Hypo-osmotic Swelling Test [HOST] identifies Individual Spermatozoon with minimal DNA Fragmentation.

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Abstract

The increasing utilization of Intracytoplasmic Sperm Injection (ICSI) has raised concerns about the selection of spermatozoa used for injection. One concern is that some 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). In the present study we report 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 (SCSA) as well as the Terminal deoxyribonucleotidyl transferase mediated dUTP Nick-End Labeling (TUNEL) assay. The HOST value was highly predictive of an abnormal DFI value by Receiver Operating Characteristic (ROC) 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, most commonly associated with high HOST values and were significantly associated with minimal DNA damage regardless of the DNA status of the ejaculate. While further research is required to demonstrate its efficacy and safety, the application of HOST may be a valuable tool in the routine identification and selection of viable, DNA-intact individual spermatozoa for ICSI.

Keywords: male infertility, ICSI, HOST, SCSA, TUNEL.

Plan Language Statement.

The Hypo-osmotic resistance to sperm as measured by the HypoOsmotic Swellling Test (HOST) was shown to strongly predict the degree of sperm DNA damage when assessed by the Sperm Chromatin Structure Assay (SCSA). The sperm that displayed only distal swelling were shown to strongly correlate to sperm with minimal DNA. The study suggests they may be a suitable candidate for sperm selection for sperm microinjection in IVF cycles.

Introduction

The development of ICSI after many years of In Vitro Fertilisation (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. 2009), the use of Preimplantation Genetic Screening and surgically collected sperm 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 conceptions may overtake IVF conception 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

centered 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, etl. 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 (Lui and Baker, 2007). It would seem therefore that the aim for sperm selection for ICSI should be to strive to emulate the sperm that facilitate fertilization in vivo and minimal DNA damage as in IVF could be a reasonable initial benchmark to apply.

Recent interest in the integrity of sperm DNA (Aitkens, 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; Gandini *et al* 2004, Sakkas, et.al. 1996) While both SCSA and TUNEL assays are standard methods for assessing sperm DNA integrity (Evenson and Wixon 2006). They nevertheless require a high degree of skill, are expensive, time consuming and above all destructive to sperm. This means it is not possible to use any of the sperm

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 sperm are self-selecting. The 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 has been applied as a useful additional test for sperm cell membrane integrity (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) commending it as potential routine method to select individual healthy spermatozoa for ICSI (Sallam *et al* 2001) particularly since the degree of swelling of sperm 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. In this report, we 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 of 36±6.22; range 24 to 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 after liquefaction and SCSA in addition to their routine testing or processing for Assisted Reproductive Technologies (ART). 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 (WHO, 1999) and morphological parameters including percentage of head, midpiece and tail defects according to Tygerberg Strict Criteria. 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 analyzed for the correlation of HOST value and the success rate of fertilization using IVF (n =11) and ICSI (n=25) techniques. 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 respectively).

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 to the SCSA values in the previous assessment. In this group, 10 samples were provided from men with low HOST/SCSA and 10 samples from men with elevated HOST/SCSA profiles.

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Hypo-osmotic swelling test (HOST)

HOST was prepared and performed on semen samples as described previously (WHO 1999). Each sample was immediately incubated at 37°C and all assessments performed after 30 minutes subject to complete liquefaction. For each patient, 0.1 ml of liquefied semen was added to 1ml of warmed 150 mOsm hypo-osmotic swelling solution containing sodium citrate (25mM) and fructose (75mM) at 37°C. Aliquots of samples were placed on a clean glass slide with a cover slip with and without HOS treatment, assessed within 5 minutes and viewed under a phase-contrast microscope. Sperm swelling was observed and graded A to G according to changes in the shape of the tail (Hossain *et al* 1998; WHO, 1999). The response to

hypo-osmotic stress resulted in spermoatozoa where there was either no tail swelling (grade A) or spermatozoa that respond with swelling that manifested in various degrees of swelling from the distal end of the flagellum (Grades B to G; Figure 1). Grades D and E, as described by WHO (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 WHO (1999). A sample of neat semen was examined and the number of sperm with natural tail swelling was counted to provide a background reference (Figure 1).

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 sperm. Based on WHO criteria (1999), 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 between 50-60% were considered as borderline.

Sperm Chromatin Structure Assay (SCSA)

SCSA was performed based on protocols by Evenson *et al* (1999). Semen samples were collected and diluted to a concentration of 10 million sperm/ml with Tris HCl buffer, pH 7.4. 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 seconds. The sperm were then stained by adding equal volume of freshly prepared solution of Acridine Orange (AO) for 3 minutes. 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 M citric acid, 0.2 M Na_2PO_4, 1 mM EDTA, 0.15 M NaCl). Fluorescent assessments using Fluorescent Activated Cell Sorting (FACS Calibur, Becton, Dickinson and Company, 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 and Company, Franklin Lakes, NJ, USA) 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) (Everson, et. al, 1999). Quantification of DNA denaturation or DFI was calculated by the parameter R3 using the following equation (R3 = red/ (red

+green fluorescence)). The average of two estimations was used for all analysis. 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.

Terminal deoxyribonucleotidyl transferase mediated dUTP nick-end labeling (TUNEL) assay on HOST selected sperm

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 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 abnormals were re-analysed in a study to include TUNEL staining. The samples displayed different sperm concentrations due to the varied initial sperm count of patients. For TUNEL purposes sperm concentration for all samples were adjusted to 20 million/ml in TNE buffer. 20 µl aliquots from each adjusted sample were smeared onto separate Superfrost Plus slides (Mezel-Glaser, Braunschweig, GER) 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, AU). The ApopTag Fluorescein In situ apoptosis detection Kit detects apoptotic cells *in situ*, utilizing an anti-digoxigenin antibody that is conjugated to a Fluorescein reporter molecule. Sperm 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 20µl of DN buffer, pH 7.2 at room temperature for 5 minutes. Samples were then treated with an equal volume of 1µg/ml DNase I (Sigma-Aldrich, Castle Hill, NSW, AU) for 10 minutes. 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 sperm in a total of 100 sperm counted. Each sample was assessed twice and the average of the two outcomes was calculated.

***Assessment of dual TUNEL staining and HOST in individual sperm*.**

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 sperm. A total of 150-400 sperm 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 sperm 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 artifact of sperm tail swelling grades due to fixation and staining while performing TUNEL assessment, a total 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 sperm 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 analyzed using Chi square, Spearman's ρ and Wilcoxon signed rank. Receiver Operator 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 (#HR46/2007). This required informed consent to be obtained from all patients at the time of collection.

Results

Hypo-osmotic swelling test (HOST)

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 (65%), twenty six samples (13%) showed a score in the abnormal range and 22% returned a borderline HOST value.

The distribution of HOST categories revealed that 29% of the sperm in this population were diagnosed as grade A while grades D/E and G were significantly more common than B, C and F. ($P<0.001$, Fig. 2A). The distribution in samples with "normal" HOST values had statistically more type D/E and less type A swelling than the samples with abnormal HOST values where the inverse was observed ($P<0.001$, Fig. 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. Our results indicated that all parameters of sperm concentration ($P<0.05$), percentage total ($P<0.001$), progressive motile count

(P<0.001) and normal sperm morphology (P<0.001) were highly correlated with the percentage of HOST scores. Of all the morphology defects, HOST was only significantly (P<0.05) negatively linked to the presence of tail morphology. Perhaps not a surprising result 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).

Sperm Chromatin Structure Assay (SCSA) and correlation with HOST

SCSA data was placed into 3 groups based on a previously reported study (Evenson and Wixon 2006), normal (<15% DFI), abnormal (>30% DFI) and borderline (15-30% DFI). One hundred and seven of the 123 samples (87%), showed low DFI level (mean = 8±4, normal, Fig. 4A). In this study, the DFI was significantly elevated in 7 samples (5.7%; mean 40±9, abnormal p<0.001) and borderline in 9 samples (13%; mean = 24±2).

In order to determine whether the HOST outcome can be used as an indirect indication of sperm DNA integrity, we correlated HOST results 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<0.001, Fig. 3A). When the average DFI

scores for all samples were 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 Receiver Operator Characteristic analysis that returned a highly significant predictability of DNA damage by the HOST score at the 30% level (Figure 7; $P<0.0001$). The predictive power of the HOST 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 sperm with affected DNA (by SCSA).

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

The above correlation confirms a relationship between HOST values and DNA fragmentation but does not clearly identify HOST grading categories were rcontributing 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 minutes 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, 1B respectively).

Comparison between the two HOST groups confirmed that significantly ($P<0.001$) more TUNEL-defined DNA fragmentation levels were observed in SCSA-abnormal (n=10) samples in comparison to the SCSA-normal samples (n=10) ($P<0.0001$, Figure 4A and Figure 5A) 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 estimate of DNA fragmentation in each of the 20 samples in this analysis ($P<0.001$; Figure 5B) 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 to grades A and G within each of the total sperm population ($P<0.0001$, Fig. 6A). This relationship was evident in both the samples classified as normal HOST (Fig. 6C) and abnormal HOST (Fig. 6D) values.

Discussion

Of all the factors influencing the fertilisation 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, J, 2008). The primary concerns regarding sperm selection are the increased risk from incidence of aneuploidy, sperm apoptosis and in particular concern about sperm DNA fragmentation. In this study, we report 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 WHO guidelines, in the 123 consecutive samples was 13% (and 25% 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 (Type A) to those with keyhole type swelling (types D/E) and to a lesser extent Type F. In samples returning a "normal" HOST value, type D/E spermatozoa constituted no more than 25% of all sperm in the ejaculate, falling to less than 10% in samples defined as "abnormal". At the same time, the incidence of Type A (HOST negative) rose form 25% to 50%. 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 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 type swelling. This association implies but does not confirm that sperm with D/E type swelling are suitable for

sperm selection. We 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.

When individual spermatozoa were assessed, HOST types D/E were confirmed as exhibiting the least proportion of TUNEL staining, regardless of whether the sample was initially diagnosed as normal or not. In the samples that originally returned a high (normal) HOST value, only one spermatozoon in this group was identified as abnormal while in the samples that had a low HOST score, less than 6% were abnormal. Effectively this result 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 largely independent of the type of sample from which it was derived (or on any other parameter) and potentially without significant impact on its viability. The guidelines for what is a normal HOST value may need to be reviewed from this study to specify the proportion of sperm in the D/E category. From these studies, type G would be considered abnormal given their level of DNA damage. We propose that the normal range be defined as greater than 15-20% type D/E and that these samples may be expected to contain normal DNA profiles.

The Hypo-osmotic swelling test was chosen for this study since this technique has previously been used to select immotile spermatozoa that have resulted in fertilisation and pregnancy. HOST values have been shown to correlate strongly to sperm morphology and motility (Jeyendran, et.a 1984), a result also observed in our sample population. Although in this small study, we could not demonstrate a relationship between IVF fertilization and pregnancy rates, it has been used to predict pregnancy rates in Intra-uterine Insemination treatment cycles (Mladenovic, et. al., 1995) and used to identify viable spermatozoa for IVF 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 fertilisation and pregnancy rates were higher (Casper,et. al., 1996; Sallan, et. al 2006, Kordus, et. al. 2008). More recently, HOST has been used to select sperm 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 sperm are likely to have significantly lower rates of aneuploidy in addition to the lower probability of DNA fragmentation identified in this study. Sperm with normal morphology also exhibit very low rates of DNA fragmentation (Liu and Baker, 2007) and aneuploidy (Celik-Ozenci, et. al. 2004). Therfore, there is an implication that type D/E swelling may select for aneuploidy in addition to DNA damage.

In this report, we have used HOST types D/E to identify DNA healthy spematozoa. However, the basis the test remains unclear. Hypotonic resistance has been used in boar sperm testing to explain the separation of cells into sub-

populations 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 types 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 sperm (type A) have high levels of dead sperm (by vital staining) whereas HOST positive sperm are always alive (data not presented). Recently, *Candida albicans* infection of spermatozoa caused deceased motility, reduced mitochondrial membrane potential, increased membrane phosphatidylserine externalization and 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 Ilius, 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 membrane have

remained healthy and functional. Regardless of whether oxidative damage is from external sources such as adjacent apoptotic spermatozoa, leucocytes or from internal metabolic activity, damage may be seen as a natural process allowing for identification and removal of redundant (damaged) cells (Storey, 2008). We have used membrane viability as a marker of DNA fragmentation but it would seem reasonable to expect that subtle effects on membrane integrity may precede DNA damage. This would explain why some samples with abnormal HOST values do not have corresponding elevated DFI but may develop DNA fragmentation over time.

Since there have been several reports detailing pregnancies from samples with immotile cilia syndrome (eg 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 zone pellucida binding has been reported in human sperm osmotically stressed (Liu, et. al 2006) and such exposure also opens K^+ and Cl^- channels in boar spermatozoa (Petrunkina, et. al. 2007). Early studies exploring HOST reported that the viability of spermatozoa is influenced by time (Tsai, et. al 1997; Smikle, et. al, 1997),

temperature (Druart, et. al. 2009) and is solution dependent (Tsai, et. al, 1997; Verheven, et. al, 1997, Yeung, et. al, 2003). These are critical observations if HOST is to be used pre-emptively for ICSI. We have used the standard HOST solution (150 mOsm/30 minutes) 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, we have used the properties of sperm membrane resistance to osmotic stress to identify a sub population of viable spermatozoa that appear to have minimal or no DNA damage and argue that this method, when fully evaluated, may form the basis of a standardized sperm selection procedure for ICSI.

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LEGENDS

Figure 1 Microscopic observation of different grades of sperm swelling

The different sperm swelling grades (A-G) as observed (A) using phase-contrast microscopy (1000x magnification) of fresh samples (upper panel) and. (B) fixed and stained samples using normal light microscopy (lower panel).

Figure 2 Distribution of HOST scores among different grades of sperm tail swelling

The mean percentages of sperm swelling scores are shown in total sample population (A), normal samples (B) and abnormal samples (C). Most of the high value swelling scores are present in grades D/E and G, in total and normal samples. In abnormal samples (C), a statistically significant ($P<0.0001$) score shift from grades D/E and F to grade A was observed. Grade G demonstrated minimal fluctuations in mean percentage of sperm swelling score (A-C).

Figure 3. Correlation between HOST and SCSA measurements.

(A) The linear correlation between the overall HOST and SCSA measurements in total 123 samples was significantly ($P<0.0001$) inverse linear.. At least 100 sperms for HOST and 5000 sperms for SCSA were analyzed. (B) The average DFI assessed by HOST scores, based on WHO criteria. Significantly ($P<0.0001$) high levels of DFI are found in abnormal samples compared to normal samples. Numbers shown above each bar represent the number of samples.

Figure 4. Correlation between TUNEL and both HOST and SCSA outcomes

(A) The average DFI assessed by TUNEL was significantly (P<0.0001) higher in abnormal samples as compared with normal samples (A). The total numbers of stained (150-400 sperms) 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; negative control omitting enzyme (1), negative sperm staining (2), induced positive control (3) and positive sperm staining (4) (1200X magnification).

Figure 5. Relationship betweenTUNEL and both HOST and SCSA outcomes

A) A significant (P<0.001) inverse linear correlation between HOST and TUNEL measurement in a total of 20 samples. B) A significantly (P<0.0001) positive linear correlation between TUNEL and SCSA in a total of 20 samples. Each dot represent an assessed individual sample. One hundred sperm for HOST and 150-400 sperm for TUNEL assessment were used.

Figure 6. The average ratio of TUNEL staining in different sperm HOST grades

(A-C) A significant (p<0.0001) lower incidence of TUNEL staining is shown in grades D/E and F in comparison to other grades in total sperm population (A, n=20), normal (C, n=10) and abnormal (D, n=10) sperm samples. B) A microscopic representation of dual TUNEL and HOST outcome showing positive staining in grade A (1) and negative staining in both grades D/E and G (2, 3 respectively) (1000x magnification).

Figure 7. Receiver Operator Characteristic (ROC) analysis for the prediction by HOST value of a positive SCSA DNA fragmentation value where the cut-off for a positive DNA Fragmentation result was set at 20% DFI**.** The area under the curve was 0.904; the calculated threshold value for %HOST having a prognostic value was 54%, the sensitivity was 100% for a specificity of 78.3%.

FIGURES

Figure 1

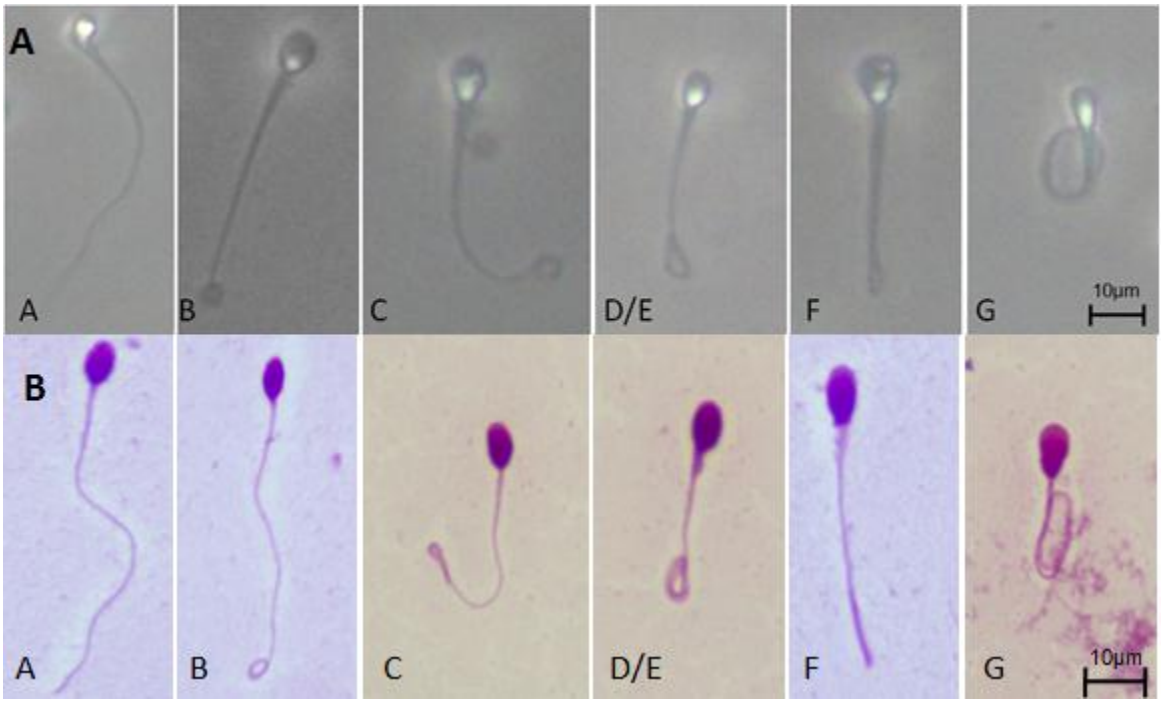
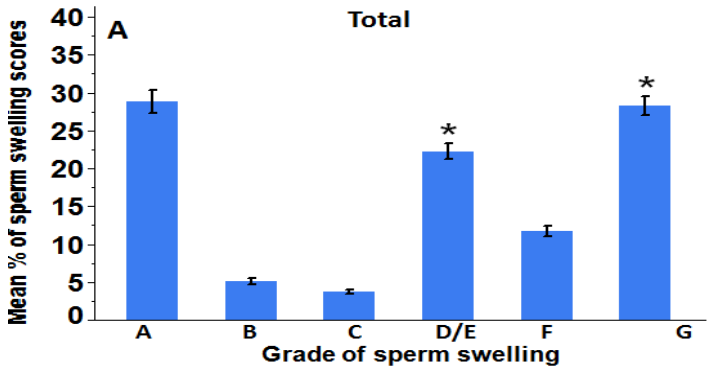
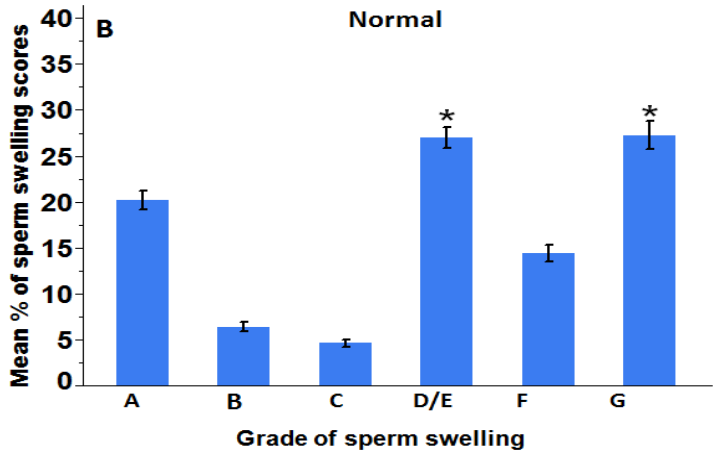


Figure 2


A
Total
Mean % of sperm swelling scores
40
35
30
25
20
15
10
5
0
A
B
C
D/E
F
G
Grade of sperm swelling
*
*


B
Normal
Mean % of sperm swelling scores
40
35
30
25
20
15
10
5
0
A
B
C
D/E
F
G
Grade of sperm swelling
*
*

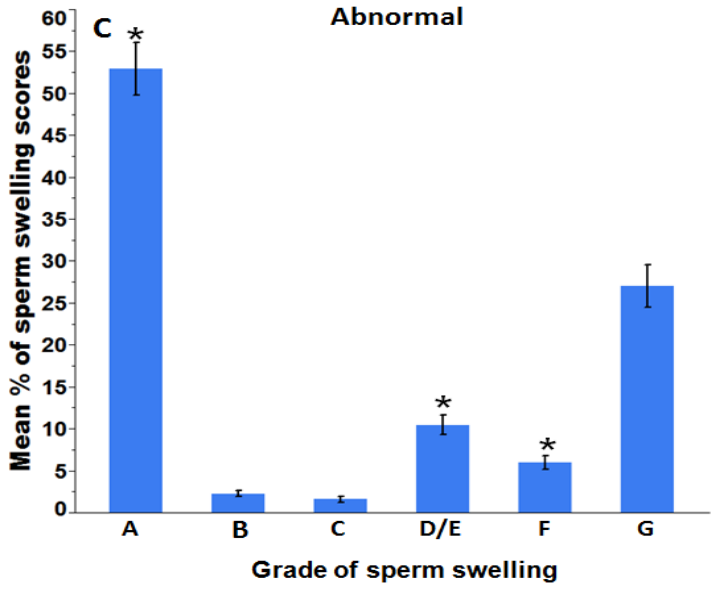
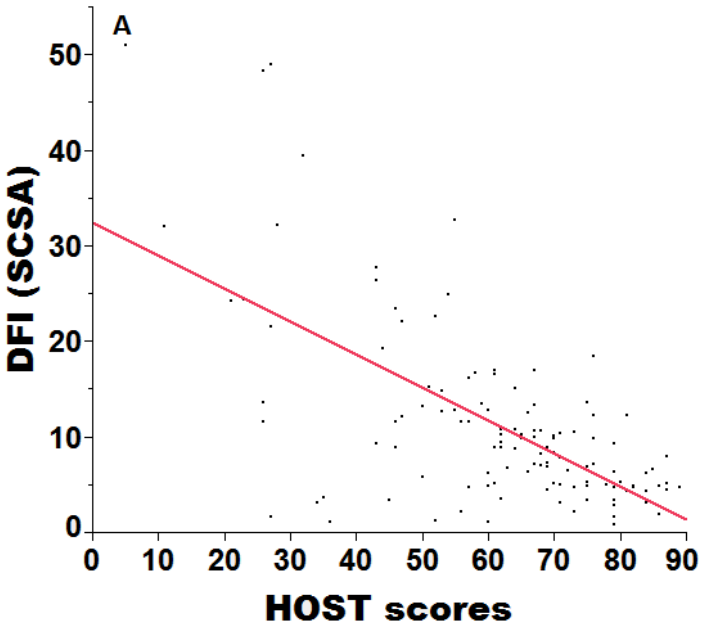

C
Abnormal
Mean % of sperm swelling scores
60
55
50
45
40
35
30
25
20
15
10
5
0
A
B
C
D/E
F
G
Grade of sperm swelling
*
*
*

Figure 3


A
DFI (SCSA)
HOST scores
0
10
20
30
40
50
60
70
80
90

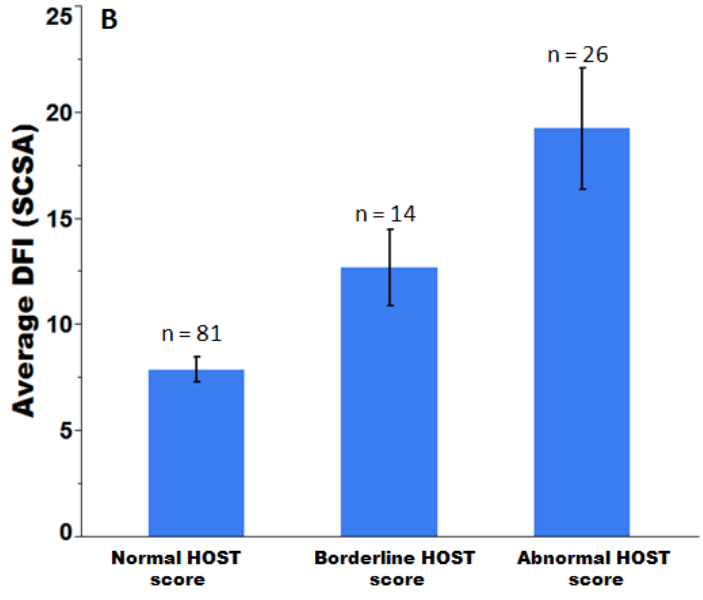

B
Average DFI (SCSA)
n = 81
n = 14
n = 26
Normal HOST score
Borderline HOST score
Abnormal HOST score

Figure 4

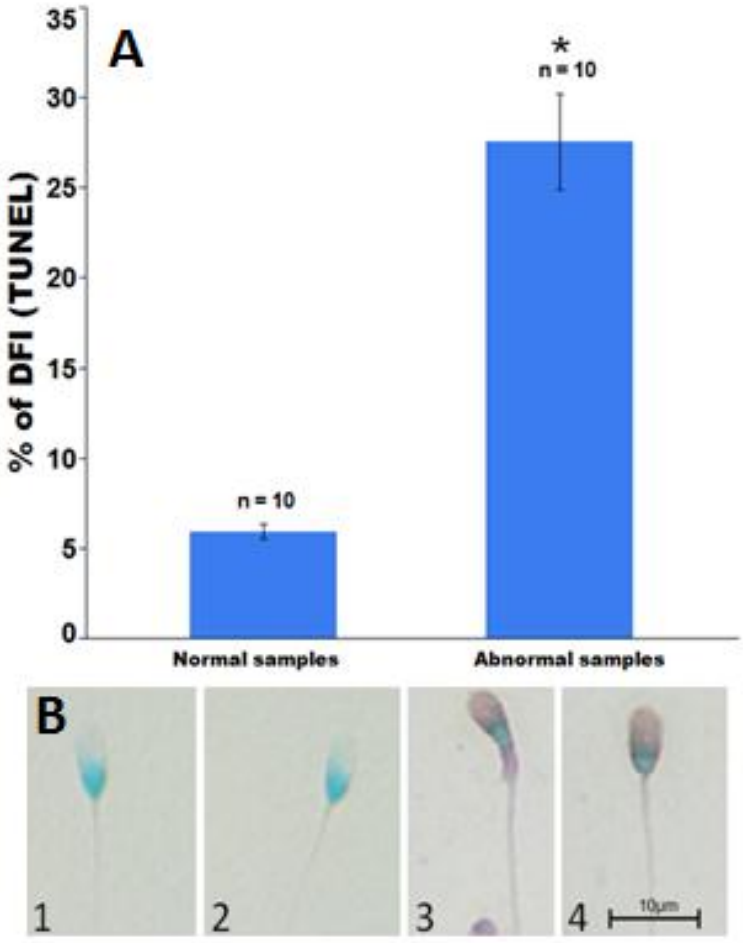

A
B
% of DFI (TUNEL)
35
30
25
20
15
10
5
0
n = 10
*
n = 10
Normal samples
Abnormal samples
1
2
3
4
10µm

Figure 5

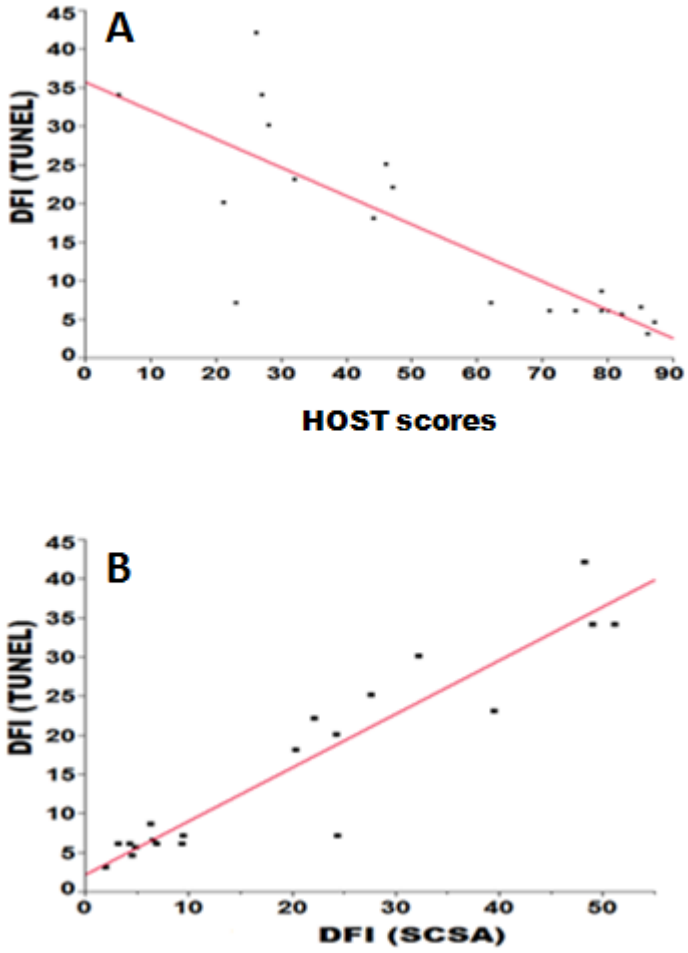


Figure 6

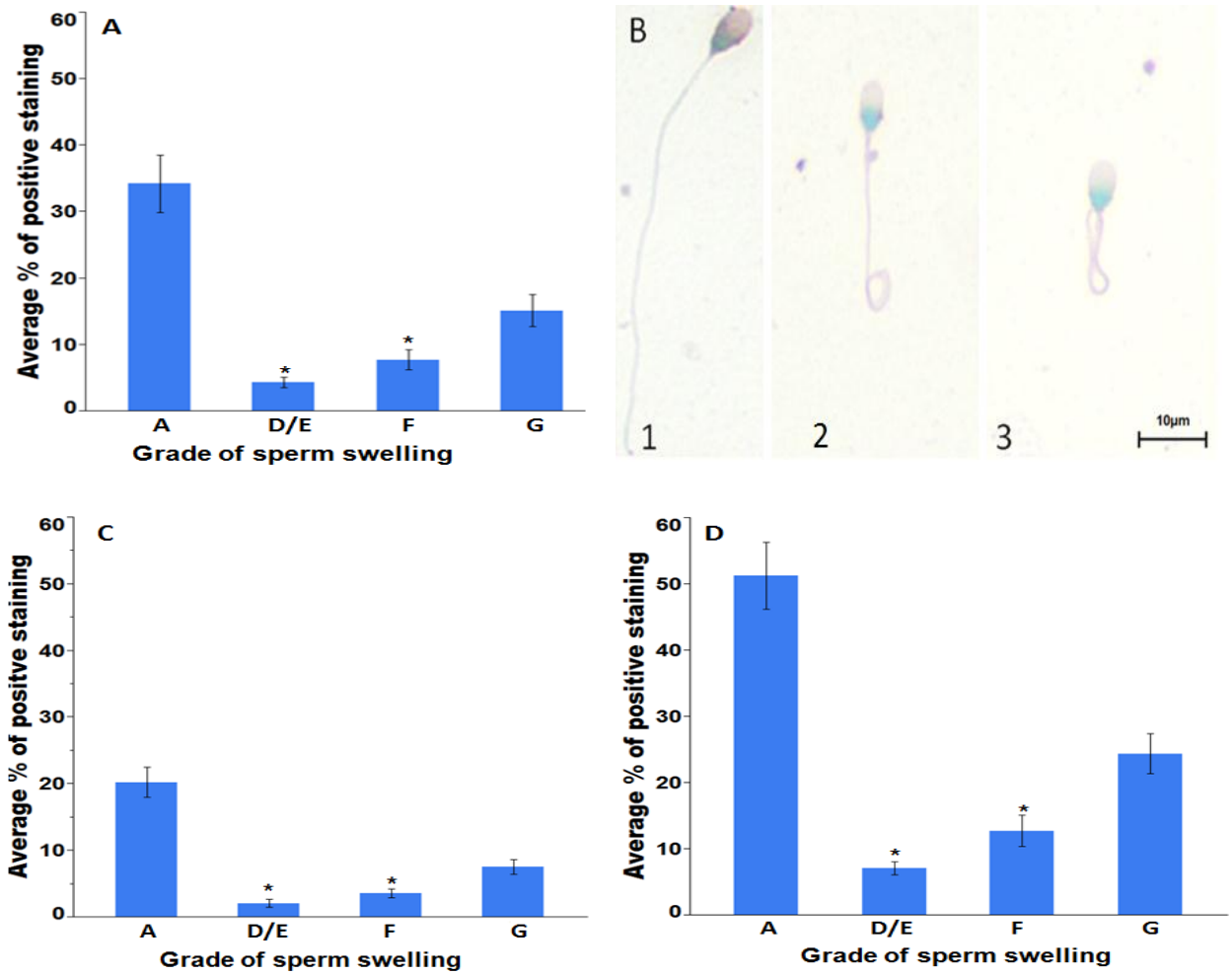

A
Average % of positive staining
60
50
40
30
20
10
0
A
D/E
F
G
Grade of sperm swelling
B
1
2
3
10µm
C
Average % of positive staining
Grade of sperm swelling
D
Average % of positive staining
Grade of sperm swelling

Figure 7.