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ARTICLE

Hypo-osmotic swelling test identifies individual spermatozoa with minimal DNA fragmentation

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Abstract One concern during intracytoplasmic sperm injection (ICSI) is that selected spermatozoa may have increased levels of DNA damage; however, the available testing for this is largely destructive in nature and therefore unsuitable as a tool for sperm selection. One alternative selection process that has previously achieved pregnancies is the hypo-osmotic swelling test (HOST). This study reports that low HOST values of neat semen samples were significantly ($P < 0.001$) associated with increased DNA damage identified by the DNA fragmentation index (DFI) from the sperm chromatin structure assay as well as the TdT-mediated dUTP nick-end labelling (TUNEL) assay. The HOST value was highly predictive of an abnormal DFI value by receiver operating characteristic curve analysis ($P < 0.001$). Furthermore, when individual spermatozoa were assessed for both HOST status and DNA fragmentation by TUNEL, the key HOST-induced tail-swelling grades D, E and F were most commonly associated with high HOST values and were significantly ($P < 0.001$) associated with minimal DNA damage regardless of the DNA status of the ejaculate. The application of HOST may be a valuable tool in the routine identification and selection of viable, DNA-intact individual spermatozoa for ICSI after further research to demonstrate its efficacy and safety.

KEYWORDS: HOST, ICSI, male infertility, SCSA, TUNEL

Introduction

The development of intracytoplasmic sperm injection (ICSI) after many years of IVF (Yovich and Stanger, 1984) revolutionized the management of male factor infertility (Palermo et al., 1992). The use of minimal ovarian stimulation and low egg numbers (Borini et al., 2006), the use of preimplantation genetic screening and surgically collected spermatozoa in IVF has further increased the incidence of ICSI over and above its original application for severe male-factor patients. Despite the current debate over whether ICSI should be used routinely (Aitken, 2008), in time, ICSI

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conceptions may overtake IVF conceptions as the primary vehicle for insemination (Wang et al., 2009). One of the hurdles for its acceptance is that ICSI methodology has not changed since first described by Palermo et al. (1992) and there appears to be minimal imperative to explore alternative approaches to the technique and in particular to sperm selection (Van Voorhis, 2007).There is no standardized methodology for sperm selection that has been defined and validated and ensuring that spermatozoa of similar quality are being selected by each embryologist is difficult from a quality management issue but in time may be an essential requirement.

Sperm selection for ICSI is visually based primarily upon motility and, to a lesser extent, morphology. Recent reports aimed at improving sperm selection have centred either on increasing the visual selection process (Berkovitz et al., 2005) or functional criteria such as hyaluronic acid binding capabilities (Huszar et al., 2007), the net surface charge (Ainsworth et al., 2007) or the identification of apoptotic markers (Said et al., 2008). Yet spermatozoa used in ICSI are not necessarily the same as those that achieve fertilization in IVF since the latter have been shown to conform to very specific morphological criteria associated with binding to the zona pellucida and that these have minimal DNA damage (Liu and Baker, 2007). The aim for sperm selection for ICSI should be to emulate the in-vivo process whereby the spermatozoon that facilitates fertilization has minimal DNA damage.

Recent interest in the integrity of sperm DNA (Aitken, 2008) has raised concerns that, while spermatozoa may appear motile, those with fragmented or incomplete protamination of DNA may still be selected in ICSI and these have been linked to poor embryonic development, reduced conception rates and increased possibility of miscarriage (Borini et al., 2006; Evenson and Wixon, 2006, 2008; Gandini et al., 2004; Sakkas et al., 1996) While both the sperm chromatin structure assay (SCSA) and the TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling (TUNEL) assay are standard methods for assessing sperm DNA integrity (Evenson et al., 1999), they nevertheless require a high degree of skill and they are expensive, time consuming and, above all, destructive to spermatozoa. This means it is not possible to use any of the spermatozoa processed by these tests for the purpose of ICSI and therefore the tests are of minimal clinical value.

An alternative approach is to use a non-destructive test for sperm viability where spermatozoa are self-selecting. The hypo-osmotic swelling test (HOST) is one sperm function test that maintains sperm viability (Jeyendran et al., 1984). Initially introduced for the diagnosis of male infertility, it has consequently been applied as a useful additional test for sperm cell membrane vitality (Rogers and Parker, 1991; Rossato et al., 2004). This technique is simple, cost effective, quick and, above all, non-invasive (Hossain et al., 1998; Tartagni et al., 2002), which commends it as potential routine method to select individual healthy spermatozoa for ICSI (Sallam et al., 2001) particularly since the degree of swelling of spermatozoa is associated with sperm viability, fertilization and pregnancy rates (Check et al., 2001; Tartagni et al., 2002). There is less information on the status of the HOST score and on differences between individual categories of tail swelling to DNA fragmentation. The current report sought to clarify whether the HOST value may predict the degree of sperm DNA damage in an ejaculate and whether the degree of tail swelling may predict the likelihood of DNA damage in individual spermatozoa, such that their identification by HOST may facilitate their routine selection for ICSI.

Materials and methods

Patients and semen samples

This is a prospective study conducted on 123 semen samples obtained from males (mean age 36 ± 6.22 years; range 24—64 years) attending for preliminary assessment of a couple's infertility or for IVF/ICSI treatment. Semen samples were provided by means of masturbation following an instructed abstinence of 2—5 days and collected on site at PIVET. Collected samples were kept at 37°C and aliquots were used for HOST and SCSA assessments after liquefaction in addition to their routine testing or processing for assisted reproductive technologies. Eighty-seven samples were referred for an initial semen analysis and 36 samples were produced for IVF/ICSI insemination. All samples were assessed for sperm concentration, percentage total motile count, progressive motile count (World Health Organization, 1999) and morphological parameters including percentage of head, midpiece and tail defects according to Tygerberg strict criteria (Menkveld et al., 2001). An aliquot from each sample was assessed immediately by HOST and another aliquot prepared for SCSA and cryostored until the weekly batch testing was performed. The samples produced as part of an IVF/ICSI treatment cycle were analysed for the correlation of HOST value and the success rate of fertilization using IVF (n = 11) and ICSI (n = 25) techniques. The proportions of IVF to ICSI cases reflect the current incidence in Australia and do not necessarily reflect severe male infertility or a bias in sample selection. Samples marked with successful fertilization were followed up after embryo transfer for the investigation of pregnancy rate outcomes (n = 9 for IVF and n = 22 for ICSI).

In a second series of experiments, a further 20 patients whose HOST value had been determined in a previous ejaculate were invited to produce another sample. These samples were assessed by the TUNEL assay and the results compared with the SCSA values in the previous assessment. In this group, 10 samples were provided from men with normal HOST/SCSA and 10 samples from men with abnormal HOST/SCSA profiles.

Hypo-osmotic swelling test

HOST was prepared and performed on semen samples as described previously (World Health Organization, 1999). Each sample was immediately incubated at 37°C and all assessments performed after 30 min subject to complete liquefaction. For each patient, 0.1 ml of liquefied semen was added to 1 ml of warmed 150 mOsm hypo-osmotic swelling solution containing sodium citrate (25 mmol/l) and fructose (75 mmol/l) and incubated for 30 min at 37°C. Aliquots of samples were placed on a clean glass slide with a cover slip with and without HOST treatment and assessed

within 5 min under phase-contrast microscopy. Sperm swelling was observed and graded A to G according to changes in the shape of the tail (Hossain et al., 1998; World Health Organization, 1999) The response to hypo-osmotic stress resulted in spermatozoa where there was either no tail swelling (grade A; Figure 1) or spermatozoa that respond with swelling that manifested in various degrees of swelling from the distal end of the flagellum (grades B—G; Figure 1). Grades D and E, as described by World Health Organization (1999) as having significant, discernable cytoplasmic swelling were observed in this study to be sufficiently similar that they were grouped and are referred to as grade D/E. All other gradings were as defined by World Health Organization (1999). A sample of neat semen was examined and the number of spermatozoa with natural tail swelling was counted to provide a background reference.

For each sample, a total of 200 spermatozoa were examined and the final HOST score was calculated by subtracting the percentage of swollen spermatozoa in the control samples from that of samples treated with hypo-osmotic buffer. HOST scores of each swelling grade were expressed as a percentage of total swollen spermatozoa. Based on (World Health Organization, 1999) criteria, sperm samples were considered normal if >60% of the spermatozoa exhibited tail curling and abnormal if <50% of the spermatozoa exhibited tail curling. Samples with scores 50—60% were considered as borderline.

Sperm chromatin structure assay

SCSA was performed based on protocols by (Evenson et al., 1999). Semen samples were collected and diluted to a concentration of 10 million spermatozoa/ml with Tris HCl buffer, pH 7.4 (TNE buffer; Evenson et al., 1999). Sperm DNA denaturation was induced by mixing 100 μl aliquots of diluted samples with acid detergent containing 0.1% Triton-X100 (pH 1.2) at a ratio of 1:2 for 30 s. The spermatozoa were then stained by adding an equal volume of freshly prepared solution of acridine orange (AO) for 3 min. The AO solution was prepared by adding 300 μl of AO stock solution (Polysciences, Warrington, PA, USA) to 50 ml of staining buffer (0.1 mol/l citric acid, 0.2 mol/l $Na2PO_4$, 1 mmol/l EDTA, 0.15 mol/l NaCl). Fluorescent assessments using fluorescent activated cell sorting (FACS Calibur, Becton Dickinson, Franklin Lakes, NJ, USA) were measured on 5000 sperm cell events per sample. The instrument was linked to a MAC computer with CELLQuest Pro software (Becton Dickinson) for analysis. For quality-control purposes, the FACS machine was calibrated using known positive and negative control samples before and after testing patient samples.

Computer-gated areas were placed on the central area of the cytogram to separate areas of green fluorescence (double-stranded DNA) and red fluorescence (single-stranded DNA; (Evenson and Wixon, 2008). Quantification of DNA denaturation, or the DNA fragmentation index (DFI) was calculated as the proportion of red to the sum of red and green fluorescence. The average of two estimations was used for all analyses. PIVET participates in an external quality assurance program for DNA fragmentation (www.fertaid.com) and its performance was within 1 SD of the mean replies over all specimens.

TUNEL assay on HOST selected spermatozoa

From the pool of 123 samples, material was prepared as described below in anticipation of further investigation by TUNEL staining. Once the HOST and DFI values were known, samples in the normal and abnormal ranges were selected. A total of 20 repeat samples were subjected to HOST, 10 of which showed normal sperm swelling and normal DFI (<10%) and another 10 showed abnormal sperm swelling and elevated/high (>20%) levels of DFI as assessed by SCSA. These 10 abnormal samples were re-analysed in a study to include TUNEL staining. The samples displayed different sperm concentrations due to the varied initial sperm count of

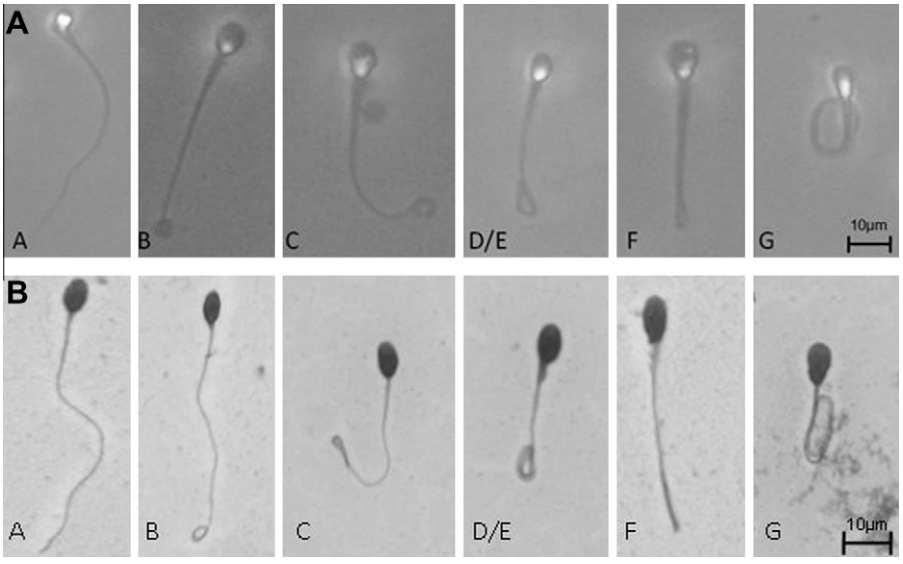

Figure 1 Microscopic observation of different grades of sperm swelling. The different sperm swelling grades (A—G) as observed using (**A**) phase-contrast microscopy (×1000 magnification) of fresh samples and (**B**) fixed and stained samples using normal light microscopy.

patients. For TUNEL purposes, sperm concentration for all samples was adjusted to 20 million/ml in TNE buffer. Aliquots (20 μl) from each adjusted sample were smeared onto separate Superfrost Plus slides (Mezel-Glaser, Braunschweig, Germany) using the side of the pipette tip.

TUNEL analysis of individual spermatozoa was performed according to the manufacturer's instructions using the ApopTag *In situ* Apoptosis Detection Kit (Millipore, North Ryde, NSW, Australia), which detects apoptotic cells *in situ*, utilizing an anti-digoxigenin antibody that is conjugated to a fluorescein reporter molecule. Spermatozoa were classified as positive when any shade of brown colouration was visually discernable under bright field microscopy.

A total of 10 slides were randomly used for the purpose of negative controls ($n = 5$) by omitting TdT enzyme and induced positive controls ($n = 5$). Positive controls were prepared by pre-treating slides with 20 μl of DN buffer (30 mmol/l Tris, 140 mmol/l sodium cacodylate, 8 mmol/l magnesium chloride, 0.1 μmol/l DTT), pH 7.2 at room temperature for 5 min. Samples were then treated with an equal volume of 1 μg/ml DNase I (Sigma-Aldrich, Castle Hill, NSW, AU) for 10 min. Samples were air dried and kept in a cool dry place until analysis. Positive and negative controls were tested in each TUNEL experiment. The positive control exhibited >95% staining in all experiments whilst the negative control exhibited no positive staining.

Counterstaining of slides was performed using methyl green (Sigma-Aldrich, Castle Hill, NSW, AU). Microscopic examination of slides was performed using a 100X oil immersion lens. DFI for each sample was calculated as a percentage of stained apoptotic spermatozoa in a total of 100 spermatozoa counted. Each sample was assessed twice and the average of the two outcomes was calculated.

Assessment of dual TUNEL staining and HOST in individual spermatozoa

All samples used for TUNEL ($n = 20$) analysis were also assessed for dual observations with HOST scores. Ten samples were obtained from men who have previously returned a normal SCSA value (termed SCSA-normal) and a further 10 samples from men who had previously demonstrated elevated SCSA values (termed SCSA-abnormal). As HOST scores were assessed in fixed stained samples, outcomes of both TUNEL and HOST were recorded for each individual spermatozoa. A total of 150—400 spermatozoa per sample were examined for this purpose in each normal and abnormal SCSA sample groups, based on HOST and SCSA scores. In order to quantify sperm TUNEL staining within individual HOST grades, the ratio of apoptotic spermatozoa as indicated by positive TUNEL staining within individual HOST sperm grade was calculated.

In a preliminary set of experiments designed to exclude the possibility of artefacts of sperm tail swelling grades due to fixation and staining while performing TUNEL assessment, a total of 10 samples including those used for dual observations were assessed before and after fixation. The HOST values were found to be the same before and after fixation and staining, indicating that the HOST status of each spermatozoon was not changed during the fixation and staining process.

Statistical methods

Statistical analysis was performed using the JMP 7 statistical discovery software (SAS Institute, Cary, NC, USA). Statistical significance was set at $P < 0.05$. Correlations between HOST, SCSA and TUNEL data were analysed using chi-squared, Spearman's ρ and Wilcoxon signed rank. Receiver operating characteristic (ROC) curve analysis for predicting the DNA fragmentation from HOST values was performed using Medicalc software (version 10.0.1.0; www.medcalc.be).

Ethical approval

All of the samples were obtained for clinical purposes as part of the patient medical evaluation and treatment. However specific ethical approval for the HOST assessment part of this study was obtained from the Human Research Ethics Committee of Curtin University which acts as PIVET's institutional ethics committee in a mandatory process required by the Reproductive Technology Accreditation Committee (no. HR46/2007). This required informed consent to be obtained from all patients at the time of collection.

Results

HOST scores

The mean HOST value for the study group was 62 ± 10.1%. Eighty-one out of a total of 123 samples assessed showed HOST scores in the normal range (66%), 26 samples (21%) showed a score in the abnormal range and 13% returned a borderline HOST value.

The distribution of HOST categories revealed that 29% of the spermatozoa in this population were diagnosed as grade A while grades D/E and G were significantly more common than B, C and F ($P < 0.001$; Figure 2A). The distribution in samples with normal HOST values had statistically more grade D/E and less grade A swelling than the samples with abnormal HOST values, where the inverse was observed ($P < 0.001$; Figure 2B, C). However, the proportions of spermatozoa with grades B, C or G swelling did not show significant fluctuations in relation to the sample's HOST value.

Correlations between HOST scores with diagnostic and clinical outcomes

There was strong inverse correlation between the HOST value and the routine semen analysis parameters. These results indicated that all parameters of sperm concentration ($P < 0.05$), percentage total motile ($P < 0.0001$), progressive motile count ($P < 0.0001$) and normal sperm morphology ($P < 0.001$) were highly correlated with the percentage of HOST scores (Table 1). Of all the morphology defects, HOST was only significantly ($P < 0.05$) negatively linked to the presence of tail morphology. Forward-entry multiple regression analysis, however, identified independent significant relationships only between HOST score and concentration ($P < 0.001$), motility ($P < 0.001$) and DFI ($P < 0.01$). All other parameters were not included in

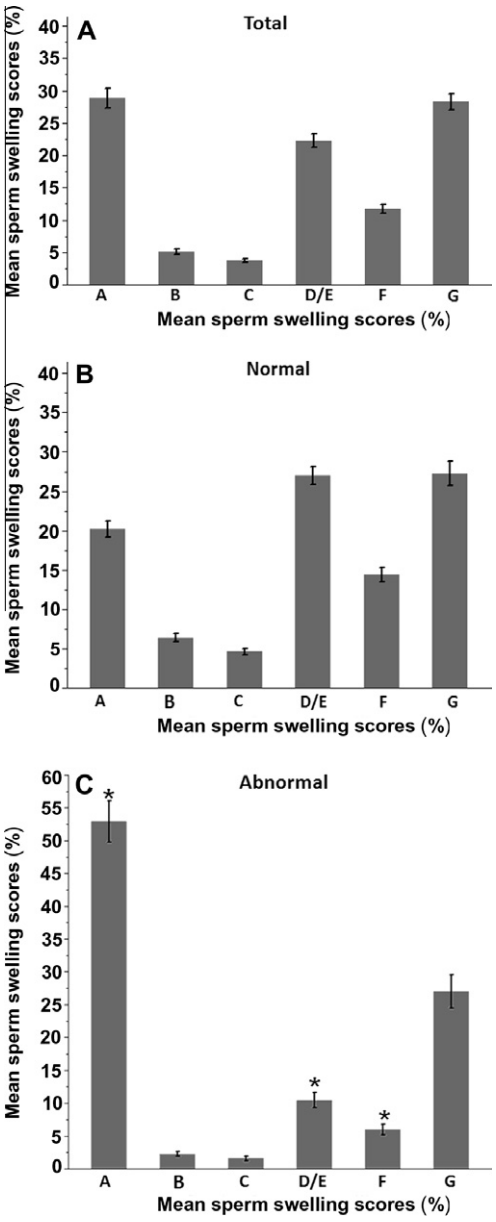

Figure 2 Distribution of hypo-osmotic swelling test (HOST) scores among different grades of sperm tail swelling. The mean percentages of sperm swelling scores are shown in (**A**) total sample population, (**B**) normal samples and (**C**) abnormal samples. Most of the high swelling scores are present in grades D/E and G, in total and normal samples. In abnormal samples, a statistically significant decease in the proportion of D/E and F grade ($P < 0.001$) and an increase in grade A ($P < 0.001$) was observed. Grade G demonstrated minimal fluctuations in mean percentage of sperm swelling score. *Indicates a significant change in the proportion between normal and abnormal HOST samples.

the model. There was no significant association between HOST values and the incidence of vacuolated spermatozoa in this sample population; however, with increased sample size this may be expected to reach statistical significance. Perhaps not surprising that given the limited sample size, there was no significant correlation observed between the HOST outcome with fertilization rates after IVF or ICSI or with pregnancy rates (data not shown).

Table 1 Relationship between semen parameters and HOST score ($n = 123$).

Parameter	R^2	*P-value*
Concentration (million/ml)	0.2179	<0.05
Motility (%)	0.7103	<0.0001
Progressive motility (%)	0.6912	<0.0001
Grade A motility (%)	0.5542	<0.0001
Grade B motility (%)	0.1657	NS
Grade C motility (%)	−0.2571	<0.05
Grade D motility (%)	−0.7086	<0.0001
Normal morphology (%)	0.3401	0.0017
Abnormal head morphology (%)	−0.1584	NS
Abnormal midpiece morphology (%)	0.1469	NS
Abnormal tail morphology (%)	−0.1965	0.0450
Abnormal cytoplasmic droplet morphology (%)	−0.1127	NS
DNA fragmentation index (%)	−0.5262	<0.0001
No. of germ cells/high power field)	−0.1498	NS
No. of leukocytes/high power field)	0.1318	NS
Days of abstinence	0.002161	NS

NS = not statistically significant.

SCSA results and correlation with HOST scores

SCSA data was categorized into three groups based on previously reported study limits (Evenson and Wixon, 2006). One hundred and seven of the 123 samples (87.0%), showed low DFI (mean 8 ± 4; normal DFI). In this study, an elevated DFI was observed in seven samples (5.7% of samples; mean 40 ± 9; abnormal DFI) and a borderline DFI in nine samples (7.3% of samples; mean 24 ± 2; borderline). This indicates that the study population was similar to the general population mix attending PIVET Medical Centre.

In order to determine whether the HOST outcome can be used as an indirect indication of sperm DNA integrity, HOST results were correlated with those of SCSA using all the samples of the study population. A statistically significant inverse correlation was found between spermatozoa with cell membrane swelling and the level of DNA fragmentation ($P < 0.0001$; Figure 3A). When the average DFI scores for all samples was distributed among the three HOST groups of samples, based on their respective HOST score range, it was evident that samples with normal HOST scores showed the least average DFI and the abnormal HOST samples the highest DFI (Figure 3B) and the borderline HOST samples also returned a borderline DFI. This observation was confirmed by ROC curve analysis that returned a highly significant predictability of DNA damage by the HOST score at 20% DFI (Figure 4; $P < 0.0001$) and 30% DFI ($P < 0.0001$). The predictive power of the HOST score could be demonstrated down to 10% DFI ($P < 0.01$). In other words, the HOST values may be used to imply even low levels of spermatozoa with affected DNA (by SCSA).

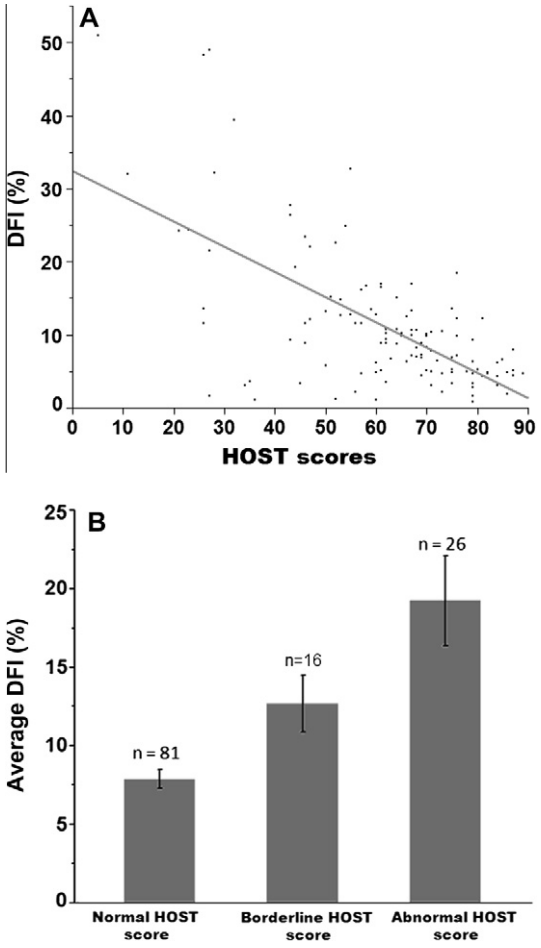

Figure 3 Correlation between hypo-osmotic swelling test (HOST) and DNA fragmentation index (DFI) measurements. (**A**) The negative linear correlation between the overall HOST and DFI measurements in the total 123 samples was significant ($P < 0.0001$; $R2 = 0.526$). At least 100 spermatozoa for HOST and 5000 spermatozoa for DFI were analysed. (**B**) The average DFI assessed by HOST scores, based on World Health Organization criteria. Significantly ($P < 0.0001$) high levels of DFI are found in abnormal samples compared with normal samples. Numbers shown above each bar represent the number of samples.

Correlation between TUNEL, HOST and SCSA outcomes in relation to HOST grades

The above correlation confirms a relationship between HOST values and DNA fragmentation but does not clearly identify which HOST grading categories were contributing to the degree or absence of DNA damage. To resolve this question, further assessments of the HOST grade and the DNA fragmentation by TUNEL in-situ testing was performed for individual spermatozoa. In these experiments, spermatozoa from neat semen samples were exposed to hypotonic solutions for 30 min and then slide preparations were made with staining for TUNEL and counted. Preliminary studies had shown that the tail swelling was not affected as a result of preparing a smear of the sperm sample since the HOST value was the same both before and after fixation by phase-contrast microscopy and by standard morphology staining procedures (Figure 1A, B, respectively).

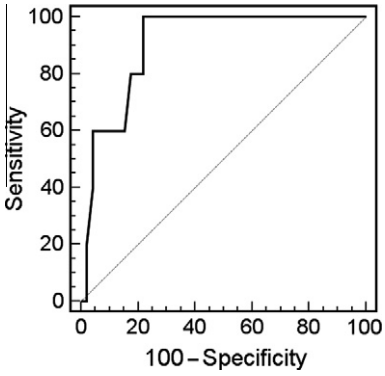

Figure 4 Receiver operating characteristic (ROC) curve analysis for the prediction of DNA fragmentation by hypo-osmotic swelling test (HOST) score where a positive (abnormal) sperm chromatin structure assay value was set at 20% DNA fragmentation. The area under the curve was 0.904; the calculated threshold value for HOST score having a prognostic value was 54%; the sensitivity was 100% for a specificity of 78.3%.

Comparison between the two HOST groups confirmed that significantly more TUNEL-defined DNA fragmentation levels were observed in HOST/SCSA-abnormal ($n = 10$) samples in comparison to the HOST/SCSA-normal samples ($n = 10$; $P < 0.0001$; Figure 5 and Figure 6A) confirming the validity of the original grouping into normal and abnormal specimens and providing some validation of the TUNEL technique. There was a significant positive correlation between the SCSA and the TUNEL estimates of DNA fragmentation in each of the 20 samples in this analysis ($P < 0.001$; Figure 6B) further validating the TUNEL procedure. In this study, a value of 30% DFI correlated with a TUNEL value of 22%.

When individual spermatozoa were assessed for their HOST and TUNEL status, grades D/E and F of sperm tail swelling showed significantly less DNA fragmentation compared with grades A and G within each of the total sperm population ($P < 0.0001$; Figure 7A,B). This relationship was evident in both the samples classified as normal HOST (Figure 7C) and abnormal HOST (Figure 7D) values. In fact, only one spermatozoon with D/E swelling exhibited TUNEL staining from the normal samples and <6% from the abnormal HOST samples.

Discussion

Of all the factors influencing the fertilization rate following ICSI, the selection of spermatozoa remains the one aspect amendable to improvement yet this has, until recently, remained largely unchanged since the technique was first described (Palermo et al., 1992). The selection process is the primary criticism of the technique and until better methods are developed to ensure that the process starts to mirror natural selection, ICSI will remain controversial and its use limited (Aitken, 2008). The primary concerns regarding sperm selection are the increased risk from

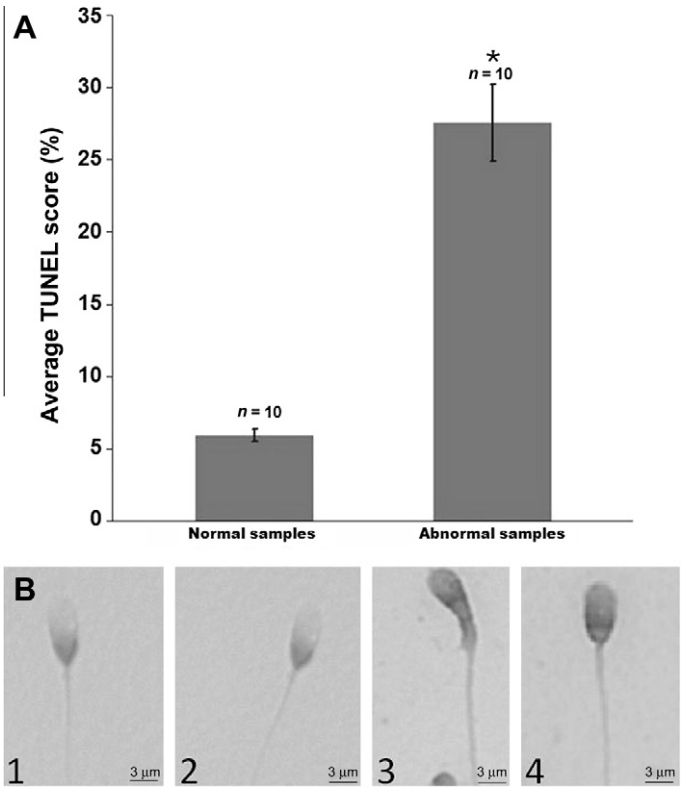

Figure 5 Correlation between TdT-mediated dUTP nick-end labelling (TUNEL) assay scores and both hypo-osmotic swelling test and sperm chromatin structure assay outcomes. (**A**) The average TUNEL score was significantly higher in abnormal samples as compared with normal samples ($P < 0.0001$; indicated by asterisk). The total numbers of stained spermatozoa (150—400) were counted for each sample. The numbers above the bars represent the number of samples assessed in each group. (**B**) Representation of TUNEL staining of different samples: (1) negative control omitting enzyme, (2) negative sperm staining, (3) induced positive control and (4) positive sperm staining (1200 magnification).

incidence of aneuploidy, sperm apoptosis and in particular concern about sperm DNA fragmentation. This study reports that individual spermatozoa with very low rates of DNA fragmentation are identifiable using hypo-osmotic-induced swelling methodology and propose that, subject to further studies and refinements, the technique may provide a low-cost real-time routine tool to improve sperm selection for ICSI.

The incidence of abnormal HOST values, based upon World Health Organization (1999) guidelines, in the 123 consecutive samples was 21% (and 34% if the borderline samples are included). The difference between the normal and abnormal sperm groups (including those described as borderline) was in the comparative incidence of spermatozoa exhibiting little or no tail swelling (grade A) to those with keyhole type swelling (grades D/E) and to a lesser extent grade F. In samples returning a 'normal' HOST value, grade D/E spermatozoa constituted 27% of all spermatozoa in the ejaculate, falling to 10% in samples defined as 'abnormal'. At the same time, the incidence of grade A (HOST negative) rose from 27% to 53%. Therefore the HOST category that is associated with normal samples was grade D/E, which incidentally is the form that is most easily identifiable and manageable under the magnification used for ICSI.

The HOST value for each sample was significantly ($P < 0.001$) related to the absence of DNA fragmentation, with all samples in the 'normal' range exhibiting DFI values also in the normal range (mean DFI 7.0%). ROC curve analysis demonstrated significant predictability of DNA damage using HOST such that HOST may well be a valuable tool to screen samples for future DNA testing. Conversely, the ejaculates with HOST values less than 60% were associated with elevated DFI and all the samples with abnormal SCSA results were from samples considered abnormal by HOST. Therefore it may be concluded that normal HOST samples contain spermatozoa with low likelihood of DNA fragmentation and that this is most likely related to the incidence of D/E grade swelling. This association implies but does not confirm that spermatozoa with D/E grade swelling are suitable for sperm selection. This study therefore sought to demonstrate that an individual spermatozoon in this category actually has no discernable DNA degeneration.

To do this, spermatozoa from 10 'normal' and 10 'abnormal' samples, identified by HOST status in previous analyses, were subjected to hypo-osmotic swelling and then individually assessed for DNA damage by TUNEL. The same significant relationship identified between SCSA and HOST was confirmed between TUNEL-negative spermatozoa and HOST. The high correlation between TUNEL and SCSA assessments indicated that while both tests are measuring different properties of DNA damage, they are effectively categorizing the samples in the same way. Furthermore it provides validation for the identification of DNA Fragmentation by TUNEL.

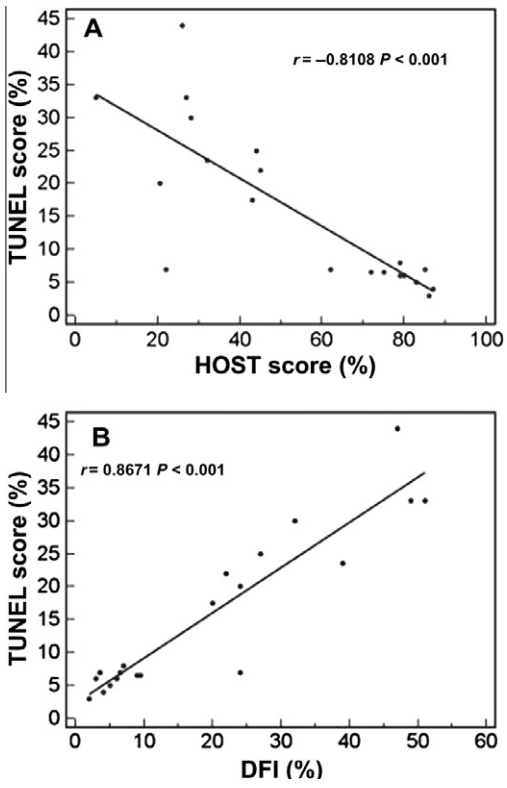

Figure 6 Relationship between TdT-mediated dUTP nick-end labelling (TUNEL) assay score and both hypo-osmotic swelling test (HOST) and DNA fragmentation index (DFI) outcomes. (**A**) A significant ($P < 0.001$) inverse linear correlation between HOST and TUNEL measurements in a total of 20 samples. (**B**) A significantly ($P < 0.001$) positive linear correlation between TUNEL score and DFI in a total of 20 samples. Each dot represents an assessed individual sample. One hundred spermatozoa for HOST and 150–400 spermatozoa for TUNEL assessment were used.

When individual spermatozoa were assessed, HOST grades D/E were confirmed as exhibiting the least proportion of TUNEL staining, regardless of whether the sample was initially diagnosed as normal or not by HOST assessment. Effectively, this observation demonstrates that individual spermatozoa, when exposed to hypo-osmotic stress, can be selected for minimal DNA damage based upon the presence of keyhole type tail swelling. This observation applies to DNA damage assessed by either SCSA or TUNEL and to whether the sample had normal or abnormal levels of HOST-positive spermatozoa.

The guidelines for what is a normal HOST value may need to be reviewed to specify the proportion of spermatozoa in the D/E category. From this study's observations, grade G would be considered abnormal given the level of DNA damage. It is proposed that the normal range be defined as greater than 15–20% grade D/E and that these samples may be expected to contain normal DNA profiles.

HOST was chosen for this study since this technique has previously been used to select viable immotile spermatozoa that have resulted in fertilization and pregnancy. HOST values have been shown to correlate strongly to sperm morphology and motility (Cincik et al., 2007; Jeyendran et al., 1984), a result also observed in the current sample population. Although this small study did not set out to identify a relationship between IVF fertilization and pregnancy rates, it has been linked to pregnancy in intrauterine insemination treatment cycles (Mladenovic et al., 1995) and early pregnancy loss (Bhattacharya, 2010) and used to identify viable spermatozoa for ICSI in cases with totally immotile spermatozoa due to some form of primary ciliary dyskinesia such as with Kartagener's syndrome or related conditions with distinct ultrastructural flagellar abnormalities. While just selecting immotile spermatozoa can result in fertilization and pregnancy (Kahraman et al., 1997; Olmedo et al., 1997; Westlander et al., 2003), most studies have recommended the use of the HOST where fertilization and pregnancy rates were higher (Casper et al., 1996; Kordus et al., 2008; Sallam et al., 2005). More recently, HOST has been used to select spermatozoa from severe male factor patients with lower rates of aneuploidy (Pang et al., 2009; Zeyneloglu et al., 2000). The relationship between DNA fragmentation and aneuploidy has been reported elsewhere (Muriel et al., 2007) especially in cases of oligoasthenoteratozoospermia where aneuploidy, DNA fragmentation and mitochondrial dysfunction were strongly correlated (Liu et al., 2004). Together they argue that HOST-positive spermatozoa are likely to have significantly lower rates of aneuploidy in addition to the lower probability of DNA fragmentation identified in this study. Spermatozoa with normal morphology also exhibit very low rates of DNA fragmentation (Liu et al., 2006) and aneuploidy (Celik-Ozenci et al., 2004). Therefore, there is an implication that grade D/E swelling may select for aneuploidy in addition to DNA damage. While in this study there was a weak association between HOST values and vacuolated spermatozoa, other studies have shown a link between the presence of vacuoles and DNA damage (Franco et al., 2008; Stanger et al., 2008).

The association between HOST and DNA damage was stronger than a link to the presence of vacuoles (Stanger et al., 2008) arguing that the presence of vacuoles is more an endpoint visual expression of chromatin degeneration while HOST may be a more precocious indicator. This observation is reinforced by multiple regression analysis that identified relationships between HOST and motility but not to head or midpiece abnormalities. It would seem reasonable therefore to conclude that HOST is identifying membrane events rather than terminal nuclear degeneration.

This report has used HOST grades D/E to identify DNA intact spermatozoa. However, the underlying basis for the test remains unclear. Hypotonic resistance has been used in boar sperm testing to explain the separation of cells into subpopulations of varying fertility and in that study, the population with good fertility was reported to decrease after ejaculation and after hydrogen superoxide treatment (Druart et al., 2009). These and other studies have demonstrated that hypotonic resistance (which is a better term than HOST) reflects the function of the Na^+/K^+ and Na^+/H^+ exchange capabilities of the membrane (Peris et al., 2000). Minimal tail swelling as in grades D/E implies membrane competency along the majority of the flagellum, competency that may be important in countering osmotic stress both *in vivo* and *in vitro* whereas complete swelling signifies impaired Na^+/K^+ ATP-ase function. Non-motile spermatozoa (grade A) have high levels of dead spermatozoa (by vital

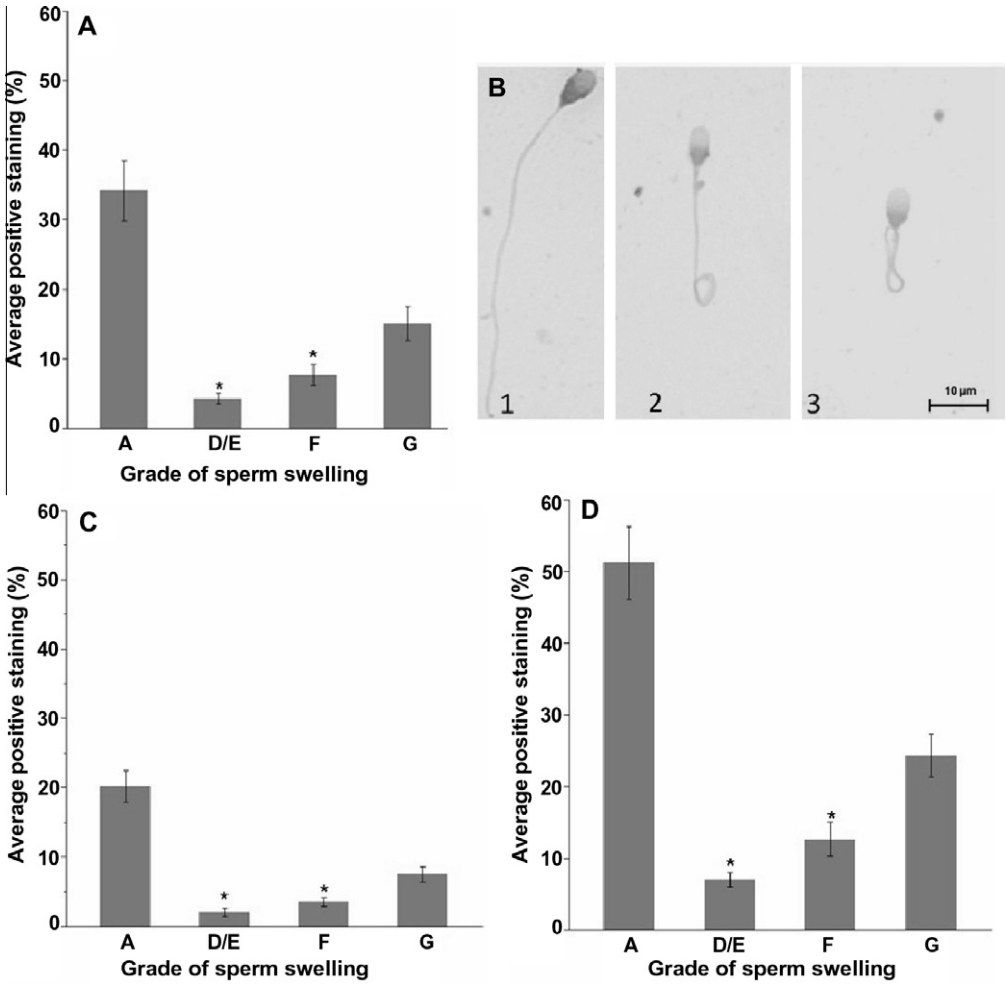

Figure 7 The average ratio of TdT-mediated dUTP nick-end labelling (TUNEL) staining in different sperm hypo-osmotic swelling test (HOST) grades. A significant ($P < 0.0001$) lower incidence of TUNEL-positive staining is shown in grades D/E and F in comparison to other grades in (**A**) total sperm population ($n = 20$), (**C**) normal ($n = 10$) and (**D**) abnormal ($n = 10$) sperm samples. (**B**) A microscopic representation of dual TUNEL and HOST outcome showing positive staining in (1) grade A and (2 and 3) negative staining in both grades D/E and G, respectively (×1000 magnification).

staining) whereas HOST-positive spermatozoa are always alive (data not presented). Recently, *Candida albicans* infection of spermatozoa was shown to decrease motility, reduce mitochondrial membrane potential, increase membrane phosphatidylserine externalization and cause DNA fragmentation (Burrello et al., 2009). It should not be surprising that the membrane properties of spermatozoa reflect the incidence of DNA damage or other changes, e.g. capacitation. Oxygen-free radical exposure that causes DNA damage has been cited as the primary cause of membrane damage (De Iuliis et al., 2009). Human spermatozoa have a high level of polyunsaturated fatty acids rendering them susceptible to oxidative damage. Motility may be one of the first functions to reflect membrane damage and it is most probable that HOST acts to identify spermatozoa whose plasma membranes have remained viable and functional. Regardless of whether oxidative damage is from external sources such as adjacent apoptotic spermatozoa, leukocytes or from internal metabolic activity, damage may be seen as a natural process allowing for identification and removal of redundant (damaged) cells (Storey, 2008). The current study used membrane viability as a marker of DNA fragmentation but it would seem reasonable to expect that subtle effects on membrane integrity may precede or be independent of DNA damage. This would explain why some samples with abnormal HOST values do not have corresponding elevated DFI but these spermatozoa may develop DNA fragmentation over time.

The few samples assessed for DNA damage and HOST that were produced as part of a treatment cycle did not show an association with fertilization rates or pregnancy. These observations were anecdotal rather than structural but other studies have also not shown that HOST values predict IVF outcomes. (Cincik et al., 2007). Recently HOST has been linked to early pregnancy loss (Bhattacharya, S, 2010), an observation in keeping with a relationship between DNA damage and pregnancy. Other studies using hyaluronan-selected spermatozoa did not show improved pronuclear development (Van Den Berg et al., 2009). However, current ICSI techniques already select on strong motility

and as such new techniques are unlikely to improve IVF fertilization rates. Rather, the issue of sperm selection during ICSI relates to easily and quickly identifying the most viable, competent spermatozoa similar to that reported by Paes Almeida Ferreira de Braga et al. (2009) using zona-selected spermatozoa.

Since there have been several reports detailing pregnancies from samples with immotile cilia syndrome (Kordus et al., 2008) resulting in live births, it implies that hypo-osmotic-induced swelling is not detrimental to the fertilization process and subsequent embryo development. Notwithstanding such HOST-mediated pregnancies, hypo-osmotic-induced swelling is not without potential negative effects. For instance, decreased tyrosine phosphorylation and sperm zona pellucida binding has been reported in human spermatozoa osmotically stressed (Liu et al., 2006) and such exposure also opens K^+ and Cl^- channels in boar spermatozoa (Petrunkina et al., 2007; Tsai et al., 1997). Early studies exploring HOST reported that the viability of spermatozoa is influenced by time (Smikle and Turek, 1997; Tsai et al., 1997) and temperature (Druart et al., 2009) and is solution dependent (Tsai et al., 1997; Verheyen et al., 1995; Yeung et al., 2003) These are critical observations if HOST is to be used pre-emptively for ICSI. Furthermore, while the association between HOST and SCSA was very strong, it was not exclusive. Whether this implies that DNA damage may occur in the presence of normal membrane function is less clear and illustrates that HOST-sensitive membrane features are linked and not causal. Studies are currently in progress to resolve the impact of varying the degree of osmotic stress and the efficacy of sperm separation techniques on the incidence of HOST-positive spermatozoa in samples used for insemination. The standard HOST solution (150 mOsm/30 min) has been used to identify cells with little DNA fragmentation. However, further studies are required to facilitate the identification of healthy spermatozoa without diminishing their competency by such testing and it may well be that modifications to the degree and timing of hypo-osmotic exposure may minimize any detrimental effects whilst retaining the facility of identification of optimal spermatozoa.

In summary, the properties of sperm membrane resistance to osmotic stress has been used to identify a subpopulation of viable spermatozoa that appear to have minimal or no DNA damage. It is argued that this method, when fully evaluated, may form the basis of a standardized sperm selection procedure for ICSI.

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References

Ainsworth, C., Nixon, B., Jansen, R.P., et al., 2007. First recorded pregnancy and normal birth after ICSI using electrophoretically isolated spermatozoa. Hum. Reprod. 22, 197–200.

Aitken, R., 2008. Just how safe is assisted reproductive technology for treating male factor infertility? Expert. Rev. Obestr. Gynaecol., 267–271.

Berkovitz, A., Eltes, F., Yaari, S., et al., 2005. The morphological normalcy of the sperm nucleus and pregnancy rate of intracytoplasmic injection with morphologically selected sperm. Hum. Reprod. 20, 185–190.

Bhattacharya, S.M., 2010. Hypo-osmotic swelling test and unexplained repeat early pregnancy loss. J. Obstet. Gynaecol. Res. 36, 119–122.

Borini, A., Tarozzi, N., Bizzaro, D., et al., 2006. Sperm DNA fragmentation: paternal effect on early post-implantation embryo development in ART. Hum. Reprod. 21, 2876–2881.

Burrello, N., Salmeri, M., Perdichizzi, A., et al., 2009. Candida albicans experimental infection: effects on human sperm motility, mitochondrial membrane potential and apoptosis. Reprod. Biomed. Online 18, 496–501.

Casper, R.F., Cowan, L., Meriano, J.S., et al., 1996. The hypo-osmotic swelling test for selection of viable sperm for intracytoplasmic sperm injection in men with complete asthenozoospermia. Fertil. Steril. 65, 972–976.

Celik-Ozenci, C., Jakab, A., Kovacs, T., et al., 2004. Sperm selection for ICSI: shape properties do not predict the absence or presence of numerical chromosomal aberrations. Hum. Reprod. 19, 2052–2059.

Check, J.H., Katsoff, D., Check, M.L., et al., 2001. In vitro fertilization with intracytoplasmic sperm injection is an effective therapy for male factor infertility related to subnormal hypo-osmotic swelling test scores. J. Androl. 22, 261–265.

Cincik, M., Ergur, A.R., Tutuncu, L., et al., 2007. Combination of hypoosmotic swelling/eosin *Y* test for sperm membrane integrity evaluation: correlations with other sperm parameters to predict ICSI cycles. Arch. Androl. 53, 25–28.

De Iuliis, G.N., Thomson, L.K., Mitchell, L.A., et al., 2009. DNA damage in human spermatozoa is highly correlated with the efficiency of chromatin remodeling and the formation of 8-hydroxy-2'-deoxyguanosine, a marker of oxidative stress. Biol. Reprod. 81, 517–524.

Druart, X., Gatti, J.L., Huet, S., et al., 2009. Hypotonic resistance of boar spermatozoa: sperm subpopulations and relationship with epididymal maturation and fertility. Reproduction 137, 205–213.

Evenson, D., Wixon, R., 2006. Meta-analysis of sperm DNA fragmentation using the sperm chromatin structure assay. Reprod. Biomed. Online 12, 466–472.

Evenson, D.P., Wixon, R., 2008. Data analysis of two in vivo fertility studies using Sperm Chromatin Structure Assay-derived DNA fragmentation index vs. Pregnancy outcome. Fertil. Steril. 90, 1229–1231.

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.

Franco Jr., J.G., Baruffi, R.L., Mauri, A.L., et al., 2008. Significance of large nuclear vacuoles in human spermatozoa: implications for ICSI. Reprod. Biomed. Online 17, 42–45.

Gandini, L., Lombardo, F., Paoli, D., et al., 2004. Full-term pregnancies achieved with ICSI despite high levels of sperm chromatin damage. Hum. Reprod. 19, 1409–1417.

Hossain, A.M., Rizk, B., Barik, S., Huff, C., Thorneycroft, I.H., 1998. Time course of hypo-osmotic swellings of human spermatozoa: evidence of ordered transition between swelling subtypes. Hum. Reprod. 13, 1578–1583.

Huszar, G., Jakab, A., Sakkas, D., et al., 2007. Fertility testing and ICSI sperm selection by hyaluronic acid binding: clinical and genetic aspects. Reprod. Biomed. Online 14, 650–663.

Jeyendran, R.S., Van der Ven, H.H., Perez-Pelaez, M., et al., 1984. Development of an assay to assess the functional integrity of the

human sperm membrane and its relationship to other semen characteristics. J. Reprod. Fertil. 70, 219–228.
Kahraman, S., Isik, A.Z., Vicdan, K., et al., 1997. A healthy birth after intracytoplasmic sperm injection by using immotile testicular spermatozoa in a case with totally immotile ejaculated spermatozoa before and after Percoll gradients. Hum. Reprod. 12, 292–293.
Kordus, R.J., Price, R.L., Davis, J.M., et al., 2008. Successful twin birth following blastocyst culture of embryos derived from the immotile ejaculated spermatozoa from a patient with primary ciliary dyskinesia: a case report. J. Assist. Reprod. Genet. 25, 437–443.
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.
Liu, D.Y., Baker, H.W., 2007. Human sperm bound to the zona pellucida have normal nuclear chromatin as assessed by acridine orange fluorescence. Hum. Reprod. 22, 1597–1602.
Liu, D.Y., Clarke, G.N., Baker, H.W., 2006. Hyper-osmotic condition enhances protein tyrosine phosphorylation and zona pellucida binding capacity of human sperm. Hum. Reprod. 21, 745–752.
Menkveld, R., Wong, W.J., Lombard, C.J., et al., 2001. Semen parameters, including WHO and strict criteria morphology in a fertile and subfertile population: an effort towards standardization of in-vivo thresholds'. Hum. Reprod. 16, 1165–1171.
Mladenovic, I., Micic, S., Genbacev, O., et al., 1995. Hypoosmotic swelling test for quality control of sperm prepared for assisted reproduction. Arch. Androl. 34, 163–169.
Olmedo, S.B., Nodar, F., Chillik, C., et al., 1997. Successful intracytoplasmic sperm injection with spermatozoa from a patient with dysplasia of the fibrous sheath and chronic respiratory disease. Hum. Reprod. 12, 1497–1499.
Paes Almeida Ferreira de Braga, D., Iaconelli, A., Cássia Sávio de Figueira, R., Madaschi, C., Semião-Francisc, E., 2009. Outcome of ICSI using zona pellucida-bound spermatozoa and conventionally selected spermatozoa. Reprod. Biomed. Online 19, 802–8077.
Palermo, G., Joris, H., Devroey, P., et al., 1992. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet 340, 17–19.
Pang, M.G., You, Y.A., Park, Y.J., et al., 2009. Numerical chromosome abnormalities are associated with sperm tail swelling patterns. Fertil. Steril. 94, 1012–1020.
Peris, S., Solanes, D., Pena, A., et al., 2000. Ion-mediated resistance to osmotic changes of ram spermatozoa: the role of amiloride and ouabain. Theriogenology 54, 1453–1467.
Petrunkina, A.M., Harrison, R.A., Tsolova, M., et al., 2007. Signalling pathways involved in the control of sperm cell volume. Reproduction 133, 61–73.
Rogers, B.J., Parker, R.A., 1991. Relationship between the human sperm hypo-osmotic swelling test and sperm penetration assay. J. Androl. 12, 152–158.
Rossato, M., Galeazzi, C., Ferigo, M., et al., 2004. Antisperm antibodies modify plasma membrane functional integrity and inhibit osmosensitive calcium influx in human sperm. Hum. Reprod. 19, 1816–1820.
Said, T.M., Agarwal, A., Zborowski, M., et al., 2008. Utility of magnetic cell separation as a molecular sperm preparation technique. J. Androl. 29, 134–142.
Sakkas, D., Urner, F., Bianchi, P.G., et al., 1996. Sperm chromatin anomalies can influence decondensation after intracytoplasmic sperm injection. Hum. Reprod. 11, 837–843.
Sallam, H.N., Farrag, A., Agameya, A.F., et al., 2001. The use of a modified hypo-osmotic swelling test for the selection of viable ejaculated and testicular immotile spermatozoa in ICSI. Hum. Reprod. 16, 272.
Sallam, H.N., Farrag, A., Agameya, A.F., et al., 2005. The use of the modified hypo-osmotic swelling test for the selection of immotile testicular spermatozoa in patients treated with ICSI: a randomized controlled study. Hum. Reprod. 20, 3435–3440.
Smikle, C.B., Turek, P.J., 1997. Hypo-osmotic swelling can accurately assess the viability of nonmotile sperm. Mol. Reprod. Dev. 47, 200–203.
Stanger, J.D., Vo, L., Yovich, J., Almahbobi, G., 2008. DNA Fragmentation Index is relative to vacuoles in the head of human spermatozoa. In: Fertility Society of Australia Scientific Meeting 2008, Abstract 105. Brisbane.
Storey, B.T., 2008. Mammalian sperm metabolism: oxygen and sugar, friend and foe. Int. J. Dev. Biol. 52, 427–437.
Tartagni, M., Schonauer, M.M., Cicinelli, E., et al., 2002. Usefulness of the hypo-osmotic swelling test in predicting pregnancy rate and outcome in couples undergoing intrauterine insemination. J. Androl. 23, 498–502.
Tsai, Y.L., Liu, J., Garcia, J.E., et al., 1997. Establishment of an optimal hypo-osmotic swelling test by examining single spermatozoa in four different hypo-osmotic solutions. Hum. Reprod. 12, 1111–1113.
Van den Bergh, M.J., Fahy-Deshe, M., Hohl, M.K., 2009. Pronuclear zygote score following intracytoplasmic injection of hyaluronan-bound spermatozoa: a prospective randomized study. Reprod. Biomed. Online 19, 796–801.
Van Voorhis, B.J., 2007. Clinical practice. In vitro fertilization. N. Engl. J. Med. 356, 379–386.
Verheyen, G., De Croo, I., Tournaye, H., et al., 1995. Comparison of four mechanical methods to retrieve spermatozoa from testicular tissue. Hum. Reprod. 10, 2956–2959.
Wang, Y., Chambers, G., Dieng, M., 2009. Assisted reproduction in Australia and New Zealand 2007. Assisted Reproduction Technology Series.
Westlander, G., Barry, M., Petrucco, O., et al., 2003. Different fertilization rates between immotile testicular spermatozoa and immotile ejaculated spermatozoa for ICSI in men with Kartagener's syndrome: case reports. Hum. Reprod. 18, 1286–1288.
World Health Organization, 1999. WHO Laboratory Manual for the examination of human semen and sperm-cervical mucus interaction. fourth ed. Cambridge University Press, Cambridge, UK.
Yeung, C.H., Anapolski, M., Depenbusch, M., et al., 2003. Human sperm volume regulation. Response to physiological changes in osmolality, channel blockers and potential sperm osmolytes. Hum. Reprod. 18, 1029–1036.
Yovich, J.L., Stanger, J.D., 1984. The limitations of in vitro fertilization from males with severe oligospermia and abnormal sperm morphology. J. In Vitro Fert. Embryo Trans. 1, 172–179.
Zeyneloglu, H.B., Baltaci, V., Ege, S., et al., 2000. Detection of chromosomal abnormalities by fluorescent in-situ hybridization in immotile viable spermatozoa determined by hypo-osmotic sperm swelling test. Hum. Reprod. 15, 853–856.

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