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REVIEW ARTICLE

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Assessing sperm chromatin and DNA damage: clinical importance and development of standards

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SUMMARY

Over the past 20 years, numerous new methods have been developed to identify changes in the organization and composition of sperm chromatin as well as to determine the extent of DNA damage in the nuclei of spermatozoa. Although these methods are being used effectively in assessing how toxicants act on sperm chromatin quality in agricultural settings, their use as complementary biomarkers of sperm quality in assessing male fertility remains controversial. We review some key aspects of the assessment of sperm chromatin quality and DNA damage and identify some of the most widely used tests to monitor these endpoints. An approach to validate three tests by standardizing methodology and determining interlaboratory variation for each test using a standard set of samples is outlined.

Sperm chromatin and DNA tests measure defects in nuclear chromatin compaction and damage to DNA respectively. The evaluation of sperm chromatin and DNA structure was initially undertaken to improve our understanding of spermatogenesis, sperm physiology, sensitivity to reproductive toxicants and reproductive biology (Evenson *et al.*, 1980a,b, 1986; Balhorn, 1982; Gatewood *et al.*, 1987, 1990; Hecht, 1987; Ward & Coffey, 1991; Perreault, 1992; Ward, 1993; Zalensky *et al.*, 1993; Barratt *et al.*, 2010). More recently, sperm chromatin and DNA tests have been used in the evaluation of the infertile man in the hope that these tests may provide a more accurate diagnosis than standard sperm parameters alone. The conventional sperm parameters include sperm concentration, motility and morphology; they show a high degree of biological variability and are only fair measures of fertility potential (Guzick *et al.*, 2001; Zini & Sigman, 2009). Sperm chromatin and DNA integrity tests have also been studied in the context of assisted reproductive technologies (ARTs) to assess their ability to predict pregnancy outcome after assisted reproduction because conventional sperm parameters are poor predictors of ART outcomes (Lewis *et al.*, 2008; Simon *et al.*, 2010; Zini, 2011).

The aetiology of human sperm DNA damage is probably multifactorial. Human sperm DNA damage may be caused by primary or intrinsic defects in spermatogenesis (e.g., genetic or developmental abnormalities) or caused by secondary or extrinsic factors causing testicular or post-testicular injury (e.g., gonadotoxins, hyperthermia, oxidants, endocrine abnormalities) (Fossa *et al.*, 1997; Sailer *et al.*, 1997; Potts *et al.*, 1999; Erenpreiss *et al.*, 2002; Saleh *et al.*, 2003; Banks *et al.*, 2005; Bungum *et al.*, 2007; O'Flaherty *et al.*, 2008, 2010; Zini & Sigman, 2009). It has been suggested that protamine deficiency (with consequent aberrant chromatin remodelling), reactive oxygen species and abortive apoptosis may be responsible for sperm DNA damage (Cho *et al.*, 2001; Sakkas *et al.*, 2003; Aoki *et al.*, 2005, 2006; Aitken & De Iuliis, 2007; Tarozzi *et al.*, 2007; Leduc *et al.*, 2008; Gregoire *et al.*, 2013). De Iuliis *et al.* (2009) have proposed a two-step model to explain the development of sperm DNA damage. Based on this model, poorly protaminated spermatids/spermatozoa, that is, with incomplete replacement of histones by protamines and poor chromatin compaction, are formed as a result of defective spermiogenesis (1st step); they then become more sensitive to oxidative stress/damage (2nd step).

Experimental (animal) models of sperm chromatin and DNA damage have demonstrated that sperm DNA fragmentation (both inherent and experimentally induced damage) is associated with reduced male fertility potential (Evenson *et al.*, 1980a; Doerksen & Trasler, 1996; Cho *et al.*, 2001; Delbes *et al.*, 2007).

Moreover, studies of sperm DNA damage in animals have also shown that this type of damage is associated with adverse reproductive outcomes after ARTs, lower pregnancy rates, chromosomal abnormalities, pregnancy loss, reduced longevity and birth defects) (Ahmadi & Ng, 1999; Fernandez-Gonzalez *et al.*, 2008; Perez-Crespo *et al.*, 2008a,b). These experimental studies have raised concerns regarding the use of DNA-damaged sperm in the context of assisted reproduction in humans. However, these important experimental observations have not been fully appreciated by clinicians, probably because the nature of sperm DNA damage in animals with induced sperm injury (typically, these animals will have damage present in all spermatozoa) is unlike the nature of DNA damage in human sperm chromatin; typically, sperm damage in humans is heterogeneous with varying degrees of DNA damage in different sperm subpopulations (Sakkas *et al.*, 2000). Yet, there is good evidence to show that inherent sperm DNA damage is also associated with poor reproductive outcomes (Evenson *et al.*, 1980a).

Several clinical studies have demonstrated that infertile men have substantially higher levels of sperm chromatin and DNA damage than do fertile men (Gatewood *et al.*, 1990; Hughes *et al.*, 1996; Evenson *et al.*, 1999; Irvine *et al.*, 2000; Spano *et al.*, 2000; Carrell & Liu, 2001; Zini *et al.*, 2001, 2002; Zhang *et al.*, 2006). These studies have also shown that specific clinical parameters (e.g. advanced paternal age, varicocoele, gonadotoxin exposure, genital tract infection, spinal cord injury and febrile illness) are associated with a higher prevalence of a positive or abnormal sperm DNA test (Moskovtsev *et al.*, 2010). Prospective studies of couples with unknown fertility status indicate that sperm DNA damage is associated with a lower probability of conception (odds ratio = ~7) and a prolonged time to pregnancy (Evenson *et al.*, 1999; Spano *et al.*, 2000; Loft *et al.*, 2003; Giwercman *et al.*, 2010). These studies also reveal that sperm DNA testing is a better predictor of pregnancy than conventional sperm parameters in this context (Giwercman *et al.*, 2010). Taken together, these data suggest that it may be reasonable to test couples with unknown fertility status when the men present with clinical characteristics predisposing them to sperm DNA damage (e.g. prior exposure to gonadotoxins or advanced paternal age).

A systematic review of studies correlating sperm DNA test results and reproductive outcomes after ARTs has shown that sperm DNA damage is associated with lower intrauterine insemination (IUI) (odds ratio = ~9) and conventional in vitro fertilization (IVF) pregnancy rates (odds ratio = ~1.6 to 1.9), but not with intracytoplasmic sperm injection (ICSI) pregnancy rates (Bungum *et al.*, 2007; Collins *et al.*, 2008; Zini, 2011; Practice Committee of the American Society for Reproductive Medicine, 2013). However, the widespread clinical application of sperm DNA tests in predicting IUI and IVF pregnancy has not been firmly established despite an already large number of clinical studies (40–50 relevant studies), because most studies are relatively small (each study has reported on roughly 100–200 ART cycles), the study characteristics are heterogeneous and the precision of the different assays remains uncertain (Zini, 2011; Practice Committee of the American Society for Reproductive Medicine, 2013).

Over the past decade, several clinicians have observed a higher rate of spontaneous pregnancy loss in men with sperm DNA damage and two systematic reviews suggest that this damage is indeed associated with an increased risk of pregnancy loss (after an established natural and IVF or ICSI pregnancy) (Zini, 2011; Robinson *et al.*, 2012). Although most studies are relatively small with heterogeneous study characteristics and uncertain assay precision, the higher rate of spontaneous pregnancy loss in couples with sperm DNA damage has been observed with near consistency in all of the available studies. The mechanism(s) responsible for the pregnancy loss is unknown, but these data are cause for concern because similar results have been reported in experimental studies and there is uncertainty regarding the long-term reproductive outcomes (e.g. post-natal health) when a pregnancy is established with DNA-damaged sperm. Furthermore, what is remarkable about these data is that, to date, no other sperm test has been linked to pregnancy loss and/or post-natal health.

In spite of the large number of studies examining the relationship between sperm chromatin and DNA damage with pregnancy rate and progeny outcome, sperm chromatin tests, as part of the assessment of a man's fertility potential, have met resistance. This stems from various factors. One hurdle to the clinical acceptance of DNA damage tests is that they do not yield a result that has an associated applicable intervention (other than possibly antioxidant supplementation) as all current DNA damage tests require the functional destruction of the individual spermatozoa whose chromatin damage is being determined. However, the lack of standardized protocols shown to provide reproducible results across a range of laboratories, that is, unknown precision regarding reproducibility and repeatability of the various assays, and the fact that the thresholds for many of these tests have not been validated, stand out as the leading factor (Practice Committee of the American Society for Reproductive Medicine, 2013). To address this concern, a consortium of 10 international laboratory leaders gathered immediately after the 5th Florence-Utah Symposium on the Genetics of Male Infertility to determine which tests should be considered for cross-laboratory testing and to establish a consensus for standardized protocols for performing these tests.

A large number of tests have been developed to measure sperm chromatin and DNA damage (Delbes *et al.*, 2010; Zini, 2011). A survey of this group of laboratories revealed that 80% or more of the participants were using the sperm chromatin structure assay (SCSA), the COMET assay (single-cell gel electrophoresis) and the TUNEL assay (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling). Although many other assays, such as chromomycin A3 test (CMA3), aniline blue assay, sperm chromatin dispersion (SCD) test, 8-hydroxy-2′-deoxyguanosine (8-oxo-dG) slide and FACS- based assays, were occasionally used by one to three laboratories, the decision was made to focus on the development of standard procedures and cross-laboratory testing using the three most prevalent tests. It is important to note that the selected assays do not measure DNA single nucleotide variants (point mutations), epigenetic modifications or aneuploidy and do not selectively differentiate clinically important from insignificant DNA damage. Each of these three assays is briefly described below.

SCSA

Based on the differential staining of chromatin single- and double-strand breaks by acridine orange (Neville & Bradley, 1961), SCSA allows the measurement of both the extent of sperm DNA fragmentation and sperm chromatin nuclear protein

alterations, such as a lack of protamination. Typically, frozen sperm samples are thawed and exposed to a 30-sec low-pH acid denaturation, immediately exposed to acridine orange and processed for flow cytometry. The Florence consortium agreed on the strict observance of the protocol established and published by Evenson (2013). Those include specific guidelines on (i) sample concentration and handling, (ii) DNA denaturing conditions and (iii) data acquisition.

Comet

The COMET assay (single-cell gel electrophoresis) is a simple method to assess sperm DNA integrity (Singh *et al.*, 1988). Briefly, a mix of spermatozoa and low melting point agarose is spread on a two-well slide, and submitted to various treatments meant to induce DNA unwinding. The cells undergo an electrophoresis and, using a fluorescent dye, the DNA forms a structure resembling a comet; the head consists of intact DNA and the tail is made of broken DNA or strands with heterogeneous molecular weights, with the intensity of the comet representing the proportion of DNA that has been broken off and the distance travelled by the comet, the relative sizes of those pieces of DNA. The alkaline COMET assay was chosen because it gives a comprehensive measure of DNA damage as it reveals multiple DNA damage subtypes (i.e. single and double DNA strand breaks, and, at higher pH conditions, alkali-labile sites). The COMET assay involves the collection of data at the level of the single cell; therefore, very few cells are needed to complete the procedure, allowing the use of samples from men having very low sperm count. However, minor modifications in test conditions can result in large variations in the results obtained (Speit *et al.*, 2009), further requiring the need for standardized operating procedures. The Florence consortium settled on several critical steps of the alkaline COMET assay, namely, (i) sample concentration and handling, (ii) DNA unwinding conditions, (iii) alkaline treatment conditions, (iv) electrophoresis critical parameters (e.g. pH, temperature, voltage/amperage and duration) and (v) staining conditions.

TUNEL

The TUNEL assay is a common method to detect DNA breaks resulting from apoptotic cascades. Although spermatozoa cannot undergo apoptosis per se, as they do not have the machinery for new protein synthesis, an essential element of the apoptotic process, this assay does accurately identify open 3′-OH ends in DNA. The assay relies on the incorporation and detection of fluorescent UTPs at both blunt and single 3′-OH ends. Although either immunohistochemistry or flow cytometry can be used for this assay (Sailer *et al.*, 1995), the Consortium decided that greater accuracy would be obtained with the FACS assay as far more cells can be assayed. Briefly, frozen sperm samples are thawed, fixed, permeabilized and stained before being processed by flow cytometry. Because of the absence of cell lysis and DNA denaturation steps, the TUNEL assay is considered a direct approach. However, this feature may impede its effectiveness because of limited access to 3′-OH nicks by highly compacted sperm DNA (Lewis *et al.*, 2008). Because of the widespread use of commercial kits in TUNEL, the Florence consortium selected the one that appeared most frequently in the protocols of participants (i.e. Roche Applied Science In Situ Cell Death Detection Kit, Fluorescein, IN, USA). However, the terms of use were specified regarding (i) sample concentration and handling, (ii) centrifugation parameters and (iii) fixation, permeabilization and staining conditions.

All 10 laboratories from the Florence consortium will be participating in the Sperm Chromatin Quality Project. Using agreed upon standardized protocols and identical samples from pools of semen, it will be possible to determine the degree of variation among laboratories for a given chromatin quality test and the extent of correlation among these three most prevalent tests.

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