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REVIEW

Sperm DNA damage and its impact on male reproductive health: a critical review for clinicians, reproductive professionals and researchers

Ashok Agarwal[a], Manesh Kumar Panner Selvam[a], Saradha Baskaran[a] and Chak-Lam Cho[b]

[a]American Center for Reproductive Medicine, Cleveland Clinic, Cleveland, OH, USA; [b]Department of Surgery, Union Hospital, Sha Tin, Hong Kong

ABSTRACT

Introduction: Sperm DNA damage is the major molecular cause of male infertility, which has a negative effect on reproductive outcomes in couples. Sperm DNA damage originates either during production/maturation or transport of spermatozoa through male genital tract. Though several assays have been used to assess the sperm chromatin integrity and sperm DNA fragmentation (SDF), routine application of SDF testing in semen analysis is generally not reinforced by professional societies. SDF testing is now emerging as a valuable tool and recent clinical practice guidelines (CPG) published by the Society for Translational Medicine recommends SDF testing in various clinical scenarios.

Areas covered: This review discusses the origin and factors contributing to sperm DNA damage, the molecular changes, especially proteomic alterations caused due to SDF, risk factors associated with SDF, methods used to analyze SDF, clinical implications of SDF, and CPG recommendations for SDF testing.

Expert opinion: Recent clinical practice recommendations suggest the potential role of SDF testing in specific clinical scenarios. This would expand the horizon of SDF testing globally as a prognostic and diagnostic tool in various male infertility scenarios and their treatment management.

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1. Background

In the current scenario, infertility is one of the pressing issues affecting 15% of couples globally with male component being the causative factor for about 50% of all reported cases [1,2]. The cornerstone of male infertility assessment is the routine semen analysis; however, they are poor indicators of reproductive outcome [3]. Different etiologies have been ascribed to male infertility, of which, sperm DNA damage has gained the utmost attention with extensive research on the structural and functional aspects of sperm [4]. Mounting evidence clearly indicates a crucial role of sperm DNA and mitochondrial integrity on male fertility status [5,6]. The successful transmission of genome to oocyte relies heavily on the precise structural integrity of the sperm DNA. Protamination imparts compaction of genetic material with in an extremely restricted space of the nucleus and facilitates protection during its voyage through female reproductive tract. Sperm exhibits unique pattern of histone modifications that includes activation and silencing marks in the promoters of genes associated with development. The growing evidence indicates that the paternal epigenome plays an important role in fertilization and early embryonic development [7]. Environmental factors as well aging are the most prominent causes for epigenetic alterations in sperm. Furthermore, advanced paternal age is associated with poor reproductive outcomes due to increased sperm aneuploidy rate and genome decay [8]. Therefore, successful fertilization, healthy embryo development, implantation, and pregnancy are extremely reliant on the integrity of sperm DNA [9]. Based on these facts, assessment of sperm genome integrity has significant clinical implications as sperm with aberrant DNA may have adverse consequences on the offspring due to the transmission of defective paternal genome [10].

Emerging evidence clearly indicates the influential effect of sperm DNA fragmentation index (DFI) on the reproductive outcomes, both naturally and via assisted reproductive technique (ART) [11,12]. Development of various sperm DNA fragmentation (SDF) assay has enhanced the understanding of male infertility at molecular level and recognizing the importance of sperm DNA integrity assessment in diagnosis and treatment management decisions of infertile couples [4,13]. Increased incidence of SDF has been reported in normozoospermic male partners of couples with unexplained recurrent pregnancy loss [14]. These reports clearly demonstrated the deficit of the standard semen analysis in assessing the male gamete and lead to an increased focus on SDF among clinical andrologists and reproductive specialists. Though, an increasing number of reports substantiate the practice of SDF testing in a certain clinical scenario, the routine application of SDF testing is generally not recommended by professional societies [15]. However, recently the American Society for Reproductive Medicine (ASRM), American Urological Association (AUA), and European Association of Urology (EAU) guidelines on male infertility have acknowledged the significance of SDF testing in male infertility cases [16,17]. Furthermore, a recent article on clinical practice guidelines (CPG), published by the Society for Translational Medicine (STM), provides recommendations of expert panel for SDF testing in various clinical scenarios on an evidence-based approach [5].

CONTACT Ashok Agarwal agarwaa@ccf.org American Center for Reproductive Medicine, Cleveland Clinic, Mail Code X-11, 10681 Carnegie Avenue, Cleveland, OH 44195, USA

In order to provide a complete outlook on sperm DNA damage as a reference article for clinicians, reproductive professionals, as well as researchers, this review covers the various aspects of sperm DNA damage and their implications in male infertility assessment. First, we provide a comprehensive insight into the etiology and molecular changes associated with sperm DNA damage. Also, we summarize the studies indicating the risk factors contributing to sperm DNA damage. Furthermore, we also discuss the different direct and indirect techniques employed to evaluate sperm DNA damage while highlighting the clinical utility of terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay in evaluating the ART outcomes. Finally, this review also discusses, in brief, the recommendations on the clinical utility of SDF testing presented by consensus of the CPG expert panel as well as the current challenges and future directions of sperm DNA testing.

2. Types of sperm DNA damage

DNA damage is noticed in the germ cells during late spermatogenesis due to dysfunctional DNA repair system [18]. Both the nuclear and mitochondrial DNA of the spermatozoa are susceptible to damage. Oxidative stress (OS), as a result of high reactive oxygen species (ROS) generation, attacks both the nuclear and mitochondrial DNA [18]. In general, the sperm DNA damage is classified as (a) DNA fragmentation, (b) mitochondrial DNA damage, (c) telomere attrition, (d) Y chromosome microdeletions, and (e) epigenetic abnormalities (Figure 1).

DNA fragmentation can occur either on single- or double-stranded DNA. ROS-mediated DNA fragmentation is induced by the modification of DNA bases and results in the production of 8-hydroxy- 2′- deoxyguanosine (8-OHdG). Increased levels of 8-OHdG serve as the markers of DNA fragmentation [19,20].

Mitochondria are considered the power houses of cell and spermatozoa contains 70–80 mitochondria in the midpiece region of the flagellum. Unlike nuclear DNA, mitochondrial DNA is circular and is not well protected by histones and protamines. Mitochondria generate ATPs and play a vital role in oxidative phosphorylation process essential for sperm motility [6]. Mitochondrial dysfunction results in excess generation of ROS which has 100 times direct impact on mitochondrial DNA compared to nuclear DNA. The DNA mutation rates are also higher (two times) in mitochondrial DNA than that of nuclear DNA [18,21].

Telomere attrition is also considered a reason for DNA damage. It contains a noncoding DNA repeats (5′-TTAGGG3′) and protects the chromosomal DNA being attacked and degraded by ROS. Telomerase reverse transcriptase (TERT) and TERC molecules are involved in the synthesis of new telomeric repeats [22]. Physiological levels of ROS are essential to maintain the telomere length [23]. Shortening of telomere repeats is a sign of aging and cell entering the apoptotic phase. Increased OS accelerates the telomere shortening and is linked with SDF [22].

Epigenetics plays a major role in male infertility and alteration in the epigenetic makeup affects the gametogenesis and gamete maturation [24,25]. Epigenetic abnormalities in methylation and acetylation process mediated by ROS have deleterious effects on sperm function. Hypomethylation of sperm DNA is associated with hypospermatogenesis [26,27]. In addition, hypermethylation also has adverse effect on the spematogenesis [28]. Global hypermethylation was reported in infertile males [29] and 143 hypermethylated sites were noticed in nonobstructive azoospermia [30]. Apart from epigenetic changes, Y chromosome microdeletions are also the cause of male infertility [31,32]. Increased levels of ROS result in SDF-associated promutagenic changes that could lead to AZF microdeletion of Y chromosome [33]. Other factors that induce Y chromosome microdeletion are abortive apoptosis and defective chromatin packaging.

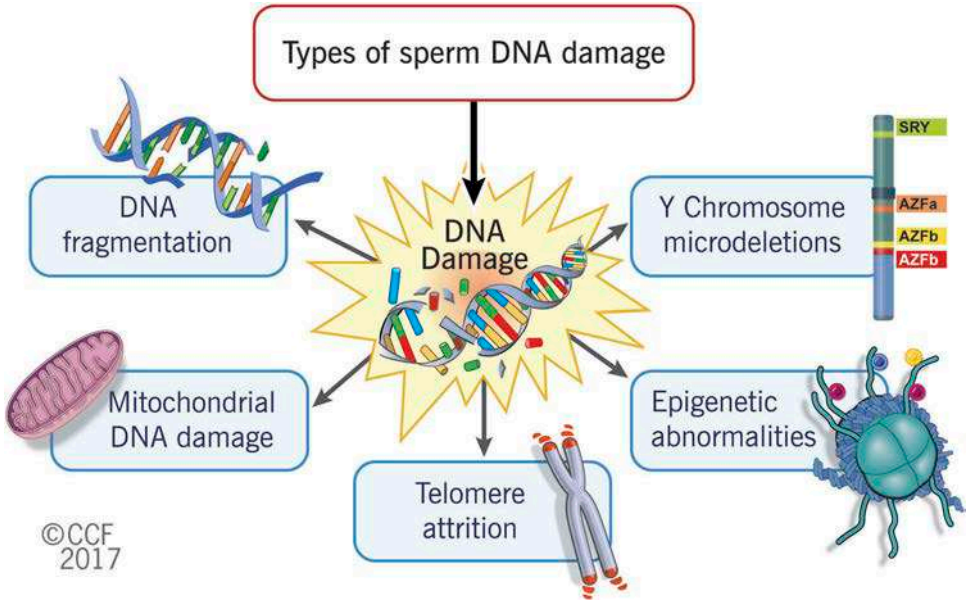


Figure 1. Types of sperm DNA damage.

3. Etiology of sperm DNA damage

The origin of sperm DNA damage can be either testicular during production/maturation or post-testicular during the transport of spermatozoa in the male genital tract. Abortive apoptosis and defective maturation are the intrinsic factors, while OS is thought to be the major extrinsic factor contributing to sperm DNA damage (Figure 2).

3.1. Abortive apoptosis

Spermatogenesis is a complex process involving mitotic proliferation, meiotic maturation, and differentiation of immature germ cells into haploid spermatozoa. In testis, the continuously proliferating and differentiating germ cells are nurtured by restricted number of Sertoli cells [34]. Therefore, maintaining the germ cells to Sertoli cell ratio is a crucial aspect of spermatogenesis, and it is achieved by a subtle balance between spermatogonial proliferation and apoptosis. A significant number of germ cells are lost due to overproduction, genetic aberrations, or accidental damage [35]. The Fas/FasL, a member of tumor necrosis factor-nerve growth factor (TNF/NGF), plays a central role in germ cell apoptosis [36]. About 50–60% of all germ cells that enter meiosis-I are tagged with apoptotic marker (Fas), which are then phagocytosed and efficiently eliminated by the Sertoli cells [37]. However, this checkpoint is not very stringent all the time as a proportion of these tagged defective germ cells undergo subsequent maturation and remodeling, finding its way to ejaculate. Increased expression of Fas receptor in the spermatozoa was demonstrated in men with abnormal sperm parameters, which led to the postulation of 'abortive apoptosis.' This theory projected the origin of DNA fragmented sperm to be the defective germ cells whose apoptotic process in testis was incomplete [38]. Though this theory was validated by numerous studies [39,40], the correlation between the percentage of SDF and expression of apoptotic markers in the ejaculated sperm was inconsistent [41], indicating that abortive apoptosis alone cannot explain the origin of DNA fragmentation.

3.2. Defective maturation

The paternal genome undergoes precise packing during spermiogenesis that leads to a unique architectural compactness to the nucleus, which is the key for fertilization and embryogenesis [9]. A drastic change in the DNA topology is a prerequisite to relieve the torsional stress and facilitate the replacement of histones by protamines [9,42]. Topoisomerase II, an endogenous nuclease, plays a crucial role in chromatin remodeling by inducing double-strand break and its subsequent religation, which aids protamination [43,44]. Therefore, DNA fragmentation is a part of normal spermiogenesis; however, the failure of religation by topoisomerase results in unresolved nicks that could have adverse effect on the genomic integrity of male gamete. In testis, the endogenous nicks have been demonstrated during the transition from round to elongated spermatids but not detected after the completion of chromatin packing [42,43]. Therefore, the presence of DNA strand breaks in the ejaculated sperm signifies defective maturation during spermiogenesis.

3.3. Oxidative stress

High rates of SDF have been demonstrated in the cauda epididymis and ejaculate when compared to testicular sperm, which

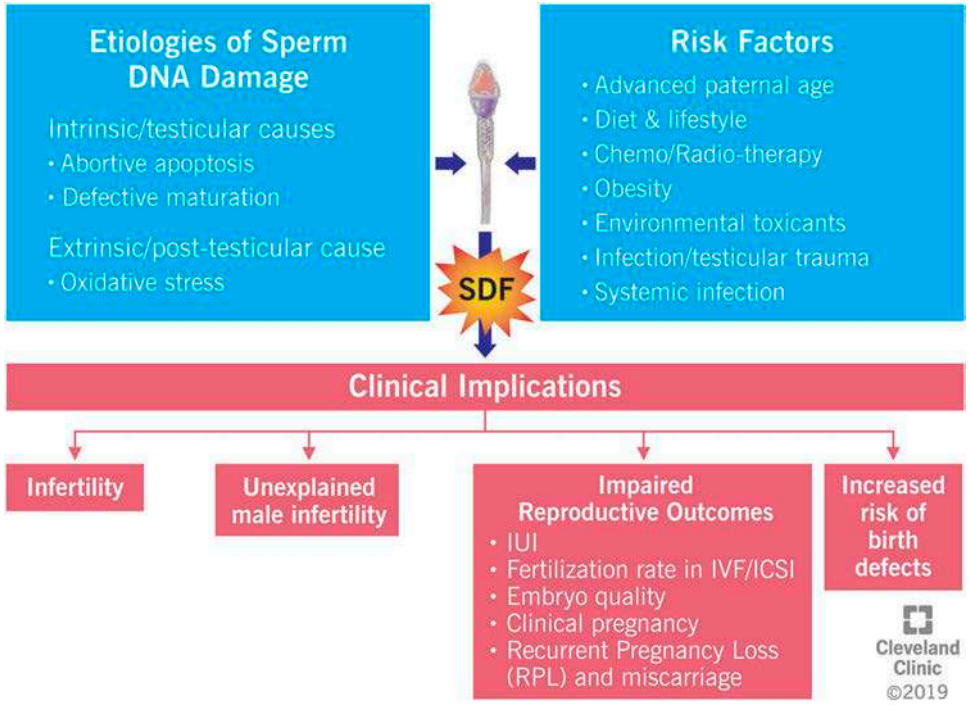


Figure 2. Etiology, risk factors, and clinical implications of SDF. SDF sperm DNA fragmentation; UMI unexplained male infertility; IUI intrauterine insemination; IVF *in vitro* fertilization; ICSI intracytoplasmic sperm injection.

indicates the major contributory role of post-testicular damage in the origin of SDF [45]. A plethora of evidences suggests OS to be the primary causative factor for post-testicular sperm DNA damage [45–47]. An imbalance between the production of ROS and scavenging ability of the antioxidant defense system results in a state of OS. The excess ROS induces DNA damage either directly resulting in base oxidation, strand breaks, and chromatin cross-links or indirectly via activation of sperm caspases and endonucleases. Augmented intrinsic production of ROS by immature spermatozoa that retains cytoplasmic droplets is the main cause of sperm DNA damage [48]. Studies have demonstrated the strong association between high seminal levels of ROS and SDF as well as poor chromatin packing in infertile men [49].

4. Sperm DNA damage and molecular changes

Sperm morphological abnormality such as teratozoospermia and ultra-structural changes such as vacuolation in nucleus are associated with SDF [50]. Furthermore, the sperm functions such as hyperactivation, capacitation, and acrosome reaction, critical for the fertilization process, are defective in sperm with high DNA damage [51,52]. Both nuclear and mitochondria DNA damages are reflected on the molecular machinery at the subcellular level [53,54]. Sperm and seminal plasma proteome are altered in the patients with high SDF [55]. Molecular processes associated with energy production, protein folding, triacylglycerol metabolism, and cellular detoxification are dysregulated in the spermatozoa with high nuclear DNA fragmentation [54]. Spermatogenesis process is disrupted in men with high SDF due to alteration in the expression of prolactin induced protein and its precursor protein. In addition, proteins associated with DNA binding (such as sperm protein associated with nucleus in the X chromosome and histone proteins), OS, and mitochondrial functions were reported to be differentially expressed in men with high SDF [56]. Post-genomic pathways associated with sperm metabolism and function and protection against OS are also dysregulated in men having high SDF [54].

Pathology associated with SDF brings change in the seminal plasma proteome depending upon the extent of sperm DNA damage [54]. Enzymes linked to DNA binding mechanisms were altered in the seminal plasma of infertile patients exhibiting high levels of ROS along with SDF [57]. Deterioration of semen quality and sperm DNA integrity in unexplained male infertity (UMI) subjects have been associated with ROS-induced alterations in methylation status of H19-Igf2 genes [58]. Post-genomic pathways were altered in the seminal plasma of normozoospermic men with low and high DNA fragmentation. These include fatty acid binding and prostaglandin biosynthesis functions in DNA damaged spermatozoa [59]. Intasqui et al. also proposed CRISPLD1, CRISPLD2, and RARRES1 as biomarkers for low SDF, and PSMA5 as biomarker in case of high SDF [59]. Studies related to cigarette smoking cohort showed association between decreased acrosome integrity and mitochondrial activity in patients with high SDF. In the same patients, high SDF led to the activation of pathways associated with positive regulation of prostaglandin secretion, protein kinase A signaling, cytokine mediated signaling, and acute inflammatory responses [59].

Molecular protein signature of both spermatozoa and seminal plasma are altered in high SDF conditions. Differentially expressed proteins may serve as potential biomarkers in pathology of sperm with compromised DNA integrity.

5. Risk factors associated with sperm DNA damage

Several studies have demonstrated the link between male factor infertility and SDF [60–62]. SDF is not only reported in men with abnormal semen parameters, but is also considered as one of the major causes of UMI [62]. A subset of infertile men with normozoospermic semen parameters were reported to have elevated levels of SDF [63]. Several male infertility factors are associated with SDF. Table 1 lists

Table 1. Risk factors associated with sperm DNA damage in infertile men.

Studies	Observations/findings
Male age	
Alshahrani et al., 2014 [64]	Men with age > 40 years are at high risk of SDF.
Brahem et al., 2011 [65]; Luetjens et al., 2002 [66]	Increased diploidy/aneuploidy rates in spermatozoa of aged men.
Diet and lifestyle	
Harlev et al., 2015 [67]	Smoking induces sperm DNA damage.
Cui et al., 2016 [68]	Smoking activates of the checkpoint kinase 1 (Chk1) facilitates S and G2 checkpoint arrest, and increases sperm DNA damage.
Akang et al., 2017 [69]	Alcohol consumption triggers SDF.
De Iuliis et al., 2009 [70]	Alcohol consumption leads to increased release of hydrogen peroxide from sperm mitochondria resulting in SDF.
Obesity	
Dupont et al., 2013 [71]	Increased risk of sperm DNA damage and lower sperm motility.
Palmer et al., 2013 [72]	Obesity induced OS has a negative impact on sperm DNA integrity
Environmental toxicants	
Jeng et al., 2014 [73]; McPherson et al., 2015 [74]	Exposure to endocrine disrupting chemicals positively correlates with SDF.
Meeker et al., 2010 [75]	Bisphenol-A exposure leads to high SDF.
Chemo/Radio therapy	
Ahmad et al., 2017 [76]; Smit et al., 2010 [77]	Positive correlation between the radiation therapy and SDF with declining sperm quality.
Agarwal et al., 2008 [78]	Poor sperm DNA integrity in men exposed to radiations from cell phones, Wi-Fi and other radioactive sources.
Infections and testicular trauma	
Gallegos et al., 2008 [79]	In chlamydia and mycoplasma infections, SDF is high.
Erenpreiss et al., 2002 [80]	Patients with leukocytospermia exhibit high levels of sperm DNA damage.
Wang et al., 2012 [107]	Varicocele is associated with high SDF due to excessive production of ROS.

out the various risk factors and its impact on sperm DNA damage.

6. Techniques for sperm DNA damage assessment

Currently, several assays are being used to assess sperm DNA damage. Based on their ability to measure the maturity and integrity of sperm chromatin, or DNA fragmentation, they are classified as direct and indirect tests (Table 2). TUNEL and sperm chromatin structure assay (SCSA) are the most commonly used SDF tests. A cross-sectional survey across 19 countries by Majzoub et al. showed that 30.6% of SDF measurements are done using TUNEL and SCSA, while 20.4 and 6.1% using sperm chromatin dispersion (SCD) and single-cell gel electrophoresis (Comet), respectively [5]. The test results of each assay are different and are not interchangeable.

6.1. Sperm DNA integrity tests

6.1.1. Aniline blue (AB) staining

Mature spermatozoa has arginine and cysteine abundant protamine, whereas immature spermatozoa contains lysine-rich histones. *AB* is an acidic dye that reacts with the lysine and stains the immature spermatozoa blue, whereas matured spermatozoa remains unstained. The intensity of the stain is directly proportional to the integrity of the sperm chromatin [81].

6.1.2. Chromomycin A_3 (CMA3)

Protamine content determines the chromatin integrity status. CMA3 dye has high affinity to sperm DNA deficient of protamine and stains light yellow [82]. Decreased protamination of the DNA increases the intensity of color [83]. Sakkas et al., using CMA3 assay, reported that fertilization rate in intracytoplamisc sperm injection (ICSI) was significantly lower with DNA damage of >30% [84].

6.2. Sperm DNA fragmentation testing

6.2.1. Sperm chromatin structure assay (SCSA)

SCSA is used to detect breaks in the single-stranded DNA (ssDNA) of sperm. Acridine orange (AO) dye is the principal stain used in this test. AO binding to ssDNA emits red fluorescence, whereas binding to double-stranded DNA emits green fluorescence. The fluorescence signals emitted are captured using a flow cytometer [85]. SCSA can be done on both fresh and frozen sperm. DNA fragmentation index (DFI) of 30% was established as the clinical reference value of sperm DNA damage using SCSA [86,87].

6.2.2. Sperm chromatin dispersion (SCD) test

Fernández et al. introduced the SCD test also known as halo assay [88,89]. This test can be performed on both neat and washed sperms. Sperms are first embedded into the low-melting agarose-coated slides and denatured with acid solution. Subsequently, the slides are stained with DAPI (4′, 6-diamidino-2-phenylindole) and visualized under a fluorescent microscope to differentiate the fragmented (small halos/non-dispersed) form from the highly condensed chromatin (large/distinct halos). The size of the halos produced is directly proportional to the DNA damage [90]. DFI value of 30% is used to differentiate between fertile and infertile men [91].

6.2.3. Comet assay/single-cell gel electrophoresis (SCGE)

SCGE assay can be performed only using fresh semen samples and it requires a minimum of 5,000 spermatozoa. SDF can be assessed easily in oligozoospermic samples using comet assay. DNA from the lysed sperm is subjected to agarose gel electrophoresis. The intact DNA remains inside the head of the sperm, whereas the fragmented DNA migrates and appears as a tail [92]. The sperm DNA is stained using fluorescent dye SYBR Green I and the fragmented DNA is visualized under fluorescent microscope. Length of the tail (fragmented DNA) is an indicator of the extent of DNA damage [93].

Recent advancement in highthroughput platforms has lead to the development of novel high throughput comet (HT-COMET) assay [94]. Implementation of HT-COMET assay reduces the imaging and scoring time by 95% and over all experimental time by >90% [95].

6.2.4. DNA breakage detection-fluorescence in situ hybridization (DBD-FISH)

DBD-FISH identifies the alkali-liable sites and detects the DNA breaks in the spermatozoa [96,97]. Sperm cells are subjected to DNA denaturation to convert the DNA breaks into ssDNA. Subsequently, ssDNA binds to FISH probes and emits fluorescence signals [98]. The main advantage of this technique is that it can be used to scan the whole-cellular DNA or specific DNA sequences of the sperm cells. The DBD-FISH is a reliable technique for determination of DNA breaks but the procedure is complex, expensive, and time-consuming.

6.2.5. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)

TUNEL assay is a robust and highly reliable assay to identify both the single- and double-strand DNA breaks in the spermatozoa from neat, washed, and cryopreserved semen samples. It is gaining clinical importance in andrology laboratories to measure SDF, for its rapid and easy procedure. We have established TUNEL protocol for the measurement of SDF using Accuri C6 benchtop flow cytometer for clinical laboratories [99]. Recently, benchtop flow cytometer was used to measure SDF in large cohort of infertile patients (n = 261) and compared with proven fertile donors. The assay had a high positive predictive value (91.4%) and specificity (91.6%) with a reference value of 16.8% [100]. Our center had collaborated with another reference laboratory at Basel, Switzerland, to determine the inter-laboratory variation for TUNEL assay using Accuri C6 benchtop. Results from both the centers showed a high correlation of r = 0.94 [101] for TUNEL assay. Further, Sharma et al. evaluated newer version of Accuri C6 benchtop flow cytometer (C6 plus) with the standard (C6) flow cytometer. They have reported that calibration of the C6 plus model is essential

Table 2. Different techniques to measure sperm DNA fragmentation.

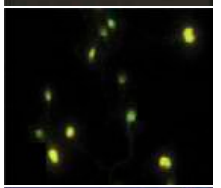
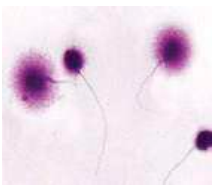
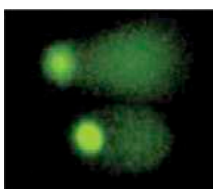
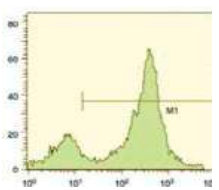
Test	Principle	Method	Result		Advantage	Disadvantage
Acridine orange (AO) test	Metachromatic shift in fluorescence of AO when bound to DNA breaks	Acid denaturation, followed by staining by AO Uses fluorescent microscopy	Normal DNA fluoresces green Denatured DNA fluoresces orange-red		Rapid and simple Inexpensive	Inter-laboratory variations Lack of reproducibility
Chromomycin A3 (CMA3) staining	Compete with protamine for the same binding site in DNA	Staining by CMA3	Highly positive test reflects a low DNA protamination state associated with poorly packaged sperm chromatin		Strong correlation has Been demonstrated with other SDF assays	Inter-observer variability Inter-laboratory variability not tested Technically demanding
SCSA	Measures the susceptibility of sperm DNA to denaturation	Acid denaturation, followed by staining by AO Measurement by flow cytometry Uses fluorescent microscopy	Normal DNA fluoresces green Denatured DNA fluoresces orange-red Result presented as DNA fragmentation index (% DFI) and high DNA stain ability (% HDS)		Standardized protocol available Rapid evaluation of large number of spermatozoa Correlations with results of other SDF assays Established clinical thresholds Can be performed on fresh or frozen samples	Indirect assay involving acid denaturation Proprietary protocol with no commercial assay Requires expensive instrument and highly skilled technicians
SCD/Halo test	Assess dispersion of DNA fragments after denaturation	Agarose-embedded sperm are subjected to a denaturing solution to remove nuclear proteins Uses fluorescent microscopy to observe chromatin dispersion after staining	Sperm with fragmented DNA do not produce halo Characteristic halo of dispersed DNA loops are observed in sperm with non-fragmented DNA Result presented as percentage of sperm with non-dispersed chromatin		Relatively simple test with commercial kit available	Indirect assay involving acid denaturation Inter-observer variability Time-consuming and labor intensive if using microscopic evaluation
SCGE/Comet assay	Electrophoretic assessment of DNA fragments of lysed DNA	Gel electrophoresis performed in alkaline or neutral conditions	Size of comet tail represents the amount of DNA fragments that stream out of the sperm head Result presented as mean amount of DNA damage per spermatozoon		Direct assay Can be performed on few sperm Detect multiple types of DNA damage of individual spermatozoon Result correlates well with other SDF assays	Requires fresh Sample Inter-observer variability Time consuming Requires experienced observer
TUNEL	Quantifies the enzymatic incorporation of dUTP into DNA breaks as percentage of fluorescent sperm	Labeled nucleotides are added to site of DNA fragmentation Fluorescence is measured by flow cytometry or fluorescence microscopy	Sperm with fragmented DNA showed fluorescence Result presented as percentage of fluorescent sperm		Direct assay Can be performed in fresh or frozen samples Can performed on few sperm Detects both single- and double-strand DNA breaks Commercial assay available Reference sample is not required	Requires standardization among laboratories Time-consuming Immature spermatozoa are not evaluated Variable clinical thresholds reported in the literature

Table adapted from Cho and Agarwal, Arab Journal of Urology (2018) 16, 21–34

to obtain a strong agreement between the sperm samples assessed for SDF on two different models of flow cytometer [102]. A standardized, simple, and easy protocol had been proposed for SDF testing using TUNEL technique in clinical laboratories [99,101,103,104].

7. Clinical implications of sperm DNA damage

7.1. Varicocele

The negative impact of clinical varicocele on semen parameters and pregnancy rate is well known. Although varicoceles are considered the most common surgically correctable cause of male factor subfertility, a substantial number of affected individuals are able to conceive naturally without difficulty. Therefore, selection of the best candidates for varicocele treatment remains an unanswered question in clinical practice. Over the last few decades, the pivotal role of SDF in the pathophysiology of varicocele-associated infertility has been increasingly recognized and provides insight into the highly controversial subject [105]. A systematic review and meta-analysis has illustrated the close relationship between high SDF and presence of clinical varicocele irrespective of fertility status [106]. Another meta-analysis echoed by reporting a significantly higher SDF of 9.84% in patients with varicocele than normal healthy controls without varicocele with inclusion of seven studies [107]. The association between SDF and varicocele was further validated by examining the effect of varicocelectomy on SDF and pregnancy outcome. A reduction in SDF by 3.37% after varicocelectomy was reported by a meta-analysis [107]. More recent studies further assess the impact of SDF reduction on pregnancy outcomes. In addition to the significant decrease in SDF after varcicocelectomy, lower postoperative SDF predicted a higher chance of pregnancy both naturally and with assisted reproduction [108,109]. Moreover, the potential benefit of varicocelectomy prior to assisted reproduction has been summarized recently. Outcomes of ICSI cycles in men with treated and untreated varicocele have been reviewed in a meta-analysis and a significant increase in pregnancy and live birth rates was revealed [110].

7.2. UMI

The high prevalence of unexplained infertility in infertile couples may signify the limitations of semen analysis in identifying the underlying etiologies of male infertility. A search for new diagnostic tools is required and SDF represents a potential marker [111]. It has been reported that up to 40% of infertile men with normal semen parameters had high SDF [112]. SDF in men with unexplained infertility were also significantly higher compared to men with proven fertility [113]. Moreover, high SDF in men with UMI had negative implication on clinical pregnancy following *in vitro* fertilization (IVF) treatment [114]. These findings suggest that SDF may attribute to the fertility problems in a significant proportion of men even when they are presented with normal semen parameters. The application of SDF assays in UMI may complement semen analysis and serves as an additional marker of sperm quality in assessment and counseling of these couples.

7.3. SDF and clinical outcomes

7.3.1. Natural conception

The strong correlation between SDF and natural pregnancy outcomes forms the basis of clinical utilization of SDF testing in evaluation of infertile men. Good quality data on time-to-pregnancy has been reported by the Danish First Pregnancy Planner Study. A high SDF index in an unselected population of unknown fertility potential was associated with a longer time to achieve natural pregnancy, in addition to lower fertility potential, compared to low SDF [115]. The association between SDF and fecundity was further supported by the Longitudinal Investigation of Fertility and the Environment Study [116]. A meta-analysis summarized the findings, involving three studies and 616 couples, and suggested that high SDF was associated with failure to achieve natural conception with an odds ratio of 7.01 [117].

7.3.2. Intrauterine Insemination (IUI)

The association between high SDF and poor IUI outcomes is not without debate. The decline in the use of IUI worldwide limited data acquisition. Several studies reported strong relationship with a higher probability of successful pregnancy in couples whose male partner has low SDF [118]. Another study reported significantly lower biochemical pregnancy, clinical pregnancy, and delivery rates after IUI in patients with SDF index >30% compared to <30% [112]. In addition, a higher level of SDF has been demonstrated in patients with failed IUI cycles [119]. A meta-analysis showed a slight but significant predictive ability of SDF testing on IUI success. An odds ratio of 5.61 and relative risk of 1.17 were reported after analyzing 1135 IUI cycles [120].

7.3.3. Fertilization rate in IVF/ICSI

The relationship between SDF and IVF/ICSI outcomes has been extensively investigated. Notwithstanding, the interpretation of results is limited by the heterogeneity in study populations, SDF assays, protocols, and thresholds. Furthermore, the involvement of multiple confounding factors and poorly controlled female factors affect the quality of data. The effect of high level of chromatin abnormalities in sperm would impede the initiation or completion of sperm DNA decondensation in oocyte, therefore leading to failure of fertilization [121].

Clinical studies on SDF and IVF/ICSI outcomes were not lacking. But the correlation between SDF and fertilization rate after IVF/ICSI is not without debate. In general, a higher proportion of IVF studies demonstrated a significant inverse relationship between SDF and fertilization rate compared to ICSI studies (60 vs 23%) [120]. However, it should be noted that a significant number of studies failed to demonstrate the inverse correlation. Earlier study has revealed a decrease in fertilization rate as SDF increased in prepared sperm. Patients with SDF ≤20% and 21–40% by alkaline Comet assay were associated with higher fertilization rate compared to those with SDF 61–100% [122]. Currently, the largest and most recent study included 1633 IVF and ICSI cycles. Patients were divided into four groups according to different levels of SDF as measured by SCSA and fertilization rate among the groups were compared. The study showed that fertilization rates for all groups with SDF >10% were

lower compared to the reference group with SDF 0–10%, but no such differences were seen in the ICSI group [123]. These results were in concert with findings of meta-analysis which demonstrated higher fertilization rate during ICSI compared to IVF in patients with unexplained infertility and normozoospermic men with elevated SDF [124].

The difference in implication of SDF on IVF and ICSI fertilization may be explained by the fact that ICSI bypassed all natural selection process during fertilization including sperm-oocyte interaction. Selection of morphologically normal spermatozoa during ICSI may increase the probability of selecting sperm with lower SDF [125]. Another explanation is the technical differences between the two techniques. Gametes in IVF are subjected to prolonged culture in laboratory setting and the resultant OS resulted in more significant impact on fertilization [126]. As a result, ICSI has been suggested as a treatment strategy for infertile couples with increased SDF.

7.3.4. Embryo quality

The negative implication of high SDF on embryo development has been well demonstrated. It has been observed that predamaged paternal genome led to high proportions of zygotes with abnormal pronuclear morphology. The abnormal zygotes cleaved slowly resulting in arrested growth during early embryonic development [127]. In addition, a late paternal effect of high SDF on the formation of blastocysts has been postulated. The developmental program of embryo would be badly affected in case a defective paternal genome was activated at day 3 [128].

Although the impact of SDF on embryo quality appears promising from experimental models, results from clinical studies seems contradictory. A systematic review including 28 studies and 3226 IVF/ICSI cycles indicated no relationship between DNA damage and embryo quality and/or development [129]. Indeed, only 36% of IVF and 21% of ICSI studies have demonstrated significant correlation between SDF and embryo quality [130] However, it is of note that the heterogeneity of outcome measurement and SDF assays hinders the interpretation of data. Differential association between SDF and embryo quality/development by application of different SDF assays and ART techniques has been suggested. As such, well-designed prospective studies are needed to evaluate the relationship between SDF with both embryo development and quality.

7.3.5. Clinical pregnancy

Earlier systematic reviews have reported a modest relationship between SDF and pregnancy rates with IVF. Lower pregnancy rates in patients with high SDF with a combined OR of 1.57 (95% CI 1.18, 2.07) was observed by evaluating nine studies [12]. Likewise, a statistically significant association between SDF and pregnancy rates after IVF was reported in 553 patients with an OR of 1.27 (95% CI 1.05, 1.52) [131]. In contrast, compelling evidence suggests that SDF has a negligible effect on ICSI outcomes. A systematic review failed to identify a significant association between SDF and pregnancy rates after ICSI [132]. Another meta-analysis also reported a lower pregnancy rate in context of high SDF in patients undergoing IVF but not ICSI [133]. More recent systematic reviews and meta-analyses still failed to reach a consensus. In a meta-analysis including 56 studies with a total of 8068 ART cycles, a statistically significant OR estimates of IVF and ICSI (1.65 and 1.31, respectively) indicated that high SDF predicts low clinical pregnancy rates after IVF and ICSI [130]. On the other hand, another systematic review and meta-analysis showed a fair-to-poor predictive value of various SDF assays in the prediction of pregnancy outcomes after IVF or ICSI. High sensitivity and low specificity were generally reported for all SDF tests [134].

7.3.6. Recurrent pregnancy loss (RPL) and miscarriage

Early systematic reviews and meta-analyses have reported a relationship between SDF and pregnancy loss, which did not depend on the method of fertilization used. It was reported 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 [12]. Another review of 16 cohorts confirmed a similar result where a significant increase in pregnancy loss was noticed in patients with high SDF compared to those with low DNA damage (risk ratio 2.16). The review included 2969 couples undergoing ART treatment resulting in 1252 pregnancies and 252 pregnancy losses [135]. More recently, a positive correlation between recurrent spontaneous abortion and high SDF has also been reported [136]. Moreover, a recent systematic review and meta-analysis involving 16 studies with 3106 couples undergoing IVF or ICSI found a significant increase in miscarriage rates for men with high SDF undergoing ICSI (OR 2.68), but not for those undergoing IVF [133]. Although most studies indicate an association between high SDF and pregnancy loss, drawing a robust conclusion remains difficult in view of the heterogeneity of studies.

In summary, high SDF is strongly associated with decreased pregnancy rates in natural conception and IUI. The association between high SDF and impaired fertilization and clinical pregnancy after IVF is suggestive, but not conclusive. The implication of SDF on ICSI outcomes remains less clear. Although there is no solid evidence on negative impact of SDF on embryo quality, risk of pregnancy loss and miscarriage seems to have increased in the context of high SDF after IVF and/or ICSI.

8. Clinical practice guidelines (CPG) for SDF testing

While the correlation between SDF on male infertility has been increasingly revealed, there seems to be insufficient evidence to suggest routine application of SDF assays in the evaluation of infertile men. Although the latest AUA and EAU guidelines on male infertility [16,17] acknowledge the value of SDF testing, the role of SDF assays in clinical practice remains poorly defined and specific indications for the test still await further clarification. A recently published practice recommendation by Agarwal et al. represents the first attempt to propose specific clinical indications for SDF testing [137]. The expert panel presented four clinical scenarios where SDF testing is most indicated, based on the best current evidence. The consensus statement was later endorsed by the Society for Translational Medicine and CPG for SDF testing in male fertility was published in 2017 [5]. The CPG summarized the updated information on clinical utility of SDF and reviewed the management of

Table 3. Clinical indications for sperm DNA fragmentation testing.

(1) Clinical varicocele
• SDF testing is recommended in patients with grade 2/3 varicocele with normal conventional semen parameters
• SDF testing is recommended in patients with grade 1 varicocele with borderline/abnormal conventional semen parameter results
(2) Unexplained infertility/IUI failure/RPL
• SDF testing should be offered to infertile couples with RPL or prior to initiating IUI
• Early IVF or ICSI may be an alternative to infertile couple with RPL or failed IUI
(3) IVF and/or ICSI failure
• SDF testing is indicated in patients with recurrent failure of assisted reproduction
• The use of testicular sperm rather than ejaculated sperm may be beneficial in men with oligozoospermia, high SDF, and recurrent IVF failure
(4) Borderline abnormal (or normal) semen parameters with risk factor
• SDF testing should be offered to patients who have a modifiable lifestyle risk factor of male infertility

SDF, sperm DNA fragmentation; IUI, intrauterine insemination; RPL, recurrent pregnancy loss; IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection

elevated SDF in each indication. It is a useful reference for reproductive medicine specialists to identify the circumstances in which SDF testing should be of the greatest clinical value.

The CPG proposed four potential clinical indications for SDF testing which are summarized in Table 3. The CPG mainly targets at the current drawbacks in clinical practice and limitation of conventional semen parameters. In addition, development of potential treatment strategies in managing high SDF forms an essential component in the clinical utilization of SDF testing in the management of male infertility. The adoption of CPG facilitates a better selection of varicocelectomy candidates, identification of underlying etiologies in couples with unexplained infertility and recurrent pregnancy loss, prediction of assisted reproductive outcomes, and reinforcement of lifestyle modification. This represents an important step forward in promoting wider application of SDF clinically and facilitating future research in andrology.

9. Strategies to reduce SDF

Several studies have extensively focused on recognizing strategies to reduce SDF or select sperm with high chromatin quality for ART. The short ejaculatory abstinence, sperm processing techniques, oral antioxidant therapy, varicocelectomy, and sperm selection strategies including use of testicular sperm for ART have been documented to alleviate SDF [138–144].

9.1. Short ejaculatory abstinence

Duration of sexual abstinence has been reported to influence SDF and pregnancy outcomes [145,146]. In fact, repetitive ejaculation with shorter abstinence has been reported to reduce SDF significantly [138]. This is due to substantial reduction in the duration of epididymal transit of spermatozoa and its exposure to the deleterious effects of ROS. Ejaculatory abstinence for 1–2 days resulted in the least amount of SDF without any detrimental effect on semen characteristics [110]. Furthermore, studies on the impact of short ejaculatory abstinence period on assisted reproductive procedures demonstrated a positive association between short-term abstinence and improved ART outcomes [146].

9.2. Sperm processing and preparation

Sperm processing techniques have been reported to impact sperm DNA integrity [139]. Shorter incubation, storage at room temperature, low-speed centrifugation, and addition of antioxidant or cryoprotectants to culture media are the few strategies that have been reported to ameliorate the negative effects of sperm preparation on SDF [139,140,147]. Density gradient centrifugation (DGC) is a common sperm preparation procedure prior to ART and has been reported to significantly increase SDF, causing lower pregnancy rate [148]. This negative effect of DGC on SDF was note when higher centrifugation force and Percoll gradients were used [149]. Contradictorily, attenuation in SDF following DGC has also been reported [141].

9.3. Oral antioxidant therapy

Substantial evidence indicates the deleterious effects of OS and associated ROS on sperm DNA integrity [150,151]. Several studies have been conducted to elucidate the possible beneficial effect of antioxidant supplementation in alleviating SDF [142,152]. A systematic analysis of 48 randomized controlled trials involving 4179 subjects compared the effectiveness of antioxidant supplementation (single or combined) in subfertile men with placebo or no treatment. The results of analysis suggested that antioxidant supplementation may improve the live birth rate and reduce SDF in subfertile subjects [152]. However, these indications were based on only four randomized controlled trails that reported live birth rate and two trials that reported DNA fragmentation as outcome. Contradictorily, adverse effect of antioxidant supplementation on sperm DNA integrity has also been reported [153]. Further studies must be conducted to validate the role of antioxidant supplementation in improving male fertility as well as standardizing treatment regimen.

9.4. Varicocelectomy

Varicocelectomy has been shown to significantly improve SDF and pregnancy rates [107,154]. A prospective study conducted by Smit et al. revealed a significant decrease in DFI from 35.2 to 30.2% with associated increase in spontaneous (37%) and assisted pregnancy (24%) rates [108]. Another study demonstrated a significant decrease in protamine-1/2 mRNA ratio and DFI in 23.81% men after varicocelectomy and their female partners conceived naturally 6 months post-surgery [155]. Several evidence support the effectiveness of varicocele repair in both reduce the oxidatively induced sperm DNA damage as well as improving the fertility potential in varicocele subjects [154]. A recent prospective study examined the relationship between SDF and ROS in 60 infertile men with clinical varicocele subjected to varicocelectomy [143]. An elevated level of DFI% was noted in varicocele patients with significant positive

correlation with ROS levels and negative correlation with total motile sperm count (TMSC). Following varicocelectomy, >50% increase in TMSC was observed in 73% of the patients with significant diminution in DFI% and ROS levels at ≥3 months postoperatively. Furthermore, regression analysis showed preoperative DFI % as a predictor of improvement after varicocelectomy [143].

9.5. Sperm selection strategies

Sperm selection techniques include magnetic cell sorting (MACS), intracytoplasmic morphologically selected sperm injection (IMSI), and physiological ICSI with hyaluronic acid binding assay (PICSI), and testicular sperm extraction (TESE)/ testicular sperm aspiration (TESA). Controversial reports have been published on the impact of sperm selection strategies on ART outcomes and their beneficiary effect on patients with high SDF. A retrospective study investigated the ICSI outcomes of 1924 infertile patients with or without an intervention (PICSI, IMSI, and TESE/TESA) [144]. In patients with high SDF, analysis of intervention subgroups revealed highest improvement in live birth rates with TESE/ TESA (49.8%), and modest improvement with IMSI (28.7%) and PICSI (38.3%) when compared with no intervention (24.2%) [144]. This report clearly demonstrates the advantage of using testicular sperm over other sperm selection strategies, which could be linked to the fact that testicular sperm demonstrates significantly lower levels of SDF than ejaculated sperm.

Studies have compared the reproductive outcome of ICSI with ejaculatory sperm (EJA-ICSI) or testicular sperm (TESTI-ICSI) in oligospermic and high SDF subjects [156,157]. Higher clinical pregnancy rate and live birth rate were reported in TESTI-ICSI group suggesting it as an effective option to overcome infertility in men with oligospermia and high SDF in the ejaculate. In line with these reports, a recent prospective study involving 36 men with high SDF compared the reproductive outcome of TESA-ICSI against EJA-ICSI group [158]. Significantly higher clinical pregnancy rate was noted with TESA-ICSI group (38.89%) compared to EJA-ICSI group (13.8%). Furthermore, live births in TESA group were documented to be 17 when compared to three in EJA group [158]. The basis behind the use of testicular sperm is that it bypasses post testicular DNA damage induced by OS during epididymal transit of spermatozoa. The results of SWOT (strength, weaknesses, opportunities, and threats) analysis support the use of testicular sperm while performing ICSI in infertile couples with post-testicular SDF [159]. However, due to intrinsic risks associated with sperm retrieval, TESTI-ICSI should be considered only when other less invasive strategies for reducing SDF was unsuccessful.

10. Current challenges and future directions of SDF testing

In the era of IVF and ICSI, SDF testing is considered as a part of semen analysis. Selection of healthy spermatozoa is one of the main challenges faced in the ICSI procedure to attain better fertilization rate. Sperm DNA integrity determines the fertilization rate in ART procedures. Decreased implantation and pregnancy rates are observed with spermatozoa having poor DNA integrity [160]. Therefore, SDF testing can be very helpful for the couples seeking ART procedures. Each technique has its own advantages and disadvantages (Table 2). Comparatively, TUNEL assay is an easy and highly reliable assay to determine the SDF rates in semen sample with high sensitivity and specificity [161]. It is recommended that expanding the use of SDF testing in clinical practice will help the physicians to identify the cause of infertility related to sperm DNA as well as to treat male infertility.

11. Conclusion

Sperm DNA integrity plays an indispensable role in fertilization. A strong association between SDF and male infertility has been documented. Recently, SDF testing has gained utmost attention and its potential clinical implication in assessing specific male infertility scenario has been acknowledged by the expert panel. Lack of well-defined reference range is the major hindrance for its routine application in evaluating male fertility potential. Standardization of various SDF assays will aid in establishing reference range and expanding the clinical utility of SDF testing as a comprehensive prognostic and diagnostic tool in assessing male infertility and optimizing its treatment management options.

12. Expert opinion

The potential of SDF testing as a clinical and diagnostic tool, as presented in this review, has culminated from the efforts over the past four decades by researchers around the globe. The recent publication of the CPGs for SDF testing [162] has summarized using an evidence-based approach, the available data on SDF testing as an initial step toward translating SDF assays from bench to bedside. This transition would not be feasible without adequate knowledge of the pathophysiology of SDF, its testing methods, implication on reproductive outcomes, and treatment strategies. The CPGs clearly demonstrate the potential role of SDF testing in clinical andrology and cautions against the current practice of relying solely on conventional semen parameters in the evaluation of infertile men.

Sperm DNA plays a critical role in embryo development and therefore influences the chances of establishing a pregnancy, both naturally and assisted, leading up to delivery of a healthy baby. The predictive value of SDF testing on the outcomes of natural conception has been convincingly supported by well-designed longitudinal studies. However, the routine use of SDF assays in male factor evaluation currently lacks the support of professional societies [16,17]. Criticisms of SDF assays include lack of standardization and clear cut-off values. Multiple efforts have been made to standardize the SCSA, TUNEL, and SCD tests. For instance, inter- and intra-laboratory precision, and inter- and intra-observer variation have improved significantly. In addition, the threshold value for several SDF assays have been proposed, and its validity in predicting natural and assisted

pregnancy outcomes has been demonstrated. The major obstacle impeding clinical application of SDF testing is the unknown site of DNA damage as well as the nature of DNA breaks detected in different SDF assays. None of the currently available SDF assays can discriminate if the DNA strand break affects coding or non-coding DNA domains. Moreover, the results obtained from one assay do not necessarily match those provided by a different test due to the distinct characteristics of each test. Different assays show different properties in identification of single-/double-stranded DNA breaks and chromatin structure.

Clearly, well-designed studies with adequate power and standardized techniques would be invaluable. It is equally important to be aware that randomized studies in human reproduction are often impractical. We believe that there is no single magic test for diagnosing male infertility considering the complexity of the human reproductive system. Correct interpretation of results from a panel of laboratory tests is often essential in the investigation of intricate systems involving multiple factors. In this context, SDF would be a useful adjunct to conventional semen analysis. In fact, SDF testing should be considered as one of the tests in a panel of male fertility assessment rather than a standalone test. A combination of a selected panel of tests, when appropriately applied, could offer additional complementary information for making a clinical diagnosis while taking into account various male and female factors in a clinical scenario.

The increasing number of SDF-related publications along with the continuing efforts of researchers could potentially revolutionize the field of male infertility, which in turn will drastically change clinical management in the upcoming years. We are looking forward to a new evidence in expanding the scope of SDF testing by exploring the correlation between SDF and assisted reproduction outcome, and new treatment strategies in alleviating SDF. As reported recently, SDF assays have been adopted by many andrology laboratories worldwide [5]. The timely revision of CPGs by various professional societies will firmly establish the role of SDF testing in the management of infertile couples.

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•• **This article provides recommendations of clinical utility of the SDF tests supported by current evidence.**