

What should be done for men with sperm DNA fragmentation?

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In an age when a small quantity of sperm can lead to pregnancy through *in vitro* fertilization or intracytoplasmic sperm injection, selecting healthy sperm is important. Sperm DNA fragmentation (SDF) is known to be higher in infertile men. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) and the alkaline comet test are SDF tests that directly measure DNA damage and have shown closer correlations with assisted reproduction results than indirect tools such as the sperm chromatin structure assay or the sperm chromatic dispersion test. It is difficult; however, to endorse a single test as the best test overall; instead, it is best to select a testing method based on each patient's clinical condition and goals. In a couple struggling with infertility, if the male partner has a high level of SDF, he should aim to decrease SDF through lifestyle modifications, antioxidant treatment, and ensuring an appropriate duration of abstinence, and physicians need to treat the underlying diseases of such patients. If sperm DNA damage continues despite the patient's and physician's efforts, other methods, such as micromanipulation-based sperm selection or testicular sperm extraction, should be used to select healthy sperm with nuclear DNA integrity.

Keywords: Assisted reproductive technique; DNA fragmentation, Infertility; Sperm

Introduction

Over 15% of married couples worldwide experience fertility problems, and 50% of these cases are caused by male factor infertility [1]. Semen analysis is the most important test for evaluating male infertility, but it does not provide information regarding all functions of sperm, nor is it sufficient for predicting male fertility potential and the likelihood of success of assisted reproductive technology (ART) [2]. Furthermore, the standards for normal values in semen analysis do not reflect average values, but are instead determined using the bottom 5% as a cut-off point. In other words, it is the minimum standard for pregnancy. In fact, 15% of patients with male infertility were

found to have normal semen analysis results [3]. Therefore, additional tests should be performed due to the limitations of using semen analysis results alone to evaluate male fertility potential. Research on new testing methods to evaluate sperm abnormalities has been conducted in the past 30 years, and sperm DNA integrity has emerged as an area of interest. Sperm DNA fragmentation (SDF) testing measures the quality of sperm as a DNA package carrier, and it therefore is more significant than the parameters analyzed in previous semen analyses [4]. DNA damage, such as fragmentation and denaturation, can have adverse effects on fertilization and embryo development and can cause infertility [5]. Infertile men have a greater extent of sperm DNA damage and poorer sperm DNA integrity than fertile men, and the fertilization of DNA-damaged spermatozoa can increase the risk of genetic diseases in the offspring [6]. SDF can be observed even in men with normal semen analysis results [7]. The value of SDF as an independent index for the evaluation of semen quality has led it to be incorporated into semen analysis procedures [8]. However, as intracytoplasmic sperm injection (ICSI) has become more common worldwide, cases of successful fertilization after ICSI

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despite poor semen analysis results or sperm DNA damage have led to questions regarding the clinical value of SDF testing [9]. We are living in the age of ICSI, which overcomes many of the barriers of natural selection. The selection of sperm with damaged DNA when using ART can result in undesirable results, such as lower pregnancy success rates, increased rates of miscarriage, chromosomal abnormalities, and other genetic or birth defects in the offspring. Therefore, it is important in the age of ART to accurately understand and manage sperm nuclear DNA integrity.

This review will discuss the clinical utility of current tests of sperm DNA damage, the effects of sperm DNA damage on offspring conceived through natural pregnancy and as a result of ART, and examine possible treatment strategies for extensive sperm DNA damage.

SDF test

Various techniques have been used to measure damage to human sperm DNA. Currently, the most widely used testing methods are sperm nuclear DNA integrity assessment and abnormal sperm chromatin packaging assessment. Direct sperm DNA integrity assessments include the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay and *in situ* nick translation (ISNT), which use reagents that attach directly to the damaged area. Indirect types of sperm DNA integrity assessments include the sperm chromatin structure assay (SCSA) and the sperm chromatin dispersion (SCD) assay, which indirectly measure the damaged area through protein denaturation in an acidic solution, as well as the comet assay. Sperm chromatin packaging assessment is a staining method that uses methyl green, aniline blue, toluidine blue, and chromomycin A3. This review will explain the currently most widely used DNA fragmentation assessments: the TUNEL assay, ISNT, SCSA, the comet assay, and the SCD assay.

1. TUNEL assay

The TUNEL assay is a method that directly measures sperm DNA damage through the attachment of deoxyuridine triphosphate (dUTP) to single- and double-strand DNA breaks using terminal deoxynucleotidyl transferase [10]. Quantification by flow cytometry and qualitative observation through fluorescence or light microscopy are possible [11]. The measured value is used as an index to evaluate male infertility [12], and it is known to be able to predict ART results [13-17]. Duran et al. [15] suggested that pregnancy is difficult if the SDF shown in a TUNEL assay is higher than 12%. The assessment can be performed with a small amount of sperm (200 spermatozoa or fewer), and like testicular biopsy, an assessment can be done with very few sperm cells, since individual cells can be distinguished from each other [18]. This method is advantageous because no additional

costs are needed, since most fertility clinics can conduct fluorescent antibody tests and have high-quality microscopes. However, this method is more expensive than other methods, results can vary depending on researcher and lab (i.e., it has high intra-assay and inter-laboratory variability), and it is limited in evaluating immature cells such as those with high DNA stainability (HDS) in SCSA [18].

2. *In situ* nick translation

ISNT is a variant of TUNEL assessment that quantifies biotinylated dUTP that attaches to single-strand DNA breaks through DNA polymerase [19]. Single-strand DNA breaks have a higher probability of being repaired by an embryo than double-strand breaks [9], and their correlation with fertilization in *in vivo* studies was not proven [4]. Therefore, the clinical value of ISNT, which only measures single-strand breaks, is extremely limited [4], and it also lacks sensitivity compared to other assessments [9].

3. Sperm chromatin structure assay

Developed in the 1970s, SCSA was revised a few times before becoming the most common commercially used test for investigating causes of male infertility related to sperm DNA damage and chromatin abnormalities. The degree of DNA denaturation is determined by measuring the changes in color of acridine orange in the DNA, from green fluorescence to red fluorescence, after heat or acid treatment [20]. The most important parameters are the DNA fragmentation index (DFI, %), which indicates the number of cells with DNA damage [21], and HDS (%), which indicates the proportion of immature sperm with defects in the histone-to-protamine transition [11]. In earlier research, Evenson et al. [21] and Spano et al. [22] suggested that a DFI of 30% or higher and an HDS rate of 15% indicated that the likelihood of *in vivo* fertilization would be close to zero. Virro et al. [23] reported that HDS rates of 15% or higher were associated with lower fertilization rates in *in vitro* fertilization (IVF), meaning that the threshold of 15% could be an indication for ICSI, that men with high levels of SDF (30% or higher) were at greater risk of low blastocyst transfer, causing difficulties for ongoing pregnancies, and that these parameters could provide valuable prognostic information to couples planning IVF or ICSI. The advantages of this test are that a large number of sperm cells and a large sample can be analyzed quickly, reproducibility is high, and freezing does not affect the test, making it more flexible [18,24,25]. The disadvantages, however, are that the testing requires expensive equipment and a high concentration of sperm, and that the reference range for the sample needs to be calibrated [18,26].

4. Comet assay

The comet assay is a simple and affordable testing method that measures the degree of sperm DNA damage qualitatively by visualiz-

ing single- and double-strand breaks using electrophoresis [27]. An image that looks like a comet with a full head and tail emerges [28], and double-strand DNA appears at the head of the comet, while damaged double- and single-strand DNA fragments move towards the tail part [29]. The higher the level of damage to the DNA, the brighter and longer the comet tail. Additional parameters such as the diameter of the nucleus, olive tail moment, and comet length have been used to improve the efficiency of the test [30]. Double-strand DNA damage can be measured in a neutral buffer, but an alkaline buffer can detect all single- and double-strand DNA damage, as well as alkali-labile sites [11]. The alkaline comet assay can be used for all cell types, including sperm, and only a few cells are needed for analysis [30]. The clinical significance of the comet assay in male infertility assessment has been confirmed by many authors [4,31-33]. However, the protocols of the comet assay have not been standardized to the point that it is possible to fully understand and compare research results from various researchers [11,18]. It is difficult to distinguish between endogenous DNA breaks and induced DNA breaks in the alkaline comet assay, and the extent of DNA damage can be underestimated due to entanglement of DNA strands. Some do not recommend clinical use of this assay because the small piece of the tail may be lost or difficult to visualize [34]. Enciso et al. [35] introduced the two-tailed comet assay, which can distinguish between single-strand DNA breaks and double-strand DNA breaks in the same sperm cell, and used the method to research the structure and function of sperm DNA.

5. SCD assay

The SCD assay is a testing method based on the characteristic halo that is formed when nuclear proteins are removed after acid denaturation [36]. In other words, sperm nuclei with severe DNA damage will form a very small halo or no halo at all, while sperm with less DNA damage will disperse DNA loops and form a large halo. This is an economically feasible method that is commonly used in male infertility laboratories since it is simple, quick, has high reproducibility, and does not require complex instruments; furthermore, quality management is also straightforward [18]. However, the peripheral border of the halo with low chromatin density may sometimes not be distinguishable from the background, not all halos are on the same focal plane, which can cause errors, and since the sperm tail is not preserved, the sperm needs to be distinguished from other contaminant cells.

Clinical significance of sperm DNA damage for pregnancy

In the following section, the importance of SDF testing in assessments of couples struggling with fertility issues and counseling strategies for patients with high SDF will be reviewed, on the basis of ob-

servations regarding the relationship between sperm DNA integrity and pregnancy outcomes.

1. Natural conception

Many studies have demonstrated that sperm DNA damage affects natural conception. Zini [37] reported that based on an SCSA analysis, high SDF made natural conception difficult. Evenson et al. [21] and Spano et al. [22] also stated that natural conception was almost impossible if SCSA found SDF of more than 30%. Furthermore, it has been reported that a higher extent of sperm DNA damage in couples planning a pregnancy without prior knowledge of their fertility could cause them to take longer to conceive naturally and reduce the likelihood of a successful pregnancy [22]. Therefore, SDF testing is relevant for male fertility through natural conception and can be applied to couples struggling with infertility with an unknown cause and used when counseling such couples regarding future methods of becoming pregnant.

2. Intrauterine insemination

Bungum et al. [38] reported that the possibility of pregnancy using intrauterine insemination (IUI) was close to zero when the DFI was higher than 30% in SCSA and that elevated DFI levels (>30%) were a predictor of decreased pregnancy and delivery rates after IUI. Duran et al. [15] reported in another study that pregnancy was difficult when the TUNEL method indicated a proportion of sperm DNA damage of 12% or higher. On the contrary, Murie et al. [39] suggested that there was no correlation between sperm DNA damage and clinical pregnancy rates after IUI when samples were analyzed using the SCD test. Measuring sperm DNA damage is beneficial in predicting IUI results ahead of time, and when sperm DNA damage is extensive, other methods such as IVF or IVF/ICSI should be considered.

3. In vitro fertilization

Much research has been conducted into the influence of SDF on the results of IVF and IVF/ICSI, but the results of these studies are complex and varied. This is due to the challenges of interpreting results from studies with heterogeneous designs and mixed protocols. Most studies reported that SDF did not affect fertilization or embryo quality, most likely because maternal regulation plays a predominant role during blastocyst development, while the effects of paternal genes are seen after the four-cell stage [40,41]. Recent studies confirmed that paternal factors and sperm DNA damage affect embryo development and early pregnancy [31,42]. Zini [37] conducted a meta-analysis of 11 studies and found a correlation between abnormal sperm DNA damage test results and low pregnancy rates. Zhang et al. [43] observed, in a meta-analysis of nine studies on IVF research, that a DFI lower than 27% was associated with higher clinical pregnancy rates.

4. Intracytoplasmic sperm injection

Collins et al. [44] conducted a meta-analysis of 13 studies on IVF and ICSI and found that sperm DNA damage had a significant influence on pregnancy rates. A meta-analysis of 14 studies conducted by Zini [37] showed that the difference in the pregnancy rate between a group with high sperm DNA damage and a group with low sperm DNA damage was 11%, but the result was not statistically significant. However, a meta-analysis of five studies conducted by Zhang et al. [43] suggested that sperm DNA damage did not lead to a difference in the clinical pregnancy rate after ICSI. Anifandis et al. [45], in a prospective cohort study, suggested that when clinical and ongoing pregnancy rates and cleavage-stage embryo quality were measured by SCD, there was no correlation with the degree of sperm DNA damage. Wdowiak et al. [46] discovered through continuous time-lapse monitoring that the degree of sperm DNA damage was related to the clinical pregnancy rate after elective single blastocyst transfer and an assessment of embryo development dynamics. The greater the extent of sperm DNA damage, the longer it took for the embryo to reach the blastocyst stage and lower the possibility of pregnancy through ICSI. However, the live birth rate is more important than the clinical and ongoing pregnancy rates. Osman et al. [47] reported that couples with low levels of male sperm DNA damage had high live birth rates after IVF and/or ICSI. However, most studies did not include the actual live birth rate after ICSI in their research, so care should be taken in interpreting their results appropriately, without overestimation or underestimation of their clinical significance.

5. Effect on assisted reproduction results

An analysis of the previous literature showed conflicting results regarding the correlation between sperm DNA damage and ART outcomes. According to some studies, sperm DNA damage has a major impact on pregnancy, meaning that sperm DNA testing should be included in routine clinical examinations [48]. In contrast, some other clinical reviews did not support the clinical use of sperm DNA damage tests [44,49]. In a recent study, Simon et al. [50] conducted a meta-analysis of 120 studies that analyzed ART results and sperm DNA damage. Of the 92 studies that analyzed the relationship between SDF and ART, 35 observed a significant inverse relationship between SDF and the fertilization rate, but the other 57 studies did not find significant relationships. The inverse relationship between sperm DNA damage and the fertilization rate for each procedure was stronger in IVF (59% or 19 of 32) than in ICSI (24% or 10 of 42) or IVF and ICSI (33% or 6 of 18). An adverse effect of DNA damage on the fertilization rate was more commonly observed in IVF procedures; in contrast to ICSI, in which the most morphologically normal and motile spermatozoon is injected into the egg, the sperm that fertilizes the egg in IVF is randomly selected by sperm-oocyte interactions [51]. Of

the 80 studies that analyzed SDF and embryo quality, 27 indicated that SDF had a significant effect on embryo DNA, while the other 53 studies did not show a significant relationship between these parameters. Reviewing these studies by the type of analysis, 64% of the studies that used the comet assay stated that sperm DNA damage had an adverse effect on embryo quality, and similar results were shown for 25% of the TUNEL studies, 24% of the SCSA studies, and 40% of the SCD studies. When classified by ART type, 36% of the IVF studies, 24% of the ICSI studies, and 50% of the mixed IVF and ICSI studies observed adverse effects of DNA damage. Therefore, if the studies are divided based on the type of analysis, we can see a differential association between sperm DNA damage and embryo quality. In particular, the degree of sperm DNA damage detected by the alkaline comet assay was higher than that detected using other methods, which may have been due to the sensitivity of the comet assay, which measures both single- and double-strand DNA damage through complete decondensation of sperm chromatin [31]. In an analysis of 70 studies, the odds ratio (OR) for the effect of sperm DNA damage on clinical pregnancy after ART in all studies was 1.15 (95% confidence interval [CI], 1.08–1.54; $p < 0.0001$), while the corresponding OR for IVF studies was 1.15 (95% CI, 1.05–1.27; $p = 0.0033$), that for ICSI studies was 0.89 (95% CI, 0.80–0.99; $p = 0.0254$), and that for mixed IVF and ICSI studies was 2.00 (95% CI, 1.66–2.41; $p < 0.0001$), indicating that sperm DNA damage is related to clinical pregnancy after ART. The ORs for the studies classified by testing method were as follows: TUNEL, 1.85 (95% CI, 1.52–2.26; $p < 0.0001$); SCD, 1.16 (95% CI, 1.02–1.32; $p = 0.0233$); comet, 4.15 (95% CI, 3.04–5.68; $p < 0.0001$); and SCSA, 0.88 (95% CI, 0.80–0.96; $p = 0.0041$), indicating that the TUNEL and alkaline comet tests, which directly measure DNA damage, showed a closer correlation with pregnancy outcomes than SCSA and SCD, which measure DNA damage indirectly [50].

6. Pregnancy loss

Current research on the effects of SDF on miscarriage after ART is limited; however, a recent meta-analysis investigated the correlation between high levels of DNA damage and an increased risk of miscarriage. Zini et al. [52] reported that regardless of the ART type (IVF or ICSI), sperm DNA damage was a predictive factor for pregnancy loss after ART. Robinson et al. [53] reported that IVF or ICSI using sperm with high levels of DNA damage had a 2.16 times higher risk of early pregnancy loss. Carrell et al. [54] suggested that when using the TUNEL assay, sperm DNA damage was higher in the male partners of couples that had experienced recurrent miscarriages (35%) than in the general population (22%) or normal fertile men (12%). In a study researching 106 men in couples struggling with fertility issues who had miscarriages in the past, Check et al. [55] found that a DFI of 30% or higher was related to high miscarriage rates and low ongoing

pregnancy rates. Khadem et al. [56] also reported that recurrent miscarriages and high levels of sperm DNA damage had a positive correlation. In conclusion, SDF measurements can be seen as a useful tool in predicting miscarriages related to paternal factors.

Treatment strategies for patients with high levels of SDF

Low pregnancy success rates and high miscarriage rates are predicted even with IVF or IVF and ICSI when SDF is high in men; therefore, methods for improving sperm DNA damage prior to ART procedures and techniques for selecting sperm with better chromatin are reviewed below.

1. Lifestyle modifications

Physical agents such as radiation and heat, cigarette smoke, airborne pollutants, chemical agents such as anticancer drugs, sexually transmitted infections, and biological factors such as increasing male age, elevated body mass index, and diabetes are environmental and lifestyle factors known to affect sperm DNA integrity [57]. Lifestyle modification is the most fundamental, important, simple, and easy way to improve sperm quality. Men with impaired sperm quality should quit smoking and drinking; engage in exercise and manage their weight; wear loose underwear; avoid environments with high temperatures such as saunas, lower-body bathing, and high temperature workspaces; and abstain from ejaculation for an appropriate duration.

2. Infection control

Studies have observed male genital tract infection and inflammation in 8%–35% of cases of male infertility [58], and infections of the male genital organ are known to cause sperm DNA damage. Inflammatory cells produce reactive oxygen species, which are known to cause DNA base modifications and DNA damage [59]. White blood cells detected in semen originate from the epididymis, and male genital tract, and antioxidants in the seminal plasma scavenge the reactive oxygen species produced by the white blood cells. However, when a large quantity of reactive oxygen species is produced, the sperm DNA is damaged by oxidative stress. Such patients can be treated with medication for 2–12 weeks to decrease the amount of reactive oxygen species produced by the white blood cells in order to improve the fertility of sperm.

3. Oral antioxidant therapy

Fifteen percent of reproductive-age couples experience fertility issues, and 50% of those cases involve issues associated with the male partner [1]. However, many male patients do not know the causes of

oligoasthenoteratozoospermia. Oxidative stress is known to be an important factor that causes male infertility by damaging sperm DNA [60–62]. Greco et al. [63] found that patients who took antioxidants before an ICSI procedure did not show differences in the fertility rate, cleavage rate, or embryo morphology, but had higher clinical pregnancy and implantation rates. However, Tremellen et al. [64] argued that the positive effects of antioxidant therapy on SDF caused by reactive oxygen species are limited and that sperm DNA damage remains high in many patients even after treatment. Imamovic Kumalic and Pinter [60] conducted a meta-analysis of 32 studies published between 2000 and 2013 related to male infertility and antioxidants and found vitamin E, vitamin C, selenium, coenzyme Q10, N-acetylcysteine, zinc, and L-carnitine to be effective. Among these, vitamin C and vitamin E were most effective in reducing DNA fragmentation, and zinc and selenium had similar effects as well. However, additional research is needed since there are no standardized treatment guidelines for male infertility patients with high levels of oxidative damage.

4. Varicocele repair

Varicocele is caused by abnormal dilatation of the pampiniform plexus veins and occurs in 15% of men. This is one of the most common causes of male infertility that can be repaired through surgical procedures. It is found in 35% of patients with male infertility and in 70% of secondary infertility patients [65]. Higher levels of SDF were observed in patients with varicocele [66]. Elevated levels of reactive oxygen species damage both the nuclear and mitochondrial DNA, causing base modifications, strand breaks, and chromatin cross-links, as well as increasing sperm DNA damage [67]. Surgical repair improved semen parameters and was more cost-effective than IVF/ICSI [65]. Among the surgical methods, microsurgical varicocelectomy through the subinguinal approach was the most effective method [65].

5. Micromanipulation-based sperm selection

Many studies have investigated techniques for selecting sperm with less DNA damage when performing ART. Some such methods include density gradient centrifugation, electrophoretic sperm isolation using a cell sorter, a hyaluronic acid-binding method, sperm magnetic sorting, and high-magnification microscopy. Sperm and embryo treatment and selection are known to decrease adverse ICSI reproductive outcomes caused by sperm DNA damage, and many fertility clinics currently perform these methods. However, there is no definitive clinical evidence that any of these methods can avoid the potentially harmful effects of abnormal sperm on ART outcomes. Sperm selection technologies face limitations because none of the current techniques can completely prevent the selection of sperm with DNA damage or aneuploidy [68].

6. Testicular sperm

Testicular sperm tend to have less DNA damage and better DNA integrity than ejaculated sperm. However, due to some genetic and epigenetic risks, doubts have been raised regarding the use of testicular sperm [69]. A retrospective analysis of neonatal data on births by sperm injection from obstructive and nonobstructive azoospermic men showed no significant differences in short-term neonatal outcomes; likewise, there were no significant differences in the congenital malformation rate in offspring of ICSI from testicular sperm [70]. Esteves et al. [71] found testicular sperm to have a three to five times lower proportion of DNA fragmentation than ejaculated sperm. In a recent prospective comparative study on the use of testicular sperm for ICSI in 172 patients with high levels of sperm DNA damage, it was found that the proportion of testicular sperm DNA damage (8.3%) was five times lower than that of ejaculated sperm DNA damage (40.9%). Better reproductive outcomes were shown when testicular sperm was used for ICSI in the patient group that continued to show oligozoospermia and high levels of sperm DNA damage even after antioxidant treatment. The birth rate in the ICSI group that used testicular sperm was 46.7%, while the birth rate in the ICSI group that used ejaculated sperm was 26.4%; moreover, the relative risks for miscarriage and birth were 0.29 and 1.76, respectively, suggesting that testicular sperm had better outcomes [71]. Furthermore, based on several recent meta-analyses, clinical pregnancy rates were low and miscarriage risks were high when IVF/ICSI was performed with ejaculated sperm with high levels of DNA damage; therefore, using testicular sperm for the next ICSI cycle may be more effective for couples that have high levels of SDF and a history of failed IVF/ICSI [72].

Conclusion

Sperm DNA damage testing methods do not have clearly standardized cut-off levels and each method has its advantages and disadvantages, making it difficult to proclaim one as a universally preferable method. The human reproductive system is complex, which makes it impossible to define a clear and universal cut-off level for various testing methods, and it is difficult to predict the outcome of dynamic interactions among several factors that may disturb fertility using a single test. Thus, it is best to select a testing method based on a patient's clinical characteristics and goals. Since sperm DNA damage tests provide genetic information from male reproductive cells, in contrast to the simple information that previous semen analysis parameters provide, more research on methods for selecting sperm with undamaged DNA in ART should be conducted in the future. Furthermore, physicians and researchers working with ART must continue to make efforts to obtain healthy sperm with nuclear DNA integrity to minimize the adverse effects that may arise in offspring

conceived from sperm with DNA damage.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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