

The impact of sperm DNA damage in assisted conception and beyond: recent advances in diagnosis and treatment

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Astract:

Sperm DNA damage is a useful biomarker for male infertility diagnosis and prediction of assisted reproduction outcomes. It is associated with reduced fertilization rates, embryo quality and pregnancy rates, and higher rates of spontaneous miscarriage and childhood diseases. This review provides a synopsis of the most recent studies from each of the authors, all of whom have major track records in the field of sperm DNA damage in the clinical setting. It explores current laboratory tests and the accumulating body of knowledge concerning the relationship between sperm DNA damage and clinical outcomes. The paper proceeds to discuss the strengths, weaknesses and clinical applicability of current sperm DNA tests. Next, the biological significance of DNA damage in the male germ line is considered. Finally, as sperm DNA damage is often the result of oxidative stress in the male reproductive tract, the potential contribution of antioxidant therapy in the clinical management of this condition is discussed. DNA damage in human spermatozoa is an important attribute of semen quality. It should be part of the clinical work up and properly controlled trials addressing the effectiveness of antioxidant therapy should be undertaken as a matter of urgency.

Introduction

Male factor infertility is implicated in more than 40% of couples presenting for assisted reproduction treatment. Conventional semen analysis continues to be the only routine test to diagnose this condition even though it is known that such descriptive assessments cannot discriminate between the spermatozoa of fertile and infertile men (Guzick et al., 2001). The shifting values for normality (all 'normal' values now lower) in the fifth edition of the WHO manual (World Health Organization, 2010) compared with the previous WHO editions may result in even less men being classified as infertile (Murray et al., 2012).

A growing number of studies report sperm DNA damage to be useful as a diagnostic tool for male infertility. This criterion of sperm quality is useful as a predictor of treatment success as suggested by its associations at numerous points in the reproductive process including impaired fertilization, disrupted preimplantation embryo development, miscarriage and birth defects in the offspring (Bungum et al., 2012; Lewis and Aitken, 2005; Simon et al., 2011; Zini, 2011; Zini et al., 2008). Childhood cancers have been also

been associated with oxidative damage to sperm DNA as a consequence of paternal smoking (Fraga et al., 1996; Ji et al., 1997).

DNA damage in spermatozoa is primarily from oxidative stress (Aitken et al., 2010). In any semen sample, the vast majority of spermatozoa are abnormal (WHO, 2010). The spermatozoa that reflect such stress most profoundly are those morphologically abnormal cells that were destined for apoptosis but the process was incomplete or those cells that have experienced defective chromatin remodelling during spermiogenesis. Such defective spermatozoa are also thought to retain excess residual cytoplasm, allowing them to generate excessive reactive oxygen species (ROS), which, given their incomplete chromatin packaging, induces DNA damage (Aitken et al., 2010).

DNA damage has long been the recognized universal indicator of cell lethality in toxicology laboratories in the pharmaceutical industry. Spermatozoa are no different. Sperm DNA damage is a robust indicator of cellular ill health. Now, with the aid of advances in assisted reproduction, fertilization can be achieved *in vitro* with spermatozoa that would have been rejected *in vivo*. However, by using spermatozoa with compromised DNA for assisted conception, the long-term health and wellbeing of children conceived by assisted reproduction treatment are being put at risk. As a matter of 'best practice', the DNA quality of male gametes should be tested before they are used clinically.

Unfortunately, as spermatozoa have few repair mechanisms, DNA damage is commonly encountered in human spermatozoa, even within the fertile donor population (Simon et al., 2010). However, what is important clinically is the level of damage that adversely impacts on treatment outcomes. For any test to be useful diagnostically or prognostically, it must have a threshold value which provides adequate discriminatory power in the clinical situation. Routine semen analysis does not meet these standards (Guzick et al., 2001; Lefièvre et al., 2007; reviewed by Lewis, 2007; Barratt et al., 2011), so improved assays are needed.

Benefits and limitations of current semen tests

Sperm numbers and quality, along with normal seminal plasma constituents, are crucial for fertility *in vivo*, so a semen analysis evaluating these parameters has long been the cornerstone of male infertility diagnosis. WHO provides guidance for semen analysis via a detailed laboratory manual and associated reference values (Cooper et al., 2010; WHO, 2010). The current minimum standard is assessment of seminal plasma by volume, appearance and liquefaction of the ejaculate, and, for spermatozoa, measurement of concentration, motility and morphology (WHO, 2010). The recently revised WHO reference values are based on samples analysed according to WHO guidelines from 1953 men in nine countries on three continents with time to pregnancy 12 months or less. This represents a significantly better reference population than previous editions of the guidelines, although it has been argued that there is still room for improvement (Cooper et al., 2010; Esteves et al., 2012; WHO, 1999). Analysis of semen samples is complicated by intersample variability caused by both technical and biological factors (reviewed in WHO, 2010) and WHO recommends the analysis of two or three samples. Consistency can be improved by implementation of quality assurance and staff training (Björndahl et al., 2002) but a significant issue is that many laboratories do not

work to WHO guidelines (Keel et al., 2002; Penn et al., 2011; Riddell et al., 2005). It is recommended that semen analysis should be complemented by other clinical assessments (physical examination, history, endocrine and genetic investigations) as appropriate (Esteves et al., 2012). However, in practice this is often overlooked (Jequier, 2008).

In both natural and assisted conception (with the exception of intracytoplasmic sperm injection (ICSI)), motility is a vital function for transit to the oocyte and penetration of the oocyte vestments. The strict Tygerberg criteria for assessing sperm morphology recommended by WHO (2010) are based on the morphology of spermatozoa capable of penetrating cervical mucus and binding to the zona pellucida and therefore have a basis in biology. There is no known link between normal head morphology and the genetic quality of a spermatozoon (Menkveld et al., 1990, 1991; Ryu et al., 2001; Simon et al., 2010). This suggests that sperm DNA testing will add further information not available through a conventional semen analysis. Both motility and morphology measurements are highly sensitive to operator subjectivity/variability and further complicated by the highly heterogeneous nature of human sperm populations (Jørgensen et al., 1997).

Although both sperm motility and morphology scores have repeatedly been found to positively correlate with fertilization rate (Kruger et al., 1987; Eggert-Kruse et al., 1996; Bonde et al., 1998; Coetzee et al., 1998; Larsen et al., 2000; Van Waart et al., 2001; Ombelet et al., 2003; Haugen et al., 2006; Nallella et al., 2006; Merviel et al., 2010; Simon and Lewis, 2011), a detailed analysis of a large cohort of fertile and subfertile men by Guzick and colleagues (2001) showed an extensive overlap in the semen profiles of the two groups. They concluded that sperm morphology, motility and concentration reference values could be no more than a guide to reproductive potential, aligning with reports that the (1999) WHO reference values were not clinically predictive (Nallella et al., 2006; Van der Steeg et al., 2011), although it is clearly too soon to judge whether the new WHO values provide a correlation with outcome. Very few (0.0041%) spermatozoa (Williams et al., 1993) reach the site of fertilization *in vivo*, This is supported by animal data where considerably fewer than 1% of spermatozoa reach the ampulla of the oviduct at the time of fertilization. Thus to expect an analysis of the gross parameters of the whole ejaculate to give strong discriminatory information is not realistic. There are many obstacles that a spermatozoon must overcome on its long journey to fertilize an oocyte and what is required are tests of sperm function to replace, or supplement, the surrogate measures currently being used (Lefièvre et al., 2007). It is estimated that a significant proportion of men with unexplained infertility have a cause attributed after sperm genomic testing (Bungum et al., 2007). In its current form, semen analysis should be considered only as a means of identifying men whose chance of achieving a natural pregnancy is reduced.

Current sperm DNA damage tests

In recent years, the Comet, SCSA, Halo and TUNEL assays have been studied extensively to analyse sperm chromatin integrity. Criticisms have been made that the results from these tests are not equivalent. This is to be expected because, although each test evaluates sperm DNA quality, each may be elucidating a different aspect of DNA damage. Further, the parameter, DNA fragmentation index (DFI), seems to have become the generic term for all 'DNA' tests but is only applicable to the SCSA test.

The Comet assay

The Comet assay is a single-cell gel electrophoretic assay that quantifies broken strands of DNA in individual spermatozoon. Within an agarose gel, the sperm membranes are lysed and the DNA decondensed in a high-concentration salt solution. Disulphide bridges are broken down and then the spermatozoon is placed in an electrophoretic field where strands of charged broken DNA stream towards the cathode. As the mass of DNA fragments stream out from the 'head' of unbroken DNA they resemble a 'comet tail', hence the name of the assay. One major advantage of this assay is that only 5000 spermatozoa from a clinical sample are needed so it is suitable for assessment of small portions of semen left over after clinical use and also for oligozoospermic samples or testicular samples where only a few spermatozoa are available.

The Comet assay is sensitive (Irvine et al., 2000; Trisini et al., 2004; Aitken and De Iuliis, 2007) and is able to detect degrees of DNA damage in an individual spermatozoon rather than a percentage of damaged spermatozoa in a whole sample. The Comet assay also measures single- and double-strand breaks, as well as altered bases. This is useful as it is not yet known which types of DNA damage are most deleterious to male fertility. A further advantage is that, unlike some other tests which detect primarily breaks in protamine-associated chromatin, the Comet assay has a broader use in detecting breaks in both protamine- and histone-bound chromatin equally. The clinical thresholds for diagnosis of male infertility and prediction of success with IVF (Simon et al., 2010,2011,2013) have now been established by studies of over 500 couples. Unlike the SCSA that gives a DFI value based on the number of spermatozoa in an ejaculate with detectable damage (subdivided into categories of non-detectable-, moderate- and high-damage groups), the Comet can quantify damage from 0 to 100% for each individual spermatozoon. The Comet is so sensitive that DNA damage is observed in every spermatozoon; even from fertile donors. The threshold values from the Comet assay are measures of the mean damage from groups of individual spermatozoon, above which spontaneous conception or success with IVF is less likely (Simon et al., 2010, 2013). Analysis of repeatability was performed using an analysis of repeatability with the residual variance from the analysis of within-laboratory variance for single DNA damage measurements. It is 3.7% but decreases 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 5000 spermatozoa assayed is 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 using the Comet assay, the effects of male infertility alone on assisted reproduction 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 (>50%) to be identified.

Most recently, live birth data has been 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 the Comet assay's sensitivity, almost half 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 the markedly lower live birth rates following IVF in both men and couples with idiopathic infertility.

The usefulness of progressive sperm motility compared with DNA damage as predictive tools for IVF rates has also 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 (95% CI 5.21–154.51) to determine fertilization outcome compared with 4.81 (95% CI 1.89–12.65) for progressive motility.

The sperm chromatin structure assay

The pioneering work describing flow cytometry measurements of sperm nuclear DNA fragmentation was published by Evenson et al. in 1980. A significant advantage of this assay, the sperm chromatin structure assay (SCSA), is the simultaneous measure of two important fertility-dependent factors (Evenson et al., 1980): DFI and the presence of immature sperm nuclei with abnormal proteins and/or altered protamine/histone ratios (high DNA stainability, HDS; reviewed by Evenson et al., 1999, 2007). The SCSA is technically much less demanding than any other DNA fragmentation test and can be conducted on fresh or frozen–thawed semen within minutes rather than hours. It has only two straightforward biochemical steps: (i) treat the raw semen dilution with pH 1.2 buffer for 30 s; and (ii) stain the spermatozoa with acridine orange, a dye that reveals broken DNA as red fluorescence and unbroken DNA strands as green fluorescence (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 acridine orange and DNA/chromatin are precisely repeatable. This is proven by comparing cytogram scatter plots with 1024 channels for both X (red) and Y (green) fluorescence values in repeat measures of individual semen samples (Evenson et al., 1991). The 5000 dots, each representing single spermatozoon, were in virtually identical X, Y coordinates (\pm five channels). This scale ranges from 0–1000 and includes normal spermatozoa with no detectable DNA fragmentation to those with 100% DNA fragmentation. Thus, the mean DFI is the mean amount of DNA fragmentation for 5000 spermatozoa. The frequency histogram of DFI allows a precision determination of the DFI. Such observations on over a hundred thousand SCSA measurements negate statements such as ‘the acid treatment tends to denature the DNA’, as if the SCSA protocol were poorly specific. The controversy regarding lack of unity in the utilization and interpretation of the SCSA data is due to incorrect implementation of the protocol. Furthermore, publications suggesting that the SCSA is of little value for clinical diagnosis and prognosis have been the result of low numbers of patients in some studies, lack of laboratory experience with the SCSA and especially the lack of screening out couples where the female partner had infertility issues.

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 spermatozoa with fragmented DNA. The SCSAsoft frequency histogram of DFI allows a precision determination of DFI. The standard deviation of DFI is a highly sensitive measure of animal infertility (Ballachey et al., 1988; Didion et al., 2009) and genotoxicant-induced DNA damage (Sailer et al., 1997; Rubes et al., 2005).

SCSA data are not correlated with the extent of free nuclear –SH groups (Evenson et al., 2000), freezing and thawing (Evenson et al., 1999) or sonication and gradient purification of sperm nuclei (Evenson et al., 1991). In a study with 182 men, the percentage HDS was significantly correlated with the efficiency of chromatin remodelling, as measured by chromomycin A₃ staining ($r = 0.610$, $P < 0.0001$). Interestingly, when men were given an antioxidant, their percentage DFI was reduced but HDS was increased (Ménézo et al., 2007). Pregnancies were not observed with HDS above 35%. 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 $\leq 25\%$ DFI has been adopted.

The sperm chromatin dispersion

The sperm chromatin dispersion (SCD) or Halo test is marketed in a simple and inexpensive kit (Halotech DNA), available to fertility laboratories in kit form. The technique involves a simple differential chromatin decondensation step after which spermatozoa with DNA fragmentation are visually smaller than those with intact DNA, which appear to have a ‘halo’ around the sperm head. It takes about an hour to prepare slides prior to counting individual cells manually with bright-field microscopy, available in all fertility laboratories. Unlike all the other tests, in its most commonly used form, it measures the absence of damage rather than the damaged DNA in spermatozoa. The halo is unique with no relationship observed between it and the SCSA (Balasuriya et al., 2011). To date, correlations have been observed between DNA and other sperm parameters (Gosálvez et al., 2008; Nasr-Esfahani et al., 2008) although few correlations between sperm DNA damage and treatment outcomes have been established using this method. Some recent studies have shown no significant associations using ejaculated spermatozoa ($n = 100$, Muriel et al., 2006); $n = 622$, Velez de la Calle et al., 2008); $n = 60$, Yilmaz et al., 2010)) or testicular spermatozoa (Meseguer et al., 2009). However, in an interesting study by Meseguer et al. (2008), sperm DNA damage as measured by the Halo assay had a negative impact on pregnancy if the oocytes were from infertile women ($n = 98$) but not if donor oocytes ($n = 112$) were used. Using the Halo test, the dynamics of DNA fragmentation have been measured with time and fragmentation has been shown to increase with time after thawing (Gosálvez et al., 2008). This is an important clinical finding, suggesting that immediate use of thawed spermatozoa for clinical purposes would be advisable.

The TUNEL assay

Among the different types of assays available to determine real, ‘actual’ DNA damage, the TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling (TUN-

EL) assay is one of the oldest. It was developed by Ausubel et al. (1992) and was first applied to spermatozoa by Gorczyca et al. (1993). The principle of this widely used assay is based on the ligation of dUTP to the 3⁰-OH phosphate ends including blunt-ended and 5⁰-recessed DNA fragments at single- and double-strand DNA breaks (Gold et al., 1994; Nakamura et al., 1995), thus measures definite end-points, which is referred to as 'actual' DNA damage. In contrast, the SCSA detects DNA damage induced by acidic conditions.(Alvarez, 2005; Henkel, 2007). The TUNEL and SCSA assays correlate well (Evenson et al., 2007), although they determine different aspects of sperm function (Henkel et al., 2010; Mitchell et al., 2011).

Since the TUNEL assay is specific for phosphodiester-strand breaks, it is often used to determine apoptosis. However, this specificity has neither been demonstrated for spermatozoa (Manicardi et al., 1998) nor can this endpoint be used because it can also be reached by other mechanisms such as necrosis. Thus, by using the TUNEL assay, one should rather refer to this DNA damage as 'DNA fragmentation' than 'apoptosis' (Henkel et al., 2004; Muratori et al., 2010).

Principally, the TUNEL assay can be performed using flow cytometry or microscopically with either fluorescent or colorimetric labels. The latter, fluorometric or colorimetric, should preferably be performed in cases with very low sperm count, for example in cases of oligozoospermia or if spermatozoa are retrieved from the epididymis or testis. Although not very sensitive due to technical specifics in the original protocol (Mitchell et al., 2011), the assay has been regarded as being precise and reproducible (Muratori et al., 2009). A number of different factors such as preparation, fixation and permeabilization of samples negatively affect its clinical application (Muratori et al., 2010). The latter factor is due to the highly compact nature of sperm chromatin by and its reinforced structure in protamines due to inter- and intramolecular bonds (Balhorn et al., 1992; Brewer et al., 2003; Vilfan et al., 2004), which prevent TdT from directly accessing the DNA strand breaks. All these factors contribute to the fact that the TUNEL assay has not yet been standardized to the same extent as the SCSA.

Only recently, solutions to these standardization problems have been found. For example, M540 bodies, which represent an interference in flow cytometry and contain apoptotic markers (Marchiani et al., 2007), were excluded and a standardized fixation protocol (Muratori et al., 2009) was introduced. Additionally, pretreating the samples with the disulphide-bridge-reducing agent dithiothreitol resulted in relaxation of the compact chromatin structure for TdT to access the DNA (Codrington et al., 2007). Using this technique, Mitchell and co-workers (2011) significantly enhanced the sensitivity of the TUNEL assay. Apart from its general clinical value, which has been shown repeatedly (Aitken et al., 2010; Henkel et al., 2004; Sharma et al., 2010), the technical standardization and proper clinical evaluation of the TUNEL assay are essential. Sharma et al. (2010) and Aitken et al. (2010) evaluated the flow cytometric TUNEL assay for clinical use and established reference ranges for DNA damage in patients and fertile donors. Nevertheless, despite the advantages of the TUNEL assay and the efforts in its standardization, more work remains to be done in order to eventually establish a robust, clinical test system to determine male infertility.

The value of sperm DNA adduct analysis

Biological and environmental factors which are known to elevate DNA adduct formation in spermatozoa have been shown to produce marked effects on embryonic development and the health of the offspring (Adler, 2000; Anderson, 2001). Further studies have clearly indicated that DNA adduct formation in human spermatozoa has direct, negative impacts on fertility and is a good predictor of treatment outcome (Tyla et al., 2000). The current understanding of the molecular nature of the DNA damage is limited to mainly a handful of aryl/alkyl adducts (Witt and Bishop, 1996) and oxidative DNA modifications (De Iuliis et al., 2009). Nevertheless, this knowledge is aiding efforts to understand the underlying aetiology of sperm DNA damage.

DNA fragmentation can arise through two independent mechanisms: direct enzymic cleavage and oxidative breakage of the backbone. In addition to these canonical mechanisms, DNA cleavage will also occur as a result of DNA adduct formation. Upon the creation of a base adduct, the glycosyl bond linking the base to the sugar moiety is weakened, leading to a basic site formation (Lee et al., 2009). These now-vulnerable sections of DNA are favoured to produce strand breaks, either spontaneously via ribose ring opening reactions (Liu et al., 2006; Zini et al., 2008) or by targeted enzymic cleavage by nucleases (topoisomerase II; Cavalieri and Rogan, 2010). DNA fragmentation assays measure strand breaks as an end point. While this provides data on the level of breakage, these tests can provide little-to-no insights into the origins of the damage. Thus, chemical characterization of the DNA adducts present in human spermatozoa will provide clues to the aetiology of this DNA damage that will supplement the data generated by DNA fragmentation assays.

The use of mass spectrometry to detect and characterize DNA adducts is well established in cancer research (Badouard et al., 2008; Stone et al., 2011) and is beginning to permeate into gamete biology (Witt and Bishop, 1996; Verhofstad et al., 2011). The measurement of sperm DNA modifications such as 8-hydroxy-2-deoxyguanosine (Lee et al., 2009; Gharagozloo and Aitken, 2011; Thomson et al., 2011; Cambi et al., 2013) and xenobiotic adduct formation (Zenzes, 2000) including benzo[a]pyrene (Park et al., 2008), has been gaining much momentum of late, especially employing antibody-based analyses. In fact, these two lesions have been recently implicated as playing a major role in male infertility and the health of the offspring (Anderson, 2001; Bidmon et al., 2007; Lee et al., 2009). While the identification of specific xenobiotic-adducts may point toward a specific cause, the identification of oxidative stress markers can be common to several aetiologies given that oxidative stress is a major mediator of DNA damage in the male germ line (De Iuliis et al., 2009). Furthermore, the presence 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). Based on the oxidative stress data, a pertinent body of work is being generated supporting the potential therapeutic effects of antioxidant supplements (Gharagozloo and Aitken, 2011; Henkel, 2012).

It is clear that this fundamental comprehension of the origins of DNA damage in the male germ line is not only critical from a basic sperm cell biology perspective but is invaluable as a diagnostic for male infertility, as it can give information on the potential underlying causes in each case. Therefore, having now established a fundamental platform

of information on sperm DNA strand breaks, the next step in gaining a detailed understanding of DNA damage to characterize the DNA adducts that created these breaks in the male gamete.

Proxy tests for sperm DNA damage

The major limitation of testing for sperm DNA damage is that each assay renders the tested spermatozoa unsuitable for clinical purpose. In an effort to overcome this problem, a number of non-invasive tests have been developed and their correlation with DNA damage assessed. These novel tests include birefringence, intracytoplasmic morphologically selected sperm injection (IMSI) and hyaluronic acid-selection of spermatozoa for ICSI. If these tests can help embryologists choose spermatozoa with low DNA damage for use in assisted reproduction, a major step forward in sperm selection will be achieved, but presently tenuous relationships are based on a few studies with very small numbers of cases.

The implications of sperm DNA damage in the diagnosis of male infertility, treatment choice and the health of future generations Fecundity in the general population

There is a consensus based on numerous publications that men in infertile couples have a higher level of sperm DNA fragmentation compared with those in the general population and proven fertile males. Such findings are interesting from a biological point of view since they point to a new, and potentially curable, cause of male infertility. 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 (as measured by DFI using SCSA) is a useful marker in the prediction of fertility in males from couples of unknown fertility. Thus, both studies have shown that the chance of spontaneous conception starts to decline at sperm DNA damage 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, high levels of damage are indicative of severely impaired male fertility. Furthermore, the SCSA data indicate that, for men who classified as 'normal' because their conventional sperm parameters are in the normal range, the risk of infertility starts to increase at DFI above 20% (odds ratio, OR, 5.1, 95% CI 1.2–23). However, this threshold drops to 10% if the sperm concentration is below $20 \cdot 10^6$ /ml and/or there is an impairment of sperm motility or morphology (OR 16, 95% CI 4.2–60; Giwercman et al., 2010). In another study (Simon et al., 2011), using the Comet assay, there was also a strong correlation between sperm DNA fragmentation and fertility status of men, with 95% of fertile donors having DNA fragmentation below 25% and 98% of infertile men having DNA fragmentation 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 excessive.

Treatment choice

Success rates for IUI are similar to those for spontaneous pregnancies, indicating a reduction in the chance of pregnancy with sperm DNA damage above 20% according to the SCSA (Bungum et al., 2007). If a test for oxidized bases is employed (8-hydroxy-2^O-deoxyguanosine) the results are even more sensitive, with a lower threshold value of 11.5% (Thomson et al., 2011).

In terms of IVF, a recent meta-analysis of nine IVF and 11 ICSI studies by Zini and Sigman (2009) showed a modestly increased pregnancy chance after IVF (OR 1.7, 95% CI 1.3–2.2) in cases when the proportion of DNA-damaged spermatozoa was below the threshold values for SCSA or TUNEL. As a result of these data, sperm DNA testing is now employed routinely throughout south Sweden. In further support, studies using the Comet assay (Simon et al., 2010, 2013; both published after Zini and Sigman's (2009) meta-analysis) showed an OR of 76 (95% CI 8.7–1700) for clinical pregnancy if the mean DNA fragmentation per spermatozoon 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%) had a live birth rate of 33% following IVF treatment. In contrast, couples with high levels of sperm DNA fragmentation (>50%) had a much lower live birth rate of 13% following IVF treatment of couples with idiopathic infertility, 39% have high sperm DNA damage. Sperm DNA damage was also associated with lower live birth rates following IVF in couples with idiopathic infertility than couples with detectable causes.

Sperm DNA damage has not been found to be predictive for ICSI treatment (Zini, 2011) except for one exception (Bungum et al., 2007). However, in this study, couples were not randomized 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 impact adversely on 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 spermatozoon is of poor quality. This is supported by the findings of Meseguer et al. (2011) where high-quality oocytes from donors offset the negative impact of sperm DNA damage on pregnancy.

Secondly, a recent major study from Dumoulin et al. (2010) shows that even the birthweight of IVF babies can be markedly influenced by minor differences in culture conditions. In contrast to IVF, ICSI spermatozoa 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 spermatozoa from up to 40% of infertile men have high levels of ROS (Aitken et al., 2012; Henkel, 2011) and their antioxidant content is also significantly lower than 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 post fertilization.

Finally, as mentioned above, evidence is emerging that embryos with high sperm DNA damage are associated with early pregnancy loss (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 (reviewed by Robinson et al., 2012).

Early pregnancy loss, whether following spontaneous or assisted conception is another form of infertility, and one for which we need to develop an appropriate patient pathway.

Implications for future generations' health

Importantly, compounds that induce oxidative stress in the male germ line such as iron dextran have also been shown to generate positive results in dominant lethal assays (Doreswamy and Muralidhara, 2005). The importance of oxidative stress in the aetiology of the dominant lethal effect is also emphasized by the fact that polyphenolic antioxidants can reverse the dominant lethal impact of benzo[a]pyrene (Shukla and Taneja, 2001). Similarly, enhancing the concentration of reduced glutathione in the male reproductive tract following treatment with N-acetylcysteine was found to reduce the dominant lethal effects observed with ethyl methanesulphonate, while glutathione depletion with phorone had the opposite effect (Gandy et al., 1992). The literature on male-mediated toxicity in animals, and the dominant lethal assay in particular, is extensive and demonstrates beyond doubt that the induction of DNA damage in the male germ line can induce miscarriage or, if the pregnancy carries to term, morbidity in the offspring, including an enhanced susceptibility to tumour formation.

To set this fundamental point beyond doubt, Fernández-Gonzalez et al. (2008) performed ICSI with mouse spermatozoa that had their DNA damaged by a freeze–thaw cycle in the absence of cryoprotectant. Fertilization with such damaged spermatozoa allowed embryonic development to proceed to term but at a reduced rate compared with control animals. Furthermore, the offspring that were generated in this study following ICSI showed reduced longevity, increased behavioural abnormalities and a significant rise in the incidence of pathologies, including the age-dependent appearance of solid tumours (Fernández-Gonzalez et al., 2008).

These animal studies provide experimental support for the correlative associations that have been observed clinically between DNA damage in human spermatozoa and morbidity in children. One paradigm that perfectly illustrates the power of these associations is paternal ageing. Thus, numerous studies demonstrate that paternal age is associated with a high incidence of DNA damage in human spermatozoa (Schmid et al., 2007; Singh et al., 2003; Varshini et al., 2012) as well as an increase in the mutational load subsequently carried by the offspring (Kong et al., 2012). Whether there is a mechanistic link between DNA damage in the germ line on the one hand and increased mutation rates in the embryo on the other is a critical issue that remains unresolved. It is possible that advanced paternal age is associated with an increased mutation rate in the spermatogonial stem cell population as a consequence of replication errors that are completely independent of any DNA damage seen in the spermatozoa. Alternatively, the DNA damage recorded in the spermatozoa of older men may be directly responsible for

the age-dependent increase in mutational load seen in the embryo, as a result of aberrant DNA repair in the oocyte prior to the initiation of S-phase of the first mitotic division (Aitken et al., 2004). Whatever the nature the genetic damage present in the spermatozoon as a consequence of paternal age its appearance is correlated with an increase in neurological conditions in the offspring such as epilepsy, spontaneous schizophrenia, autism and bipolar disease (Sipos et al., 2004; Reichenberg et al., 2006; Aitken and De Iuliis, 2007; Frans et al., 2008). DNA lesions in the spermatozoa of ageing fathers are also associated with an increased risk of cancer in the offspring (Hemminki et al., 1999; Johnson et al., 2011), birth defects (Green et al., 2010) and the appearance of dominant genetic mutations including achondroplasia and Apert's syndrome (Crow, 2000).

A second paradigm that shows the same relationship between DNA damage in the spermatozoa and a significantly increased risk of morbidity in the offspring is smoking. Paternal smoking creates oxidative stress in the ejaculate and significantly enhanced levels of oxidative DNA damage in the spermatozoa (Fraga et al., 1996). A number of independent studies have indicated that this genetic damage to the spermatozoa is, in turn, associated with significantly increased levels of cancer in the offspring (Ji et al., 1997; Lee et al., 2009).

A third situation in which DNA damage in the spermatozoa is associated with defects in the offspring is assisted reproduction treatment. The detrimental effect of assisted conception on the health and wellbeing of the progeny has been conclusively demonstrated in a recent meta-analysis (Wen et al., 2012). In this review of the existing literature, no difference was found between the risks associated with IVF and ICSI; however, 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). Interestingly, this difference was not observed with cryostored embryos, suggesting that the stresses associated with embryo freezing might selectively eliminate any defective embryos generated as a result of the ICSI procedure. In light of these results, routine cryostorage of ICSI embryos might be considered in cases where DNA damage in the father's spermatozoa is high and a potential risk to the normality of embryonic development is therefore evident.

Finally, because the three factors known to be associated with DNA damage in human spermatozoa (ageing, smoking and infertility) are also associated with the formation of oxidative DNA adducts in the germ line, these data suggest that oxidative stress is a critical trigger for paternally mediated impacts on development. The implications of this rationale are 2-fold. Firstly, if this is the case, then any factor capable of causing oxidative stress in the male reproductive tract is capable of influencing the health and wellbeing of the next generation. The list of such factors is very extensive and, in addition to age and smoking, includes alcohol consumption, exposure to radio-frequency electromagnetic radiation, chemotherapy, diabetes, heat, testicular torsion, oestrogenic steroids, anti-retroviral drugs, anti-epileptics, phthalate esters, heavy metals, acrylamide, arsenic, pesticides, herbicides, paracetamol, hypobaric hypoxia, cryostorage and indeed idiopathic infertility, among others (Mathur and D'Cruz, 2011). Given the extensive nature of this list, it is not surprising that oxidative DNA damage in human

spermatozoa is such a widespread phenomenon (Aitken et al., 2010, 2012). The implications of such damage in terms of the incidence of disease in the offspring is therefore potentially immense. Secondly, if oxidative stress is involved in the aetiology of DNA damage then antioxidants should be part of the cure (Greco et al., 2005).

The potential of antioxidant therapy

Excess ROS chemically modify cellular components such as proteins, lipids, RNA and DNA molecules, thus impairing normal cell function. This is true for all cell types. Sperm cells are particularly vulnerable to oxidative attack for three reasons: (i) their plasma membrane is largely composed of polyunsaturated fatty acids such as docosahexaenoic acid, which, with six double bonds per molecule, creates an 'electron sink' rendering it highly susceptible to oxidation and other chemical modifications (Jones et al., 1978, 1979); (ii) spermatozoa have inherent deficiencies in intracellular antioxidant enzyme protection; and (iii) unlike most cell types, spermatozoa have a limited capacity for DNA damage detection and repair. Chronic oxidative stress in the male reproductive tract is therefore potentially detrimental to sperm health compromising motility, membrane fusion events with the oocyte and, most critically, the integrity of the DNA they transport. Nonetheless, depending on factors such as the nature, extent and location of the DNA damage, spermatozoa with such lesions can still fertilize oocytes and generate pregnancies. Such pregnancies will carry an elevated risk of miscarriage and may lead to embryonic DNA mutations with deleterious consequences for the health of the offspring. It is therefore prudent to include tests that measure sperm DNA damage as part of the patients' routine semen analysis. Patients diagnosed with moderate-to-severe oxidative DNA damage will be guided towards a more informed decision regarding the most appropriate treatment. For example, patients may be advised to skip IUI treatments altogether in favour of IVF or ICSI since IUI success rates are very poor for patients with sperm DNA damage. Moreover, the physician may recommend the use of testicular spermatozoa or high-magnification ICSI as alternative procedures. In all cases, patients with oxidative DNA damage should be encouraged to consider a simple course of antioxidants as a first-line therapy prior to undertaking any form of assisted reproduction treatment. The use of antioxidants in ameliorating sperm oxidative stress has been the subject of some 20 clinical trials over the last decade, summarized in a recent review paper (Gharagozloo and Aitken, 2011). Although, most of these trials have serious shortcomings, the results unanimously and consistently show a reduction of sperm oxidative stress with a diverse array of antioxidants. In some cases, the reported improvement is accompanied by correlations with one or more secondary clinical outcomes such as pregnancy. These studies do not delineate which antioxidant or combination of antioxidants offers the best protection against oxidative stress and there is no clear consensus regarding the doses of the antioxidants that should be administered. Future research and clinical studies should address these issues as a matter of urgency. For now, based on current studies and theoretical considerations, we offer the following opinion. Since oxidative stress is not a localized phenomenon in cells or in tissues and is caused by vastly different oxidants, it is reasonable to assume that a combination of antioxidants targeting the male reproductive tract with the appropriate oral bioavailability will tender a better protection than any single antioxidant. It should also be noted that the complexities involved in the identification of such a formulation is never a trivial one as there are no 'hard and fast' rules in developing such a formula.

While awaiting the development of efficacious formulations backed up by quality human clinical data, physicians should consider the use of one or more antioxidant(s) reported in recent review papers (Zini et al., 2009; Lanzafame et al., 2009; Ross et al., 2010; Gharagozloo and Aitken, 2011; Showell et al., 2011). Fortunately, there is little or no concern over the safety of these natural antioxidants with the following caveats. High doses and long durations of administration as well as the use of synthetic or chemically modified versions of antioxidants should be avoided. For example, the long-term use of synthetic esterified racemic vitamin E at 400 IU/d in the large cancer prevention SELECT clinical trials shows a significant rise in prostate cancer among healthy men (Klein et al., 2011). Additionally, antioxidants with no known benefit to reproductive health should not be considered. Physicians or IVF specialists should also avoid the use of arbitrary antioxidant formulations where little evidence concerning their safety and efficacy is reported. Some formulations may combine a large number of antioxidants with aggressive doses, raising the possibility of 'reductive stress' (O'Flaherty et al., 2005) by potentially depleting the physiological concentrations of ROS known to be critical for normal sperm function. It is also important to note that, according to recently published data, 40% of men seeking fertility treatment are fertile and devoid of sperm oxidative DNA damage (Cohen-Bacrie et al., 2009). These men do not require antioxidant treatment, which may in fact lower their fertility potential. For example, administering selenium to fertile men is known to reduce the number of motile spermatozoa possibly through modifying thyroid hormone metabolism (Hawkes and Turek, 2001). Similarly, in high doses, vitamin C is reported to reduce the interchain disulphide bridges in protamines opening the cysteine net and subsequently promoting DNA decondensation in spermatozoa (Donnelly et al., 1999; Ménézo et al., 2007; Giustarini et al., 2008).

Thus, based on such overwhelming considerations and as a matter of 'best practice', the current status quo governing male fertility treatment should change to include tests of sperm DNA quality as part of the routine semen analysis. To enhance fertility potential, lower the risk of miscarriage and reduce sporadic embryonic DNA mutations, patients tested positive for sperm DNA damage should be treated with one or more antioxidants for the duration of spermatogenesis. This approach will lead to improvement of patients' sperm DNA quality prior to undertaking any form of fertility treatment by assisted reproduction treatment.

Conclusion

The large body of literature informs those working in human fertility of the importance of assessing DNA damage in human spermatozoa, in terms of effectiveness of treatment and the health of offspring conceived using this technology. It is an important element of semen quality: useful in the diagnostic workup of the male and also as an additional indicator of assisted reproduction treatment success. Well-designed and powered clinical trials should be undertaken to address the effectiveness of antioxidant therapy as a matter of urgency.

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