Sperm DNA Fragmentation: Consequences for Reproduction

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Abstract

DNA fragmentation, or the accumulation of single- and double-strand DNA breaks, is a common property of sperm, and an increase in the level of sperm DNA fragmentation is known to influence natural reproduction. The effect of sperm DNA fragmentation on male infertility and assisted reproductive treatment (ART) outcomes remains controversial and is one of the most frequently debated topics of reproductive medicine. For the past 30 years, a number of assays have been developed to quantify the level of sperm DNA fragmentation. In this chapter, we review the causes of sperm DNA fragmentation, describe the commonly used tests to evaluate these abnormalities, and perform a systematic review of existing studies to determine the impact of sperm DNA fragmentation on male fertility and ART outcomes.

Keywords

Sperm DNA fragmentation · Comet assay · SCSA · TUNEL assay · SCD assay · Male infertility · ART outcomes

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Introduction

Sperm are a well-designed vehicle that facilitate the transfer of a haploid genome from the father to the oocyte (Aitken and De Iuliis 2010). To perform such a function, the spermatogonial stem cell must undergo a series of meiotic divisions and morphological and biochemical alterations resulting in the formation of a mature sperm, and this process is known as spermatogenesis. As a result of spermatogenesis, millions of sperm are produced every day. Clearly, normal embryonic development is dependent on the delivery of intact and complete genetic material to the oocyte (Simon et al. 2014a). Therefore, the sperm nucleus has adopted a unique structural architecture in which the DNA is tightly packaged with small and positively charged proteins, termed protamines, resulting in the formation of compact nuclear structure (Oliva 2006). During this process, the sperm loses its cytoplasmic content, resulting in the formation of streamline sperm structure that facilitates the motility and protection of the genetic material.

The removal of the cytoplasm leaves the sperm nucleus vulnerable to the potential negative effects of free radicals; however, the compact, toroidal organization of sperm chromatin in normally protaminated sperm is known to protect sperm DNA from most free radical-mediated damage (Aitken 2012). Additionally, the seminal plasma not only acts as a medium for the sperm

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to swim, but consists of high concentrations of antioxidants that can scavenge the free radicals to minimize the effect of oxidative stress-mediated DNA damage (Koca et al. 2009). Despite these preventive mechanisms, oxidative stress is generated in sperm when the concentration of free radicals produced exceeds the level of antioxidant activity, resulting in sperm "DNA fragmentation", or the accumulation of DNA strand breaks (Saleh et al. 2002). Recently, it has been shown that DNA fragmentation is a common property of all sperm and the level of DNA damage may vary from one sperm to another (Simon et al. 2017a).

Sperm DNA fragmentation can also occur as a result of intrinsic factors where poor structural organization of sperm chromatin leaves the sperm vulnerable to oxidative stress-mediated DNA damage (Aoki et al. 2005). Studies have suggested that there may be a cascade of events that start with seminal oxidative stress leading to apoptosis of sperm (Aitken and Koppers 2011). Other factors such as medication, heat, radiation, etc. are some of the extrinsic factors also known to cause sperm DNA fragmentation (Agarwal and Allamaneni 2005; Aitken et al. 2005; Morris 2002). Regardless of the cause, DNA fragmentation occurring in sperm is permanent, as sperm lack any ability to repair damaged DNA.

Methods of Sperm DNA Fragmentation Analysis

A number of assays are now available to measure the level of sperm DNA fragmentation. Of these methods, single-cell gel electrophoresis (commonly called as the Comet assay), in situ nick translation assay, and the terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) assays directly measure the level of DNA fragmentation, whereas the Sperm Chromatin Dispersion (SCD) assay (commonly called as the Halo test) and Sperm Chromatin Structure Assay (SCSA) are known to indirectly measure the level of DNA fragmentation in sperm. These assays differ in their ease of use, cost, and the type of DNA strand breakage measured (Fig. 6.1).

Comet Assay

The Comet assay is one of the simplest methods to measure sperm DNA fragmentation and quantifies single- and double-strand breaks (McKelvey-Martin et al. 1997). The principle of the assay is that the sperm nuclear DNA is separated in an electric field based on charge and size, which can be viewed by using a fluorescent dye. The resulting image resembles a comet, with an intact head and tail based on the amount of DNA fragmentation. The intensity of staining determines the extent of DNA fragmentation (Ostling and Johanson 1984). Additional quantitative parameters have been used to increase the efficiency of the test, such as diameter of the nucleus, olive tail moment, and the comet length (Singh et al. 1988).

One of the principles of the Comet assay is that the double-stranded DNA remains in the comet head, whereas short fragments of double- and single-stranded DNA migrate into the tail area (Klaude et al. 1996). Therefore, sperm with high levels of DNA strand breaks would show an increased comet tail which can be measured by its intensity of fluorescence (Hughes et al. 1999) and comet tail length (Singh and Stephens 1998). The Comet assay can be performed in a neutral or alkaline environment. In neutral pH buffer, only DNA with double-strand breaks are measured, while in the alkaline buffer single- and double-strand breaks, and alkali-labile sites, are detectable due to unwinding of the strands (Tarozzi et al. 2009). This is the only technique that can measure the direct level of DNA fragmentation in individual cells. The Comet assay is relatively inexpensive and one of the most sensitive techniques available to measure DNA fragmentation. However, the assay is relatively labor intensive. According to published results, Comet assay results are correlated to the results obtained from the TUNEL assay (Aravindan et al. 1997). The alkaline Comet assay can be used in all cell types and also in the sperm. The assay requires only a

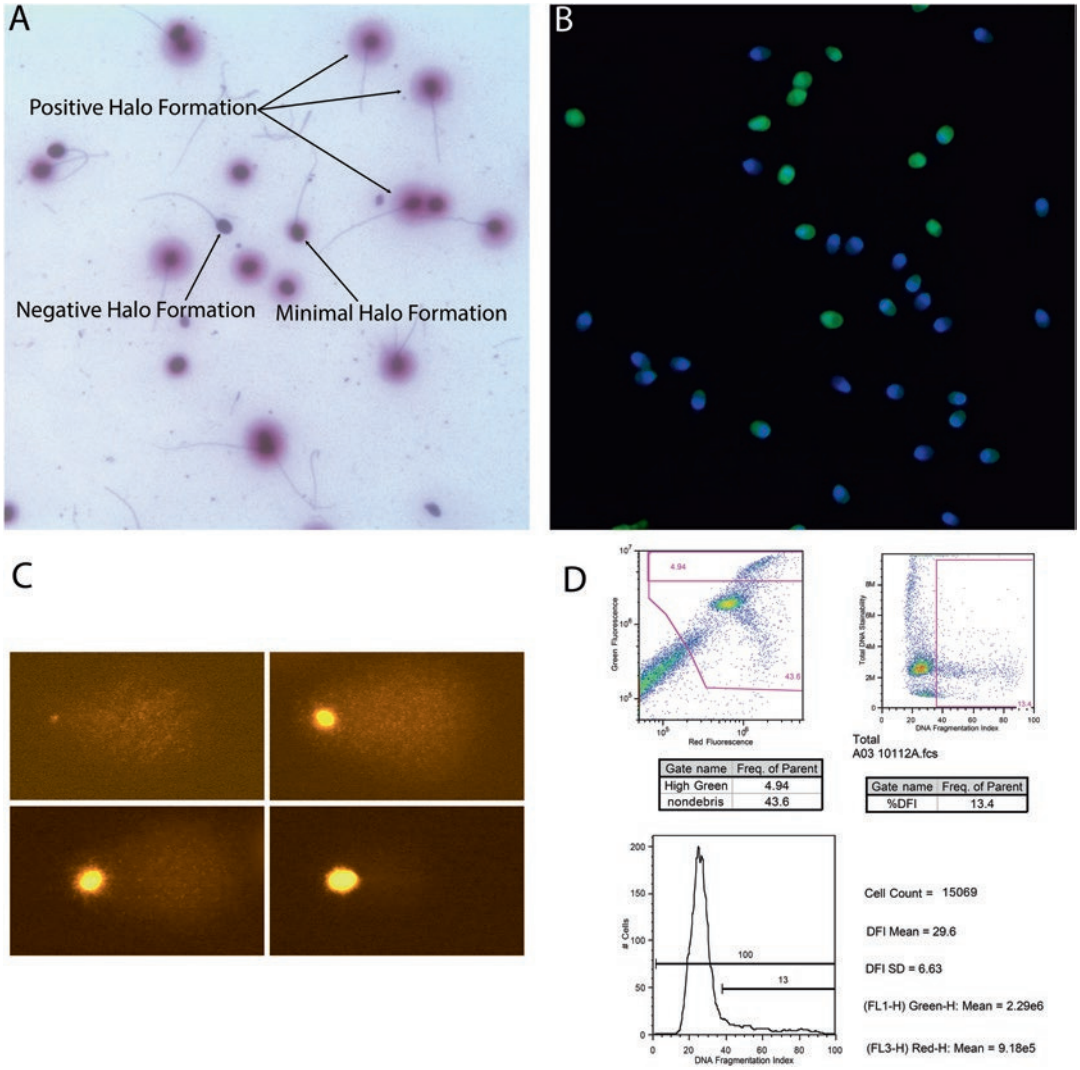

Fig. 6.1 Image of the four major assays for DNA fragmentation detection. (**a**) Micrograph of sperm analyzed using the SCD assay. (**b**) TUNEL assay micrograph identifying sperm with DNA damage (green) and non-damaged (blue). (**c**) Comet assay showing varying degrees of damage evidenced by tail length and intensity. (**d**) Printout of the data derived using the SCSA

few cells, of benefit for analysis of sperm from severely oligozoospermic men, and data can be collected at the level of individual cells.

The clinical importance of the Comet assay in assessing male infertility has been demonstrated by a number of authors (Simon et al. 2010, 2011a, 2017a, b; Irvine et al. 2000; Donnelly et al. 2001; Lewis and Agbaje 2008). The disadvantage of the assay is that it still lacks standardized protocols, which makes it difficult to fully understand and relate the results of different authors (Tarozzi et al. 2007). It is known to damage the alkaline labile sites and therefore makes it difficult to discriminate between endogenous and induced DNA breaks. The assay is also criticized for underestimation of DNA fragmentation due to entangling of DNA strands. Additionally, incomplete chromatin decondensation, in the case of sperm DNA, will not allow breaks to be revealed. Overlapping

comet tails decrease the accuracy of the assay and some extremely small tail fragments are lost or too small fragments are difficult to be visualized. The assay is laborious, has high level of inter-laboratory variation, and hence is not routinely recommended for routine clinical screening, but has been shown to be valuable in research applications (Olive et al. 2001).

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling Assay

The TUNEL assay quantifies the incorporation of deoxyuridine triphosphate (dUTP) at single- and double-strand DNA breaks in a reaction catalyzed by the template-independent enzyme, terminal deoxynucleotidyl transferase (Gorczyca et al. 1993). The incorporated dUTP which is labeling the breaks can be quantified by flow cytometry, fluorescent microscopy, or even light microscopy (Tarozzi et al. 2007). The TUNEL assay is widely known to measure direct sperm DNA fragmentation. The TUNEL assay resembles the nick translation in situ in a number of technical aspects, but can reveal both single- and double-strand breaks (Tarozzi et al. 2007). The sperm DNA fragmentation measured by TUNEL assay has good stability over time, so it is possible to measure and monitor baseline damage in both fertile and subfertile men (Sergerie et al. 2005a). The assay is broadly used to assess sperm DNA fragmentation as an indicator of male fertility (Sergerie et al. 2005b) and has been demonstrated to predict assisted reproduction outcome (Sun et al. 1997; Lopes et al. 1998; Duran et al. 2002; Benchaib et al. 2003; Borini et al. 2006).

The TUNEL assay can simultaneously detect single- and double-strand breaks, unlike other assays that either simply measure sperm susceptibility to DNA damage or require elaborate protocols to study both types of strand breakages (Lopes et al. 1998; Fraser 2004). Freezing raw or washed semen samples does not affect the results of the TUNEL assay (Sailer et al. 1995). The TUNEL assay is highly sophisticated and expensive; however, its popularity is justified by good quality control parameters, such as a low intra- and inter-observer variability (Barroso et al. 2000). This fluorescence labeling technique eliminates the problems associated with dye fading in the conventional microscopic method, thereby giving technicians more time to analyze a greater number of cells (Host et al. 1999). Due to the unique chromatin packaging of sperm, staining can be limited to the periphery of the cell; therefore, it is necessary to include techniques for relaxation of sperm DNA prior to labeling (Fraser 2004).

The use of flow cytometry protocols within the TUNEL assay makes it possible to evaluate a very high number of cells, thus enhancing reproducibility and accuracy of the technique. However, the TUNEL assay does not quantify the magnitude of DNA fragmentation within a given cell unless the measurement is conducted by flow cytometry as it only counts the number of cells within a population with DNA fragmentation as TUNEL-positive cells (Shamsi et al. 2008). The assay can be simplified to analyze cells using light microscopy, in which stained cells (with DNA fragmentation) and unstained cells (without fragmentation) are manually counted. However, in this case background staining can decrease the accuracy of the assay.

In Situ Nick Translation

The in situ nick translation (ISNT) assay is a modified form of the TUNEL assay that quantifies the incorporation of biotinylated deoxyuridine triphosphate (dUTP) at single-stranded DNA breaks in a reaction that is catalyzed by the template-dependent enzyme, DNA polymerase I (Shamsi et al. 2008). Unlike the TUNEL assay, which utilizes template-independent end labeling, nick translation can only be used for single-strand breaks, not for both single-strand and double-strand breaks as in the TUNEL assay (Irvine et al. 2000). This assay identifies variable levels of DNA strand breaks in each sperm (Manicardi et al. 1995) and is positively associated with protamine deficiency

(Bianchi et al. 1993). The clinical value of the nick translation assay is severely limited because no correlation has been proven with fertilization capacity during in vivo studies (Irvine et al. 2000), and it lacks sensitivity compared with other assays (Twigg et al. 1998). Furthermore, the assay may be less biologically relevant given that single-strand breaks can be more easily repaired by the embryo than the double-strand break (Twigg et al. 1998).

The accuracy of the DNA polymerase enzyme used in the assay is high and hence single-strand nicks are efficiently incorporated with labeled dUTP, resulting in identification of sperm with very low levels of DNA strand breaks. The assay is capable to identify a variable level (low to high) of DNA damage in individual sperm within an ejaculate (Shamsi et al. 2008). The clinical value of the nick translation assay is severely limited because no correlation has been proven with fertilization in in vivo studies (Irvine et al. 2000). When the ISNT is compared with other tests, TUNEL and Comet assays show better correlations with ART outcomes as they measure both single-strand and double-strand breaks present in the sperm DNA (Irvine et al. 2000).

Sperm Chromatin Structure Assay

The Sperm Chromatin Structure Assay (SCSA) is the most commonly used commercial test to characterize male infertility. It is a flow cytometric method to determine abnormal sperm chromatin which is highly susceptible to chemically induced in situ partial DNA denaturation. The extent of DNA denaturation is determined by measuring the metachromatic shift from green fluorescence to red fluorescence after heat or acid treatment (Evenson et al. 1980). The most important parameter of this test is the DNA fragmentation index (%), which represents the population of cells with DNA fragmentation (Evenson and Jost 2000). It also measures the High DNA stainability (%). The SCSA measures the susceptibility of sperm DNA to heat- or acid-induced DNA denaturation in situ, followed by staining with acridine orange stain where the double-strand DNA fluoresce green and the single-strand DNA fluoresce red (Evenson and Jost 2000). The use of flow cytometry makes it possible to measure a large number of spermatozoa per sample making the technique therefore simple and highly reproducible (Evenson and Jost 2000). DNA fragmentation index (DFI) represents the sperm population with detectable denaturable single-stranded DNA and the highly DNA stainable (HSD) cells describe the sperm population with increased accessibility of double-stranded DNA to the dye, mainly due to impaired replacement of histones with protamines (Tarozzi et al. 2007).

Sperm DNA fragmentation measured by the SCSA is known to be more constant over a longer period of time when compared with the traditional sperm evaluation parameters (Zini et al. 2001). The consistency of the test makes it useful in epidemiological studies (Spanò et al. 1998). Freezing of semen does not affect the test, allowing samples to be batched for convenience or used in multi-center trials and analyzed at a later date in a central facility. The assay determines the percentage of sperm with DNA fragmentation. Several clinical studies have shown its usefulness in evaluating male fertility (Evenson et al. 2002; Spano et al. 2000; Virro et al. 2004). It is simple and rapid for the analysis of thousands of human sperm (Fraser 2004). Generally, most users have defined that a threshold value above 30% DFI and 15% HSD predicts couples who are likely to be infertile. Several clinical studies have shown its usefulness in evaluating male fertility in relation with fertilization, blastocyst development, ongoing pregnancy in IVF, and ICSI (Evenson and Jost 2000; Evenson et al. 2002; Spano et al. 2000; Virro et al. 2004).

The SCSA does not give information about the extent of DNA fragmentation in individual sperm (Fraser 2004). The assay requires expensive equipment for analysis. Laboratory factors affect the test giving high variation between replicates (Boe-Hansen et al. 2005a, 2006). There is conflicting data as its usefulness in predicting fertilization rates, embryo quality, or pregnancy outcomes (Larson et al. 2000; Payne et al. 2005; Erenpreiss et al. 2006).

Sperm Chromatin Dispersion Assay

The Sperm Chromatin Dispersion (SCD) assay has been described as a simple and inexpensive method for the analysis of sperm DNA fragmentation. It is based on the principle that sperm with fragmented DNA fail to produce the characteristic halo that is seen when sperm are mixed with agarose following acid denaturation and removal of nuclear proteins (Fernandez et al. 2003). The methodology of the test includes the following steps. Sperm are immersed in an agarose matrix on a slide, treated with an acid solution to denature DNA that contains breaks, and then treated with lysis buffer to remove membranes and proteins. The agarose matrix allows working with unfixed sperm on a slide in a suspension-like environment. Removal of nuclear proteins results in nucleoids with a central core and a peripheral halo of dispersed DNA loops. Following fluorescent staining, sperm nuclei with elevated DNA fragmentation produce very small or no halos of DNA dispersion, whereas those sperm with low levels of DNA fragmentation release their DNA loops forming large halos. These results have been confirmed by DNA breakage detection-fluorescence in situ hybridization, a procedure in which the restricted single-stranded DNA motifs generated from DNA breaks can be detected and quantified (Fernández and Gosálvez 2002).

The test does not rely on fluorescence intensity, hence it is simple to analyze with light microscopy. The test does not require the use of complex instrumentation; it can be carried out with equipment normally available in andrology laboratories (microscope). The test endpoints (non-dispersed and dispersed nuclei) can be easily obtained without extensive training of laboratory technicians.

Despite its ease of use, some limitations of the assay are well known. The assay has been reported to have low-density nucleoids, which are fainter with less contrasting images. Thus, the peripheral limit of the halo, where the chromatin is even less dense, may not be accurately discriminated from the background. Furthermore, all of the halos are not necessarily in the same visual plane of the agarose; hence, the use of software to analyze can result in misreading due to unfocused halos. Lastly, sperm tails are not preserved; therefore, discriminating sperm from other contaminant cells is problematic.

Consequence of Sperm DNA Fragmentation on Male Reproductive Health

During the past few decades, a number of studies have associated DNA fragmentation with male infertility (Host et al. 1999; Zini et al. 2001; Hughes et al. 1996; Evenson et al. 1999; Saleh et al. 2003a; Simon et al. 2011b; Castillo et al. 2011). Most of these studies suggest that sperm DNA fragmentation is associated with male infertility. Additionally, DNA fragmentation in the sperm of men from the general population planning their first pregnancy, with no previous knowledge of their fertility capability, was associated with diminished fecundity associated with an increase in sperm DNA fragmentation, indicating the necessity of normal sperm chromatin for the expression of male fertility potential (Spano et al. 2000).

Men with unexplained or idiopathic infertility have been shown to have increased levels of oxidative stress in the seminal plasma compared to controls (Pasqualotto et al. 2001), resulting in sperm DNA fragmentation (Sikka et al. 1995; Alkan et al. 1997). Increased levels of sperm DNA fragmentation has also been observed in men diagnosed with idiopathic male infertility (Saleh et al. 2003a). Leukocytospermia is common in patients with infections in the male genital tract, and resulting oxidative stress can result in sperm DNA fragmentation (Agarwal et al. 2014; Erenpreiss et al. 2002) and have a negative impact on ART outcomes (Lackner et al. 2008).

Varicoceles are a common cause of diminished sperm production and/or decreased sperm quality. In patients with a varicocele, an increased level of oxidants and reduced antioxidants is observed (Abd-Elmoaty et al. 2010). The level of oxidants in the seminal plasma has also been shown to positively correlate with the degree of varicocele (Barbieri et al. 1999), resulting in

increased sperm DNA fragmentation. Sperm DNA fragmentation has been shown to be reduced after varicocelectomy treatment, concomitantly with increased in pregnancy rates (Baker et al. 2013).

Lastly, an increase in the level of DNA fragmentation in infertile men can be attributed to abnormal histone to protamine exchange (Simon et al. 2011a; Zhang et al. 2006), sometimes observed as an abnormal protamine content or ratio (Castillo et al. 2011; Aoki et al. 2006). The mechanism by which diminished or altered protamination results in DNA fragmentation may be associated with a loss of the "protective" nature that protamination confers on sperm DNA.

Systematic Analysis of the Consequences of Sperm DNA Fragmentation on Assisted Reproduction Technologies

The existing literature regarding the effects of sperm DNA fragmentation on ART outcomes are controversial. A recent study showed a strong influence of sperm DNA fragmentation on male reproductive health and suggested that sperm DNA testing should be incorporated into routine clinical use (Simon et al. 2017b). In contrast, earlier meta-analyses and reviews did not support the clinical use of sperm DNA fragmentation (Collins et al. 2008; Zini and Sigman 2009). Therefore, for this chapter, we have performed an updated literature search and analysis of the association of sperm DNA fragmentation with ART outcomes, as measured using the four most commonly used assays (TUNEL, SCSA, SCD, and Comet). Our literature search identified 70 articles that included 94 study methodologies, TUNEL assay (35 studies), SCSA (30 studies), Comet assay (10 studies), and SCD assay (19 studies). Based on the treatment type, these studies involve standard-type insemination of IVF (30 studies), ICSI (41 studies), and IVF + ICSI mixed (23 studies) studies (Table 6.1).

Studies with overlapping data, inappropriate sampling method, assays that are less commonly used (neutral Comet assay, in situ nick translation assay, and acridine orange slide-based staining method), and studies with insufficient data were excluded from this systematic analysis. A drawback of this systematic analysis is that, in some studies, there were differences in the definition of threshold values for DNA fragmentation assays, study design, lack of control for female factors, diverse patient group, nonconsecutive recruitment of patients, and variations in the protocols used to measure DNA fragmentation assays, while in some studies, the inclusion and exclusion criteria of subject selection were not clearly stated.

Effect of Sperm DNA Damage on In Vitro Fertilization Rates

Of the 94 studies that analyzed sperm DNA fragmentation with ART outcomes, 18 did not evaluate and study the relationship between fertilization rates and sperm DNA fragmentation. The remaining 76 studies (26 IVF, 32 ICSI, and 18 mixed IVF + ICSI studies) involved 8711 treatment cycles (3149 IVF, 2558 ICSI, and 3004 mixed IVF + ICSI cycles). Forty percent (30/76 studies; including 14 TUNEL, 5 SCSA, 7 SCD, and 4 Comet studies) reported a significant inverse relationship between sperm DNA fragmentation and fertilization rate, whereas the other 46 studies (16 TUNEL, 19 SCSA, 6 SCD, and 5 Comet) showed no significant relationship between these parameters (Table 6.1).

There appears to be a stronger effect in standard IVF compared to ICSI. Fifty eight percent of the studies (15/26) reported a significant inverse relationship between sperm DNA fragmentation and fertilization rate compared to ICSI (25% or 8/32) and mixed IVF + ICSI studies (39% or 7/18). One possible explanation of this effect is that during IVF fertilization, the sperm fertilizing the oocyte is randomly selected based on the sperm-oocyte interaction, in contract to the ICSI process where the most morphologically normal and motile sperm are injected into the oocytes (Ola et al. 2001). Such selection of sperm for ICSI fertilization may result in selection of sperm with low DNA fragmentation, as sperm

Table 6.1 Description of studies associating sperm DNA fragmentation with ART outcomes

Study	ART	DD test	(n)	Fertilization rate	Embryo quality	Clinical pregnancy	Miscarriage	Live birth
Anifandis et al. (2015)	IVF + ICSI	SCD	139	Nonsignificant	Nonsignificant	Nonsignificant	Nonsignificant	NA
Avendano et al. (2010)	ICSI	TUNEL	36'	NA	Significant	Significant	NA	NA
Bakos et al. (2007)	IVF	TUNEL	45	Significant	Nonsignificant	Nonsignificant	NA	NA
	IVF + ICSI	TUNEL	113	Nonsignificant	Nonsignificant	Significant	NA	NA
	ICSI	TUNEL	68	Nonsignificant	Nonsignificant	Significant	NA	NA
Benchaib et al. (2003)	IVF	TUNEL	50	Significant	Nonsignificant	Nonsignificant	NA	NA
	ICSI	TUNEL	54	Significant	Nonsignificant	Nonsignificant	NA	NA
Benchaib et al. (2007)	IVF	TUNEL	84	Nonsignificant	Nonsignificant	Nonsignificant	Significant	NA
	ICSI	TUNEL	218	Significant	Significant	Nonsignificant	Significant	NA
Boe-Hansen et al. (2005b)	IVF	SCSA	139	NA	NA	Nonsignificant	NA	NA
	ICSI	SCSA	47	NA	NA	Nonsignificant	NA	NA
Borini et al. (2006)	IVF	TUNEL	82	Significant	NA	Nonsignificant	Nonsignificant	NA
	ICSI	TUNEL	50	Nonsignificant	NA	Significant	Significant	NA
Bungum et al. (2007)	IVF	SCSA	388	Nonsignificant	Nonsignificant	NA	Nonsignificant	Nonsignificant
	ICSI	SCSA	223	Nonsignificant	Nonsignificant	NA	Nonsignificant	Nonsignificant
Caglar et al. (2007)	ICSI	TUNEL	56	Nonsignificant	Nonsignificant	Nonsignificant	NA	NA
Check et al. (2005)	ICSI	SCSA	106	NA	Significant	Nonsignificant	NA	NA
Dar et al. (2013)	ICSI	SCSA	153	Nonsignificant	NA	Nonsignificant	Nonsignificant	NA
Daris et al. (2010)	ICSI	TUNEL	20	Nonsignificant	NA	NA	NA	NA
Esbert et al. (2011)	IVF + ICSI	TUNEL	178	Nonsignificant	Nonsignificant	Nonsignificant	Nonsignificant	NA
Fang et al. (2011)	IVF	SCSA	111	Significant	Nonsignificant	Nonsignificant	NA	NA
Frydman et al. (2008)	IVF	TUNEL	117	Nonsignificant	Nonsignificant	Significant	Significant	Significant
Gandini et al. (2004)	IVF	SCSA	12	Nonsignificant	NA	Nonsignificant	NA	NA
	ICSI	SCSA	22	Nonsignificant	NA	Nonsignificant	NA	NA
Gosalvez et al. (2013)	ICSI	SCD	81	NA	NA	Significant	NA	NA
Gu et al. (2009)	IVF	SCD	136	Significant	NA	Significant	NA	NA
Gu et al. (2011)	IVF	SCD	67	Significant	Nonsignificant	Significant	NA	NA
Guerin et al. (2005)	IVF + ICSI	SCSA	100	Nonsignificant	Nonsignificant	Nonsignificant	NA	NA
Hammadeh et al. (2006)	IVF	TUNEL	26	Nonsignificant	NA	NA	NA	NA
	ICSI	TUNEL	22	Nonsignificant	NA	NA	NA	NA
Hammadeh et al. (2008)	IVF	TUNEL	26	Nonsignificant	NA	Nonsignificant	NA	NA
	ICSI	TUNEL	22	Nonsignificant	NA	Nonsignificant	NA	NA

Study	ART	DD test	(n)	Fertilization rate	Embryo quality	Clinical pregnancy	Miscarriage	Live birth
Henkel et al. (2003)	IVF	TUNEL	208	Nonsignificant	Nonsignificant	NA	NA	NA
	ICSI	TUNEL	54	Significant	Nonsignificant	NA	NA	NA
Host et al. (1999)	IVF	TUNEL	175	Significant	Significant	Nonsignificant	NA	NA
	ICSI	TUNEL	60	Nonsignificant	Nonsignificant	Nonsignificant	NA	NA
Huang et al. (2005)	IVF	TUNEL	204	Significant	Nonsignificant	Nonsignificant	NA	NA
	ICSI	TUNEL	86	Significant	Nonsignificant	Nonsignificant	NA	NA
Jiang et al. (2011)	IVF	SCSA	137	Nonsignificant	Nonsignificant	Nonsignificant	NA	NA
	ICSI	SCSA	50	Nonsignificant	Nonsignificant	Nonsignificant	NA	NA
Kennedy et al. (2011)	ICSI	SCSA	233	NA	NA	Nonsignificant	Significant	Significant
Larson et al. (2000)	IVF + ICSI	SCSA	24	Nonsignificant	Nonsignificant	Significant	NA	NA
Larson-Cook et al. (2003)	IVF + ICSI	SCSA	82	Nonsignificant	Nonsignificant	Significant	NA	NA
Lazaros et al. (2013)	ICSI	SCSA	36	Significant	Significant	Significant	NA	NA
Lewis et al. (2004)	ICSI	Comet	77	Nonsignificant	NA	Significant	NA	NA
Li et al. (2011)	IVF	SCD	51	Significant	Nonsignificant	NA	NA	NA
Lin et al. (2008)	IVF	SCSA	137	Nonsignificant	Nonsignificant	Nonsignificant	Significant	NA
	ICSI	SCSA	86	Nonsignificant	Nonsignificant	Nonsignificant	Significant	NA
Lopes et al. (1998)	ICSI	TUNEL	131	Significant	Nonsignificant	NA	NA	NA
Lopez et al. (2013)	IVF + ICSI	SCD	152	NA	NA	Nonsignificant	NA	NA
Marchetti et al. (2002)	IVF	TUNEL	111	Significant	NA	NA	NA	NA
Meseguer et al. (2011)	IVF + ICSI	SCD	210	NA	NA	Significant	NA	NA
Micinski et al. (2009)	ICSI	SCSA	60	Significant	Nonsignificant	Significant	NA	NA
Morris et al. (2002)	IVF + ICSI	Comet	60	Nonsignificant	Nonsignificant	Nonsignificant	Nonsignificant	NA
Muriel et al. (2006)	IVF + ICSI	SCD	85	Significant	Significant	Nonsignificant	NA	NA
Nasr-Esfahan et al. (2005)	ICSI	Comet	28	Nonsignificant	Significant	NA	NA	NA
Ni et al. (2014)	IVF	SCD	1380	NA	Nonsignificant	Nonsignificant	Nonsignificant	Nonsignificant
	ICSI	SCD	355	NA	Nonsignificant	Nonsignificant	Nonsignificant	Nonsignificant
Nicopoullos et al. (2008)	ICSI	SCSA	56	Nonsignificant	NA	Nonsignificant	NA	NA
Nijs et al. (2009)	IVF + ICSI	SCSA	205	Significant	NA	Nonsignificant	NA	NA
Nijs et al. (2011)	IVF + ICSI	SCSA	278	Nonsignificant	NA	Nonsignificant	NA	NA
Nunez-Calonge et al. (2012)	ICSI	SCD	70	Nonsignificant	NA	Significant	NA	NA
Ozmen et al. (2007)	ICSI	TUNEL	42	Nonsignificant	NA	Significant	Significant	Significant
Payne et al. (2005)	IVF + ICSI	SCSA	95	Significant	Nonsignificant	Nonsignificant	NA	NA

(continued)

Table 6.1 (continued)

Study	ART	DD test	(n)	Fertilization rate	Embryo quality	Clinical pregnancy	Miscarriage	Live birth
Pregl Breznik et al. (2013)*	IVF	SCD	133	Significant	Significant	NA	NA	NA
	ICSI	SCD	133	Significant	Significant	NA	NA	NA
Rama Raju et al. (2012)	IVF + ICSI	SCD	247	Nonsignificant	Significant	Significant	NA	NA
Sanchez-Martin et al. (2013)	ICSI	SCD	40	NA	NA	NA	NA	NA
Seli et al. (2004)	IVF + ICSI	TUNEL	49	NA	Significant	Nonsignificant	NA	NA
Sharbatoghli et al. (2012)	ICSI	SCD	120	Nonsignificant	Nonsignificant	Nonsignificant	NA	NA
	ICSI	TUNEL	120	Nonsignificant	Nonsignificant	Significant	NA	NA
Simon et al. (2010)	IVF	Comet	219	Significant	Significant	Significant	Nonsignificant	Nonsignificant
	ICSI	Comet	116	Nonsignificant	Nonsignificant	Nonsignificant	Nonsignificant	Significant
Simon et al. (2011a)	IVF	Comet	70	Significant	Significant	Significant	NA	NA
Simon and Lewis (2011)	IVF	Comet	73	Significant	Significant	Significant	NA	NA
	ICSI	Comet	22	Nonsignificant	Nonsignificant	Nonsignificant	NA	NA
Simon et al. (2014a)	IVF + ICSI	Comet	238	Significant	Significant	Significant	NA	NA
	IVF + ICSI	TUNEL	238	Significant	Nonsignificant	Significant	NA	NA
	IVF + ICSI	SCSA	102	Nonsignificant	Nonsignificant	Nonsignificant	NA	NA
Smit et al. (2010)	IVF + ICSI	SCSA	27	NA	NA	Significant	NA	NA
Speyer et al. (2010)	IVF	SCSA	192	Nonsignificant	Nonsignificant	Nonsignificant	Nonsignificant	NA
	ICSI	SCSA	155	Nonsignificant	Nonsignificant	Significant	Nonsignificant	NA
Stevanato et al. (2008)	IVF + ICSI	TUNEL	35	NA	NA	NA	NA	NA
Sun et al. (1997)	IVF	TUNEL	143	Significant	Significant	NA	NA	NA
Tarozzi et al. (2009)	IVF	TUNEL	82	NA	NA	Nonsignificant	NA	NA
	ICSI	TUNEL	50	NA	NA	Significant	NA	NA
Tavalaee et al. (2009)	IVF + ICSI	SCD	92	Nonsignificant	NA	Nonsignificant	NA	NA
Tomsu et al. (2002)	IVF	Comet	40	NA	Significant	Nonsignificant	NA	NA
Velez de la Calle et al. (2008)	IVF + ICSI	SCD	622	Significant	Significant	Nonsignificant	NA	NA
Virro et al. (2004)	IVF + ICSI	SCSA	249	Nonsignificant	Significant	Significant	NA	NA
Yang et al. (2013)	ICSI	SCSA	62	NA	NA	Significant	Nonsignificant	NA
Zeyad et al. (2018)	ICSI	TUNEL	84	Nonsignificant	NA	Nonsignificant	NA	NA
Zheng et al. (2018)	IVF	SCD	161	Nonsignificant	Significant	Significant	NA	NA
Zini et al. (2005)	ICSI	SCSA	60	Nonsignificant	Significant	Nonsignificant	Nonsignificant	NA

TUNEL terminal deoxyuridine nick-end labeling assay, *SCD* Sperm Chromatin Dispersion assay, *SCSA* Sperm Chromatin Structure assay, *IVF* in vitro fertilization, *ICSI* intracytoplasmic sperm injection, *NA* data not available

*Pregl Breznik et al. (2013) –IVF and ICSI cycles of treatment were not specified

motility and sperm morphology are negatively associated with sperm DNA fragmentation (Borini et al. 2006; Huang et al. 2005; Caglar et al. 2007; Lin et al. 2008; Simon and Lewis 2011).

Effect of Sperm DNA Damage on Embryo Development

From the systematic review (Table 6.1), we identified 62 eligible studies (22 IVF, 24 ICSI, and 16 mixed IVF + ICSI studies) that analyzed sperm DNA fragmentation with embryo quality, of which 21 studies (34%) showed a significant association between embryo quality and sperm DNA fragmentation. The 62 studies involved 9116 treatment cycles (4193 IVF, 2445 ICSI, and 2478 mixed IVF + ICSI cycles). In 34% (21/62) of the studies (5 TUNEL, 4 SCSA, 6 SCD, and 6 Comet), a significant inverse relationship between sperm DNA fragmentation and embryo quality was reported, whereas the remaining 41 studies (17 TUNEL, 15 SCSA, 6 SCD, and 3 Comet) showed no significant relationship between these parameters.

Studies using the Comet assay more commonly reported an adverse effect (67%), whereas 22% of TUNEL, 21% of SCSA, and 50% of SCD studies reported adverse effects of sperm DNA fragmentation on embryo quality. In terms of the type of assisted treatment, 36% of IVF, 29% of ICSI, and 38% of mixed IVF + ICSI studies reported adverse effect of sperm DNA fragmentation on embryo quality. Our analysis showed a differential association between sperm DNA fragmentation and embryo quality; when the studies were segregated into groups based on assay types, sperm DNA fragmentation detected by the alkaline Comet assay was strongly associated with poor embryo quality when compared to other assays. This association may be due to the sensitivity of the Comet assay, which measures both single- and double-stranded DNA fragmentation following complete chromatin decondensation, or may be due to the small number of studies (Simon et al. 2014b).

Effect of Sperm DNA Damage on ART Success

An extensive review of the existing literature and meta-analysis of studies testing the effect of DNA fragmentation on ART treatment were recently published by our group (Simon et al. 2017b). In this meta-analysis (56 studies), clinical pregnancy was analyzed in 3734 IVF treatment cycles from 16 studies, 2282 ICSI treatment cycles from 24 studies, and 2052 mixed IVF + ICSI treatment cycles from 16 studies. An overall relationship between sperm DNA fragmentation and clinical pregnancy outcome from 56 studies (including 8068 ART cycles) supported a strong and significant association between the two parameters [Odds Ratio (OR): = 1.68; 95% CI: 1.49–1.89, P <0.0001] (Simon et al. 2017b). The meta-analysis showed a strong relationship between sperm DNA fragmentation and clinical pregnancy outcome based on the type of treatment. A significant association between sperm DNA fragmentation and clinical pregnancy was observed for IVF treatment (OR = 1.65; 95% CI: 1.34–2.04; P <0.0001), ICSI treatment (OR = 1.31; 95% CI, 1.08–1.59; P = 0.0068), and combined IVF + ICSI treatment (OR = 2.37; 95% CI: 1.89–2.97; P <0.0001) (Simon et al. 2017b).

The meta-analysis suggested that DNA fragmentation measured by TUNEL (n = 2098 cycles from 18 studies; OR = 2.22; 95% CI: 1.61–3.05; P <0.0001), SCD (n = 2359 cycles from 8 studies; OR = 1.98; 95% CI: 1.19–3.3; P = 0.0086), and Comet (n = 798 cycles from 7 studies; OR = 3.56; 95% CI: 1.78–7.09; P = 0.0003) assays reported a significant relationship with clinical pregnancy outcome. However, the association between the two parameters using SCSA studies was not statistically significant (n = 2813 cycles from 23 studies; OR = 1.22; 95% CI: 0.93–1.61; P = 0.1522) (Simon et al. 2017b).

Our results are in contrast with previously published meta-analysis (Collins et al. 2008; Practice Committee of the American Society for Reproductive Medicine 2013; Li et al. 2006; Zhao et al. 2014) as these studies were unable to

show any relationship between sperm DNA fragmentation and clinical pregnancy outcome. Our recent meta-analysis (Simon et al. 2017b) concludes that a modest but significant association between sperm DNA fragmentation and clinical pregnancy rates is present in all three ART treatment groups (IVF, ICSI, and mixed IVF + ICSI studies) with a variable effect according to the type of sperm DNA assay. A moderate relationship between the two parameters may be due to the failure of prior studies to control for strict patient inclusion criterion, such as the failure of most studies to not exclude couples with female factors infertility. Studies in which more than half of the couples had been diagnosed with female infertility resulted in lower odds of predicting a success via DNA damage analysis (Payne et al. 2005; Frydman et al. 2008; Meseguer et al. 2011), whereas studies in which patients with female infertile factor were controlled, the odds to predicting a successful pregnancy have significantly increased irrespective of the type of DNA fragmentation testing method (Simon et al. 2011b; Giwercman et al. 2010).

Association of Sperm DNA Damage with Pregnancy Loss

Robinson et al. performed a meta-analysis evaluating the relationship between sperm DNA damage and pregnancy loss. The results of the meta-analysis suggested a significant increase in miscarriage in patients with high DNA fragmentation compared with those with low DNA fragmentation (Risk risk (RR): 2.16; 95% CI: 1.54–3.03; P <0.0001) (Robinson et al. 2012). The meta-analysis also reported a strong association of DNA fragmentation on miscarriages, observed when DNA fragmentation was measured in the raw semen (RR: 1.65; 95% CI: 1.66–2.33; P <0.0001) as well as the density gradient prepared subpopulation (RR: 3.47; 95% CI: 2.13–5.63; P <0.0001). These results are in support of previous meta-analysis (Zini et al. 2008) where a positive impact of sperm DNA fragmentation on spontaneous pregnancy loss was observed.

Although the specific mechanism(s) by which sperm DNA damage leads to increased pregnancy loss is not understood, it is well known that many factors contribute to this problem (Ford and Schust 2009). Interestingly, the negative impact of sperm DNA fragmentation is more pronounced in animal models where induced sperm DNA damage leads to abnormal embryo development, reduced implantation rate, and frequent pregnancy loss (Ahmadi and Ng 1999; Fatehi et al. 2006). Such prolonged effects of sperm DNA fragmentation, also known as the late paternal effect (Tesarik et al. 2004), may be in part due to the inability of the oocyte to repair the damaged sperm chromatin when it exceeds the threshold value (Simon et al. 2014a).

Effect of Sperm DNA Damage with Intrauterine Insemination Success

Our literature search identified ten studies that analyzed the association between sperm DNA fragmentation and IUI outcome. A total of 1673 IUI cycles were analyzed using SCSA (7 studies), TUNEL (2 studies), and SCD (1 study) assays. The results from five of the seven studies by SCSA (Saleh et al. 2003b; Bungum et al. 2004, 2007, 2008; Yang et al. 2011) and one study using the TUNEL assay (Duran et al. 2002) suggested a significant statistical difference in the level of sperm DNA fragmentation between the clinically pregnant and non-pregnant groups. Conclusive results were not published in two studies using SCSA (Boe-Hansen et al. 2006; Alkhayal et al. 2013), while no correlations were reported in two studies: using TUNEL assay (Thomson et al. 2011) and using SCD assay (Muriel et al. 2006).

Data were available to construct a two-by-two table from six of the seven studies performed using SCSA. The remaining five studies were used to construct a meta-analysis consisting of 1135 IUI cycles and with an overall pregnancy rate of 18.23%, resulting in an odds ratio of 5.61 (CI: 2.59–12.16; Z statistics: 4.37; p <0.0001) and relative risk of 1.17 (CI: 1.12–1.22; p

<0.0001) indicating a strong association between sperm DNA fragmentation and IUI outcome (unpublished data). The positive and negative predictive values were 18.96% and 96.00%, respectively. This model provided a high sensitivity (96.30%) but low specificity (17.76%) values. Our recent meta-analysis suggests a slight but significant ability of DNA fragmentation to predict IUI success, which is in contrast to the previous meta-analysis that included four of the ten studies presented above for the analysis (Practice Committee of the American Society for Reproductive Medicine 2013).

Sperm DNA Fragmentation as a Biomarker

Approximately, 30% of couples having fertility issues are diagnosed with unexplained infertility. In couples with unexplained infertility, sperm DNA fragmentation is elevated (Simon et al. 2013; Feijó and Esteves 2014). Simon et al. analyzed 147 unexplained infertile men for sperm DNA fragmentation using the Comet assay and reported that 84% of these unexplained infertile men had DNA fragmentation above the 25% cutoff value used to determine fertile from infertile men (Simon et al. 2013). In addition, the study reported that 41% of men categorized with unexplained infertility issues have sperm DNA fragmentation above the threshold of 52% fragmentation, a level previously shown to categorize the probability of a clinical pregnancy following IVF treatment (Simon et al. 2013). In another study using the SCSA assay, Oleszcuk et al. reported that 26% of men diagnosed with unexplained infertility had high DNA fragmentation index (Oleszczuk et al. 2013). Similarly, studies using TUNEL and SCD assays have reported that men with unexplained infertility have high levels of sperm DNA fragmentation (Feijó and Esteves 2014). These studies suggest that, to some extent, sperm DNA fragmentation assays may be helpful as a biomarker to identify men with fertility problems even when they are presented with normal semen analysis, as reported in unexplained infertility.

It has been shown that sperm are vulnerable to xenobiotic agents, resulting in DNA fragmentation (Aitken and De Iuliis 2007). The exposure to xenobiotics can be classified into three major types such as occupational exposure, environmental exposure, and pharmacological exposure. Workers in contact with polycyclic aromatic hydrocarbon exposure have higher sperm DNA fragmentation (Hsu et al. 2006). Elevated levels of DNA fragmentation were also observed in workers associated with waste incineration (Oh et al. 2005). Men working in the factories in contact with organic molecules such as styrene (Migliore et al. 2002), men working in the insecticide and pesticide industries (Xia et al. 2005), and men exposed to organic chemicals (Migliore et al. 2002) also have increased in sperm DNA fragmentation.

Pharmacological intervention for the treatment of diseases can result in genotoxic to sperm and male germ cells. A well-known example for such intervention is cyclophosphamide, which is used as a chemotherapeutic agent to treat cancer (Hales et al. 2005). In addition, environmental estrogens and similar compounds can have profound effects on male fertility, including affecting sperm DNA fragmentation (Anderson et al. 2003). Other environmental pollutions that have the ability to induce DNA fragmentation include organo-chlorides (Spano et al. 2005) and smog (Evenson and Wixon 2005). Therefore, sperm DNA fragmentation may not only be useful to identify male reproductive health status but also can serve as a biomarker to diagnose men exposed to xenobiotics.

Conclusion

General semen quality parameters (sperm concentration, sperm motility, sperm morphology, and total sperm count) have shown little or no correlation with fertility outcome in populations of first pregnancy planners (Bonde et al. 1998; Andersen et al. 2002; Cooper et al. 2010; Buck Louis et al. 2014). In contrast, studies correlating sperm DNA fragmentation with time to pregnancy (Spano et al. 2000; Evenson et al. 1999)

show a strong association between the two parameters. In addition, men with infertility issues are showed to have higher levels of sperm DNA fragmentation when compared with fertile men, suggesting a strong association between sperm DNA fragmentation and male infertility (Simon et al. 2011b).

The meta-analyses and systematic review presented here demonstrate that sperm DNA fragmentation is a good predictor of IUI failure and is associated with IVF pregnancy but less so with ICSI outcomes. Sperm DNA fragmentation is also negatively associated with embryo development and implantation and positively associated with miscarriage rates. Based on the evidence presented here, we suggest that sperm DNA fragmentation is closely associated with male infertility and it is independent of semen parameters. In addition, the level of sperm DNA fragmentation could influence various parameters of ART outcomes.

Controversy still exists regarding the clinical implementation of DNA fragmentation assays. Future studies should carefully consider the cost effectiveness and clinical utility of routine screening, versus targeted analysis. Furthermore, while preliminary data are intriguing, more data are still needed regarding the clinical utility of clinical interventions, such as antioxidant therapy and testicular sperm aspiration (TESE). Lastly, until testing procedures can become standardized, it will be nearly impossible to solve issues of variability and ultimate utility of the assay.

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