Reproductive BioMedicine Online (2011) 22, 428–436

www.sciencedirect.com
www.rbmonline.com

ARTICLE

Sperm chromatin dispersion test in the assessment of DNA fragmentation and aneuploidy in human spermatozoa

A Balasuriya [a,*], B Speyer [a], P Serhal [b], A Doshi [b], JC Harper [a,b]

[a] *UCL Centre for PG and D, Institute for Women's Health, University College London, UK;* [b] *Centre for Reproductive and Genetic Health, University College London Hospital, London, UK*
[*] Corresponding author. *E-mail address:* a.balasuriya@ucl.ac.uk (A Balasuriya).



Amanthi Balasuriya is a first-year PhD student at the UCL Centre for PG and D. She graduated with a first-class degree in genetics and a Master's degree with distinction in prenatal genetics and fetal medicine from University College London. Her research thus far has examined different clinical assays available to measure DNA fragmentation and aneuploidy in human spermatozoa and whether a relationship exists between these two parameters. She is currently studying whether there is variable DNA damage in cumulus and granulosa cells from patients undergoing IVF and what factors affect the damage.

Abstract Sperm DNA damage is thought to be increased in men with male factor infertility. Previous studies suggest a correlation between sperm DNA fragmentation and aneuploidy. The sperm chromatin dispersion (SCD) test was modified to produce the Halosperm Kit. The SCD-fluorescent in-situ hybridization (FISH) test allows the simultaneous detection of DNA fragmentation and aneuploidy on the same sperm cell. The objectives of this study were to validate the SCD, SCD-FISH and Halosperm tests for the analysis of sperm DNA fragmentation and compare them to the sperm chromatin structure assay (SCSA). Semen samples from 20 males undergoing IVF/intracytoplasmic sperm injection were processed using FISH, SCD-FISH, SCD and Halosperm, and compared with SCSA results. There was a significant difference between FISH and SCD-FISH results in the detection of aneuploidy ($P = 0.000$) and the level of sperm DNA fragmentation in the samples subjected to SCSA and SCD ($P = 0.001$) or SCSA and SCD-FISH ($P = 0.001$). There was no significant correlation between DNA fragmentation and aneuploidy. If sperm aneuploidy is to be determined, more reliable results will be obtained if FISH is performed rather than SCD-FISH. A lack of validation and unknown clinical significance question the value of DNA fragmentation assays.

© 2011, Reproductive Healthcare Ltd. Published by Elsevier Ltd. All rights reserved.

KEYWORDS: DNA fragmentation, male infertility, sperm

Introduction

Afflicting approximately one in twenty men of reproductive age, defective sperm function is thought to contribute towards human infertility (Hull et al., 1985; McLachlan and de Kretser, 2001). Increased frequencies of aneuploidy, a foremost cause of pregnancy loss and mental retardation in humans (Griffin, 1996; Hassold and Hunt, 2001), have been shown to draw a parallel with increasing severity of infertility (Collodel et al., 2007; Faure et al., 2007; Martin

1472-6483/$ - see front matter © 2011, Reproductive Healthcare Ltd. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.rbmo.2011.01.012

et al., 2003; Tempest and Griffin, 2004). The observation that men with severe semen defects display a higher level of chromatin damage (Sun et al., 1997) has led to studies that have associated damaged paternal DNA to detrimental effects of embryo development, ill effects on the offspring and subsequent generations (Fernández-Gonzalez et al., 2008). Although controversial, it has been suggested that 'normal' semen samples can hold significant DNA damage and those with apparently poor sperm quality can carry minimal DNA fragmentation; sperm chromatin and DNA defects can be indicative of male subfertility (Evenson et al., 1999).

Sperm karyotyping studies demonstrated higher baseline numerical and structural chromosome aberrations following exposure to mutagens (Genescà et al., 1990; Kamiguchi and Tateno, 2002). Higher frequencies of aneuploidy have been associated with semen samples of inferior quality (Rubio et al., 2001), which in turn is thought to be correlated with high levels of DNA fragmentation. In addition, similar to samples with a high level of DNA fragmentation (Virro et al., 2004), spermatozoa with a high aneuploidy rate tend to lower pregnancy and implantation rates following intracytoplasmic sperm injection (ICSI) (Rubio et al., 2001).

A close relationship between the proportion of immature spermatozoa with excessive cytoplasmic retention and sperm disomies has been suggested (Kovanci et al., 2001). Immature sperm cells assessed by the sperm chromatin structure assay (SCSA) (Ollero et al., 2001) and sperm chromatin dispersion (SCD) (Fernández et al., 2003) tests also display a higher level of DNA fragmentation. This suggests DNA fragmentation and aneuploidy tend to occur in immature arrested spermatozoa. Reportedly, the proportion of aneuploid spermatozoa is associated with the percentage of apoptotic spermatozoa, strengthening the suggested correlation between sperm DNA fragmentation and aneuploidy (Carrell and Emery, 2008; Liu et al., 2004). Whilst it has been suggested that mis-segregated chromosomes can trigger apoptotic cell death (Dobles et al., 2000), Muriel et al. (2007) proposed that aneuploidy may trigger DNA fragmentation. This may lead to an apoptotic-like process regulated by endogenous nucleases, in an attempt to genetically inactivate sperm nuclei with aberrant genetic constitutions.

Embryo competency obviously depends on the quality of the gametes involved in fertilization. Because the DNA repair capacity of the oocyte is restricted (Ahmadi and Ng, 1999; Twigg et al., 1998), although it is possible for spermatozoa with fragmented DNA to penetrate the oocyte (Henkel et al., 2004), embryo development may be compromised in terms of rate and fidelity. This can trigger cell cycle arrest (Seli et al., 2004) or sperm chromatin decondensation failure (Sakkas et al., 1996). Thus, even if aneuploid spermatozoa with fragmented DNA succeed in fertilizing the oocyte, it may result in a non-viable embryo. Unexplained recurrent pregnancy loss has been associated with a variety of DNA aberrations including sperm DNA fragmentation (Saleh et al., 2003). DNA damage in the male germ line can potentially result in poor fertilization, suboptimal development of the preimplantation embryo, miscarriage and elevated risk of morbidity in the offspring (reviewed by Aitken and De Iuliis (2010)). It has been speculated that analysis of the gametes prior to the initiation of an IVF cycle can identify defects which may alter normal embryogenesis, thus improving the quality of embryos transferred. This provides the clinician with information that can affect clinical decisions, increase pregnancy rates and minimize recurring failure following IVF. Identifying the transmission risk of chromosomal and genetic defects to early embryogenesis is fundamental in enhancing patient care. However, the heterogeneous nature of human spermatozoa raises the question of the clinical and diagnostic value of indices like morphology and DNA damage; do they tell us about an individual gamete or an underlying quality of spermatogenesis? Thus, the clinical significance behind the utilization of sperm DNA fragmentation assays needs to be scrutinized.

Currently, tests used to discriminate between infertile men whose partners have difficulty conceiving and those who do not (reviewed by Barratt (2010)) have been inconsistent. This could be attributed to flawed study designs, small sample size, complex nature of fertility and the uncertainty of damage being assessed using different assays. The ability to accurately diagnose male factor infertility may be important clinically (Adamson and Baker, 2003), but despite the routine employment of these assays in clinical practice, they have not been evaluated critically. Robust methods in the detection of DNA damage and rigorous examination of their application using standardized procedures are required, as with tests in other disciplines (Gluud and Gluud, 2005).

Over the last 20 years, a number of DNA fragmentation assays have been introduced for both clinical and research purposes. These tests include Comet (Irvine et al., 2000; Lewis et al., 2004), terminal deoxynucleotidyl transferase-mediated dUDP nick-end labelling (TUNEL) (Spanò et al., 2000; Tesarik et al., 2004), SCSA (Bungum et al., 2007; Evenson et al., 1999) and SCD (Fernández and Gosálvez, 2002). Extensively studied from a clinical point of view, the SCSA test exploits the susceptibility of damaged DNA to reach different degrees of denaturation under acidic conditions, depending on the level of the DNA breaks. It exploits the metachromatic properties of acridine orange, which only attaches to the ends of broken DNA, thus differentiating between fragmented (red fluorescence, single-stranded) and native (green fluorescence, double-stranded) DNA (Darzynkiewicz and Kapuscinski, 1990). The degree of damage is quantified by flow cytometry and expressed as a DNA fragmentation index (DFI). Studies state that a DFI $>$27% is associated with pregnancy failure in assisted reproduction treatment (Evenson et al., 1999; Payne et al., 2005; Virro et al., 2004). It must be noted that the SCSA has been scrutinized more than other tests and future studies may reveal limitations to assays such as TUNEL and the SCD/Halosperm Kit (INDAS Laboratories, Madrid, Spain; reviewed by Sakkas and Alvarez (2010)). Despite the suggestion that cytogenetic and DNA integrity tests may help predict pregnancy outcome, it is suspected that sperm DNA and chromosome integrity results differ by analysis technique.

The recently developed SCD test produces sperm nucleoids that comprise a core and peripheral halo due to the release of DNA loops, signifying the absence of DNA fragmentation (Fernández and Gosálvez, 2002). This is the only assay that claims to allow the analysis of DNA fragmentation and aneuploidy simultaneously on the same sperm cell (SCD-fluorescence in-situ hybridization (FISH)) (Fernández

et al., 2005) and was recently improved to promote the Halosperm Kit (Muriel et al., 2007).

Discrepancies between the levels of DNA fragmentation observed in a study by Fernández et al. (2005) brought to light the evident variation in the numerical values between results generated from the SCSA and SCD tests (Evenson and Wixon, 2005). The aims of this study were to validate the SCD-FISH procedure in the assessment of sperm aneuploidy and to validate the Halosperm, SCD and SCD-FISH tests in the analysis of sperm DNA fragmentation. The third was to investigate the hypothesis that a correlation exists between sperm DNA fragmentation and sperm aneuploidy. Whilst the basic principles behind these tests appear sound, compared with existing assays, they have not yet been evaluated extensively in a clinical setting. The current study attempted to determine if the Halosperm, SCD and SCD-FISH tests are reliable assays to use in the assessment of DNA fragmentation and aneuploidy in a clinical environment. Semen samples from 20 males undergoing treatment at the Centre for Reproductive and Genetic Health (CRGH) for IVF/ICSI were analysed for DNA fragmentation and FISH analysis was carried out using probes for chromosomes X, Y and 18.

Materials and methods

Research ethics committee approval

This study was approved by the Outer West London Ethics Committee (REC Number: 10/H0709/26).

Preparation of semen samples

Twenty semen samples collected from patients attending the CRGH for IVF/ICSI were used for analysis in this study. These samples were stored as 0.1 ml aliquots in Eppendorf tubes at −80°C. These samples have previously been analysed to record sperm concentration, DFI and high DNA stainability levels (Speyer et al., 2010).

Assessment of sperm aneuploidy using conventional FISH

Sperm preparation

Phosphate-buffered saline (PBS, 5 ml, P6887; Sigma) was added to 0.1 ml semen and centrifuged at 750*g* for 5 min. The pellet was resuspended in 10 ml PBS and spun again. This wash was repeated. The pellet was resuspended in 10 ml fix (methanol/acetic acid 3:1), incubated at room temperature for 10 min and centrifuged again. The pellet was resuspended in enough fresh fix to give a moderately opalescent suspension of spermatozoa and stored at −20°C.

Preparation of slides

Prepared spermatozoa (6 μl) was spread on a glass slide, delineated with a diamond marker and air dried. The slides were then dehydrated through an ethanol series (70%, 90%, 100%) for 3 min each.

Decondensation

The fresh slides were incubated in 10 mmol/l dithiothreitol (DTT) solution in 0.05 mol/l tris for 6 min, followed by two 2-min washes in 2 × SSC (0.3 mol/l sodium chloride, 0.03 mol/l tri sodium citrate, pH 7.2) at room temperature. The wet slides were then dehydrated through another ethanol series for 3 min each.

Denaturation

DNA probe mix (4 μl; probe/buffer 1:1) were pipetted onto a 13 mm coverslip and inverted onto the area defined by a diamond marker. The DNA probes used were for the alphoid centromeric regions of X chromosome (DXZ1 Locus, SpectrumGreen; Vysis, Izasa, Spain), Y chromosome (DYZ3 locus, SpectrumOrange; Vysis), and chromosome 18 (D18Z1 locus, SpectrumAqua; Vysis). The slides were denatured at 75°C for 5 min. They were transferred to a moist chamber and incubated at 37°C for 1 h, allowing hybridization of the probes.

Post-hybridization washes

All post-hybridization washes were performed in the dark. The coverslips were removed and the slides were submerged in 60% formamide/2 × SSC at 42°C, twice for 5 min each. This was followed by 5 min in 4 × SSC/20% Tween at room temperature. The slides were dehydrated through an ethanol series for 3 min each, air dried, mounted in Vectarshield (30 μl (4,6-diamidino-2-phenylindole), DAPI) and stored in the dark at 4°C.

Optimization of protocols to determine if SCD and SCD-FISH could be performed in the laboratory via first principles

A modified version of the SCD test can apparently be used to simultaneously determine the level of DNA fragmentation and aneuploidy on the same sperm cell (Muriel et al., 2007). The reproducibility of the method using first principles was assessed by replacing just one factor/reagent from the commercially available kit with a laboratory-produced substitute (i.e. lysing solution prepared in the laboratory versus lysing solution provided in the kit) whilst using all other components (i.e. agarose pre-coated slides, acid denaturant) from the kit. The results were compared with slides processed entirely using the commercial kit. This ensured that the results observed were identical regardless of whether the Halosperm kit or materials prepared in the laboratory using first principles were employed.

The level of DNA fragmentation and aneuploidy were scored on the same semen samples subjected to the SCD test only, SCD-FISH and conventional FISH. The spermatozoa were scored to determine the frequency of cells with DNA damage (demonstrated by the absence of a halo), aneuploidy and both.

Analysis of DNA fragmentation using the SCD test

An aliquot of frozen semen sample, stored at −80°C, was thawed in a water bath at 37°C for 40 s. The sample was diluted in PBS solution to a concentration of 5—10 million cells/ml. Eppendorf tubes containing gelled aliquots of low-melting point aqueous agarose (to obtain a 0.7% final agarose concentration at 37°C) were placed in an oven at 90°C for 5 min to melt the agarose and then placed at 37°C.

After 5-min incubation at 37°C to allow for equilibrium, 40 μl diluted semen sample was added to the Eppendorf tube containing agarose and mixed well. Aliquots of 20 μl of the semen/agarose mixture were pipetted onto a 22-mm glass coverslip and inverted onto a 0.65% agarose precoated glass slide and outlined by a diamond marker. The slide was placed on a cold plate at 4°C for 5 min. This allowed the agarose to produce a microgel with the trapped spermatozoa inside. The coverslip was gently removed and the slide was immediately immersed horizontally in 0.08 mol/l HCl solution at 7 min at room temperature. The slide was then immersed in 30 ml lysing solution 1 (0.4 M Tris, 0.8 M DTT, 1% SDS and 50 mmol/l EDTA, pH 7.5) for 10 min and then 30 ml of lysing solution 2 (0.4 M Tris, 2 M NaCl and 1% SDS, pH 7.5) for 5 min at room temperature. Following a 5-min wash in distilled water, the slides were dehydrated in an ethanol series of increasing concentration (70%, 90%, 100%) for 2 min each. The slides were air-dried and mounted in 30 μl of 2 mg/ml DAPI in 1 ml Vectarshield (Vectar Laboratories, CA, USA, 6 μl DAPI) and stored in a tightly closed box in the dark at room temperature.

Analysis of DNA fragmentation using the Halosperm test

The semen sample was diluted in PBS to a concentration of 5—10 million per millilitre and 25 μl was added to the melted agarose. The cell suspension from the agarose Eppendorf was placed on the super-coated slides and covered with a glass coverslip. Ensuring the slide was in a horizontal position throughout the entire process, the slide was placed on a cold surface at 4°C for 5 min. The coverslip was removed and the slide was immersed in acid denaturant for 7 min. It was then incubated in tempered lysis solution at room temperature for 25 min. Following a 5-min wash in distilled water, the slide was dehydrated in an ethanol series of increasing concentration (70%, 90%, 100%) for 2 min each before air drying and mounting in 30 μl of 2 mg/ml DAPI in 1 ml Vectarshield (6 μl DAPI; Vectar Laboratories, CA, USA). The slides were stored in a tightly closed box in the dark at room temperature (Halosperm; Halotech Dna, SL, USA).

Analysis of aneuploidy using FISH on cells processed for the SCD test (SCD-FISH)

The sperm samples were processed according to the SCD procedure, until the final dehydration series. Slides were immersed in 1% paraformaldehyde/PBS at 4°C for 10 min, washed in excess PBS and then distilled water for 1 min each. They were dehydrated in an ethanol series of increasing concentration (70%, 90%, 100%) for 2 min each and air-dried. Denaturation and post-hybridization washes were performed as with conventional FISH (Muriel et al., 2007).

Validation of the SCD, SCD-FISH and Halosperm tests

The DNA fragmentation values obtained from SCD, SCD-FISH and Halosperm were compared with the previously recorded DFI values obtained from SCSA.

Fluorescence microscopy and scoring criteria

The slides were examined with a Nikon fluorescence microscope, comprising a triple-band pass filter and with monochrome filters for DAPI, SpectrumGreen, SpectrumOrange and SpectrumAqua. Approximately 500 spermatozoa were scored per sample. Those sperm nuclei that overlapped or showed nullisomy were disregarded. FISH signals were scored as described by Muriel et al. (2007). During FISH analysis on conventional sperm spreads, spermatozoa were considered disomic when displaying duplicated domains with similar size, shape and intensity, and separated by a distance of at least one domain. Sperm nuclei were considered only if they were intact, non-overlapping, had a distinct border and had not been decondensed to more than twice the size of a condensed sperm head.

Data analysis

The data was analysed using the Statistical Package for Social Sciences version 14.0 for Windows (SPSS, Chicago, IL, USA). Pearson correlation and Wilcoxon rank tests were carried out to determine possible correlations and the significance of results generated.

Results

Validation of the SCD-FISH test in the assessment of aneuploidy in spermatozoa

To establish the accuracy of aneuploidy analysis using SCD-FISH, the level of chromosomal abnormality was compared in samples subjected to SCD-FISH and conventional FISH. The levels of aneuploidy ranged from 2.4% to 9.0% following FISH (mean ± SD, 5.6 ± 1.6) and 11.0—28.2% following SCD-FISH (17.9 ± 4.4) (**Figure 1**). The Wilcoxon rank test was used to statistically analyse the two sets of data generated ($P = 0.000$; two-tailed $P \leq 0.05$), therefore the difference between the level of aneuploidy detected when using SCD-FISH and conventional FISH was statistically significant. When comparing the level of aneuploidy between SCD-FISH and the gold-standard FISH, a significant result suggests that SCD-FISH does not give the same results as FISH. However, they could be equivalent, but on different scales. It is therefore interesting to test the correlation between these two tests.

The difference in the level of aneuploidy when the same sperm samples are assessed by the established FISH and

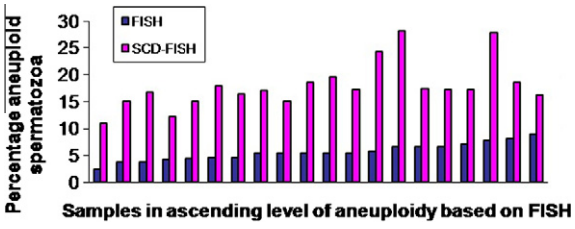


Figure 1 Comparison of the frequency of aneuploidy in spermatozoa, based on sperm chromatin dispersion (SCD)-fluorescence in-situ hybridization (FISH) and conventional FISH.

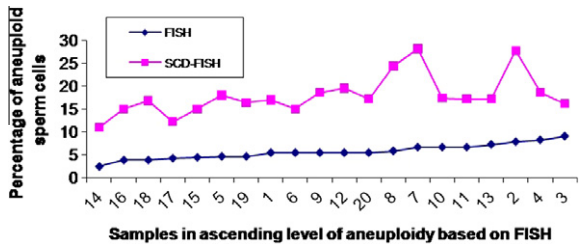


Figure 2 Correlation matrix for the level of aneuploidy when comparing sperm chromatin dispersion (SCD)-fluorescence in-situ hybridization (FISH) with conventional FISH.

SCD-FISH is illustrated in **Figure 2**. The high correlation ($r = 0.51$, $P = 0.021$; Pearson correlation $P < 0.05$) suggests that the same underlying quantity (i.e. percentage of cells displaying aneuploidy) is being measured, but not on the same scale. A lower correlation ($r < 0.2$) would imply that the same quantity is not being measured.

Validation of SCD, Halosperm and SCD-FISH in the assessment of DNA fragmentation in spermatozoa

To investigate the accuracy of DNA fragmentation analysis using SCD, Halosperm and SCD-FISH, these assays were used to compare the proportion of sperm cells without halos with samples subjected to SCSA. The levels of DNA fragmentation based on the SCSA, Halosperm, SCD and SCD-FISH tests were 4—43% (9.8 ± 5.3), 6—64% (19.6 ± 14.2), 0.4—40.6% (6.1 ± 9.1) and 7.6—99% (52.4 ± 34.0), respectively (**Figure 3**). A statistically significant difference was observed between the levels of sperm DNA fragmentation in the samples subjected to SCSA and SCD ($P = 0.001$), SCSA and SCD-FISH ($P = 0.001$) and SCD and SCD-FISH ($P = 0.000$). A statistically significant difference was not observed when comparing SCSA and Halosperm ($P = 0.211$) (Wilcoxon rank test, two-tailed $P \leq 0.05$). The correlation between the SCSA and SCD results was significant ($r = 0.628$, $P = 0.003$), but correlation between SCSA and Halosperm ($r = 0.407$, $P = 0.075$), SCSA and SCD-FISH ($r = 0.123$, $P = 0.606$) (Pearson correlation, two-tailed $P < 0.05$) (**Figure 4**) was not. It can therefore be assumed that whilst the laboratory-prepared SCD test was not accurate in assessing DNA fragmentation, the Halosperm assay produced results similar to those generated by the SCSA.

Investigating the correlation between DNA fragmentation and aneuploidy in spermatozoa

Pearson correlation tests were performed to investigate possible correlations between DNA fragmentation and aneuploidy in spermatozoa (level of significance $P < 0.05$, two-tailed). Samples processed by FISH and SCD demonstrated a significant negative correlation ($r = -0.457$, $P = 0.043$; **Figure 5A**). SCD-FISH was used on 20 samples to simultaneously determine aneuploidy and DNA fragmentation on the same sperm cells. Statistical significance was not established ($r = -0.191$, $P = 0.419$; **Figure 5B**). The correlation between DNA fragmentation and aneuploidy in sperm cells processed by Halosperm and FISH was of no statistical significance ($r = 0.355$, $P = 0.125$; **Figure 5C**).

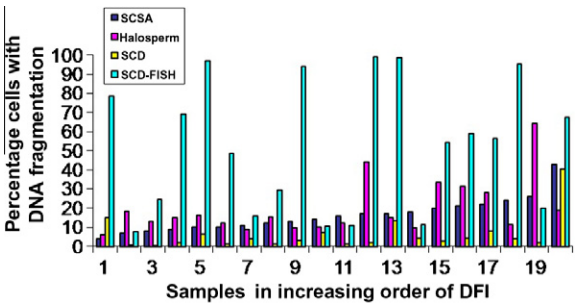


Figure 3 The levels of DNA fragmentation index (DFI) based on sperm chromatin structure assay (SCSA), Halosperm, sperm chromatin dispersion (SCD) and SCD-fluorescence in-situ hybridization (FISH).

Surprisingly, the correlation between the samples subjected to the gold standard analysis techniques of FISH and SCSA was also not significant ($r = -0.078$, $P = 0.742$; **Figure 5D**).

Discussion

The aim of this study was to validate the SCD, SCD-FISH and Halosperm tests in the analysis of sperm DNA fragmentation and compare them to SCSA. The SCD-FISH test had to first be validated in terms of its reliability when scoring aneuploidy and DNA fragmentation, preceding its utilization to study the correlation between these two parameters. The SCD test was carried out using reagents prepared in the laboratory, in addition to the Halosperm Kit (Fernández et al., 2003).

Interpretation of results

There was no significant correlation between the level of aneuploidy assessed by FISH and SCD-FISH. Because spermatozoa with fragmented DNA tend to display spotted FISH signals, discriminating between single and multiple signals proved difficult. Muriel et al. (2007) claim that FISH signals in spermatozoa without DNA fragmentation have the propensity, albeit rarely, to spread from the core to the halo in the nucleoids. This can easily cause two FISH signals to overlap, resulting in an underestimation of the aneuploidy rate. However, the present study observed that the dispersion of FISH signals led to an over-estimation of aneuploidy. A frequent occurrence, this can result in subjectivity when interpreting the signals and therefore errors during the scoring procedure.

Based on the present results, SCD, SCD-FISH and Halosperm are unsuitable to measure sperm DNA damage. Although the level of DNA fragmentation observed when using the different assays varied considerably, the results obtained using Halosperm are somewhat comparable to those observed using SCSA. Determining whether Halosperm is a reliable and sufficient test to measure DNA fragmentation however, requires further work. Despite expecting SCD and SCD-FISH to generate similar results considering they were both made using in-house solutions, the statistically significant difference between the two sets of data and the lack of a significant correlation implicates the

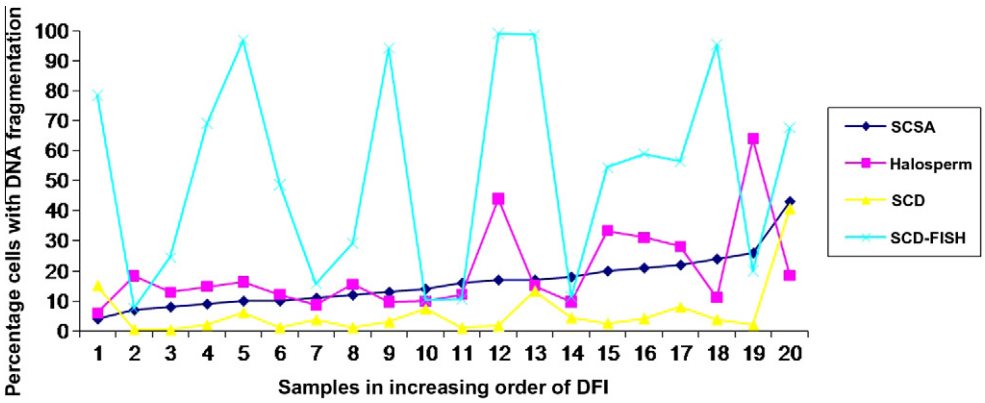


Figure 4 Correlation matrix for the levels of DNA fragmentation index (DFI) when comparing sperm chromatin structure assay (SCSA), Halosperm, sperm chromatin dispersion (SCD) and SCD-fluorescence in-situ hybridization (FISH).

influence of post-washes in the inconsistency surrounding halo visualization. The halos observed using the Halosperm Kit and reagents prepared in the laboratory were identical during the optimization process, so the inconsistency in halo preservation requires further investigation. Reproducing the SCD test using first principles generated technical complications. Preparation of the lysing solution and the effect it had on sperm cells proved problematic and the visualization of halos was poor in some cases. This may contribute to the unexpected results generated. A similar observation was noted by Muriel et al. (2007), who attributed the phenomenon to the aggressive conditions of the lysing solution. This issue was apparently rectified when using the Halosperm Kit (Muriel et al., 2007).

This study suggests that SCD-FISH is an inapt method to assess aneuploidy or DNA fragmentation, either independently or simultaneously. The lack of a significant positive correlation between the level of aneuploidy and DNA fragmentation in sperm cells analysed by these methods (**Figure 5**) does not necessarily discourage the suggestion of such a correlation, but may be more indicative of the unreliability of the methods themselves. It was also noted that the FISH signals and halos observed manually through the microscope and those captured on camera appeared different to one another. Considering the literature that supports the hypothesis that a positive relationship exists between DNA fragmentation and aneuploidy (Liu et al., 2004; Muriel et al., 2007), the negative values of the correlation coefficient are unexpected. As in the case of many commonly used statistics, r is not robust and its value can be misleading if outliers are present. The Pearson correlation in particular is not outlier resistant (Wilcox, 2005).

A double-blinded study is recommended to determine the level of subjectivity and reliability of these assays in the detection of sperm DNA fragmentation and during the simultaneous assessment of DNA fragmentation and aneuploidy on the same sperm cell. The reliability of the present data may be questioned, in terms of scoring accuracy, but this simply further highlights the ineffectual nature of the test considering the time and effort required to familiarize oneself with the scoring procedure. Tests such as these should be easy to validate in a laboratory setting. Recounting the same slides in the future is probably worthwhile, in addition to using a larger sample size.

DNA fragmentation tests: scientific fact or commercial fiction?

Many assays have been developed to measure the level of DNA fragmentation in spermatozoa from infertile men and fertile donors. Techniques used to analyse sperm DNA fragmentation in any clinical andrology or assisted reproductive technology laboratory should be simple, reproducible and ideally without the need for new, complex or expensive technology (De Jonge, 2002). The advantages of the SCD test include the fact that it does not rely on the determination of either colour or fluorescence intensity, as in the case of other semi-quantitative tests. Ideally, the endpoint measured by the SCD test consists of determining the percentage of spermatozoa with non-dispersed or dispersed nuclei, which should theoretically be accomplished by the naked eye. This is unfortunately not always as simple. Whilst the Halosperm Kit measures only ~500 sperm cells on a light microscope slide, 5000 can be analysed using the SCSA test (Evenson and Wixon, 2005). The SCD test displays a significantly higher sensitivity for detecting sperm DNA fragmentation (mean difference of 2.16%). Additionally, they deviate more in samples with higher SCSA DFI levels; a sample with a DFI of 30—35% can have a SCD result ranging between 20—50% (Evenson and Wixon, 2005). This pattern of variation and lack of correlation was consistent with the results observed when the DFI levels calculated were compared with those obtained following the SCD-FISH and SCD procedures. Furthermore, the inter-observer error can be as high as 12% (Evenson and Wixon, 2005). Therefore, the real biological value can be either negligible or nearly double the value stated. There also appeared to be a lack of significance concerning the mean sperm cells with fragmented DNA when the samples were observed by different technicians (results not shown). Whilst the promotion of the commercial Halosperm Kit continues, a significant absence of unbiased scientific literature validating the simultaneous examination of DNA fragmentation and aneuploidy brings its authenticity into question.

Clinical use of DNA fragmentation assays

Originally intended as a complementary test to those already in place prior to routine IVF procedures, it is now

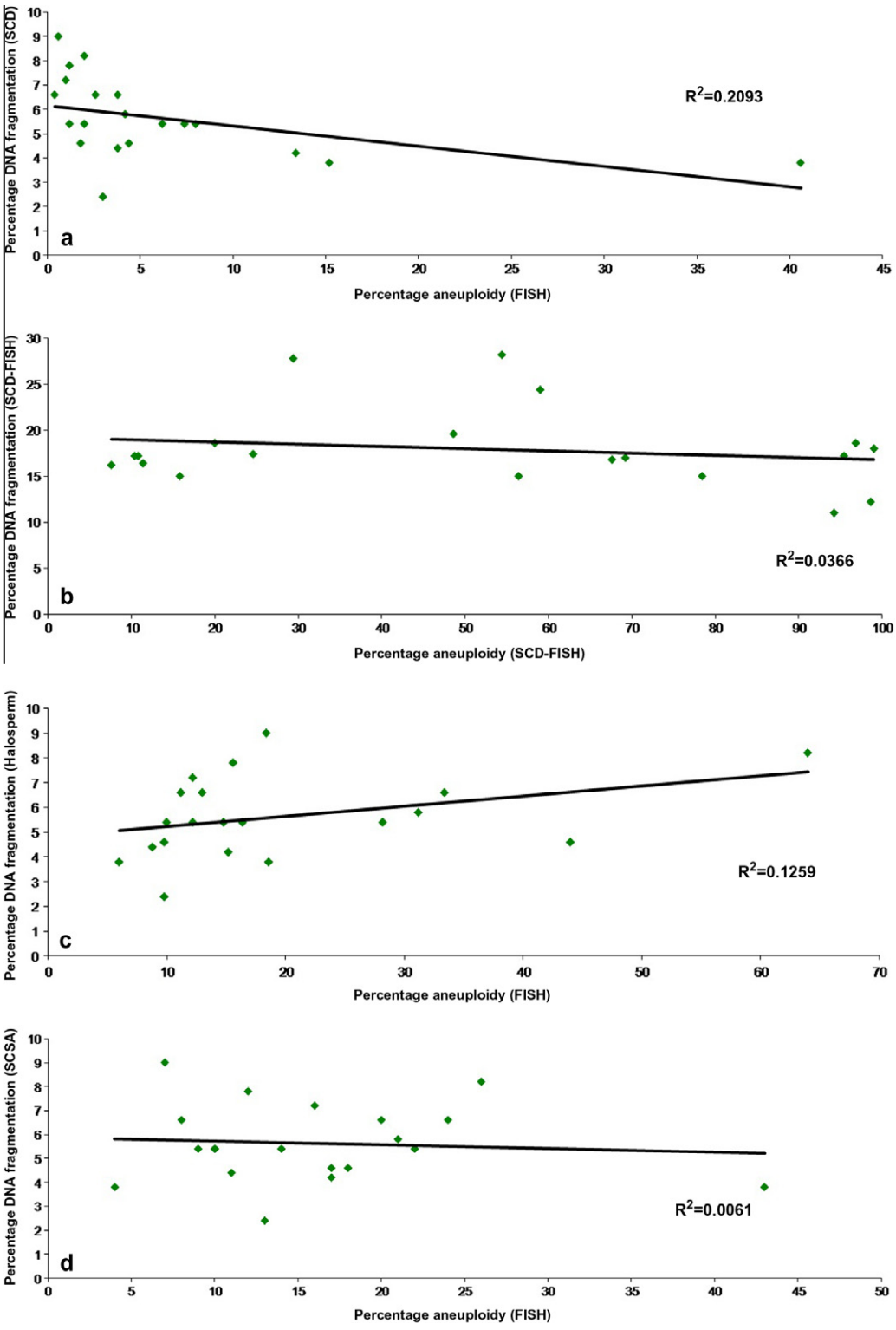


Figure 5 Correlation between DNA fragmentation and aneuploidy in sperm cells, processed by (a) sperm chromatin dispersion (SCD) and fluorescent in-situ hybridization (FISH), (b) SCD-FISH, (c) Halosperm and FISH and (d) sperm chromatin structure assay (SCSA) and FISH.

customary for patients to request tests for DNA damage. Concerns over its usefulness as an independent or complementary factor of male infertility and what it actually measures still exist. Whilst the level of damage observed is at a cellular level, it is impossible to determine which sequences of the genome are affected. The age of the female partner and her oocyte quality plays a role in her ability to repair the sperm damage during fertilization. The heterogeneous nature of spermatozoa needs to be examined carefully; it is difficult to determine whether characteristics of sperm

quality such as morphology and DNA damage represent an individual gamete or underlying spermatogenic process (Aitken and De Iuliis, 2010). This is particularly important in ICSI. Ironically, because the success rates of ICSI compete with those of IVF, there has been little incentive to develop new sperm selection tests. To accurately interpret the data, it is important to distinguish between genuine DNA damage and artefacts due to lack of reagent access to DNA (Barratt et al., 2010) and compensate for the varying changes in sperm chromatin structure following ejaculation (Kvist et al., 1988).

Until such discrepancies are ironed out, its establishment in the routine diagnostic investigations of infertile men should be shelved. Different values obtained from different assays, or even from different laboratories using the same technique, may be because of variations in conditions and protocol. Clearly defined reference values to discriminate between fertile and infertile groups have not been established (Simon et al., 2010) and should be used with caution. These issues highlight the fact that predicting fertility on the basis of a single sperm variable is extremely difficult, whether a measure of DNA damage or any other characteristic of semen quality (Aitken et al., 2009). This makes the validation of DNA fragmentation assays an even more tedious, but necessary, task.

These factors perhaps contribute to unexpected reports where successful pregnancies result despite a high level of sperm DNA damage. Among the several factors that influence the predictive value of DNA fragmentation tests are single- versus double-stranded DNA fragmentation, percentage of spermatozoa with DNA damage, primary or secondary DNA damage and capacity of the oocyte and embryo to repair DNA damage (Lewis et al., 2008). The type of DNA fragmentation test used is also important, because tests that measure the level of DNA damage under physiological conditions like Comet at neutral pH, or TUNEL, have a higher predictive value than those (SCSA and SCD) that measure DNA damage or susceptibility to damage under non-physiological conditions (Lewis et al., 2008).

In conclusion, the history of clinical andrology has had many false dawns. The reason that sperm function assays have thus far failed to have a significant impact on the clinical management of infertile couples may be due to the lack of standardized protocols. It would be unfortunate if scepticism about the clinical significance of DNA damage generates complacency among infertility specialists. DNA damage in the male germ line may result in adverse clinical outcomes and the pathophysiology and clinical consequences of sperm DNA damage are being actively researched. Despite its plausible appeal and fervour of its supporters, the benefits of widespread DNA testing that only achieves the distressing of couples with the knowledge that effectual therapeutic strategies are absent are questionable. Commercially, however, it is no doubt lucrative. The clinical and scientific community considers it a matter of urgency to translate the basic science behind how a cell prepares for fertilization into routine clinical practice (reviewed by Barratt (2010)). However, it is equally important, if not more, to allow the science behind such applications to draw level with its practice before its widespread implementation.

Acknowledgement

The authors would like to acknowledge Darren Griffin for his kind assistance in the preparation of this manuscript.

References

Adamson, G.D., Baker, V.L., 2003. Subfertility: causes, treatment and outcome. Best Pract. Res. Clin. Obstet. Gynaecol. 17, 169–185.

Ahmadi, A., Ng, S., 1999. Fertilizing ability of DNA-damaged spermatozoa. J. Exp. Zool. 284, 696–704.

Aitken, R.J., De Iuliis, G.N., 2010. On the possible origins of DNA damage in human spermatozoa. Mol. Hum. Reprod. 16, 3–13, Epub 2009 Jul 31. Review.

Aitken, R.J., De Iuliis, G.N., McLachlan, R.I., 2009. Biological and clinical significance of DNA damage in the male germ line. Int. J. Androl. 32, 46–56, Epub 2008 December 2. Review.

Barratt, C.L., 2010. Male infertility joins the translational medicine revolution. Sperm DNA: from basic science to clinical reality. Mol. Hum. Reprod. 16, 1–2.

Barratt, C.L., Aitken, R.J., Björndahl, L., et al., 2010. Sperm DNA: organization, protection and vulnerability: from basic science to clinical applications—a position report. Hum. Reprod. 25, 824–838, Epub 2010 February 6.

Bungum, M., Humaidan, P., Axmon, A., Spano, M., Bungum, L., Erenpreiss, J., Giwercman, A., 2007. Sperm DNA integrity assessment in prediction of assisted reproduction technology outcome. Hum. Reprod. 22, 174–179, Epub 2006 August 18.

Carrell, D.T., Emery, B.R., 2008. Use of automated imaging and analysis technology for the detection of aneuploidy in human sperm. Fertil. Steril. 90, 434–437, Epub 2007 October 23.

Collodel, G., Capitani, S., Baccetti, B., et al., 2007. Sperm aneuploidies and low progressive motility. Hum. Reprod. 22, 893–898.

Darzynkiewicz, Z., Kapuscinski, J., 1990. Acridine orange: a versatile probe of nucleic acids and other cell constituents. In: Melamed, M.R., Lindmo, T., Mendelsohn, M.L. (Eds.), Flow Cytometry and Sorting, second ed. Wiley-Liss, New York, NY, pp. 291–314.

De Jonge, C., 2002. The clinical value of sperm nuclear DNA assessment. Hum. Fertil. (Camb) 5, 51–53.

Dobles, M., Liberal, V., Scott, M.L., Benezra, R., Sorger, P.K., 2000. Chromosome missegregation and apoptosis in mice lacking the mitotic checkpoint protein Mad2. Cell 101, 635–645.

Evenson, D.P., Wixon, R., 2005. Comparison of the Halosperm test kit with the sperm chromatin structure assay (SCSA) infertility test in relation to patient diagnosis and prognosis. Fertil. Steril. 84, 846–849.

Evenson, D.P., Jost, L.K., Marshall, D., et al., 1999. Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. Hum. Reprod. 14, 1039–1049.

Faure, A.K., Aknin-Seifer, I., Frerot, G., et al., 2007. Predictive factors for an increased risk of sperm aneuploidies in oligo-astheno-teratozoospermic males. Int. J. Androl. 30, 153–162.

Fernández, J.L., Gosá lvez, J., 2002. Application of FISH to detect DNA damage: DNA breakage detection-FISH (DBD-FISH). Methods Mol. Biol. 203, 203–216.

Fernández, J.L., Muriel, L., Rivero, M.T., Goyanes, V., Vázquez, R., Alvarez, J.G., 2003. The sperm chromatin dispersion test: a simple method for the determination of sperm DNA fragmentation. J. Androl. 24, 59–66.

Fernández, J.L., Lourdes, M., Goyanes, V.J., et al., 2005. Simple determination of human sperm DNA fragmentation with an

improved sperm chromatin dispersion (SCD) test. Fertil. Steril. 84, 833—842.
Fernández-Gonzalez, R., Moreira, P.N., Pérez-Crespo, M., et al., 2008. Long-term effects of mouse intracytoplasmic sperm injection with DNA-fragmented sperm on health and behavior of adult offspring. Biol. Reprod. 78, 761—772, Epub 2008 January 16.
Genescà, A., Benet, J., Caballín, M.R., Miró, R., Germà, J.R., Egozcue, J., 1990. Significance of structural chromosome aberrations in human sperm: analysis of induced aberrations. Hum. Genet. 85, 495—499.
Gluud, C., Gluud, L.L., 2005. Evidence based diagnostics. Br. Med. J. 330, 724—726.
Griffin, D.K., 1996. The incidence, origin, and etiology of aneuploidy. Intl. Rev. Cytol. 167, 263—296.
Hassold, T., Hunt, P., 2001. To err (meiotically) is human: the genesis of human aneuploidy. Nat. Rev. Genet. 2, 280—291.
Henkel, R., Hajimohammad, M., Stalf, T., et al., 2004. Influence of deoxyribonucleic acid damage on fertilization and pregnancy. Fertil. Steril. 81, 965—972.
Hull, M.G., Glazener, C.M., Kelly, N.J., et al., 1985. Population study of causes, treatment, and outcome of infertility. Br. Med. J. (Clin. Res. Ed.) 291, 1693—1697.
Irvine, D.S., Twigg, J.P., Gordon, E.L., Fulton, N., Milne, P.A., Aitken, R.J., 2000. DNA integrity in human spermatozoa: relationships with semen quality. J. Androl. 21, 33—44.
Kamiguchi, Y., Tateno, H., 2002. Radiation- and chemical-induced structural chromosome aberrations in human spermatozoa. Mutat. Res. 504, 183—191, Review.
Kovanci, E., Kovacs, T., Moretti, E., et al., 2001. FISH assessment of aneuploidy frequencies in mature and immature human spermatozoa classified by the absence or presence of cytoplasmic retention. Hum. Reprod. 16, 1209—1217.
Kvist, U., Kjellberg, S., Björ rndahl, L., Hammar, M., Roomans, G.M., 1988. Zinc in sperm chromatin and chromatin stability in fertile men and men in barren unions. Scand J. Urol. Nephrol. 22, 1—6.
Lewis, S.E., O'Connell, M., Stevenson, M., Thompson-Cree, L., McClure, N., 2004. An algorithm to predict pregnancy in assisted reproduction. Hum. Reprod. 19, 1385—1394, Epub 2004 April 29.
Lewis, S.E.M., Agbaje, I., Alvarez, J., 2008. Sperm DNA tests as useful adjuncts to semen analysis. Syst. Biol. Reprod. Medicine. 54, 111—125.
Liu, C.H., Tsao, H.M., Cheng, T.C., et al., 2004. DNA fragmentation, mitochondrial dysfunction and chromosomal aneuploidy in the spermatozoa of oligoasthenoteratozoospermic males. J. Assist. Reprod. Genet. 21, 119—126.
Martin, R.H., Greene, C., Rademaker, A.W., et al., 2003. Analysis of aneuploidy in spermatozoa from testicular biopsies from men with nonobstructive azoospermia. J. Androl 24, 100—103.
McLachlan, R.I., de Kretser, D.M., 2001. Male infertility: the case for continued research. Med. J. Aust. 174, 116—117.
Muriel, L., Goyanes, V., Segrelles, E., Gosálvez, J., Alvarez, J.G., Fernández, J.L., 2007. Increased aneuploidy rate in sperm with fragmented DNA as determined by the sperm chromatin dispersion (SCD) test and FISH analysis. J. Androl. 28, 38—49.
Ollero, M., Gil-Guzmán, E., Sharma, R.K., et al., 2001. Characterization of subsets of human spermatozoa at different stages of maturation: implications in the diagnosis and treatment of male infertility. Hum. Reprod. 16, 1912—1921.
Payne, J.F., Raburn, D.J., Couchman, G.M., Price, T.M., Jamison, M.G., Walmer, D.K., 2005. Redefining the relationship between sperm deoxyribonucleic acid fragmentation as measured by the sperm chromatin structure assay and outcomes of assisted reproductive techniques. Fertil. Steril 84, 356—364.
Rubio, C., Gil-Salom, M., Simón, C., Vidal, F., Rodrigo, L., Mínguez, Y., Remohí, J., Pellicer, A., 2001. Incidence of sperm chromosomal abnormalities in a risk population: relationship with sperm quality and ICSI outcome. Hum. Reprod. 16, 2084—2092.
Sakkas, D., Alvarez, J.G., 2010. Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis. Fertil. Steril. 93, 1027—1036, Epub 2010 January 18.
Sakkas, D., Urner, F., Bizzaro, D., et al., 1996. Sperm chromatin anomalies can influence decondensation after intracytoplasmic sperm injection (ICSI). Hum. Reprod. 11, 837—843.
Saleh, R.A., Agarwal, A., Sharma, R.K., Said, T.M., Sikka, S.C., Thomas Jr., A.J., 2003. Evaluation of nuclear DNA damage in spermatozoa from infertile men with varicocele. Fertil. Steril. 80, 1431—1436.
Seli, E.S., Gardner, D.K., Schoolcraft, W.B., Moffat, O., Sakkas, D., 2004. Extent of nuclear DNA damage in ejaculated spermatozoa impacts on blastocyst development after in vitro fertilization. Fertil. Steril. 82, 378—383.
Simon, L., Brunborg, G., Stevenson, M., Lutton, D., McManus, J., Lewis, S.E., 2010. Clinical significance of sperm DNA damage in assisted reproduction outcome. Hum. Reprod.
Spanò, M., Bonde, J.P., Hjøllund, H.I., Kolstad, H.A., Cordelli, E., Leter, G., 2000. Sperm chromatin damage impairs human fertility. The Danish First Pregnancy Planner Study Team. Fertil. Steril. 73, 43—50.
Speyer, B.E., Pizzey, A.R., Ranieri, M., Joshi, R., Delhanty, J.D., Serhal, P., 2010. Fall in implantation rates following ICSI with sperm with high DNA fragmentation. Hum. Reprod.
Sun, J.G., Jurisicova, A., Casper, R.F., 1997. Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization in vitro. Biol. Reprod. 56, 602—607.
Tempest, H.G., Griffin, D.K., 2004. The relationship between male infertility and increased levels of sperm disomy. Cytogenet. Gen. Res. 107, 83—94.
Tesarik, J., Greco, E., Mendoza, C., 2004. Late, but not early, paternal effect on human embryo development is related to sperm DNA fragmentation. Hum. Reprod. 19, 611—615, Epub 2004 January 29.
Twigg, J.P., Irvine, D.S., Aitken, R.J., 1998. Oxidative damage to DNA in human spermatozoa does not preclude pronucleus formation at intracytoplasmic sperm injection. Hum. Reprod. 13, 1864—1871.
Virro, M.R., Larson-Cook, K.L., Evenson, D.P., 2004. Sperm chromatin structure assay (SCSA) parameters are related to fertilization, blastocyst development, and ongoing pregnancy in in vitro fertilization and intracytoplasmic sperm injection cycles. Fertil. Steril. 81, 1289—1295.
Wilcox, R.R., 2005. Introduction to Robust Estimation and Hypothesis Testing, second ed. Academic Press.

Declaration: The authors report no financial or commercial conflicts of interest.

Received 2 August 2010; refereed 17 November 2010; accepted 27 January 2011.