Article

Sperm nuclear DNA damage: update on the mechanism, diagnosis and treatment

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Abstract

Previous studies have shown that repeated intracytoplasmic sperm injection failures can be associated with sperm DNA damage. This paper reviews the current understanding of the mechanism of sperm DNA damage, discusses different diagnostic methods and their threshold values to discriminate between good- and poor-prognosis patients, and outlines the currently available treatment options. A rational approach to the interpretation of sperm DNA fragmentation data and to the choice of the optimal treatment method is suggested.

Keywords: diagnosis, high-magnification ICSI, male infertility, oral antioxidants, sperm DNA damage, testicular sperm retrieval, treatment

Introduction

At the beginning of the in-vitro fertilization (IVF) era, in the 1980s, it was believed that the aptitude of a sperm sample to be fertilized in vitro and to generate viable embryos can be predicted by standard sperm examination, including the determination of sperm count, motility and morphology. Below certain thresholds, these values predicted a poor IVF outcome. However, with accumulating experience, it became evident that these basic semen parameters are not sufficient to evaluate with precision sperm performance in IVF, and novel, so-called ‘advanced’ sperm examinations were suggested to evaluate sperm ability to bind and penetrate the zona pellucida and to fuse with the oocyte (reviewed in Mortimer et al., 1996). In contrast, the subsequent introduction and proliferation of intracytoplasmic sperm injection (ICSI) tended to minimize the importance of both the conventional and the functional sperm evaluations, based on the observation that success rates of ICSI appeared to be independent of both basic sperm parameters (Küpker et al., 1995; Mansour et al., 1995; Nagy et al., 1995; Svalander et al., 1996; Lundin et al., 1997; Sukcharoen et al., 1998) and the integrity of sperm functions required for oocyte penetration (Yanagimachi, 2004; Katsuki et al., 2005).

However, with increasing experience with ICSI, it became evident that spermatozoa from some patients repeatedly fail to form viable embryos, although they can fertilize the oocyte and trigger the early preimplantation development (Hammadeh et al., 1996; Sanchez et al., 1996; Shoukir et al., 1998). Sperm DNA fragmentation is associated with some of the cases showing this paternal effect, namely with those in which apparently morphologically normal preimplantation embryos initially form but they later fail to implant or are lost soon after the detection of pregnancy (Tesarik et al., 2004a; Tesarik, 2005).

These findings suggest that pathologically increased sperm DNA fragmentation is one of the main paternal-derived causes of repeated assisted reproduction failures in the ICSI era and open a series of questions concerning the mechanism of sperm DNA fragmentation, the methodology of its evaluation, the reproducibility and interpretation of different methods.
Currently in use, the definition of thresholds allowing assisted reproduction outcome prediction in different clinical scenarios, and the development of appropriate treatment approaches. Here the latest findings concerning these issues are updated.

Mechanism of sperm DNA damage

Sperm DNA damage can be revealed by the detection of multiple DNA strand breaks, similar to those resulting from programmed cell death (reviewed in Sakkas et al., 2003). Programmed cell death (apoptosis) is a physiological process in the seminiferous tubules and appears necessary for normal mature spermatogenesis to develop, probably because it maintains an optimal cell number ratio between individual germ cell stages and Sertoli cells in spermatogenesis (Rodriguez et al., 1997). In addition to this ‘physiological’ role, however, programmed cell death is also the preferred way of removing damaged germ cells from the seminiferous tubules in different pathological conditions, including gonadotrophin withdrawal (Hikim et al., 1995), cryptorchidism (Shikone et al., 1994; Henriksen et al., 1995), heat exposure (Yin et al., 1997; Lue et al., 1999) and irradiation (Henriksen and Parvinen, 1998).

The similarity between the nature of DNA damage observed in spermatozoa of some men and that resulting from programmed cell death in somatic cells (Gorczyca et al., 1993a) was at the origin of a hypothesis that an abnormality of programmed cell death regulation in the testis is responsible for this reproductive pathology (Gorczyca et al., 1993b). However, later studies failed to detect an association between sperm DNA fragmentation and the presence of markers of the classical signal transduction pathway leading to programmed cell death. For instance, Bcl-x vs p53 expression (Sakkas et al., 2002) or caspase activity (Tesarik et al., 2004b) do not coincide with the presence of fragmented DNA in human spermatozoa, and the specific sperm DNA damage imposed by irradiation did not result in the induction of depolarization of the mitochondrial inner membranes or facilitated phosphatidylserine externalization (Fatehi et al., 2006), the two phenomena typically associated with an early phase of programmed cell death (Gulbins et al., 2003). Moreover, germ cells that are undergoing programmed cell death in the seminiferous tubules of men with primary testicular failure appear to be efficiently removed by phagocytosis and are only rarely released from their contact with Sertoli cells (Tesarik et al., 2004b).

Rather than programmed cell death, the mechanism of DNA damage detected in ejaculated spermatozoa is thus related to oxidative damage through reactive oxygen species present in fluids filling the male genital tract (Twigg et al., 1998), and the susceptibility to this oxidative damage is increased by faulty nuclear remodelling during the final phase of spermatogenesis (McPherson and Longo, 1993a,b). Abnormal function of Sertoli cells in the diseased testis enables immature germ cells to be released prematurely, which augments the impact of oxidative damage to germ cells and leaves them to escape from phagocytosis, thus leading to the appearance of DNA-fragmented cells in the ejaculate (Tesarik et al., 2004b).

To look for treatment strategies for infertility due to sperm DNA damage, it is thus important to realize that (i) this pathology is mainly due to non-specific oxidative stress independent of the classical programmed cell death pathway, (ii) it develops during a relatively short period of time, especially after sperm release from Sertoli cells, and (iii) individual spermatozoa are not equally exposed to the risk of DNA damage, the most vulnerable being those with anomalies of chromatin remodelling during the final phase of spermatogenesis.

Diagnostic methods for the detection of sperm DNA damage: interpretation in terms of clinical relevance

Based on the knowledge that clinically relevant sperm DNA damage takes the form of DNA fragmentation, methods suggested to reveal this anomaly are based either on the detection of low-molecular-weight DNA fragments or on the visualization of endogenous nicks in the DNA molecule. Comet assay belongs to the former category, while sperm chromatin structure assay (SCSA) and terminal deoxyribonucleotidyl transferase-mediated dUTP fluorescein–dUTP nick-end labelling (TUNEL) assay represent the latter one (reviewed in Sergerie et al., 2005).

High ranges of the percentage of DNA-fragmented spermatozoa were described with any of these techniques both in fertile and infertile men (reviewed in Sergerie et al., 2005). Several studies, working with SCSA, TUNEL and Comet assays, were performed with the aim to define threshold values with which success or failure of natural conception and assisted reproduction treatment could be predicted. The values recommended in different studies showed a high degree of variability with a clear relationship with the type of assay used and the type of assisted reproduction treatment indicated (Table 1).

The first attempt at determining a threshold of the percentage of DNA-fragmented spermatozoa with regard to intrauterine insemination was made by Duran et al. (2002) who used TUNEL and microscopic count with 300 spermatozoa per patient and obtained no pregnancy with samples showing >12% TUNEL-positive spermatozoa (Table 1). Microscopic evaluation of TUNEL results (500 spermatozoa per patient) was also used by Benchaab et al. (2003) who constructed a receiver-operating characteristic (ROC) curve to determine the threshold values of DNA fragmentation having a prognostic role for achieving a pregnancy in conventional IVF and ICSI. They suggest a threshold of 18% TUNEL-positive spermatozoa for ICSI (Table 1) However, no relationship was found when conventional IVF was performed (Benchaab et al., 2003). Using the same approach, other authors calculated the discriminating threshold of the percentage of DNA-fragmented spermatozoa to be 24.3% for ICSI (Henkel et al., 2003) and 36.5% for conventional IVF (Henkel et al., 2004) (Table 1). A recent study (Greco et al., 2005a) suggested a threshold of 15% of TUNEL-positive spermatozoa to define patients with poor prognosis in ICSI (Table 1). All these studies used microscopic observation of TUNEL-stained sperm smears to determine the percentage of TUNEL-positive spermatozoa. Interestingly, when TUNEL outcomes were evaluated by flow cytometry, the threshold value of TUNEL-positive spermatozoa with regard to ICSI outcome was calculated to be 30% (Hazout et al., 2006), which was much higher than for TUNEL evaluated by microscopic observation (Table 1).
Table 1. Discriminating threshold values of SCSA and TUNEL suggested for prediction of assisted reproduction treatment outcome, presented in chronological order of publication.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Threshold (%)</th>
<th>Clinical context</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>TUNEL (M)</td>
<td>12; TUNEL+</td>
<td>IUI</td>
<td>Duran et al., 2002</td>
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<tr>
<td>TUNEL (M)</td>
<td>18; TUNEL+</td>
<td>ICSI</td>
<td>Benchabli et al., 2003</td>
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<tr>
<td>TUNEL (M)</td>
<td>24.3; TUNEL+</td>
<td>ICSI</td>
<td>Henkel et al., 2003</td>
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<tr>
<td>TUNEL (M)</td>
<td>36.5; TUNEL+</td>
<td>IVF</td>
<td>Henkel et al., 2003, 2004</td>
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<tr>
<td>SCSA (FC)</td>
<td>27; DFI</td>
<td>IVF, ICSI</td>
<td>Larson-Cook et al., 2003</td>
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<td>SCSA (FC)</td>
<td>30; DFI</td>
<td>IVF, ICSI</td>
<td>Virro et al., 2004</td>
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<td>SCSA (FC)</td>
<td>27–30; DFI</td>
<td>IUI, IVF</td>
<td>Evenson and Wixon, 2006</td>
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<td>TUNEL (M)</td>
<td>15; TUNEL+</td>
<td>ICSI</td>
<td>Greco et al., 2005a</td>
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<tr>
<td>TUNEL (FC)</td>
<td>30; TUNEL+</td>
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FC = flow cytometry; ICSI = intracytoplasmic sperm injection; IUI = intrauterine insemination; M = microscopy; SCSA = sperm chromatin structure assay; TUNEL = terminal deoxyribonucleotidyl transferase-mediated dUTP fluorescein-dUTP nick-end labelling. 

TUNEL+ stands for the percentage of TUNEL-positive spermatozoa determined in a sample. 

DFI is DNA fragmentation index, calculated as a product of the number of red-stained spermatozoa (single-stranded DNA) divided by the sum of red-stained and green-stained (double-stranded DNA) spermatozoa.

With the use of SCSA and flow cytometry (5000 spermatozoa evaluated per patient), the threshold DNA fragmentation index (DFI = number of spermatozoa with single-stranded DNA divided by the sum of spermatozoa with single-stranded and double-stranded DNA) discriminating between good and poor prognosis was suggested to be 27% both for conventional IVF and ICSI (Larson-Cook et al., 2003), which was later corrected by the same group to 30% (Virro et al., 2004) (Table 1). Previously, these authors proposed to divide patients into three categories with regard to their individual fertility potential according to DFI as follows: excellent <15%, good 15–24%, fair 25–30%, and poor >30% DFI (Evenson et al., 2002). Several protocol steps in the SCSA that can affect results of the assay have been determined, and precise protocol description with regard to these sensitive steps is thus expected to increase agreement within and between different laboratories (Boe-Hansen et al., 2005).

Sperm chromatin dispersion (SCD) test, based on the observation that spermatozoa with fragmented DNA fail to produce the characteristic halo of dispersed DNA loops that is observed with non-fragmented sperm DNA after acid denaturation and removal of nuclear proteins, was proposed recently as a relatively simple replacement of SCSA for sperm DNA evaluation (Fernandez et al., 2003; Fernandez et al., 2005). It was shown that SCD test gives a similar predictive value for sperm DNA fragmentation as TUNEL and SCSA (Chohan et al., 2006). However, results of a blind prospective study failed to find a correlation between DNA dispersion, as measured by SCD test, and pregnancy outcome in intrauterine insemination (Muriel et al., 2006), and the SCD test also lacks the statistical rigour of SCSA (Evenson and Wixon, 2005).

The importance of the clinical context for sperm DNA fragmentation data interpretation

The data outlined in the previous section illustrate the existence of significant differences in the threshold values of sperm DNA fragmentation suggested to be clinically relevant, which complicates the decision-making procedure for choosing the most suitable assisted reproduction technique. The co-existence of different methodologies for the detection of sperm DNA damage can partly account for this variability, but it can hardly explain the differences in the relationship between the extent of sperm DNA fragmentation and clinical outcomes of assisted reproduction treatment obtained by different authors even with the use of the same methodology. As a matter of example, a prospective clinical study, involving 34 male infertile patients, the husbands of women undergoing conventional IVF or ICSI, no differences were seen in SCSA parameter values between patients initiating pregnancies and not doing so, and normal pregnancies were obtained even with high scores of DFI, well above the thresholds shown in Table 1 (Gandini et al., 2004). A lack of correlation between IVF/ICSI pregnancy outcomes and sperm DNA fragmentation evaluated by the TUNEL assay was also reported recently (Huang et al., 2005).

How can these contradictory observations be explained? The analysis of the clinical context in which observations reported in each study were made may be a clue to the understanding of these apparent discrepancies. In fact, the same figure of sperm DNA fragmentation may be compatible or incompatible with normal pregnancy depending on other variables of both male and female origin that can be addressed by a thorough study of
the couple’s history of infertility. In this section two co-factors conditioning the clinical impact of sperm DNA fragmentation are dealt with: the number of previous ICSI failures and the maternal age.

The number of previous ICSI failures

In a recent study, reporting 29 ICSI cycles in cases in which the percentage of DNA-fragmented spermatozoa, detected by the TUNEL assay, was >15%, only two pregnancies and no births were obtained (Greco et al., 2005a). However, it has to be underscored that this patient population was not derived from an overall population of patients attending the clinic, but these patients were selected from a restricted patient subpopulation who had experienced at least two previous ICSI failures (Greco et al., 2005a). In other words, patients were enrolled in the study when they simultaneously fulfilled two inclusion criteria: the history of two or more ICSI failures and the percentage of DNA-fragmented spermatozoa >15%. To what extent was the predictive power of the threshold of 15% DNA-fragmented spermatozoa conditioned by the restriction of the analysis to patients with previous ICSI failures? To know this, it is necessary to compare the percentage of DNA-fragmented spermatozoa in selected groups of patients having undergone ICSI for the first time with groups having different numbers of previous ICSI failures.

It can be expected that DNA fragmentation data may have different clinical significance in couples with a history of previous failures of assisted reproduction treatment as compared with couples without such a history. In fact, the impact of sperm DNA fragmentation on embryo viability may be alleviated by DNA repair mechanisms. Although such mechanisms are absent in male germ cells (Chandley and Kofman-Alfaro, 1971; Gledhill and Darzynkiewicz, 1973), oocytes do possess an operative nucleotide excision repair capacity (Masui and Pedersen, 1975) that can also repair sperm DNA that was damaged before fertilization (Generoso et al., 1979; Brandriff and Pedersen, 1981). It is thus possible that the variation reported for the relationship between the extent of sperm DNA fragmentation and the outcome of assisted reproduction is at least partly due to variable ability of the oocyte to repair the existing damage. If this hypothesis is true, it could be expected to detect a higher threshold of the percentage of DNA-fragmented spermatozoa in a subgroup of cases in which oocytes of young women were used as compared with cases with higher maternal age.

Maternal age

To test the hypothesis that the impact of sperm DNA fragmentation on assisted reproduction outcomes can be conditioned by the maternal age, a study on the effect of maternal age on IVF outcomes in cases with different degrees of sperm DNA fragmentation is needed. Such a study is currently underway at the clinic.

Preliminary data (Tesarik J et al., unpublished) suggest that the percentages of TUNEL-positive spermatozoa were higher in patients who did not achieve pregnancy, irrespective of the age of the women from which oocytes were retrieved. The husbands of the patients who achieved a term pregnancy with oocytes from young donors had higher percentages of DNA-fragmented spermatozoa as compared with the patients achieving term pregnancy with their own wife’s oocytes. There was no difference in the percentage of DNA-fragmented spermatozoa between the two groups was found in the attempts that failed to establish a term pregnancy.

Associated reproductive pathologies

The degree of impairment of sperm reproductive potential by a given degree of sperm DNA damage is also likely to depend on the eventual presence of other pathological conditions that are known to negatively influence testicular function. Several molecular abnormalities that may underlie such conditions, such as mutations of the testis-specific ubiquitin protease 26 gene (Paduch et al., 2005), abnormalities of CREM gene function (Krausz and Sassone-Corsi, 2005), microdeletions concerning genes located in the AZF region in Yq11 (Vogt, 2005), and abnormalities of post-transcriptional control of genes involved in spermatogenesis (Ehrmann and Elliott, 2005). All these conditions are suspected to disturb male gamete chromatin remodelling during spermiogenesis and may thus cause an increase propensity of spermatozoa to suffer DNA damage in response to relatively low intensities of oxidative stress that would not be harmful for spermatozoa with normal chromatin structure and function.

Treatment

Three treatment approaches to the problem of elevated sperm DNA damage have been suggested recently: ICSI using surgically-retrieved testicular spermatozoa instead of ejaculated ones (Greco et al., 2005b), ICSI with ejaculated spermatozoa after two months of oral antioxidant treatment (Greco et al., 2005a), and ICSI with spermatozoa selected with the use of a high-magnification optical system (high-magnification ICSI) (Hazout et al., 2006).

ICSI with testicular spermatozoa

The recourse to testicular spermatozoa for ICSI in cases with increased sperm DNA damage was based on the finding that most of free-floating spermatozoa retrieved by testicular biopsy have normal DNA, whereas most of DNA-fragmented testicular spermatozoa are tightly associated with Sertoli cells (Tesarik et al., 2004b). In a group of 18 couples who had undergone at least two unsuccessful ICSI attempts with ejaculated spermatozoa and whose male partner had ≥15% of ejaculated spermatozoa with damagedDNA, two sequential attempts, one with ejaculated spermatozoa and the other with testicular spermatozoa, were compared (Greco et al., 2005b). The incidence of DNA fragmentation was markedly lower in testicular spermatozoa as compared with ejaculated spermatozoa and, in spite of the absence of differences in fertilization and cleavage rates and in embryo morphological grade, eight ongoing clinical pregnancies (four singleton and four twin) were achieved by ICSI with testicular spermatozoa (44.4% pregnancy rate; 20.7% implantation rate), whereas ICSI with ejaculated spermatozoa led to only one pregnancy which spontaneously aborted (Greco et al., 2005b). However, these findings are not in agreement with some other studies (reviewed in Nicopoulos et al., 2004).
Oral antioxidant treatment

Since sperm DNA damage is supposed to be mainly due to oxidative stress, 64 patients with >15% of DNA-fragmented spermatozoa were randomly assigned to an antioxidant treatment (1 g vitamin C and 1 g vitamin E daily for 2 months) or to a placebo group. After 2 months, the percentage of DNA-fragmented spermatozoa was markedly reduced in the treatment group as compared with the placebo (Greco et al., 2005c). Based on this observation, the same antioxidant treatment was administered to 38 men during 2 months before an ICSI attempt, leading to a decrease in the percentage of DNA-fragmented spermatozoa in the ejaculate in 29 (76%) of them (Greco et al., 2005a). The subsequent ICSI attempt led to a marked improvement of clinical pregnancy (48.2% versus 6.9%) and implantation (19.6% versus 2.2%) rates as compared with the pretreatment ICSI outcomes in spite of the absence of differences in fertilization and cleavage rates or in embryo morphology (Greco et al., 2005a).

High-magnification ICSI

It was shown previously that spermatozoa appearing as morphologically normal at a magnification usually employed for ICSI (×400) may in fact carry various structural abnormalities that can only be detected with the use of a higher magnification (×6600) obtained by using a ×100 oil-immersion objective lens and an inverted microscope equipped with Nomarski differential interference contrast optics combined with a digitally enhanced secondary magnification system (Bartoov et al., 2002). A prospective controlled study, performed in couples with male infertility and at least two previous failed ICSI attempts, showed that ICSI with spermatozoa selected at this high magnification resulted in a significantly higher pregnancy rate as compared with conventional ICSI (Bartoov et al., 2003). Particular attention was paid to the presence of sperm head intranuclear vacuoles that signal anomalies of sperm chromatin packaging and incomplete nuclear remodelling during the final phase of spermatogenesis (Berkovitz et al., 2005). In fact, incomplete sperm nuclear protein transition, resulting in protamine deficiency, has been shown to be positively related to sperm DNA damage (Aoki et al., 2005; Nasr-Esfahani et al., 2005).

High-magnification ICSI was recently used in 125 patients, 72 of whom were examined for sperm DNA integrity (Hazout et al., 2006). An improvement of clinical ICSI outcomes was evident both in patients with an elevated degree of sperm DNA fragmentation and in those with normal sperm DNA status. It was noteworthy that a high birth rate (6/21; 28.6%) was obtained even in the group of patients with >40% of DNA-fragmented spermatozoa (Hazout et al., 2006), which is well above the threshold values suggested for conventional ICSI (Table 1).

High-magnification ICSI thus appears to be an efficient method for patients with high sperm DNA fragmentation. However, this method requires additional investment and is time-consuming. Therefore it has been replaced with an ICSI variant using a ×40 objective lens with Hoffman modulation contrast optics, which makes part of the standard equipment in an ICSI laboratory, and a secondary digital magnification of ×4000. Even with this simple system, small nuclear vacuoles could be detected with precision (Figure 1), and a comparison with the Nomarski-based high-magnification system showed a similar efficiency with regard to the exclusion of spermatozoa with abnormal nuclear chromatin structure (Tesarik J, unpublished). An improvement of sperm selection by excluding spermatozoa with abnormal chromatin structure is thus accessible to a standard ICSI laboratory without the necessity of an additional investment.

Rational management of infertility associated with sperm DNA damage

When examining an infertile patient with elevated sperm DNA damage, the first question to be asked is: what is the probability that the DNA deficiency detected is actually the cause of the problem? The threshold values outlined in this paper can be helpful, but it has to be remembered that they have a relative rather than absolute value. They need to be interpreted with

Figure 1. A spermatozoon with three small intranuclear vacuoles (left) and a spermatozoon with normal nuclear structure (right) observed with Hoffman modulation contrast optics and a ×40 objective lens. Final magnification, ×1000.
regard to the overall clinical context including the reproductive history of the couple, the number and type of previous assisted reproduction attempts, the presumed oocyte quality and the maternal age.

If sperm DNA damage is judged to be the real cause of the existing fertility problem, the patient can be given oral antioxidant treatment during two months before an ICSI attempt. This treatment is simple, well-tolerated, and it decreases the percentage of DNA-fragmented spermatozoa in most cases. If no improvement is achieved, high-magnification ICSI is indicated. In cases that are resistant to these treatments, ICSI with testicular spermatozoa can be envisaged as the last recourse.

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