Review Article

Sperm DNA integrity and male infertility: a narrative review and guide for the reproductive physicians

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Background and Objective: Conventional semen analysis (SA) remains an essential tool in the initial male fertility evaluation and subsequent follow-up. However, it neither provides information about the functional status of spermatozoa nor addresses disorders such as idiopathic or unexplained infertility (UI). Recently, assessment of sperm DNA fragmentation (SDF) has been proposed as an extended sperm test that may help overcome these inherent limitations of basic SA. In this review, we aim to: (I) discuss the pathophysiological aspects of SDF, including natural repair mechanisms, causes, and impact on reproductive outcomes; (II) explain different assessment tools of SDF, and describe potential therapeutic options to manage infertile men with high SDF; and (III) analyse the strengths, weaknesses, opportunities and threats (SWOT) of current research on the topic.

Methods: This review was constructed from original studies, systematic reviews and meta-analyses that were published over the years up until August 2021, related to the various aspects of SDF.

Key Content and Findings: Different mechanisms lead to high SDF, including defective chromatin packaging, apoptosis, and seminal oxidative stress. The relevance of sperm DNA integrity to male fertility/infertility has been supported by the frequent observation of high levels of SDF in infertile men, and in association with risk factors for infertility. Additionally, high SDF levels have been inversely correlated with the outcomes of natural pregnancy and assisted reproduction. Terminal deoxynucleotidyl transferase dUTP nick end labelling, sperm chromatin structure assay, sperm chromatin dispersion, and Comet assay are four commonly used assays for measurement of SDF. Addressing lifestyle risks and underlying conditions, antioxidants, hormonal therapy, and advanced sperm selection techniques have all been proposed as potential therapeutic options to lower SDF.

Conclusions: The sum of literature provides evidence of detrimental effects of high SDF on both natural and assisted fertility outcomes. Standardization of the techniques used for assessment of SDF and their incorporation into the work up of infertile couples may have significant implications on the future management of a selected category of infertile men with high SDF.

Keywords: Assisted reproduction; male infertility; natural pregnancy; semen analysis (SA); sperm DNA fragmentation (SDF)

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Introduction

Infertility is a highly prevalent condition worldwide, but the exact extent of the phenomenon is difficult to assess, since it has various definitions (1). Current inconsistencies in medical approaches, in addition to individual reproductive choices, allow some cases to go undetected. An epidemiologic study, in which live birth over a 5-year period was measured, reported that the prevalence of couples suffering from infertility was estimated at 48.5 million all over the world (2). According to the definition by the World Health Organization (WHO) (i.e., absence of conception after 12 months of regular, unprotected intercourse), infertility is a condition in which male factors, female factors, or both may contribute, with males being responsible for 50% of the cases overall. A significant percentage of these cases can be attributed to unexplained infertility (UI), where no currently applied knowledge or tools are able to establish a direct diagnosis (3).

Semen analysis (SA) is the backbone of male infertility assessment, however it does not provide any insight into the functional status of spermatozoa and cannot justify adverse reproductive outcomes or UI when the baseline evaluation of both partners is unremarkable (4). The recently published 6th edition of the WHO laboratory manual for the examination and processing of human semen has acknowledged such limitations, and in an attempt to identify and underline the importance of sperm function testing, it has introduced sperm DNA fragmentation (SDF) as an extended examination and recommends this assessment in certain clinical settings, which has been quoted as a major strength of the new manual (5).

SDF occurs in male germ cells when nuclear DNA damage is induced by different factors and is not properly repaired due to dysfunctional repair systems (6). Recent evidence has pointed to the relevance of sperm DNA integrity in reproductive outcomes, since high levels of SDF have been found in infertile men (7) and in association with risk factors for infertility (e.g., drugs, pollutants, lifestyle habits and diseases), which can damage male gametes through testicular apoptosis, altered chromatin maturation and, increased reactive oxygen species (ROS) production (8).

The aim of the present narrative review is to discuss the pathophysiological aspects of SDF including physiological mechanisms that oppose SDF, its causes and significance in terms of reproduction. Additionally, we discuss the different methods of assessment of SDF and provide guidance for potential therapeutic interventions for the management of infertile men with high levels of SDF. Furthermore, we highlight the strengths, weaknesses, opportunities, and threats (SWOT) of current research in SDF and provide insights for future research that may help optimize the diagnostic and predictive potentials of this test in clinical practice. We present the following article in accordance with the Narrative Review reporting checklist (available at https://tau.amegroups.com/article/view/10.21037/tau-22-149/rc).

Methods

To construct this narrative review, a comprehensive literature search was conducted using PubMed from inception with a final endpoint in August, 2021. The search was conducted independently by three authors (AF, GS, and SK) and involved articles pertaining to all aspects of SDF. AF conducted the search for articles on the clinical impact of SDF on male fertility outcomes as well as the treatment options for SDF. Search terms included: "Sperm DNA fragmentation/damage/integrity", "male infertility", "assisted reproduction technologies", "treatment/therapy/management", and "sperm selection". GS conducted the search for articles on the molecular aspects of sperm DNA including normal repair and molecular mechanisms of SDF. Search terms included: "Sperm DNA fragmentation/damage/integrity", "male infertility", "sperm condensation", and "sperm DNA repair". SK conducted the search for articles on the causes and risk factors of SDF as well as the diagnostic tests and guidelines for SDF testing. Search terms included: "Sperm DNA fragmentation/damage/integrity", "causes/risk factors", "measurement/diagnosis", and "guidelines". All constructed formulas, using the various keywords, were applied to PubMed for a conclusive literature search and included well-designed observational studies and clinical trials, reviews and meta-analyses. Commentaries, editorials, case reports, and non-English articles were excluded. Any uncertainties were resolved by the senior authors AA, RS, and PV. The search strategy is summarized in *Table 1*.

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Table 1 Search strategy summary

Items	Specification
Date of search	August, 2021
Databases	PubMed
Search terms used	"Sperm DNA fragmentation/damage/integrity", "male infertility", "assisted reproduction technologies", "treatment/therapy/management", "sperm selection", "sperm condensation", "sperm DNA repair", "causes/risk factors", "measurement/diagnosis", and "guidelines"
Timeframe	Year 2006 to 2021
Inclusion and exclusion criteria	Included: observational studies, clinical trials, reviews and meta-analyses
	Excluded: commentaries, editorials, case reports, and non-English articles
Selection process	Independent search was conducted by the authors, selecting well-designed observational studies, clinical trials reviews and meta-analyses related to SDF pathogenesis, clinical implication, testing methods, and management

SDF, sperm DNA fragmentation.

Natural sperm DNA repair

Germ cells can actively react to DNA damage in two ways: apoptosis or DNA repair. Five different mechanisms of sperm DNA repair have been described, which are: nucleotide excision repair (NER), base excision repair (BER), DNA mismatch repair (MMR), post-replication repair (PRR), and double-strand breaks (DSBs) repair. NER acts in the presence of extensive DNA damage (e.g., pyrimidine dimers, bulky adducts, and DNA intra-strand cross-links) and consists of DNA damage detection by XPC/RAD23B proteins, helix unfolding, and cleavage of lesions by XPG and XPF/ERCC1. Conversely, BER operates to remove more limited damage such as non-helix distorting base lesions, which involves recognition and excision of the damaged base through the cleavage of the N-glycosidic bond to deoxyribose by DNA glycosylases. In both pathways, after removal of a single base or surrounding DNA, the gap is then filled by DNA polymerase. MMR, instead, is a security system that verifies the correct replication of DNA by DNA polymerase, playing a key role in maintaining genomic stability (9). On the opposite, PRR mechanisms allow spermatozoa to tolerate single stranded breaks (SSBs), by avoiding fork collapse and accumulation of DSBs, with persistence of DNA damage, but at the cost of reduced DNA replication fidelity (10). Finally, DSB repair mainly occurs via three different pathways: non-homologous end joining, homologous recombination and alternative non-homologous end joining. These mechanisms are significantly different, because homologous recombination uses Rad51 protein and an undamaged homologous template to replace the damaged one, ensuring high fidelity, whereas non-homologous end joining mechanisms act to create compatible ends in DSBs through the removal of non-ligatable termini by the MRE11 complex (9).

Interestingly, in mammals, DSBs repair kinetics in sperm cells differ from any other differentiated cell due to the extremely high grade of chromatin compaction of mature sperm cells that require specific DNA repair proteins. In addition, in cases of DNA damage, the different chromatin compositions between the various spermatogenic cell types influence the recruitment of different sets of repair proteins (11). This partly explains the decline in the ability to repair DNA damage observed in the later stages of spermatogenesis, during which genomic damage can accumulate and be transmitted to the embryo (12). It is worth mentioning as an example, that mature sperm cells are able to initiate the previously described process of BER by 8-oxoguanine DNA glycosylase 1, but they lack the downstream components of this pathway, whereby subsequent DNA repair is strictly oocyte-dependent (13).

An embryo that forms following fusion of a sperm nucleus carrying damaged DNA with an oocyte may exhibit poor developmental prospects, may fail to implant in the uterine lining or may even be aborted at later stages of the first trimester of a clinical pregnancy (14). Normally, the oocyte has its own DNA repair mechanism that are activated following fertilization, which can compensate for the damage in sperm DNA, especially for SSBs involving less than 8% of the sperm genome (15). Therefore, oocyte quality

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could play an important role in the success of DNA repair. In the process of initial embryo development, the zygote may induce DNA repair for spermatozoon containing SSBs as the complementary DNA strand is present (16-18). However, if the damage is only partially repaired, abnormal development of the embryo may occur (11).

What causes SDF?

Types of SDF

In general, DNA fragmentation is classified into SSBs and DSBs (19,20). In case of SSBs, replication is usually still possible using the other unaffected strand. DSBs are considered to be more harmful than SSBs as they could result in genetic rearrangements. Ribas-Maynou *et al.* have presented evidence that DSBs are associated with recurrent miscarriage in a couple without a female factor (21). On the other hand, SSBs were reported not to have a significant impact on embryo development or implantation rates (22). Although SSBs and DSBs may have different implications on reproductive outcomes as well as different underlying molecular mechanisms, it is important to point out that most studies investigating the effect of SDF on male infertility and poor reproductive outcomes do not distinguish between the different types of breaks. This may be attributed, at least in part, to the fact that most assays that measure SDF do not distinguish between SSBs and DSBs, as will be discussed later in this review.

Molecular mechanisms of SDF

The active replication of germ cells in the first phase of spermatogenesis can result in transcription errors that could damage the integrity of the inherited genome of the organism. Indeed, DSBs are physiologically induced in spermatocytes during prophase I of meiosis to allow DNA recombination between homologous chromosomes (14). DSBs occurring in meiosis may be potentially dangerous as they can cause chromosomal fragmentation, loss of chromosomal domains, translocations, and other genetic abnormalities (11).

In addition, during the last phases of spermatogenesis, i.e., spermiogenesis, sperm chromatin undergoes DNA condensation, where nearly all the original histones are replaced by protamines, ensuring a high level of compactness in the sperm nucleus (23). Defective chromatin condensation during spermiogenesis which results in an inappropriate protamination and insufficient chromatin packaging, could generate sperm DNA damage (24).

SSBs are caused by DNA ligase activity adjacent to the lesion or by abortive topoisomerase. Topoisomerase is an enzyme which overwinds or unwinds DNA strands. During the process of chromatin compaction in spermiogenesis, topoisomerase II facilitates the creation of breaks in DNA to reduce torsional stress for histone disassembly and chromatin packaging. If these breaks are not modified, consequent impairment of the chromatin packing results in increasing SDF (25,26). DNA ligase is another enzyme that plays a role in joining DNA strands together by catalysing the formation of a phosphodiester bond. Incomplete unwinding or fixation of DNA by abortive function of these enzymes could lead to SSBs.

Furthermore, excessive ROS, such as free radicals and hydrogen peroxide are considered as one of the main causes of SSBs (27,28). They cause DNA base modifications leading to the formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) and lipid peroxidation. The DNA structure is destabilized by those oxidized base adducts, which results in DNA breaks (29,30). Other bases, including adenosine, cytosine, and thymidine are also sensitive to oxidative damage, although to a lesser extent compared to guanosine (31).

In addition, exogenous sources such as genotoxic drugs and ionizing radiation can directly lead to DNA strand breaks, including DSBs (32). DSBs are also caused as a consequence of SSBs in the process of DNA replication, increased levels of ROS (33), or collapsed replication forks (34).

Risk factors of SDF

Oxidative stress (OS) is caused by an imbalance in homeostasis between ROS production and total antioxidant capacity in semen (35). OS may originate from exogenous sources such as toxicants, smoking, alcohol, diet, and radiation (36-38) or endogenous sources such as varicocele (39). Studies have shown that men with varicocele have significantly higher SDF levels compared to men without varicocele (40,41). A meta-analysis by Wang *et al.* showed significantly higher levels of SDF in infertile men with varicocele as compared to controls, with an average difference of 9.84% ($P<0.00001$) (42). Increased scrotal temperature is also known to increase SDF partially through apoptosis induced by OS and elevated expression of specific proteins (43,44). In animal studies, SDF is observed with 2–4 ℃ increase in physiologic scrotal temperature (45,46). Obesity and physical abnormalities such as varicocele or

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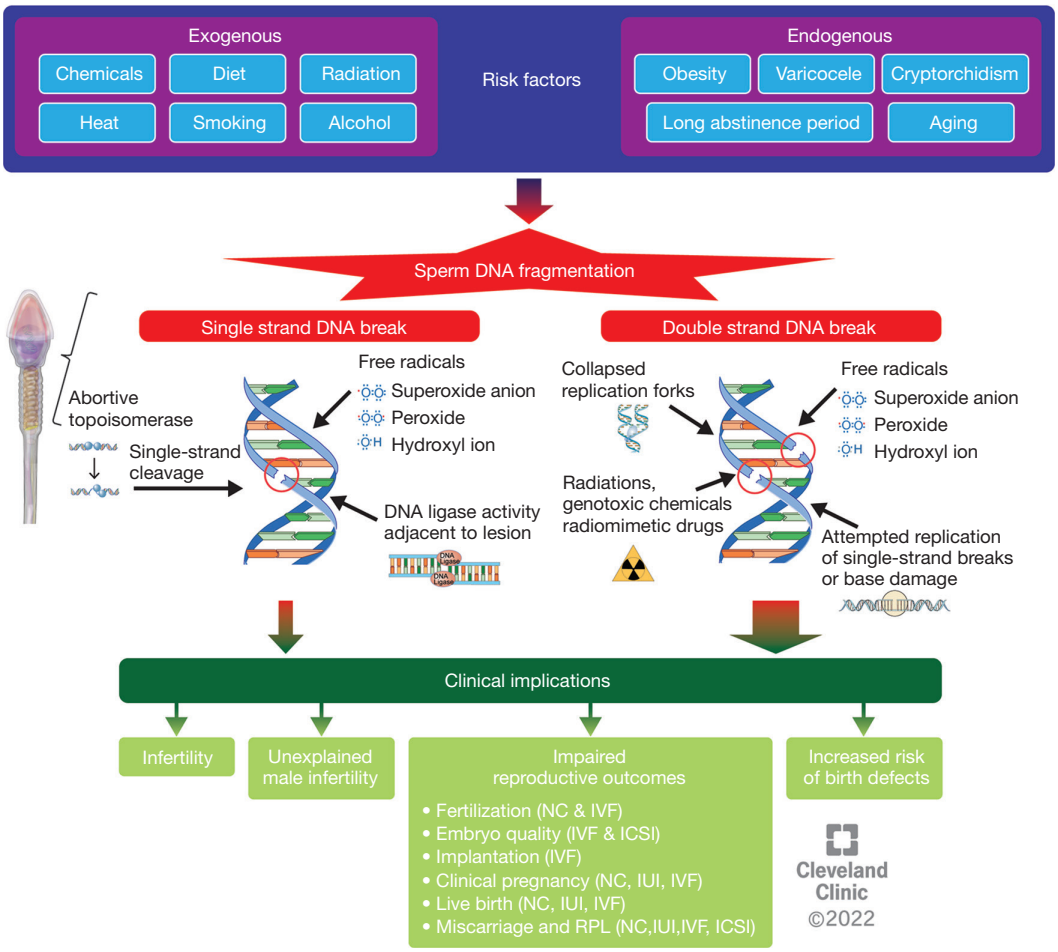

Figure 1 Summary of risk factors, molecular mechanisms of sperm DNA fragmentation, and its clinical implications. Due to exogenous and endogenous factors, SSBs are induced through several molecular mechanisms such as abortive topoisomerase, DNA ligase activity adjacent to lesion, and free radicals, while DSBs are induced by collapsed replication forks, replication in DNA strand with SSBs, and free radicals. Although both SSBs and DSBs could lead to male infertility and impaired reproductive outcomes, DSBs have been clearly linked with significant adverse effects (as demonstrated by the wider arrow). Reprinted with permission, Cleveland Clinic Foundation ©2022. All Rights Reserved. SSB, single strand DNA break; DSB, double strand DNA break; NC, natural conception; IVF, in vitro fertilization; RPL, recurrent pregnancy loss; IUI, intrauterine insemination; ICSI, intracytoplasmic sperm injection.

cryptorchidism cause an increase in scrotal temperature and moreover introduce OS. Aging is another factor associated with SDF increase (47,48). This may be attributed to the cumulative exposure to OS and disordered apoptosis that occurs with aging, as well as a decline in antioxidant capacity and a cumulative higher number of spermatogenic cycles in a context where DNA repair activities may decrease and more mutations can be acquired. Genitourinary infections and male genital tract inflammation can also increase ROS production via activation of white blood cells, contributing to SDF (35). Finally, ROS exposure during transfer of spermatozoa from testis to the epididymis after spermatogenesis may also lead to SDF. Longer abstinence could increase SDF as sperm DNA can be damaged by a biological environment of OS while spermatozoa are stored in the epididymis (35). Studies have reported that a shorter abstinence period in ejaculation contributed to decreased ROS levels in semen and sperm DNA fragmentation index (DFI) (49,50). *Figure 1* summarizes different etiologies, risk factors, and pathogenesis of SDF.

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Effect of SDF on fertility outcomes

The growing importance of SDF in the diagnosis and management of male infertility lies in the fact that SDF can have tremendous implications on male fertility and reproductive outcomes. Unexplained male infertility (UMI) refers to infertile men who have no identifiable cause for their infertility and a completely normal SA (51). Sperm from men with UMI has been found to contain significantly higher SDF levels when compared to fertile controls (52,53). In one report, 1 in 4 men with UMI have SDF levels above 20% (53). Idiopathic male infertility (IMI), on the other hand, refers to infertile men who have no identifiable cause for their infertility but do present with abnormalities in their conventional SA (51). Men with idiopathic oligoasthenoteratozoospermia (OAT) have been found to have significantly higher levels of SDF compared to men with normal semen parameters (54). A meta-analysis by Santi *et al.* has determined that SDF levels of 20% or higher are predictive of male infertility with 79% sensitivity and 86% specificity (7).

Given the large number of studies that have investigated the adverse impact of SDF on reproductive outcomes after natural conception and assisted reproductive technologies (ART), many meta-analyses have been conducted, some of which are summarized in *Table 2* (55-68) and are highlighted in the following points:

- SDF can reduce the ability of a couple to conceive naturally.
- SDF can reduce pregnancy rates and delivery rates after intrauterine insemination (IUI).
- SDF can reduce fertilization rates, implantation rates, pregnancy rates, and impair embryo quality after *in vitro* fertilization (IVF), as well as lead to higher rates of miscarriage and lower live birth rates.
- SDF has no effect on fertilization rates or pregnancy rates after intracytoplasmic sperm injection (ICSI), but it does impair embryo quality and is associated with a higher risk of miscarriage and may lead to lower live birth rates.
- Elevated SDF can lead to recurrent pregnancy loss (RPL).

When looking at pregnancy rates with different methods of conception, it is clear that elevated SDF impairs pregnancy after natural conception, IUI, and IVF. Regarding ICSI, although the meta-analysis by Simon *et al.* have shown reduced pregnancy with high SDF (62), most other analyses have demonstrated no associations or small non-significant associations between the level of SDF and pregnancy rates after ICSI (56-58,61). Regarding fertilization rates, Li *et al.* showed lower fertilization rates after IVF with high SDF, but no difference in rates of fertilization with ICSI (55). A retrospective study demonstrated a significant negative correlation between DFI and fertilization rate after IVF (69). The most recent meta-analysis by Ribas-Maynou *et al.* reported data on implantation rates being affected with elevated SDF after IVF but not significantly after ICSI (57). Conversely, it is important to point out that not all studies have reported an association between SDF and reproductive outcomes after IVF or ICSI (70).

Differences in the IVF and ICSI procedures can explain the differential effect of SDF on fertilization and pregnancy rates between the two procedures. In IVF, spermatozoa are co-incubated with the oocytes and fertilization is allowed to proceed randomly as one spermatozoon penetrates the zona of the oocyte. This also allows spermatozoa to be exposed to OS from the surrounding environment as well as the supporting cumulus and corona cells of the oocyte during culture, adding to the SDF and impairing fertilization and ability to achieve pregnancy (71,72). Furthermore, SDF level was inversely correlated to normal motility and morphology of sperm cells when used in IVF (73). In ICSI, on the other hand, the sperm cell is manually selected through micromanipulation and based on morphological and kinetic criteria and directly injected into the oocyte after all surrounding cells are removed, bypassing the random fertilization process that occurs with IVF and minimizing exposure to OS.

During the early divisions of embryonic development, fragmented male genome is unequally distributed between daughter cells, leading to a phenomenon called chaotic mosaicism; meaning cells of the developing embryo do not contain an identical and balanced genotype. This has been attributed as a major cause of miscarriage resulting in a reduced live birth rate following ART, as well as repeated miscarriage incidents with RPL (74). If the pregnancy progresses to live birth, damaged sperm leading to chromosomal abnormalities may lead to congenital anomalies in the offspring (74).

Data on the effect of SDF on live birth rate is more heterogeneous. The recent meta-analysis by Ribas-Maynou *et al.* reported non-significant association between sperm DNA damage and live birth rate after IVF and no associations between the two after ICSI (57). This is in line with the meta-analysis by Deng *et al.* that reported no

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Table 2 Summary of selected meta-analyses on reproductive outcomes and SDF

Outcome studied	Method of conception	Number of studies, sample size	Study group *vs.* comparison	Results	First author, year
Fertilization rate	IVF	4 studies, 770 IVF cycles	High *vs.* low sperm DNA damage	Lower fertilization rate with high sperm DNA damage (55.4% *vs.* 71.8%) with (RR =0.79, P=0.23)	Li, 2006 (55)
	ICSI	3 studies, 201 ICSI cycles	High *vs.* low sperm DNA damage	Fairly similar fertilization rates for both groups (79.8% *vs.* 78.2%) with (RR =1.03)	Li, 2006 (55)
Embryo quality	IVF or ICSI	8 studies, 17,879 embryos	High *vs.* low DFI	Lower rates of good quality embryos with high DFI (42.8% *vs.* 69.7%) with (RR =0.65, P<0.01)	Deng, 2019 (56)
Implantation rate	IVF	8 studies, 4,055 IVF cycles	High *vs.* low sperm DNA damage	Lower implantation rates after IVF with high sperm DNA damage (RR =0.68, P<0.01)	Ribas-Maynou, 2021 (57)
	ICSI	11 studies, 3,405 ICSI cycles	High *vs.* low sperm DNA damage	No difference in implantation rates after ICSI between both groups (RR =0.79, P=0.09)	Ribas-Maynou, 2021 (57)
Pregnancy rate	Natural	3 studies, 616 couples	High *vs.* low sperm DNA damage	Failure to achieve natural pregnancy with high sperm DNA damage (OR =7.01, P<0.001)	Zini, 2011 (58)
	IUI	10 studies, 2,839 IUI cycles	High *vs.* low SDF	Lower pregnancy rates after IUI with high SDF (RR =0.34, P<0.001)	Chen, 2019 (59)
		3 studies, 917 IUI cycles	High *vs.* low SDF	Higher pregnancy rates after IUI with lower SDF (RR =3.30, P<0.05)	Sugihara, 2019 (60)
	IVF	11 studies, 1,805 IVF cycles	High *vs.* low sperm DNA damage	Lower pregnancy rates after IVF with high sperm DNA damage (OR =1.7, P<0.05)	Zini, 2011 (58)
		5 studies, 816 IVF cycles	High *vs.* low sperm DNA damage	Lower pregnancy rates with high sperm DNA damage (27.6% *vs.* 43.1%) with (RR =0.68, P=0.0006)	Li, 2006 (55)
		9 studies, 1,268 IVF cycles	High *vs.* low sperm DNA damage	Lower pregnancy rates after IVF with high sperm DNA damage (OR =0.66, P=0.008)	Zhao, 2014 (61)
		16 studies, 3,734 IVF cycles	High *vs.* low sperm DNA damage	Higher pregnancy rates after IVF with low sperm DNA damage (OR =1.92, P=0.0005)	Simon, 2017 (62)
		7 studies, 2,130 IVF cycles	High *vs.* low DFI	Lower pregnancy rates after IVF with high DFI (RR =0.77, P=0.05)	Deng, 2019 (56)
		15 studies, 3,711 IVF cycles	High *vs.* low sperm DNA damage	Lower pregnancy rates after IVF with high sperm DNA damage (RR =0.72, P=0.02)	Ribas-Maynou, 2021 (57)
	ICSI	14 studies, 1,171 ICSI cycles	High *vs.* low sperm DNA damage	Pregnancy rates after ICSI are not related to level of sperm DNA damage (OR =1.15, P=0.65)	Zini, 2011 (58)
		3 studies, 201 ICSI cycles	High *vs.* low sperm DNA damage	Lower pregnancy rates with high sperm DNA damage (37.7% *vs.* 49.4%) with (RR =0.76, P=0.09)	Li, 2006 (55)
		10 studies, 1,047 ICSI cycles	High *vs.* low sperm DNA damage	No difference in pregnancy rates after ICSI between both groups (OR =0.94, P=0.65)	Zhao, 2014 (61)
		24 studies, 2,282 ICSI cycles	High *vs.* low sperm DNA damage	Higher pregnancy rates after ICSI with low sperm DNA damage (OR =1.31, P=0.0068)	Simon, 2017 (62)
		4 studies, 278 ICSI cycles	High *vs.* low DFI	No significant difference in pregnancy rates after ICSI between the two groups (RR =0.75, P=0.29)	Deng, 2019 (56)
		25 studies, 5,467 ICSI cycles	High *vs.* low sperm DNA damage	No difference in pregnancy rates after ICSI between both groups (RR =0.89, P=0.09)	Ribas-Maynou, 2021 (57)

Table 2 *(continued)*

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Table 2 *(continued)*

Outcome studied	Method of conception	Number of studies, sample size	Study group *vs.* comparison	Results	First author, year
Miscarriage rate	IVF	5 studies, 808 IVF cycles, 345 pregnancies	High *vs.* low sperm DNA damage	Higher rates of miscarriage with high sperm DNA damage (OR =2.17, P<0.05)	Zini, 2008 (63)
		8 studies, 339 pregnancies	High *vs.* low sperm DNA damage	Rates of miscarriage after IVF between both groups were not significantly different (OR =1.84, P=0.06)	Zhao, 2014 (61)
		7 studies, 1,339 couples	High *vs.* low DFI	Higher miscarriage rates after IVF with high DFI (RR =1.68, P=0.003)	Deng, 2019 (56)
	ICSI	6 studies, 741 ICSI cycles, 295 pregnancies	High *vs.* low sperm DNA damage	Higher rates of miscarriage with high sperm DNA damage (OR =2.73, P<0.01)	Zini, 2008 (63)
		7 studies, 301 pregnancies	High *vs.* low sperm DNA damage	Higher rates of miscarriage with high sperm DNA damage (OR =2.68, P=0.003)	Zhao, 2014 (61)
		4 studies, 96 couples	High *vs.* low DFI	Higher miscarriage rates after ICSI with high DFI (RR =3.38, P=0.005)	Deng, 2019 (56)
	Variable	16 studies, 1,252 pregnancies	High *vs.* low sperm DNA damage	Significantly higher rates of miscarriage with high sperm DNA damage (RR =2.16, P<0.00001)	Robinson, 2012 (64)
Delivery rate/live birth rate	IUI	2 studies, 518 IUI cycles	High *vs.* low DFI	Lower delivery rates after IUI with high SDF (RR =0.14, P<0.001)	Chen, 2019 (59)
	IVF	4 studies, 553 couples	High *vs.* low SDF	Higher live birth rates after IVF with low SDF (RR =1.27, P=0.01)	Osman, 2015 (65)
		6 studies, 1,634 IVF cycles	High *vs.* low sperm DNA damage	Lower live birth rates after IVF with high sperm DNA damage but non-significant (RR =0.48, P=0.06)	Ribas-Maynou, 2021 (57)
	ICSI	5 studies, 445 couples	High *vs.* low SDF	Higher live birth rates after ICSI with low SDF (RR =1.11, P=0.04)	Osman, 2015 (65)
		9 studies, 3,017 ICSI cycles	High *vs.* low sperm DNA damage	No relationship between sperm DNA damage and live birth rate after ICSI (RR =0.92, P=0.62)	Ribas-Maynou, 2021 (57)
	IVF or ICSI	10 studies, 1,785 IVF or ICSI cycles	High *vs.* low DFI	No difference in live birth rate between both groups (61.9% *vs.* 62.3%) with (RR =0.89, P>0.05)	Deng, 2019 (56)
Recurrent pregnancy loss	Not specified	13 studies, 517 cases and 384 controls	Couples with RPL *vs.* Fertile couples	Higher rates of SDF among couples with RPL with a mean difference of 10.7%, P<0.0001	McQueen, 2019 (66)
	Natural	13 studies	Couples with RPL *vs.* Fertile couples	Higher rates of SDF among couples with RPL with a mean difference of 11.98%, P<0.001	Tan, 2018 (67) yet the relationship between DNA fragmentation in the male gamete and idiopathic recurrent pregnancy loss (RPL
	Not specified	18 studies, 1,181 cases and 925 controls	Couples with RPL *vs.* Fertile couples	Higher DFI among couples with RPL with a standard mean difference of 1.6, P<0.00001	Li, 2021 (68)

SDF, sperm DNA fragmentation; DFI, DNA fragmentation index; IUI, intrauterine insemination; IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection; RPL, recurrent pregnancy loss; RR, relative risk; OR, odds ratio.

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association (56), but in conflict to the results of a previous analysis by Osman *et al.* that reported significantly reduced live birth rates with high SDF after both IVF and ICSI (65).

The majority of the meta-analyses presented in this review and in *Table 2*, have indicated as a limitation, the variability of the different observational studies, investigating the effects of elevated SDF on reproductive outcomes, that were included and analysed. This weakens the conclusions set forth by these meta-analyses. Therefore, well-designed studies that account for different patient factors and control for heterogeneity are needed to reach more solid conclusions on the effect of SDF on the various reproductive outcomes.

How to select a test for sperm DNA fragmentation?

Implementing SDF into clinical practice: Guidelines of Professional Societies and the WHO 6th Edition

Currently, the American Society for Reproductive Medicine (ASRM) guidelines do not recommend routine SDF test in evaluation of male infertility due to lack of information (75-77). On the other hand, the European Society of Human Reproduction and Embryology (ESHRE) guidelines states that SDF testing could be considered for explanatory purposes in couples with RPL (78). Similarly, the European Association of Urology (EAU) guidelines in 2020 also recommend SDF test in couples with RPL following natural pregnancy and male partners with UI (79).

In the latest WHO Manual, SDF is recognized as an important indication in the practice of male infertility, becoming one of the most discussed and promising biomarkers in andrology (80). The WHO also picked up supportive evidence between SDF and embryo development, implantation, spontaneous pregnancies and pregnancies in assisted reproduction (62,64,67,81). In the section of extended testing, the WHO manual provides the detailed procedures of the four commonly used assays for determining the percentage of SDF in any given semen sample; Terminal deoxynucleotidyl transferase deoxyuridine triphosphate (dUTP) nick end labelling (TUNEL), acridine orange flow cytometry (AO-FCM) also known as sperm chromatin structure assay (SCSA), sperm chromatin dispersion test (SCD), and single cell gel electrophoresis assay (Comet assay) (82). The manual also states that the diagnostic thresholds of these assays should be determined and validated by the performing laboratory and according to the procedure utilized.

Summary of common tests for assessment of SDF

Currently there is no globally accepted guideline regarding the choice of assays for SDF testing. The choice of assay in clinical practice depends on trained personnel, availability for instrumentation including cost, and running-time in each clinic. Major practical points for each test are reviewed in *Table 3*.

TUNEL

TUNEL is currently the most commonly used SDF assay worldwide. It is based on technique to label free 3'-OH nicks with dUTP at the DNA break (83). The 3'-OH-termini of the DNA strand breaks work as primers and become labelled in this procedure with bromodeoxyuridine (Brd-U) in a reaction catalysed by Terminal deoxynucleotidyl Transferase (TDT), which is a template-independent DNA polymerase (84). The method can directly assess both SSBs and DSBs. d-UTP is the substrate that is added by the TDT enzyme to the free 3'-OH-termini. The added d-UTP can be labelled directly and plays a role as a direct marker of breaks in DNA, or its signal can be amplified by using a modified d-UTP to which labelled anti-d-UTP antibody can be adsorbed (85). Although this method needs flow cytometry, TUNEL is able to analyse up to 2.5×10^6 spermatozoa, which is a higher number of cells compared to other methods (86). A DFI threshold for infertile men as measured by TUNEL was found to be 17% in one report, with a specificity of 91.6% and a sensitivity of 32.6% (87). As a limitation, TUNEL cannot detect DNA fragmentation produced by nucleases generating 3'-PO_4/5'-OH breaks, because the TdT enzyme used in TUNEL does not react with 3'-PO_4 ends and the T4 DNA ligase in the ligation assay is unable to attach the probe to 5'-OH ends in DNA (88).

SCSA

The method of SCSA is based on the action of AO. It emits green fluorescence when it is introduced between double-stranded DNA, and it also emits red fluorescence if it is related to single-stranded DNA (82). This method can be used to assess fresh or frozen samples and comprises only two main methodological steps. In the first step, spermatozoa are treated with a low pH (1.2) detergent solution to denature their chromatin and allow its subsequent staining. In the second step, the sperm suspension is neutralized,

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Table 3 Summary of the principle, advantages and disadvantages of the four commonly used SDF assays

Assay	Direct or indirect	Principle	Type of DNA fragmentation	Advantages	Disadvantages
TUNEL	Direct	Labelling of free 3'-OH nicks with dUTP at the DNA break	SSBs & DSBs simultaneously	• Highly standardized protocol • Minimal inter-observer variability • Testing can be done on both fresh and frozen specimens	• More technically demanding–equipment training • No clear cut-off values
SCSA	Indirect	Evaluation of DNA integrity with acid denaturation followed by Acridine Orange staining	SSBs & DSBs simultaneously	• Simultaneous examination of a large number of cells • Highly standardized protocol • Testing can be done on both fresh and frozen specimens	• More technically demanding–equipment training • Commercial kits are not available
SCD	Indirect	Evaluation of the dispersed chromatin ("halo") after lysis of sperm membranes	SSBs & DSBs simultaneously	• Highly standardized protocol • No special equipment training required • Both fresh and frozen-thawed samples can be used	• Inter-observer variability
Comet	Direct	Single-cell gel electrophoretic separation	SSBs and/or DSBs	• Possibility to detect SSBs and DSBs separately at the same time • Both fresh and frozen-thawed samples can be used	• Poor repeatability • High inter-observer variability • Non standardized protocols and thresholds

SDF, sperm DNA fragmentation; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling; SCSA, sperm chromatin structure assay; SCD, sperm chromatin dispersion; SSBs, single-stranded breaks; DSBs, double-stranded breaks.

and the cells are stained with a second solution containing AO. Data for red and green fluorescence obtained from analyzing 5,000–10,000 spermatozoa per sample are collected by flow cytometry and transformed. The degree of red fluorescence in the sperm population corresponds to DFI, which represents the percentage of cells with broken DNA. The analysis of the collected data can be done in any flow cytometric software. SCSA also needs flow cytometry, similar to TUNEL assay, and is also able to analyse many (5,000–10,000) spermatozoa within few seconds and thus provides a less subjective result (89). Moreover, this method also reports % high DNA stainability (HDS) which indicates the extent of stainability of the sperm nucleus, representing immature spermatozoa (90). The DFI threshold for male infertility diagnosis using SCSA is reported as 20–25% (7). In a prospective study on 1,316 infertile couples who underwent IVF and 266 who underwent ICSI, a DFI threshold using SCSA of <11.3% was predictive of pregnancy success after IVF with 56.1% sensitivity and 60% specificity, and a threshold of <30.3% was predictive of pregnancy success after ICSI with 50.6% sensitivity and 68.8% specificity (91). In this study, thresholds were determined using ROC analysis.

SCD

SCD test is a method to evaluate the sensitivity of sperm DNA to acid denaturation. The principle of SCD is based on the fact that intact DNA loops expand following denaturation and extraction of proteins in the nucleus, whereas when DNA is fragmented, dispersion does not develop or is minimal. This method relies on the capacity of the intact sperm chromatin to create dispersion halos after exposure to acid and a lysing solution; the halos correspond to loosened DNA loops attached to the residual structure of nuclear, which are released after the removal of

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nuclear proteins. The DNA breaks prevent this dispersion as they are susceptible to denaturation (92). Different from TUNEL and SCSA, this method does not need any advanced equipment and can be performed in a private clinic or office setting using commercially available assay kits. However, SCD analyses only 300–500 spermatozoa manually under the microscope, which can create inter-observer variability (20). DFI thresholds to diagnose male infertility using SCD range from 16–30%, but 20% is considered to be the best (7). In one report, a threshold of 22.5% SDF using SCD, with 76.8% sensitivity and 92.9% specificity was predictive of achieving pregnancy after natural conception (21).

Comet assay

The name of the test is related to the 'comet' appearance under fluorescence microscope of the stained unwound DNA fragments that are detached from the sperm head after electrophoretic movement. It is a method to evaluate SDF in individual sperm based on the differential migration of broken DNA strands influenced by an electric field depending on the size and charge of the strands (19). In Comet, the spermatozoa are migrated in an agarose matrix, wherein a detergent-induced lysis is promoted under high salt conditions. During an electrophoresis step, the broken DNA strands migrate towards the anode, generating the characteristic dispersion pattern that resembles a comet tail. The intact DNA constitutes the comet's head, while the fragmented strains of DNA constitute the comet's tail. The relative fluorescence of this last element compared to the head serves as a measure of the amount of DNA damage. The average amount of DNA fragmentation across is shown as the average Comet score, which is a calculated by the analysis of 100 individual comets (spermatozoa) (93). Neutral Comet assay uses only controlled protein depletion to detect DSBs selectively while the 2-dimensional Comet assay applies two electrophoretic runs to map SSBs and DSBs simultaneously (94). Alkaline Comet Assay, where a buffer of pH >13 is used, can detect SSB more sensitively compared to neutral (95), and it is now more commonly used in andrology labs than the neutral assay. In one report, a threshold of 26% by alkaline comet (average comet score) is reported to discriminate fertile from infertile men with an AUC of 0.925 (sensitivity: 73%; specificity: 100%) (96). Similar to SCD, Comet assay does not need advanced equipment but only commercially available assay kits. As descried above, among the four currently available methods, Comet has the ability to distinguish between SSBs and DSBs. However, only a small number [100] of spermatozoa can be analysed manually, the same as SCD, which creates a chance for inter-observer variability (20).

SDF testing thresholds

SDF thresholds to enable precise diagnosis and management are lacking. The cut-off values reported in the literature demonstrate an increased variability, ranging between the extremes of 4% to 56% and this uncertainty persisted over the last 20 years despite significant data contribution from several research groups (20). This situation has been proven difficult to resolve due to multiple factors surrounding SDF testing such as; the variability of the techniques, the study population, the infertility factor, the reproductive outcomes tested and the laboratory specialization and experience. Additionally, potential intrinsic and extrinsic factors that may impact the interpretation of SDF test results such as; female factor infertility, patient's age, basic and advanced semen variables, genetic repair mechanisms, semen processing and conditions, ART method are not fully addressed. Furthermore, the standardization of SDF assessment was not resolved in the latest 6th Edition of the WHO manual for human SA (5). Although the manual highlights the indications of SDF testing and provides an updated guide on laboratory protocols of the few techniques, it lacks threshold definition for each test and recommends adopting individual reference ranges according to each laboratory operational procedures and clinical experience on representative values, echoing fertility dynamics.

In light of this heterogeneity in SDF testing methods and thresholds, a recent meta-analysis by Santi *et al.*, suggested a universal cut-off value of 20% for the discrimination between fertile and infertile males (7). A recent study by Agarwal *et al.* also suggested a unanimous 20% cut-off for all laboratory assessment methods (20). Similarly, Esteves *et al.* suggested a 20% threshold for SCSA, TUNEL, and SCD, but differed in the assessment through Comet method by suggesting a cut-off value of 26% (97). In terms of reproductive outcome prediction, Esteves *et al.*, concluded that SDF values of 20–30% appear to correlate with adverse pregnancy outcomes through either natural conception or assisted reproduction (97). Nevertheless, formal guidelines are warranted to alleviate the existing discrepancies and that can be clinically implemented to utilize the crucial information offered through this diagnostic utility (98).

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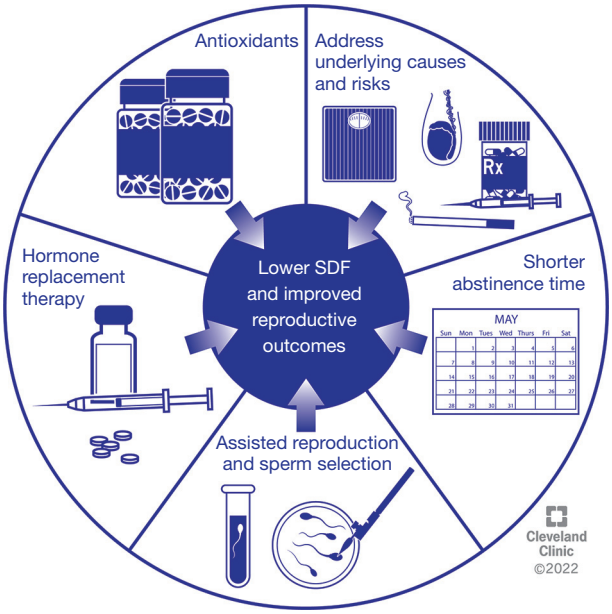

Figure 2 Many therapeutic approaches exist to help men with elevated SDF improve their reproductive outcomes. Underlying causes and etiologies, such as varicocele, male genital tract infections, obesity, and lifestyle risk factors should be addressed. Antioxidants can be supplied to counteract the oxidative stress implicated in the pathogenesis of SDF. Reduced ejaculatory abstinence has also been shown to reduce SDF. Hormonal therapy may be given, and finally men can be directed towards assisted methods of reproduction as well as advanced sperm selection techniques. Reprinted with permission, Cleveland Clinic Foundation ©2022. All Rights Reserved. SDF, sperm DNA fragmentation.

Management of elevated SDF

Several strategies can be employed to reduce SDF among infertile men before natural or assisted reproduction to improve reproductive outcomes. Known underlying causes and risk factors of high SDF such as smoking and environmental exposures should be addressed (98). Also, suitable antibiotic therapy should be considered for male genital tract infections which are associated with elevated SDF (99). The different strategies that can be used to improve sperm DNA integrity are summarized in *Figure 2* and are discussed in the subsequent sections.

Weight loss

SDF was found to be significantly higher among obese men [body mass index (BMI) ≥30 kg/m^2] and men with metabolic syndrome (100-102). Therefore, weight loss as a means to reduce SDF, has been studied. In a prospective study, 105 infertile men with above than normal BMI were given a diet and exercise weight loss program for 12 weeks, and reported significant reductions in their mean SDF after they completed the program (103). The impact of surgical weight loss on SDF has also been studied with promising results (104,105).

Varicocele repair

Men with clinical varicocele were found to have significantly elevated SDF levels compared to healthy controls (106). Varicocelectomy can reduce SDF levels and improve fertility outcomes in this population of infertile men. A prospective study that included 60 infertile men with clinical varicocele reported significant reductions in their mean SDF from 29.5% to 18.8% (P<0.001), 3–6 months after varicocelectomy (107). A recent meta-analysis including 394 infertile men with clinical varicocele indicated significant reduction of SDF following varicocele repair (108). Furthermore, significantly improved clinical pregnancy and live birth rates after ICSI were reported in the group of infertile men with clinical varicocele that underwent repair (109).

Antioxidants

Given the important role of seminal OS in the pathogenesis of SDF (8), many researchers have investigated the use of different antioxidants to improve sperm DNA integrity and fertility outcomes. For example, Jannatifar *et al.* supplied 50 infertile men with n-acetyl cysteine for 3 months and reported a significant decline in their mean SDF from 19.3% to 15.1% (P=0.01) (110). Alahmar *et al.* studied the impact of co-enzyme Q10 supplementation to 65 infertile men with idiopathic oligoasthenozoospermia, and also reported significant reductions in SDF from 35.6% to 30.9% (P<0.001) after treatment, as well as improvement in conventional semen parameters and markers of OS (111). In 2005, Greco *et al.* treated infertile men who have failed an initial cycle of ICSI and had ≥15% SDF with a combination

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of daily vitamin C and vitamin E, and reported significantly improved pregnancy and implantation rates with the second ICSI attempt after two months of treatment (112).

On the other hand, some studies have published contradicting results. The MOXI trial was a multi-center randomized clinical trial that included 171 infertile men with at least one abnormal conventional semen parameter or SDF ≥25%, who received either a combination antioxidant regimen for 3–6 months or a placebo. After 3 months of treatment, there was no significant difference in percentage DNA fragmentation between the antioxidant and the placebo group. Furthermore, reproductive outcomes including clinical pregnancy rates, first trimester miscarriage rates and live birth rates were fairly similar between both groups (113). Another randomized clinical trial supplied infertile men with a combination of zinc and folic acid or a placebo for 6 months, and reported significantly higher DFI among the group that received antioxidants with a statistically significant mean difference of 2.4% (114). To explain this adverse outcome of antioxidants on SDF, it is important to keep in mind that not all SDF is caused by OS, with other pathophysiologic mechanisms playing a role as discussed earlier in this review. In fact, any disruption of the redox balance within the male reproductive system may lead to infertility, and supplying excessive or unwarranted antioxidants may tilt the scale towards reductive stress which, similar to OS, can also cause infertility by affecting the physiologic functions of endogenous ROS such as sperm capacitation and sperm DNA integrity (115). Supplying antioxidants may impair adequate maturation of the sperm nucleus, as excessive antioxidants prevent the creation of disulfide bridges between protamines, making sperm DNA less condensed and more susceptible to exogenous insults and damage, therefore increasing SDF (31).

Overall, several studies that explore the effects of antioxidants therapy on SDF have been published (116,117). Various antioxidant regimens, whether single agents or combination therapy, were supplied to the populations of infertile men by the different studies and for different treatment durations, mostly ranging from two to six months. The population of infertile men included also differed among the different studies, for example some included infertile men with abnormal conventional semen parameters, others have also looked into seminal OS, while others have included infertile men of couples who have failed ART or infertile men with known underlying conditions such as subclinical varicocele, leukocytospermia, and environmental exposure and studied the impact of antioxidant supplementation in them. The heterogeneity of the various studies in terms of patient selection, antioxidant regimen and dose, duration of treatment, and different conditions that may be associated with SDF, together with the scarcity of well-designed randomized clinical trials and the contradicting evidence published, as well as the potential harms have precluded the ability to make specific recommendations on the appropriate antioxidant treatment regimen to infertile men in general, and in those with elevated SDF in particular.

Shorter abstinence

A shorter ejaculatory abstinence is associated with lower levels of SDF, with semen collected after 1–2 days of abstinence containing significantly less SDF (9–10%) as measured by TUNEL assay, and semen collected after 9–11 days of abstinence containing significantly higher SDF (15–21%) (P<0.001) (118). This has also been demonstrated on clinical grounds with improved reproductive outcomes after IUI with an improved clinical pregnancy rate when an ejaculatory abstinence of two or less days was used compared to three or more days (11.3% *vs.* 6–7.3%; P<0.05) (119) and significantly improved pregnancy rates after ICSI, when recurrent ejaculation was performed in the last four days before the procedure and a final abstinence period of 12 hours was used (120). Therefore, reduced ejaculatory abstinence and recurrent ejaculation can be recommended to men with elevated SDF in an attempt to improve ART outcomes (98).

Hormonal therapy

Follicle stimulating hormone (FSH) is a commonly prescribed medical therapy for men with IMI and has been associated with improved semen parameters and fertility outcomes (121). FSH has also been shown to improve DNA integrity, as demonstrated by a meta-analysis that included 6 studies and reported a small but significant improvement in SDF after FSH therapy (7). However, the evidence presented by this analysis is not reliable, as the included studies are very heterogeneous in terms of the patients included, the regimen of hormonal therapy, and the methods of measuring SDF (122). Furthermore, a pharmacogenetic study found that FSH treatment significantly improves SDF only in men with IMI that show NN genotype of the FSH receptor, but not the SS genotype (123). Given the poor

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quality of evidence and the possible genetic contribution to response, FSH therapy was not included in the recently published guidelines regarding the approach and management of SDF (20,97).

Advanced sperm selection

Given the less impact of SDF on pregnancy rates after ICSI, couples with persistently elevated SDF may be directed to this ART procedure (20). However, elevated SDF may still be associated with miscarriage, even after ICSI. Several methods of advanced sperm selection can be used to reduce SDF before sperm is used for any ART procedure. These include:

- Magnetic activated cell sorting (MACS): uses Annexin V beads to bind phosphatidylserine on the surface of apoptotic sperm and removes them, thereby lowering SDF (124). In a retrospective study, the outcomes of ICSI performed for infertile men with SDF >20% as measured with TUNEL, were compared between 366 men for whom MASC was performed after density-gradient centrifugation (DGC) and 358 men who underwent ICSI after DCG without MACS (125). Although fertilization rates were similar for both groups, the MASC group had significantly higher clinical pregnancy (60.7% *vs.* 51.5%; P=0.014), and live birth rates (47.4% *vs.* 31.2%; P=0.001), and lower miscarriage rate (14.7% *vs.* 20.6%; P=0.034) compared to the DCG only group.
- Intracytoplasmic morphologically selected sperm injection (IMSI): uses microscopy to choose motile sperm devoid of vacuoles in their head, which have been found to contain less SDF (126). In a trial that included 80 infertile men, SDF measured by TUNEL was significantly lower in the IMSI group compared to the ICSI group (P<0.0001) (127). A meta-analysis that included studies comparing IMSI and ICSI outcomes for infertile men, reported significantly improved implantation rates (OR =1.56; P=0.01) and pregnancy rates (OR =1.61; P=0.004) for IMSI compared to ICSI, but no difference in miscarriage rates (128).
- Physiologic ICSI (P-ICSI): uses hyaluronic acid binding to choose mature sperm which were found to contain significantly lower SDF compared to initial unprocessed sperm (5.3% *vs.* 16.5%; P<0.001) (129). When compared to MACS, no significant differences were found for implantation, clinical pregnancy, and ongoing pregnancy rates overall, but when stratified for female age, outcomes were better when P-ICSI was used for women older than 30 versus MACS, although not statistically significant (130).
- Microfluidic sperm sorters: passes sperm through microchannels using fluid dynamics to choose motile sperm, which have been found to have significantly lower DFI compared to sperm processed by conventional techniques of centrifugation and swim-up (0.8% *vs.* 10.1%; P=0.0014) (131).
- Testicular-derived sperm: a meta-analysis that included studies comparing SDF and reproductive outcomes between testicular sperm and ejaculated sperm concluded that SDF is significantly lower in testicular sperm (8.9% *vs.* 33.4%, P<0.0001) and when used for ICSI, is associated with significantly improved reproductive outcomes including clinical pregnancy (OR =2.42, P<0.001) and live birth rates (OR =2.58, P<0.001) and lower miscarriage rates (OR =0.28, P=0.005) (132).

When discussing sperm selection, it is important to point out that little evidence is available regarding the benefit in improving SDF and reproductive outcomes. Furthermore, conflicting evidence exists, for example in a retrospective study on IMSI, sperm head vacuoles were not associated with SDF or live birth rates (133). Well-designed clinical trials that investigate the effect of the various sperm selection methods for infertile men and the impact on ART outcomes are needed in order to make strong recommendations. As for testicular sperm, although it has been recommended for use as a last resort in cases of elevated SDF by the recent guidelines, the evidence behind the use of this invasive method is of poor quality and with no valid SDF testing for testicular sperm (98). Furthermore, a prospective study investigating the outcomes of ICSI with testicular sperm compared to ejaculated sperm, in infertile men with elevated SDF and previous ICSI failure, reported no benefit of testicular sperm in improving reproductive outcomes (134).

Future of SDF research: a SWOT analysis

Recently, two different guidelines on SDF, based on high-quality evidence, have been published by two research groups (20,97). In order to evaluate the current state of research on SDF, we analysed these sources by applying the SWOT (strengths, weaknesses, opportunities, and threats) analysis

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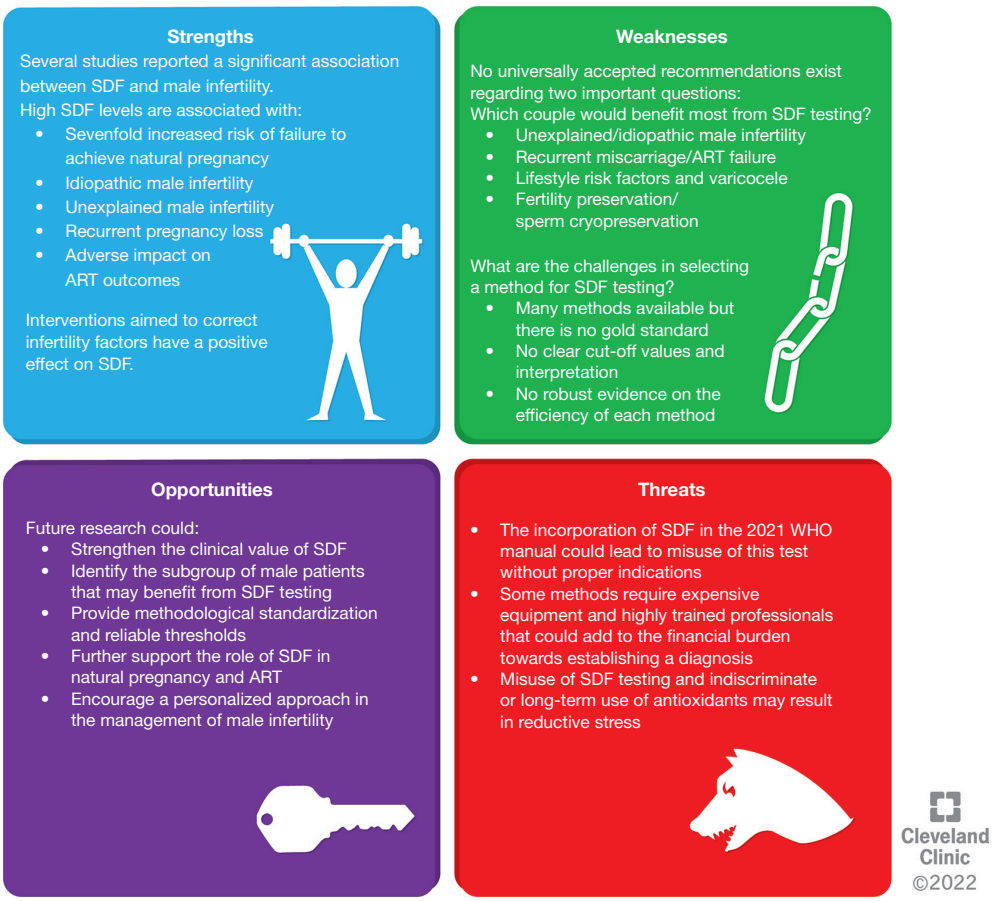

Figure 3 A SWOT analysis into the use of SDF testing in clinical practice. Reprinted with permission, Cleveland Clinic Foundation ©2022. All Rights Reserved. SWOT, strengths, weaknesses, opportunities and threats; SDF, sperm DNA fragmentation; ART, assisted reproductive technologies.

framework, a model derived from the business world that has already been used in the field of reproductive medicine research (135). The results are presented in *Figure 3*.

Strengths

Several studies reported that SDF negatively impacts male fertility. In particular, SDF levels inversely correlate with probability of natural pregnancy and high SDF levels are associated with a 7-fold increased risk of failure to achieve natural pregnancy (20). Moreover, high SDF can be found in up to 20% (97) or 30% (20) of idiopathic infertile couples and in subjects with varicocele (20). Higher levels of SDF were also observed in couples with RPL than in fertile couples (20,97). Interventions aimed to correct infertility factors such as varicocelectomy and changes in lifestyle habits have a positive effect on OS and SDF (20,97).

Weaknesses

Unfortunately, despite the validity of SDF as an infertility marker, there are uncertainties about its actual clinical application. In particular, two important questions remain unanswered: which couples would benefit most from SDF testing and how should the test be conducted? To answer the first question, Agarwal *et al.* suggested SDF testing in men with UMI, IMI, recurrent miscarriage, lifestyle risk factors, varicocele and recurrent ART failure (Grade C recommendation—low quality of evidence). Esteves *et al.* offer similar indications (recommendation grade based on levels of evidence B-D—moderate-very low quality of evidence) and suggest SDF testing also for men undergoing sperm cryopreservation (Grade D recommendation). Regarding the best investigation technique and optimal cut-offs, Agarwal *et al.* conclude that the TUNEL assay is

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the most commonly used and it is reliable and accurate, but high heterogeneity of published data exists to establish clear cut-off values. Esteves *et al.* provide a review of all the limitations and advantages of the different methods, but do not express a preference for any of them (20,97).

Opportunities

Given that the association between SDF and male infertility is strong, future efforts should focus on investigating the possible practical uses of this parameter. In particular, it is necessary to establish which subgroup of infertile couples can benefit most from the identification of high levels of SDF. Also, standardisation of the testing methods and providing reliable references values are warranted. Since reducing SDF levels could be a valid objective in the treatment of infertility, it could be an excellent opportunity for high-quality interventional research.

Threats

Despite the introduction of SDF assay in the 6th edition of WHO laboratory manual as an important extended test of semen (136), there are still many uncertainties about this type of investigation. Its indiscriminate use may not bring benefits outside specific contexts that should be identified. Furthermore, some tests such as the TUNEL assay require expensive equipment and need highly trained laboratory personnel.

Conclusions: summary of key points

- Currently, SDF is recognized as an important parameter in the practice of male infertility. There is growing evidence that high SDF negatively impacts reproductive outcomes under natural and ART conditions.
- Defects in sperm chromatin packaging, apoptosis, and excessive seminal OS are underlying pathophysiological mechanisms that lead to SDF, which is associated with many conditions and risk factors including: aging, varicocele, genital tract infections, poor lifestyle habits, and noxious environmental or occupational exposure.
- The procedures that are commonly used for SDF assessment include the TUNEL, SCSA, SCD, and Comet. The choice of assay depends on trained personnel and availability of instrumentation, and the diagnostic thresholds should be determined and validated by the performing laboratory.
- The ASRM guidelines do not recommend routine SDF test in the evaluation of male infertility due to lack of information. On the other hand, the ESHRE and EAU guidelines consider SDF in couples with RPL. The WHO 2021 manual describes SDF testing as a potentially important addition in the work-up of infertile men.
- Several strategies are suggested to reduce SDF among infertile men before natural or assisted conception including: (I) control of body weight; (II) reduce smoking and environmental exposures; (III) treatment of genital tract infection; (IV) varicocele repair; and (V) antioxidant therapy. In addition, several methods of sperm selection can be used in ART procedure including MACS, IMSI, P-ICSI or testicular sperm.
- Strong evidence is available on the negative impact of SDF on male fertility potential. However, the quality of evidence and the heterogeneity of the available studies prevent the ability to make strong recommendations regarding indications of SDF testing, assays for SDF testing and their cut-off values, and treatment of infertile men with elevated SDF.

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