INVITED REVIEW

Clinical implications of sperm DNA damage

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Abstract
Traditionally, the diagnosis of male infertility has relied upon microscopic assessment and biochemical assays to determine human semen quality. These tests are essential to provide the fundamental information on which clinicians base their initial diagnosis. However, none of these parameters addresses sperm function and their clinical value in predicting fertility is questionable. The advent of intracytoplasmic sperm injection (ICSI) has further reduced the significance and perceived need for sperm quality tests since ICSI requires only one sperm for the procedure to be successful. Even the conventional measures of sperm quality in terms of normal morphology or motility are not necessary for successful ICSI. Funding of andrological research has been neglected and improvement in assisted reproductive technology (ART) success has suffered as a consequence. Testing of sperm DNA damage shows much promise both as a diagnostic test for male infertility and a prognostic test for ART outcomes. It has been shown to be closely associated with numerous fertility outcomes including negative relationships with fertilization, embryo quality, implantation and positive relationships with miscarriage and childhood diseases. Here we report the relationships between in vitro fertilisation, ICSI pregnancy rates and sperm DNA damage, using the Comet assay to measure DNA fragmentation and also a novel test to measure modified bases, as a indication of oxidative DNA injury.

Keywords: Sperm DNA

Introduction
Falling birth rates are becoming a public health issue as they continue in a sustained decline across Europe. Over the last fifty years, they have plummeted to reach an unprecedented low of 1.4 children per couple (Commission of the European Communities, 2009). Last year, the European Parliament (European Parliament, 2008) acknowledged that falling birth rates were a major cause of its demographic decline. Reduced family size is the major determinant of the future population number and composition in Europe (Maccheroni, 2007). One solution to the problem of reduced birth rates is to lessen the decline through assisted reproductive technology (ART). Europe already performs about 60% of all ART treatments in the world (Andersen & Erb, 2006) and in European countries between 1 and 6% (RAND, 2006) of births are currently aided by ART. Hence, ART in Europe has the potential and capacity to make a significant contribution in redressing the adverse economic and demographic factors by reducing falling birth rates. The European Parliament has now recommended that ART should be incorporated into the proposed population policy mix (European Parliament, 2008; Ziebe & Devroey, 2008). In a resolution adopted by the Parliament on 21 February 2008, it called on member states to ensure the right of couples to universal access to infertility treatment. If implemented, this would be a major step forward since the majority of infertility provision is currently performed in the private sector (except in Scandinavia and Belgium) with only those who can afford it able to avail of such services.

Those in the Reproductive Medicine Community must work together to design novel tests to improve ART success rates. Mean European ‘take-home baby’ rates still have room for improvement as they are only 30.1% (Andersen et al., 2008) compared to 27.0% a decade ago (Land & Evers, 2003) and there is disparity between European countries (van den Bergh et al., 2006). The UK national live birth rate...
for fresh cycles to women less than 35 years is 32.3% (Human Fertilization and Embryology Authority, 2002; 2007). If ART is to be included as a substantive part of the new population policy, there will need to be government led and funded demands for ART enhancement. Male infertility has been long neglected and this is the area where most rapid progress could be made. However, this will force ART personnel to re-examine assessment of male fertility potential and agree on improved prognostic sperm function tests with clinical relevance for each type of ART treatment. Ironically, implementing appropriate sperm function tests has been delayed by the success of intracytoplasmic sperm injection (ICSI) for men with male infertility. The studies of Nagy et al. (1995) and Svalander et al. (1996) had enormous influence in ART clinics worldwide when they reported that ICSI was successful even with semen samples destined for ICSI being performed simply to estimate sperm concentration in the ejaculate. Since they reported that ICSI was successful even with semen well below World Health Organization recommended classification of normozoospermia.

Their reports resulted in semen analysis of poor samples destined for ICSI being performed simply to estimate sperm concentration in the ejaculate. Since this approach has led to ‘take-home baby’ rates with ICSI that are as good or better than those with in vitro fertilisation (IVF) where the best sperm are isolated and presented to the oocyte, there has been little incentive for ART centres to invest in the research and development of sperm selection tests. However, this must change in order to raise ART success rates by finding a robust test with high prognostic strength to ensure choice of the best sperm, not just for high implantation rates but for the long term health of subsequent generation.

Conventional semen analysis is now recognized to be of limited value in the determination of a couple’s fertility status (reviewed by Lewis, 2007). In contrast, sperm DNA testing has been hailed as a more promising test (Aitken & de Iuliis, 2007; Evenson et al., 2007; Barratt et al., 2010). As a diagnostic tool, sperm DNA damage has been shown to be more reproducible (Zini et al., 2001; Evenson et al., 2002; Loft et al., 2003) with studies reporting high monthly repeatability within donors (Evenson et al., 1999; Smit et al., 2007) although another study has shown that just 37% of patients with a DNA fragmentation index 30% in one test had a similar result in a second test so recommending repeated testing (Erenpreiss et al., 2006).

Measurement of sperm DNA damage is also a useful prognostic biomarker with numerous studies showing its association with longer times to conceive compared with fertile couples (Spano et al., 2000), impaired embryo cleavage (Morris et al., 2002), higher miscarriage rates (Evenson et al., 1999), and most closely with increased risk of pregnancy loss after both IVF and ICSI (Zini et al., 2008). However, the implications of sperm DNA damage may be even more far reaching. As sperm have few repair mechanisms (Aitken & Baker, 2006) and oocytes can only repair a limited amount of sperm DNA damage (Ahmadi & Ng, 1999; Derijck et al., 2008) such damage may remain in the germ line for generations. Nature does not prevent a sperm with oxidative DNA damage reaching the oocyte, achieving fertilisation and thus contributing to mutations during embryonic development (Fraga et al., 1991) or even causing the loss of the foetus. Damaged sperm DNA may be incorporated into the embryonic genome, thus leading to errors in DNA replication, transcription and translation during embryogenesis, contributing to a range of human diseases (Cooke et al., 2003) in not just one but subsequent future generations (reviewed by Aitken et al., 2008). In particular, sperm DNA can impact on the short and long term health of children born by ART. Children conceived by ART, particularly ICSI have a higher incidence of disease than children spontaneously conceived (Basatemur & Sutcliffe, 2008; Katari et al., 2009). Continuing into childhood, there is a strong association between poor sperm DNA integrity and a range of diseases ranging from childhood cancers, leukemias to autism. Literature is rapidly accumulating (reviewed by Aitken et al., 2008) to show that the link is through DNA damage to the father’s sperm.

The clinical usefulness of a test (i.e. its ability to predict the chance of sperm populations achieving a clinical pregnancy) is based on its odds ratio which in turn is based on threshold values which vary enormously (Collins et al., 2008) depending on the assay, sperm preparation and scientific choice. A threshold value has been established for the Sperm Chromatin Structure Assay (SCSA, Bungum et al., 2007). It is drawn from a study by Evenson et al., 1999 of 165 presumed fertile couples. None of these couples with > 30% DNA damage achieved a clinical pregnancy and so 30% was taken as the threshold above which DNA damage was not considered compatible with fertility. The threshold levels for the SCSA assay vary from 20% (Boe-Hansen et al., 2006), 27% (Larson et al., 2000; Larson-Cook et al., 2003) to 30% (Evenson et al., 1999; Virro et al., 2004; Payne et al., 2005; Zini et al., 2005). However, these thresholds may or may not be appropriate for infertile couples undergoing IVF or ICSI and may also differ for sperm from native, unprocessed and sperm prepared for ART by density centrifugation (DCG).

The threshold levels for which the Terminal Transferase dUTP Nick End Labeling (TUNEL) assay has clinical relevance show greater variation; from 4% (Host et al., 2000; Huang et al., 2005), 10% (Borini et al., 2006), 15% (Benchabib et al., 2007) to 20% (Host et al., 2000). There is a lack of understanding of the clinical significance of a TUNEL assay result.
20% (Benchaib et al., 1993; Seli et al., 2004) to 35% (Frydman et al., 2008). The wide variation is a reflection of the range of different laboratory protocols for the Tunel assay (Mitchell et al, 2010).

Until now, there have been no clinical thresholds for the Comet assay (Lewis et al., 2004), in either its neutral or alkaline forms, although it is recognised to be more sensitive than other DNA damage tests. (Klaude et al., 1996; Irvine et al., 2000; Barratt et al., 2010) It is the only technique that allows the quantitative measurement of DNA damage in individual cells; particularly useful in a heterogeneous population like sperm. The Comet assay measures both single and double DNA strand breaks using alkaline pH (Hughes et al., 1996; Donnelly et al., 2001) or neutral pH conditions (Morris et al., 2002). It is highly reproducible (Hughes et al., 1997) and as it requires a much smaller number of cells (Hughes et al., 1996) for analysis than other tests, it is suitable for measures of testicular and oligozoospermic sperm samples where cells are scarce. A further advantage is that, unlike the TUNEL and SCSA which detect primarily breaks in histone associated chromatin, the Comet assay has a broader use as it can detect breaks in both protamine and histone bound chromatin.

Two recent systematic reviews have shown that the impact of sperm DNA damage on ART outcomes decreases from intrauterine insemination IUI to IVF and is least useful in ICSI (Collins et al., 2008; Zini & Sigman, 2009). In IVF, using TUNEL and SCSA assays, the combined odds ratio is 1.57 (95% confidence interval 1.18, 2.07; p < 0.05). We have recently (Simon et al., 2010) reported two thresholds to calculate odds ratios for IVF, using the Comet assay: 56% for native, unprocessed sperm and 48% for the DGC sperm. These high values (relative to the SCSA and TUNEL) are due to the sensitivity of the Comet assay in that following lysis and decondensation, all double and single strand breaks and alkali labile sites are revealed in contrast to other assays where perhaps only peripheral DNA damage is determined. In our study, the odds ratios for clinical pregnancy following IVF were 7.79 (1.74–48.6) in native semen and 6.58 (1.46–41.11) in sperm after DGC indicating its promise as a prognostic test. In contrast, the odds ratio for clinical pregnancy following ICSI was not significant at 2.27 (0.94–5.5) in the native semen and 1.61 (0.67–3.89) in the DGC sperm showing less robustness and supporting the combined odds ratio of 1.14 reported by Zini and Sigman (2009).

In ICSI cycles, in contrast to IVF, we did not obtain a significant association between DNA fragmentation of native or DGC sperm and clinical pregnancy using the Comet without formamidopyrimidine-DNA glycosylase (FPG) (Figures 1 and 2). This is in agreement with many studies (Host et al., 2000; Morris et al., 2002; Benchaib et al., 2003; 2007; Bungum et al., 2004; 2007; Gandini et al., 2004; Greco et al., 2005; Huang et al., 2005; Payne et al., 2005; Zini et al., 2005; Boe-Hansen et al., 2006; Muriel et al., 2006; Lin et al., 2008). As before, the literature is divided and, in contrast to our results, there are studies showing a significant decrease in clinical pregnancy with increase in DNA fragmentation (Larson et al., 2000; Larson-Cook et al., 2003; Saleh et al., 2003; Virro et al., 2004; Borini et al., 2006; Bakos et al., 2008; Bungum et al., 2008). However, our data supports the hypothesis that ICSI is able to compensate for existing DNA strand breaks as well as inadequate conventional sperm parameters (Ozmen et al., 2007; Bungum et al., 2008). Our data supports the belief that ICSI

![Figure 1. Correlation between pregnancy and DNA fragmentation measured by Comet and FPG in the IVF cycles, values expressed as mean ± SEM, *p < 0.05, **p < 0.01.](image-url)
bypasses genetic, as well as functional defects, although this is difficult to comprehend.

Oxidative stress has long been implicated as the major etiological factor in sperm DNA damage. Physiological levels of reactive oxygen species (ROS) are necessary to maintain normal sperm function but if reactive oxygen levels increase they lead to deteriorating function or reduced survival (Aitken & Baker, 2002). In contrast to somatic cells, sperm are very vulnerable to oxidative stress (Sies, 1993) due to their unique membrane structures combined with limited antioxidants (Lewis et al., 1995) or protective enzymes. Oxidative stress instigates deoxyribose damage, loss of bases or modifications to bases such as seven, 8-dihydro-8-oxo-2'-deoxyguanosine (8-OHdG), an oxidative adduct of the purine guanosine (Croteau & Bohr, 1997). Furthermore, such base modifications may also lead to discrete DNA strand breaks (Croteau & Bohr, 1997). Compared to other cell types, sperm exhibit much greater oxidative DNA damage (Kodama et al., 1997), and higher levels of 8-OHdG have been observed in sperm from infertile compared to healthy subjects (Kodama et al., 1997; Shen et al., 1999).

In our recent study (Simon et al., 2010) we also used the alkaline Comet assay with the addition of FPG, a bifunctional DNA glycosylase (base excision repair enzyme) that recognizes and removes oxidized purines such as 8-OHdG thereby converting modified bases (MB) into strand breaks measurable by the Comet (Collins, 2004).

Using the Comet, we have reported sperm DNA fragmentation was significantly higher in sperm from non pregnant couples compared with that from pregnant couples undergoing IVF in both native semen and in DGC sperm for clinical use (Figure 1) and addition of the FPG enzyme enhanced the difference between non pregnant compared with that from pregnant couples in the native semen and in DGC sperm in IVF (Figure 1).

A major cause of sperm DNA damage is oxidative stress, due to the generation of the ROS from contaminating leukocytes, defective sperm and antioxidant depletion (Lewis et al., 1995; Garrido et al., 2004). In addition to damage caused by creating strand breaks, we measured, for the first time, additional oxidative damage by exciting modified bases (MB) to make them measurable by the Comet assay. When we converted oxidised purines into strand breaks in both IVF and ICSI (n = 126) an increase in damage of 15.9 ± 1.3% was observed in native semen and 16.7 ± 1.4% in DGC sperm. By including MB, a strong association emerged between DNA fragmentation and clinical pregnancy rates following ICSI (Figure 2) as well as increasing the sensitivity in IVF. This shows the importance of including MB in potential prognostic tests rather than focusing on existing strand breaks alone for male infertility. Earlier studies had reported that the measurement of 8-OHdG (Horak et al., 2003a, Ni et al., 1997) is an important biomarker to investigate DNA damage and human infertility. MB are also known to increase in embryos of smoking couples (Zenzes et al., 1999). Recently, Horak et al. (2007) reported that sperm MB impair fertilization rates following ICSI. Horak et al. (2003a) showed fertile individuals and patients with male-factor infertility differed significantly with respect to the level of alteration. A significant negative correlation was obtained between MB (Horak et al., 2003b), 8-OHdG (Ni et al., 1997) and semen quality in patients with an impaired fertility. Further, Horak et al. (2003b) showed the level of bulky MB sperm is positively associated with amounts of leukocytes in semen, and also higher in semen of infertile subjects.

![Figure 2. Correlation between pregnancy and DNA fragmentation measured by Comet and FPG in the ICSI cycles, values expressed as mean ± SEM, *p < 0.05, **p < 0.01.](image-url)
FPG sensitive sites in human sperm, we increased the prognostic value of the Comet test. Since this study shows that a significant proportion of DNA damage is specifically due to oxidative stress, it highlights the possibility of antioxidant therapy to protect sperm DNA prior to ART treatment.

In ICSI couples, when MB were included, the DNA damage between pregnant and non-pregnant couples was markedly different in contrast to Comet without FPG where there was no significance (Figure 2). Thus, by including MB, a strong association emerged between DNA damage and clinical pregnancy in ICSI as well as increasing the sensitivity of the test in IVF suggesting that this approach may prove to enhance the prognostic usefulness of current sperm DNA damage testing.

In conclusion, it is our opinion that we are making excessive demands on current sperm DNA testing. Infertility is a couple’s problem but one single test for the male cannot take into consideration the contribution of the female, in terms of oocyte or embryo competence. Here, we are expecting a test of one measure of gamete dysfunction from just one partner of the couple to give an impossible predictive precision before introducing it into clinical practice. Conversely, we seem content to continuing using more conventional semen analysis tests long shown to be imperfect and thus limit the success of ART.

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