OR) of 7.01 (95% CI, 3.68–13.36) (79).

Furthermore, a few studies have linked SDF to recurrent miscarriage, defined as three consecutive pregnancy losses prior to 20 weeks' gestation (71). Using SCD, Khadem *et al.* compared 30 couples with RSA to another 30 control couples (72). The SDF was higher in the RSA group than in the control group (43.3% *vs.* 16.7%, $P=0.024$) (72). Another study using the SCD test similarly demonstrated a significantly higher SDF in the RSA group compared to the control group ($P\leq 0.05$) (73).

SDF and IUI success rates

High levels of SDF may be associated with lower IUI pregnancy rates. Duran *et al.* evaluated semen samples from

154 IUI cycles. SDF was measured using TUNEL or AO testing. The SDF level was significantly higher among the failed cycles, where no woman inseminated with a sample having >12% of sperm with fragmented DNA, by TUNEL, achieved a pregnancy (74). Another study by Bungum *et al.* (70) measured SDF using SCSA in 387 IUI cycles. They reported significantly lower biochemical pregnancy (3% *vs.* 24%), clinical pregnancy (3% *vs.* 23.7%) and delivery rates (1% *vs.* 19%) in patients with an SDF index >30% *vs.* ≤30%, respectively (70).

Recommendation

A high DNA fragmentation index in clinical scenario #2 patient would provide a possible explanation for RSA and IUI failure. Therefore, it is reasonable to offer SDF testing to infertile couples with RSA or prior to initiating IUI (Table 2) as these couples may be better served by IVF or ICSI sooner rather than later (Table 3, grade C recommendation).

IVF and/or ICSI failure

Clinical scenario # 3: a 33-year-old man presents with secondary infertility. A previous SA revealed oligoasthenoteratozoospermia, and the couple was counseled to undergo IVF. After an unsuccessful IVF cycle, they were subjected to an ICSI cycle that resulted in a clinical pregnancy. However, 10 weeks after pregnancy initiation, his wife had a miscarriage.

SDF effect on IVF success rate

The relationship between SDF and the outcomes of conventional IVF has been extensively investigated. Major controversies exist in this particular topic and are principally related to the heterogeneous nature of the conducted studies. Multiple factors may affect the outcome measures such as the assays used to measure DNA fragmentation, female age and fertility status and the source of the utilized sperm. Two systematic reviews have reported a modest relationship between sperm DNA damage and pregnancy rates with IVF (75,107). Zini and Sigman evaluated 9 IVF studies (6 using TUNEL and 3 SCSA) and reported lower pregnancy rates in patients with a high SDF with a combined OR of 1.57 (95% CI, 1.18–2.07; $P < 0.05$). Likewise, another review involving 553 patients who underwent conventional IVF showed a statistically significant association between SDF (measured by TUNEL, SCSA and COMET) and pregnancy rate with an OR of 1.27

(95% CI, 1.05–1.52; $P = 0.01$) (76). However, delivery rates were not analyzed, and subgroup analyses indicated that the SDF measurement method influenced the magnitude of effect size.

Aiming to understand female factor contribution, Jin *et al.* assessed the influence of SDF on the clinical outcomes of assisted reproductive technology (ART) in women with normal ovarian reserve versus reduced ovarian reserve (77). SDF, measured with SCD, significantly affected IVF outcome only in the patients with reduced ovarian reserve. The authors concluded that oocyte quality may be the pivotal determinant for the negative effect of SDF (77).

SDF effect on ICSI success rate

The impact of SDF on ICSI has also been studied. While controversy remains, compelling evidence suggests SDF has a negligible effect on ICSI outcome measures. The systemic review by Zini and Sigman failed to find a significant association between SDF and ICSI pregnancy rates (combined OR, 1.14; 95% CI, 0.86–1.54) (75). Another meta-analysis of 2,756 couples revealed that a lower pregnancy rate in the context of high SDF was noted only in the patients undergoing conventional IVF but not ICSI (78). This difference in outcome measures between conventional IVF and ICSI cycles may be explained by technical differences between the two methods of ART. In IVF, the gametes are subjected to prolonged culture, which may augment the effect of SDF. Indeed, culture media can significantly influence IVF outcomes as reported by Dumoulin *et al.* (108) who demonstrated that the birth weight of IVF babies can be markedly influenced by minor differences in culture conditions. Conversely, ICSI sperm are injected directly into the optimal environment of the oocyte soon after ejaculation, which may protect them from culture media or laboratory-induced damage. Furthermore, during IVF, the oocytes are exposed to marked oxidative stress (resulting from ROS accumulation *in vitro* and the absence of endogenous defense mechanisms) that is thought to be the principle cause for SDF in the first place (109). Meanwhile in ICSI, the oocyte is protected from this attack and uses its energy to repair the damage in the sperm immediately following fertilization (110).

SDF and risk of pregnancy loss after ART

Several studies have reported a relationship between SDF and pregnancy loss after both conventional IVF and ICSI (78–82). The systematic review by Zini and Sigman showed

that SDF was associated with a significant increase in the rate of pregnancy loss after IVF and ICSI with a combined OR of 2.48 (95% CI, 1.52–4.04; $P < 0.0001$) (75). Another review of 16 cohorts utilizing AO, TUNEL and COMET for DNA fragmentation measurement confirmed a similar result where a significant increase in pregnancy loss was noticed in patients with high DNA damage compared with those with low DNA damage [risk ratio (RR) = 2.16 (95% CI, 1.54–3.03; $P < 0.001$)] (82).

Several strategies have been proposed to minimize the influence of abnormal chromatin integrity on ART outcomes. They include: intake of oral antioxidants (111), varicocele ligation (66), frequent ejaculation (112) and sperm selection methods such as magnetic cell sorting (113) or intracytoplasmic morphologically selected sperm injection (114). While varying success rates for these strategies have been reported to reduce SDF, their effect on ART outcomes is still unknown, thus questioning the clinical value of routine application in cases of high SDF before ART (115). Another more promising maneuver is the utilization of sperm harvested from the testes instead of ejaculated sperm. It is believed that most DNA damage occurs during the epididymal transit of sperm (116,117). This is mainly because the sperm is more vulnerable to DNA damage before disulphide cross-linking of its chromatin occurs in the epididymis (118). A few reports have confirmed this phenomenon by finding significantly higher levels of SDF in ejaculated sperm compared with testicular sperm (24,83,84).

Greco *et al.* observed that SDF levels, as assessed by TUNEL, were lower in testicular sperm ($4.8\% \pm 3.6\%$) than in ejaculated sperm ($23.6\% \pm 5.1\%$; $P < 0.001$), and they reported higher pregnancy rates by ICSI using testicular sperm (44.4% *vs.* 6%; $P < 0.05$) (84). In a recent prospective observational study, Esteves *et al.* compared 81 testicular sperm cycles to 91 ejaculated sperm cycles in couples whose male partners had oligozoospermia and high SDF (24). Despite reporting a significantly higher fertilization rate with ejaculated sperm, the live birth rate was significantly higher and the miscarriage rate significantly lower in the testicular sperm cycles (24). The authors also found that SDF assessed by the SCD method was 5-fold lower in testicular sperm than in ejaculated sperm ($40.7\% \pm 9.9\%$ *vs.* $8.3\% \pm 5.3\%$; $P < 0.001$). In another study of similar design but involving normozoospermic men with high SDF, the fertilization rate and miscarriage rate did not differ; however, a

significantly higher pregnancy rate was reported in testicular sperm cycles (85).

Recommendation

While further research in this area is still warranted, DNA fragmentation testing in patients with recurrent ART failure is indicated as it can provide useful prognostic information on subsequent ART cycles (Table 2). Several studies have shown some benefit in using testicular sperm rather than ejaculated sperm in men with oligozoospermia, high SDF and recurrent IVF failure (Table 3, grade B–C recommendation).

Borderline abnormal (or normal) SA with risk factors

Clinical case #4: a 48-year-old man working at a pesticide factory presents with primary infertility of 6 years' duration. He has smoked 1 pack of cigarettes per day for the past 25 years. His body mass index is 46 kg/m^2 while the rest of his physical examination is otherwise unremarkable. His SA reveals mild oligoasthenoteratozoospermia.

This scenario highlights the influence different modifiable lifestyle factors have on male fertility. SDF is influenced by a number of factors that disrupt the balance between oxidants and reductants. As stated previously, oxidative stress is the key to the pathophysiology of male infertility. Like any other cell in the body, spermatozoa produce small amounts of ROS during mitochondrial energy production (119). These are generally counterbalanced by antioxidants in the mitochondria and in seminal fluid (120). An imbalance between ROS and antioxidants triggers a state of oxidative stress, which may damage sperm DNA.

Lifestyle influence on SDF

A number of lifestyle factors have been linked with oxidative stress-induced SDF. Age is a non-modifiable example (18,121). Advancing male age has been associated with increased frequency of sperm DNA defects (86–88). The most commonly accepted hypothesis is that it occurs secondary to age-associated increases in oxidative stress (18).

Smoking is the primary cause of preventable illness—it affects nearly every organ of the body. Cigarette smoke contains more than 7,000 chemicals (122) that can adversely affect fertility in a number of ways. Studies have confirmed that smoking has a detrimental effect on conventional semen parameters (123), sperm fertilizing capacity (124)

and risk of infertility (89). SDF is also shown to be consistently higher in smokers than in nonsmokers (35). In one study, patients with idiopathic infertility were categorized into three groups: fertile non-smokers (n=16), infertile non-smokers (n=36), and infertile smokers (n=34). All patients underwent SA and SDF testing by SCSA. The percentage of DNA fragmentation was significantly higher in the infertile smokers than in the infertile nonsmokers. Moreover, significant negative correlations were noticed between the degree of DNA damage and worsening of semen parameters (90). Chemicals such as nicotine (125), cadmium (126), lead (127) and benzopyrene (128) were specifically investigated and found to cause sperm DNA damage.

Obesity is another factor associated with male infertility and abnormal semen parameters secondary to endocrine dysregulation, increased scrotal temperatures, fatty accumulation of toxins and/or altered sexual health (129). Its influence on SDF has been investigated in several studies with conflicting results. While a few reports failed to detect a significant association (91,92), larger studies did confirm its presence (93,130,131). Kort *et al.* evaluated 520 male partners of infertile couples using SCSA (93) and found a positive correlation between body mass index and SDF, with the mean SDF rising from 19.9% in men with a normal body mass index (20–24 kg/m²) to 27.0% in obese men (>30 kg/m²) (93). Similar results were also reported in studies utilizing COMET (131) or TUNEL (130) for DNA fragmentation measurement.

Occupational exposure is yet another subject that is considerably linked to male infertility. Many toxins and/or pollutants have been described, which cannot possibly be completely covered in this publication. Wijesekara *et al.* (94) interviewed 300 men who were undergoing infertility evaluation looking for the duration of and physical distance from exposure to environmental and occupational chemicals. They reported lower sperm parameters in the exposed group when compared to the non-exposed group. Lead and cadmium were detected in 38.3% and 23% of exposed men, respectively, and their levels were inversely related to the distance from the source of environmental or occupational exposure (94).

Exposure to organochlorine pollutants such as polychlorinated biphenyls and metabolites of dichlorodiphenyltrichloroethane has been associated with DNA fragmentation in spermatozoa. Using SCSA, Sánchez-Peña *et al.* reported a significant influence from exposure to organophosphorus pesticides by which 75% of exposed workers had a SDF >30% whereas

unexposed controls had mean SDF of 9.9% (95).

Bisphenol A is another compound widely utilized in plastic containers used in food and drink industries. Rahman *et al.* recently revealed that high concentrations of BPA can alter sperm function, fertilization, and embryonic development via regulation and/or phosphorylation of fertility-related proteins in spermatozoa (96). Bisphenol A was also found to alter sperm DNA integrity. Wu *et al.* incubated semen samples in 1 and 10 μM of bisphenol A and reported a significant direct correlation between SDF and bisphenol A concentrations *in vitro* (P<0.001) (132).

Recommendation

Infertile men with evidence of exposure to pollutants or those found to have a modifiable lifestyle risk factor during evaluation should be offered SDF testing (Table 2). The sperm DNA test can help reinforce the importance of lifestyle modification (e.g., cessation of cigarette smoking, antioxidant therapy), predict fertility and monitor the patient's response to intervention (Table 3, grade C recommendation).

Conclusions

Sperm DNA is an integral element in the success of human reproduction. There is fair evidence indicating that SDF testing is a useful diagnostic tool in male fertility evaluation. While it has been extensively researched over the past two decades, newer studies help us clarify the role of SDF testing and its indications. SDF should be included in the evaluation of male factor fertility along with SA.

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Footnote

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