Chapter 7
Sperm DNA Fragmentation and Base Oxidation

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Abstract Sperm DNA damage has been shown to be a valuable diagnostic and prognostic biomarker for male infertility and assisted reproductive treatment (ART) outcome. It is linked to every fertility checkpoint from reduced fertilization rates, lower embryo quality and pregnancy rates to higher rates of spontaneous miscarriage and childhood diseases. It is more robust than conventional semen parameters.

The aim of this chapter is to provide an overview of current laboratory tests and relationships between sperm DNA damage and clinical outcomes. The conclusion is that sperm DNA damage is an important indicator of semen quality, and its routine use in the fertility clinic would improve ART success rates.

Keywords Sperm DNA damage • Art outcomes • Male infertility diagnosis

Introduction

Infertility affects approximately 15 % of couples of reproductive age (Cates et al. 1985; Hull et al. 1985; Kols and Nguyen 1997; Rutstein and Shah 2004), with male infertility contributing nearly 50 % of all cases (Irvine 1998; Niederberger et al. 2007; Vela et al. 2009; WHO 2010). As a result of population ageing and adverse lifestyle changes, infertility continues to increase, but with only marginal improvement in pregnancy and birth rates after assisted reproductive treatment (ART), in the developed world (Dupas and Christine-Maitre 2008; HFEA 2008; Povey and Stocks 2010; Ferraretti et al. 2012). In the last 30 years ART has become increasingly utilized with the number of cycles (de Mouzon et al. 2010) increasing by up

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to 7 % per year in Europe. Still, pregnancy and live birth rates remain disappointingly low (average 27–33 %) (HFEA 2008; Ferraretti et al. 2012). One reason for this is that little has been done to resolve the causes and potential therapies for male infertility at the molecular level. Furthermore, there are currently no routine pharmaceutical therapies for male infertility.

Sperm DNA damage is a substantial indicator of ill health at the cellular level. It has been identified as a major contributor to male infertility as well as outcomes following ART, including impaired embryo development, miscarriage and birth defects in offspring (Freour et al. 2010; Koskimies et al. 2010; Bungum et al. 2011; Ebner et al. 2011; Gu et al. 2011; Simon et al. 2011; Zribi et al. 2011). Childhood health-related issues, such as childhood cancers, have also been linked to sperm DNA damage resulting from oxidative stress caused to sperm by smoking (Fraga et al. 1996; Ji et al. 1997). Since using sperm with damaged DNA for assisted conception means risking the long-term health and wellbeing of children conceived by ART, it is simply 'best practice' to test sperm DNA before using them clinically.

Current Semen Tests: Benefits and Limitations

Conventional semen analysis remains the gold standard for the initial investigation of male infertility. However, despite being the universal battery of tests, semen analysis is today considered of only limited value in predicting a couple's chance of pregnancy with ART. The World Health Organization (WHO) provides guidance for semen analysis through measures of concentration, motility and morphology (WHO 2010). Sperm motility is probably the most useful of these parameters since it is a real-time indicator of sperm metabolism (see later). Sperm concentration and morphology have minimal relation to ART success. In addition, both motility and morphology measurements are susceptible to inter- and intra-laboratory variation, and their wide ranges reflect the fact that sperm are amongst the most heterogeneous of all human cells (Jörgensen et al. 1997).

In 2001, Guzick's group (Guzick et al. 2001) examined semen from a large cohort of fertile and infertile men and reported a significant overlap in the semen profiles of the two groups. They concluded that sperm morphology, motility and concentration reference values were a blunt instrument in assessing male reproductive potential. This was subsequently confirmed by reports that the WHO (1999) reference values were not clinically predictive (Nallella et al. 2006; Van der Steeg et al. 2011). Since a very small proportion of sperm get to the site of fertilisation in vivo (Williams et al. 1992), expectations that information about the wider ranging properties of a complete ejaculate is unrealistic. In summary, a semen analysis is only useful in identifying those men with very few or no sperm.

For any test to be useful diagnostically or prognostically, it must have a threshold value which provides adequate discriminatory power in a clinical situation. Routine semen analysis does not meet these standards [Lefièvre et al. 2007; Guzick et al. 2001; reviewed by Lewis (2007) and Barratt et al. (2011)], so improved assays are needed.

Unfortunately, the success of intra-cytoplasmic sperm injection (ICSI) has allowed those in the field of infertility to become complacent, choosing to bypass rather than address the problem of male infertility. ICSI has reduced the significance and perceived need for sperm quality tests; ICSI requires only one sperm – even if morphologically abnormal and immotile – for the procedure to be around 25 % successful in most European clinics. ICSI is now the most widely used means of fertilisation in ART, but should the infertility speciality be satisfied with this modest level of success? Will adoption of a better test than semen analysis improve results?

Tests Currently Available to Assess Sperm DNA Damage

Since sperm have few repair mechanisms, DNA damage is ubiquitous in human sperm, even within donor populations (Simon et al. 2010). However, what is important clinically is the level of damage that adversely impacts ART outcomes.

The tests most often used today are the comet assay, SCSA, the terminal transferase dUTP nick end labelling (TUNEL) assay and the sperm chromatin dispersion (SCD or halo) test.

Comet Assay

The comet assay is a single-cell gel electrophoretic test that quantifies broken strands of DNA in individual sperm. As the mass of DNA fragments streams out from the head of unbroken DNA, it resembles a comet tail, hence the name of the assay. The comet is sensitive, repeatable and capable of detecting both high and low levels of damage in sperm (Irvine et al. 2000; Trisini et al. 2004; Aitken and De Iuliis 2007). A major advantage of this assay is that it requires only 5,000 sperm and so is suitable for the assessment of small samples left over from clinical use or for samples where only a few sperm are available. The comet assay can measure both single- and double-strand breaks and, with an additional step, can detect oxidised bases (Simon et al. 2010). This is important because we do not yet know which types of DNA damage are most deleterious to male fertility. A further advantage of the comet assay is that, unlike other tests which detect primarily breaks in histone-associated chromatin, it has a broader use in detecting breaks in both protamine- and histone-bound chromatin equally.

Clinical thresholds for diagnosis of male infertility and prediction of success with in vitro fertilisation (IVF) (Simon et al. 2010, 2011, 2013) have now been established by studies including over 500 couples. Unlike other sperm DNA fragmentation tests that give a DNA fragmentation index (DFI), which is the proportion of sperm in an ejaculate with some damage, the comet can detect damage in all individual sperm, even from fertile donors. The threshold values from the comet assay are measures of the actual damage in individual sperm above which spontaneous conception or success with IVF is less likely (Simon et al. 2010, 2013).

Analysis of repeatability was performed using the S_r^2, repeatability variance of the within-laboratory variances for single DNA damage measurements. It was 3.7 % but decreased to 2.6 % and 2.2 % for duplicates and triplicates respectively [ISO 5725:1994(E) guidelines for determination of repeatability of a standard measurement method, as described in Simon et al. (2013)]. In light of these results, analysis of just 50 of the 5,000 sperm included in the assay was sufficient to provide a measurement of DNA damage in the total sperm population with a coefficient of variation lower than 4 %.

In a recent study, the effects of male infertility alone on ART were evaluated by excluding all couples presenting with female factors or without detectable fertility problems from either partner (idiopathic infertility) (Simon et al. 2011). This study design allowed clinical thresholds for male infertility (25 %), success with IVF (25–50 %) or the need for ICSI (over 50 %) to be identified.

Most recently, live birth data were reported for the first time using the comet assay. Couples whose pregnancy resulted in a live birth had significantly lower sperm DNA fragmentation than those couples who did not achieve a live birth following IVF treatment (Simon et al. 2013). With the benefits of comet assay sensitivity, 80 % previously unexplained couples now have a diagnosis in the form of sperm DNA damage (Simon et al. 2013). In this latest study, high levels of sperm DNA damage were also associated with markedly lower live birth rates following IVF in 80 % couples with idiopathic infertility.

The usefulness of progressive sperm motility compared with DNA damage as predictive tools for in vitro fertilization rates has also been reported using the comet assay (Simon et al. 2011). Progressive motility is the only semen parameter that correlates with sperm DNA damage. This may be explained as a real-time functional test of sperm vitality. However, while fertilization rates are directly dependent upon both sperm progressive motility and DNA fragmentation, the latter is a stronger test, with an odds ratio of 24.18 (5.21–154.51) to determine fertilization outcome compared with 4.81 (1.89–12.65) for progressive motility (Simon et al. 2011).

Sperm Chromatin Structure Assay

The SCSA is a fluorescence cell sorter test which measures the susceptibility of sperm DNA to denature after exposure to acid conditions.

Neat semen is diluted with a pH 1.2 buffer for 30 s, and then the sperm are stained with acridine orange (AO) (Darzynkiewicz et al. 1975). Both the 30 s, low-pH-induced opening of the DNA strands at sites of DNA breaks and the biochemical interaction between AO and DNA/chromatin are precisely repeatable. This is proven by comparing cytogram scatter plots with 1,024 channels for both X (red) and Y (green) fluorescence values in repeat measures of individual semen samples (Evenson et al. 1991). The software SCSAsoft computes the raw red versus green fluorescence data as red/red + green fluorescence (Evenson et al. 2002).

This produces a vertical dot pattern for non-denatured DNA and a horizontal dot pattern for sperm with fragmented DNA. The SCSAsoft frequency histogram of DFI allows a precision determination of percentage DFI. Following repeated studies (Evenson et al. 1999; Spano et al. 2000; Evenson and Wixon 2006a, b; Bungum et al. 2007) an internationally accepted statistical threshold for natural and intrauterine insemination (IUI) conception of approximately 25 % DFI was adopted. The SCSA has robust statistical power, but it is unsuitable for samples with low counts. In addition, it measures only single-stranded fragments and has demonstrated associations between native, but not prepared, sperm and ART outcomes.

Sperm Chromatin Dispersion (Halo) Test

The halo test is a simple and inexpensive assay, available to fertility labs in kit form. Unlike all the other tests, it measures relaxed intact DNA associated with only peripheral histones rather than the damaged DNA in sperm. The test is convenient in that it does not rely on either colour or fluorescence intensity and is simple to analyse in a routine laboratory with light microscopy. One limitation of the assay is that its low-density nucleoids are relatively faint, with less contrasting images. To date, correlations have been observed between DNA damage and other sperm parameters, although few correlations between sperm DNA damage and ART outcomes have been established with the halo test, even in large ($n = 600$) studies. However, Meseguer et al. (2009) reported that sperm DNA damage as measured by halo has a negative impact on pregnancy.

TUNEL Assay

The TUNEL assay detects 'nicks' (free ends of DNA) by incorporating fluorescently stained nucleotides. This allows the detection of single- and double-stranded damage. The cells can be assessed either microscopically or by flow cytometric (FCM) analysis. This gives the assay the flexibility to be used for small numbers with microscopic analysis and in small laboratories which do not have dedicated and expensive FCM facilities. However, it can also be analysed by FCM, giving it the advantage of robust numbers with reduced time and labour. A disadvantage of the assay is its many protocols, which makes comparison between laboratories almost impossible and explains its many clinical thresholds. Recently, Aitken's group (Mitchell et al. 2010) improved the TUNEL assay by including a preliminary step of DDT to relax the whole chromatin structure and allow access to all nicks. They also added a viability stain so that DNA damage is measured only in live sperm. This has eliminated a previous inaccuracy of measuring damage (often at high levels) in dead cells. Robust clinical thresholds have yet to be established.

Novel Tests for Oxidised Bases

DNA damage tests usually measure strand breaks. While this provides data on the final stage of damage, these tests give little information about how the damage came about. Knowledge about the DNA adducts present in human sperm will also provide information about earlier stage DNA damage and thereby enhance the prognostic value of our current tests. DNA damage in sperm is primarily from oxidative stress (OS) (Aitken et al. 2010). A low physiological level of reactive oxygen species (ROS) is considered necessary to maintain normal sperm function, but ROS levels above physiological norms may cause deteriorating function or reduced survival (Aitken et al. 1989). The sperm most susceptible to OS are those that survived incomplete or abortive apoptosis in the testis and sperm that underwent flawed chromatin remodelling during spermiogenesis (Aitken and De Iuliis 2007). A number of biological and environmental factors that create DNA adduct formation in sperm are associated with impaired embryonic development and the health of the offspring (Adler 2000; Anderson 2001).

The measurement of sperm DNA modifications such as 8-hydroxy-2-deoxyguanosine (Lee et al. 2009; Makker et al. 2009; Gharagozloo and Aitken 2011; Thomson et al. 2011) and xenobiotic adduct formation (Zenes 2000) including benzo[a]pyrene (Park et al. 2008), are the latest area of research. Already, these two lesions have been reported as significant in male infertility and childhood health (Anderson 2001; Lee et al. 2009). The characterisation of OS markers can result from a number of infertility aetiologies suggesting that OS is a major mediator of DNA damage in the male germ line (De Iuliis et al. 2009). The incidence of these markers together with the detection of others, such as advanced glycation end products, may confirm specific pathologies such as diabetes (Agbaje et al. 2008).

Sperm DNA Damage in Male Infertility Diagnosis

Infertile males have greater sperm DNA fragmentation compared to those in the general population or men with recently proven fertility (Schulte et al. 2010). Two independent, population-based studies, one from the USA (Evenson et al. 1999) and one from Denmark (Spano et al. 2000), have shown that sperm DNA damage is a useful marker in the prediction of fertility in males from couples of unknown fertility. Both of these studies have shown that the chance of spontaneous conception declines at sperm DNA damage (DNA fragmentation index, DFI; this parameter relates to SCSA tests only) values above 20 % and approaches zero for readings over 30–40 %. This means that although low sperm DNA damage (<20 %) does not guarantee normal male fertility, higher levels of damage suggest more substantial male infertility. Furthermore, the SCSA data indicate that for men who have been

classified as normal by a semen analysis the risk of infertility starts to increase at DFI levels above 20 % [odds ratio (OR) 5.1, 95 % confidence interval (CI): 1.2–23]. The threshold becomes even lower (10 %) if the man's semen has a subnormal semen analysis as well (Giwercman et al. 2010). In another study (Simon et al. 2011), this one using the comet assay, there was also a strong correlation between sperm DNA fragmentation and the fertility status of men, with 95 % of fertile donors having DNA fragmentation below 25 % and 98 % (mean DNA damage per sperm) of infertile men having DNA fragmentation values above 25 %. The prognostic value of sperm DNA fragmentation in relation to infertility showed an OR for infertility of 120 (95 % CI: 13–2700) in men with DNA damage above 25 % (Simon et al. 2011). Thirdly, a comparison between male infertility patients and sperm donors using a flow cytometric TUNEL assay gave 19.25 % as the cut-off value with no donors but 65 % patients having DNA damage above this level (Sharma et al. 2010). Thus there is robust evidence from all the DNA fragmentation tests that the chance of spontaneous pregnancy is reduced when DNA damage is high.

Sperm DNA Damage and Assisted Reproduction

Success rates for IUI are similar to those for spontaneous pregnancies, indicating a reduction in the chances of pregnancy with sperm DNA damage values above 20 %, according to the SCSA (Bungum et al. 2007). If a test for oxidised bases is employed (8-hydroxy-2`-deoxyguanosine; 8-OHdG), the results are even more sensitive, with a lower threshold value of 11.5 % (Thomson et al. 2011). Lewis' group recently reported that 80 % of couples with unexplained infertility, and therefore those couples likely to be offered IUI as a first treatment, although they have significant sperm DNA damage (Simon et al. 2013).

For IVF, Zini and Sigman (2009) published a meta-analysis showing an increased chance of pregnancy (OR: 1.7; 95 % CI: 1.3–2.2) in cases where the proportion of DNA damaged sperm was below the threshold values for SCSA or TUNEL. As a result of these data, sperm DNA testing is now employed routinely throughout southern Sweden. Support for these data is given in two studies using the comet assay (Simon et al. 2010, 2013), both published after Zini and Sigman's (2009) meta-analysis with an OR of 76 (95 % CI: 8.7–1700) for clinical pregnancy if the mean DNA fragmentation per sperm was below 52 % (Simon et al. 2011). The latest study using the comet assay showed that couples with low levels of sperm DNA fragmentation (<25 %) have a live birth rate of 33 % following IVF treatment. In contrast, couples with high levels of sperm DNA fragmentation (greater than 50 %) had a much lower live birth rate of 13 % following IVF treatment. Thirty-nine percent of couples with idiopathic infertility have high (greater than 50 %) sperm DNA damage. Sperm DNA damage was also associated with lower live birth rates following IVF in couples with idiopathic infertility than in couples with detectable causes.

When considering the reports to date, this author is of the opinion that our expectations of sperm DNA testing tend to be excessive. How can a single parameter (from only one of the two gametes) provide an absolute criterion for fertility or infertility? A successful ART outcome will depend on many other traits of sperm quality and function, as well as the influences of the oocyte, uterine receptivity and maternal immune system competence.

Implications of DNA Damage for the Health of Future Generations

Animal studies provide compelling evidence that the induction of DNA damage in the male germ line can induce miscarriage and morbidity in offspring (Fernández-Gonzalez et al. 2008). A higher risk of morbidity in the offspring is presented by smoking, and again paternal smoking induces sperm DNA damage (Fraga et al. 1996) as well as childhood cancers in the offspring (Ji et al. 1997; Lee et al. 2009). Paternal age is also linked to a high incidence of DNA damage in human sperm (Singh et al. 2003; Schmid et al. 2007; Varshini et al. 2012). Paternal age is also linked to a higher incidence of epilepsy, schizophrenia, autism, and bipolar disease (Sipos et al. 2004; Reichenberg et al. 2006; Aitken and De Iuliis 2007; Frans et al. 2008). It is also linked to an increased risk of cancer in the offspring (Hemminki et al. 1999; Johnson et al. 2011) and congenital anomalies (Green et al. 2010). This suggests that adverse paternal effects on the offspring health are passed on by DNA damaged sperm. This may be even more important in men seeking infertility treatment. In a recent meta-analysis (Wen et al. 2012) it was reassuring to note that no difference was found between the risks associated with IVF and those associated with ICSI. However, in contrast, a recent analysis of pregnancies in South Australia revealed a significantly enhanced chance of birth defects in ICSI compared with IVF children (Davies et al. 2012).

Potential of Antioxidant Therapy

If OS is involved in the aetiology of DNA damage, then antioxidant therapy should be part of the cure (Greco et al. 2005). Men who have been diagnosed with oxidative sperm DNA damage by one of the tests described earlier might be helped by taking antioxidants before ART is begun. A recent review paper (Gharagozloo and Aitken 2011) summarised 20 clinical trials of ART outcomes following antioxidant use over the last decade. All the trials showed a reduction in sperm OS, and some also reported improvement in clinical outcomes such as pregnancy. Clinicians routinely recommend the use of antioxidant(s), as was recently reported (Lanzafame et al. 2009; Zini et al. 2009; Ross et al. 2010; Showell et al. 2011; Gharagozloo and Aitken 2011). Regimes of concentration, constituents or duration of therapy have not been carefully considered, but these dietary supplements are probably not unsafe, although their benefits may be limited. In contrast, higher doses and long

durations of administration as well as the use of synthetic or chemically modified versions of antioxidants should be avoided. One example of danger to health was reported in the large cancer prevention SELECT clinical trials (long-term use of vitamin E at 400 IU/d) where a significant rise in prostate cancer among 35,533 healthy men was found (Klein et al. 2011). Future research and clinical studies should address these issues as a matter of urgency.

Why Does ICSI Work with Poor Sperm?

Sperm DNA damage has not been found to be predictive for ICSI treatment (Zini 2011), with one exception (Bungum et al. 2007). However, in this study, couples were not randomised for IVF or ICSI, so the impact of other factors contributing to the choice of treatment cannot be excluded. A number of reasons have been put forward to explain the finding that poor sperm DNA does not appear to adversely affect ICSI outcomes. Firstly, unlike IVF, up to 30 % of women (with subfertile partners) having ICSI have no detectable problems. They may be fertile and their oocytes may have more capacity to repair DNA damage, even if the injected sperm is of poor quality. This is supported by the findings of Meseguer et al. (2011) where high-quality oocytes from donors offset the negative effect of sperm DNA damage on pregnancy.

Secondly, a recent major study (Dumoulin et al. 2010) showed that even the birth weight of IVF babies can be markedly influenced by minor differences in culture conditions. In contrast to IVF, ICSI sperm are injected into the optimal environment of the ooplasm within a few hours of ejaculation. This may protect them from laboratory-induced damage.

Thirdly, it is well documented that sperm from up to 40 % of infertile men have high levels of ROS (Henkel 2011; Aitken et al. 2012), and their antioxidant content is also significantly lower than in fertile men (Lewis et al. 1995). During the IVF process, oocytes can be exposed to an overnight oxidative assault from 0.5 million spermatozoa releasing ROS. This may well impair the oocyte's functional ability, including its capacity to repair sperm DNA fragmentation after fertilization.

Finally, as mentioned earlier, evidence is emerging that embryos with high sperm DNA damage are associated with early pregnancy loss, as reviewed by Zini et al. (2008) using 11 studies composed of 808 IVF and 741 ICSI cycles, so ICSI success rates are sometimes affected adversely by sperm DNA damage, but at a later stage. In fact, high levels of sperm DNA damage are associated with increased risk of pregnancy loss (OR: 2.5; 95 % CI: 1.5–4.0), regardless of the in vitro technique applied, as reviewed by Robinson et al. (2012).

Conclusion

Thus, as a matter of best practice, to improve ART outcomes, sperm DNA damage testing should become part of routine semen analysis (Fig. 7.1).

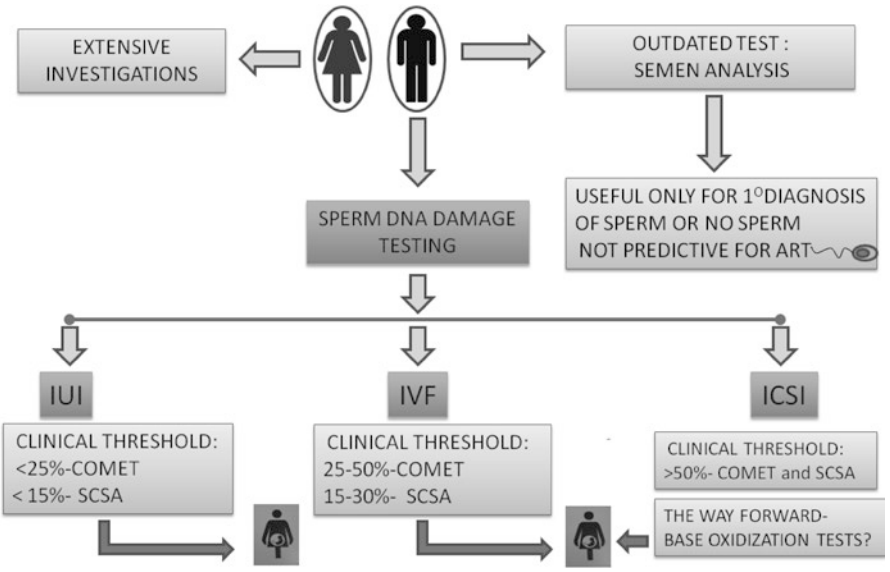

Fig. 7.1 Summary of clinical use of sperm DNA damage testing

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