Chapter 6

Hypo osmotic swelling test enables selection of sperm with superior DNA integrity

Introduction

Successful fertilization in humans follows a complex series of events, including the completion of meiotic maturation of the oocyte with the extrusion of the second polar body, the decondensation of the sperm nucleus and the maternal chromosomes into male and female pronuclei, the restoration of the sperm centrosome, and the nucleation of microtubule-mediated motility necessary to bring the male and female pronuclei into close apposition (Navara et al., 1997).

Although widely acknowledged that all that fathers essentially contribute half their genome, to the next generation, recent progress towards understanding biological processes such as sperm maturation and fertilization indicates that the paternal contribution has been largely underestimated (Krawetz, 2005). In contrast to the traditional view which regards mammalian fertilization as a simple blending of two gametes and the spermatozoon as a mere vector that transfers DNA to the egg, research over the past decade indicates the crucial contribution of the
male gamete to embryogenesis. Normal fertilization and subsequent early embryo development has now been shown to be dependent on several structures/organelles and molecules present in the spermatozoon (Sutovsky and Schatten, 2000).

The fertilizing human spermatozoon is essential for contributing at least three components: (1) the paternal haploid genome, (2) the signal to initiate metabolic activation of the oocyte (a putative oocyte-activating factor, OAF), and (3) the centriole, which directs microtubule assembly leading to the formation of the mitotic spindles during the initial zygote development. It is now well established that there is extensive cross-talk between the fertilizing sperm and the egg, leading to activation of the egg on one hand and sperm head decondensation on the other. This is orderly followed by female and male pronuclear formation, syngamy, and the first cleavage divisions (Barroso et al., 2009).

ICSI, a form of ART which is routinely employed in the treatment of severe male factor infertility, involves injection of a mature spermatozoon into a metaphase II oocyte. As such, this technique bypasses multiple steps of the natural fertilization process by introducing an apparently intact spermatozoon into the ooplasm. The impact of the microinjection technique on fertilization and post fertilization events should be definitely established to determine the immediate safety of ART as well as any possible long-term consequences, including embryonic anomalies that result in developmental arrest or transgenerational effects carried over to the offspring. Clinical evidence now derived from the use of ART points to the fact that defective sperm contributions may extend
well beyond fertilization, and that early and late paternal effects may even be
determinants of abnormal development (Barroso et al., 2009).

In this regard, sperm DNA damage is gaining interest as a potential cause of infertility
(Evenson et al., 1980; Irvine et al., 2000; Shen and Ong, 2000; Spano et al., 2000; Zini et
al., 2001, 2002). The critical role played by normality of sperm nuclear DNA in
mammalian fertilization and subsequent embryonic development has been established
(Tesarik et al., 2004; Bungum et al., 2007; Zini et al., 2008; Aitken et al., 2009). Interestingly, it has been demonstrated that sperm cells with damaged or fragmented-
DNA can still fertilize oocytes in vitro (Ahmadi and Ng, 1999a). Although the past
decade has witnessed a surge in the number of articles published regarding the presence
of DNA damage in sperm (Lopes et al., 1998b; Larson et al., 2000; Evenson et al., 2002;
Morris et al., 2002; Benchabib et al., 2003; Kovalevsky and Patrizio, 2005; Evenson and
Wixon, 2008; Zini et al., 2008; Zini and Sigman, 2009), very little attention has been paid
to the identification of the DNA damaged sperm and selection individual or populations
of ‘‘normal’’ sperm, with superior DNA integrity.

Given the fact that infertility is no longer solely a personal problem; and is evolving into
more of a public health concern, we urgently require tests of sperm function that not only
ensure normalcy of sperm DNA, but also a generation of healthy children from ART. The
Hypo Osmotic Swelling Test (HOST) has been routinely used in the identification of
viable sperm in a non-destructive manner (Jeyendran et al., 1984). The present study has
been planned to ascertain if viable sperm selected by HOST also possess superior DNA
integrity. If successful, this strategy could in turn, pave way for the specific selection of healthy, DNA competent spermatozoa for clinical utilization in ICSI.

**Materials and Methods**

**Subjects:**

Patients visiting the infertility clinic of Kasturba Medical College for fertility evaluation and treatment participated in this prospective study. A total of 16 subjects were included in the present study, after provision of a written, informed consent. All subjects were asked to provide semen samples after 3-5 days of ejaculatory abstinence. Semen specimens were produced by masturbation directly into a sterile plastic container, in a room specially provided for this purpose and located adjacent to the laboratory.

**Semen Analysis:**

After liquefaction, routine semen analysis was performed according to the recommendations of the WHO (WHO, 1999). Seminal volume was determined in a graduated tube and sperm concentration was assessed by conventional method using Makler counting chamber (Sefi Medical Instruments, Israel) and expressed in millions/mL. The sperm motility was assessed in at least 100 sperm and expressed as percent of motile sperm (sum of rapid progression plus slow progression sperm). Sperm morphology was assessed by Shorr staining and sperm viability by Eosin-Nigrosin stain. The reference values of semen variables and nomenclature for some semen variables used throughout the thesis has been presented in Chapter 3.
Sample Handling:
Followed by routine andrological evaluation, a part of the sample was subjected to sperm preparation by swim-up and the supernatant with a high proportion of motile and viable sperm was used for the study. DNA damage in the sperm was induced using hydrogen peroxide (Sigma-Aldrich, St.Louis) at a concentration of 70.4 µM for 15 minutes at 4°C. Subsequent to hydrogen peroxide exposure, a part of the sample was assessed for DNA damage using the alkaline comet assay. This formed the internal control group against which the effectiveness of HOST in sperm selection could be compared. The rest of the hydrogen peroxide exposed spermatozoa from the supernatant were subjected to HOST and HOST positive and negative spermatozoa were selected as described below, to be followed by DNA damage assessment using alkaline comet assay.

Hypo osmotic swelling test:
The swelling solution consisted of 0.0735 g of sodium citrate dihydrate and 0.351 g of fructose in 10 ml of distilled water (WHO, 1999). Aliquots of this solution were frozen at -20°C, in closed Eppendorf tubes. Prior to use, the swelling solution was warmed by placing it in a water bath maintained at 37°C for 5 minutes. About 0.1 ml of the supernatant fraction (also exposed to hydrogen peroxide) was then added to the solution and mixed gently with a pipette. The tube was placed in a water bath at 37°C for 45 minutes. Following this incubation, the sample was subjected to centrifugation at 100 x g for 5 minutes and HOST negative spermatozoa (no tail coiling; with straight tail) were selected from the supernatant fraction. Micromanipulation was employed to pick up HOST positive spermatozoa (with tail coiling), manually.
**Comet Assay:**

Assessment of DNA damage by alkaline comet assay (Singh et al., 1988) was performed as adapted for sperm, with some modifications. Normal melting agarose (NMA) (1.0%) was prepared by heating agarose in phosphate buffered saline (PBS). Following successive cleaning of the slide with soap water and methanol, 200 µl of 1% NMA was spread uniformly and the slide was allowed to dry at room temperature. The slides were stored in a dry place at room temperature until further use. The cell suspension containing sperm (approximately 5 µl) was thoroughly mixed with low melting point agarose (LMPA) (0.5%), prepared in PBS and maintained at 37°C. 200 µl of the cell suspension with LMPA was layered onto the slide. A cover slip was placed over the cell suspension and slide was placed on ice until the agarose layer hardened. Subsequently, the cover slip was gently slid off and a third agarose layer (150 µl LMPA) added, to the slide. Followed by hardening of agarose on ice and removal of the cover slip, the slides were slowly lowered into cold, freshly made lysing solution (2.5 M sodium chloride, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, 10% dimethylsulphoxide, pH:10). Following overnight incubation, 15 mM dithiothreitol was added to the lysing solution and the slides were incubated for additional 4 hours. The slides were then carefully removed from the lysing solution and transferred to an electrophoretic chamber and placed side by side. The buffer reservoirs were filled with freshly made electrophoresis buffer (1 mM EDTA and 300 mM NaOH buffer, pH>13) and left to stand for 20 minutes to allow for alkaline unwinding of DNA. Electrophoresis was carried out at 25V (~0.74 V/cm) and 300 mA for 30 minutes. The slides were then neutralized with the neutralization buffer (0.4M Tris, pH 7.5) at 4°C for 10 minutes. The slides were then dehydrated in absolute ethanol.
for 2 hours and immersed for 5 minutes in 70% ethanol. Slides were air-dried at room temperature. Immediately before scoring, slides were stained with 100 µl of ethidium bromide stain (EtBr, 20 µg/ml), rinsed once in PBS, coverslipped and analysed within 3 hours. Slides were examined at 40X under a fluorescent microscope, equipped with a 490 nm excitation filter (Imager-A1, Zeiss, Germany). While scoring, random images of 6-7 spermatozoa were obtained, from different regions of the slide. Approximately fifty cells were scored from each specimen. A computerized image analysis system (Komet 6.0, Kinetic Imaging, Nottingham, UK) was used to measure the different comet parameters.

**Statistical Analysis**

Basic descriptive statistics were calculated for standard semen parameters and different comet assay parameters using Statistical Package for Social Sciences (SPSS 16.0). The value represents Mean ± SEM. Statistical analysis of the means between different study groups was performed using paired T test, since the values obtained represented a single sample. A P value < 0.05 was considered statistically significant.
Reagents

**Sigma Aldrich, St. Louis, MO, USA**

Normal melting agarose (NMA)  Cat No A9539
Low melting point agarose (LMPA)  Cat No A9414
Potassium chloride  Cat No P5405
Tris base  Cat No T4661
Dithiothreitol  Cat No D9760
Triton X-100  Cat No T8532
Dimethylsulphoxide (DMSO)  Cat No D5879
Nigrosin  Cat No N4754
Sodium citrate dihydrate  Cat No C7254
Fructose  Cat No F3510
Ethidium Bromide  Cat No E8754

**Himedia, India**

Sodium chloride  Cat No MB023
Disodium Ethylene diamine tetra acetic acid  Cat No MB011
Disodium hydrogen phosphate  Cat No RM1154
Potassium dihydrogen phosphate  Cat No RM249
Tris Hydrochloride  Cat No RM613
Sodium hydroxide  Cat No RM467
Ethidium bromide  Cat No E8754

**Merck & Co, USA**

Shorr stain  Cat No UN1993
Xylene  Cat No 1330-20-7
Hydrogen peroxide solution 30%  Cat No 7722-84-1
Ammonia solution (about 25% pure)  Cat No 1336-21-6
Fisher Scientific, UK
Methanol Cat No 32407

Hayman, UK
Absolute Alcohol (Ethanol)

BDH Chemicals, UK
Eosin Cat No 3419720

Sisco Research Laboratories, India
DPX Mountant for histology Cat No 42848
Results

The average age of the men and seminal volume was \(37.81 \pm 1.25\) years and \(3.06 \pm 0.43\) ml respectively. The average sperm count, percentage total motility and percentage viability was \(47.62 \pm 6.2\) millions/ml; \(60.69 \pm 3.65\) and \(64.31 \pm 3.76\), correspondingly. The percentage of sperm with normal morphology, head defects, tail defects and cytoplasmic droplets in the unprocessed fraction (neat fraction) were \(30.38 \pm 1.64\); \(48.25 \pm 2.12\); \(21.38 \pm 2.08\); \(7.44 \pm 1.53\) respectively. Following sperm processing by swim up, there was a significant (\(P<0.001\)) increase in the percentage of total motile sperm to \(81.94 \pm 1.31\) (Data not shown). In addition, following the HOST, all the samples included in the present study exhibited a tail swelling of greater than the established threshold of 60% (WHO, 1999).

Assessment of sperm DNA damage by alkaline comet assay

Komet 6, comet analysis system by Kinetic Imaging (Nottingham, UK), is a simple and most advanced and powerful software solution for analysis, data management and presentation of comet assay samples. 24 parameters are computed from the comet image based on intensity and migration patterns for comparison between control and experimental groups. Among the various parameters available, percent of tail DNA, olive tail moment and tail length were analysed as a measure to compare the extent of DNA damage and is presented in Table 6.1.
The representative images obtained by alkaline comet assay in sperm exposed to hydrogen peroxide, HOST positive and HOST negative sperm is given in Figure 6.1a, 6.1b and 6.1c respectively. The control group, only subjected to exposure of hydrogen peroxide, had the least DNA integrity by alkaline comet assay, to be followed by HOST negative spermatozoa (Table 6.1). The HOST positive spermatozoa had the highest DNA integrity, revealed in terms of percent of tail DNA, Olive tail moment (OTM) and tail length. However, there was no statistically significant difference between HOST positive and HOST negative spermatozoa (Table 6.1). In addition, although HOST positive spermatozoa exhibited lowest percent of tail DNA, Olive tail moment (OTM) and tail length, compared to the control group, no significant difference was observed between the groups with respect to the comet parameters mentioned above (Table 6.1).
Figure 6.1 DNA damage assessment by alkaline comet assay
# Table 6.1: Association between sperm selection by HOS test and DNA damage by alkaline comet assay

<table>
<thead>
<tr>
<th>Comet assay parameter</th>
<th>Control sperm exposed to H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;; N= 840</th>
<th>HOST negative sperm with straight tail; N=844</th>
<th>HOST positive sperm with coiled tail; N=828</th>
</tr>
</thead>
<tbody>
<tr>
<td>Head DNA (%)</td>
<td>82.90 ± 2.15</td>
<td>82.76 ± 1.57</td>
<td>83.67 ± 1.61</td>
</tr>
<tr>
<td>Tail DNA (%)</td>
<td>17.08 ± 2.15</td>
<td>17.26 ± 1.56</td>
<td>16.31 ± 1.61</td>
</tr>
<tr>
<td>Olive Tail Moment (OTM)</td>
<td>2.40 ± 0.61</td>
<td>2.02 ± 0.27</td>
<td>1.73 ± 0.24</td>
</tr>
<tr>
<td>Tail length (µm)</td>
<td>10.38 ± 1.23</td>
<td>9.46 ± 0.95</td>
<td>8.28 ± 0.91</td>
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</tbody>
</table>
Discussion

Fertilization *in vivo* requires the coordination of a number of highly regulated processes, to be concomitantly followed by a complex series of selection events that ensure the participation of only the fittest gametes, giving rise to a viable embryo (Van Soom *et al*., 2007). Clearly, when these go awry, infertility can occur. Defects in any step required for fertility will profoundly influence a couple's life plan and their vision together for a family. Although the development of ART has allowed otherwise hopelessly infertile couples to experience the joy of parenthood, these technologies also traverse the natural barriers preventing the transmission of genetic defects (Matzuk and Lamb, 2008).

The new methods of assisted fertilization, represented by ICSI and round spermatid injection, bypass multiple steps of natural fertilization by introducing an intact spermatozoon or spermatogenic cell into oocyte cytoplasm. Consequently, the carryover of sperm accessory structures that would normally be eliminated before or during the entry of sperm into oocyte cytoplasm persist therein and may interfere with early embryonic development, thus decreasing the success rate of assisted fertilization and possibly causing severe embryonic anomalies (Sutovsky and Schatten, 2000).

Human male gametes pass over more to the oocyte than just the haploid male genome. Recent research now provides evidence of important cytoplasmic contributions made by the fertilizing spermatozoon to the zygotic makeup, to the organization of preimplantation development, and even reproductive success of new forms of assisted fertilization (Sutovsky and Schatten, 2000; Ostermeier *et al*., 2004). It has been suggested that failure to complete the fertilization process,
syngamy, or early cleavage might be the result of an early paternal effect. It is speculated that an abnormal release of a putative OAF and/or dysfunctions of the centrosome and cytoskeletal apparatus may mediate these effects. On the other hand, a later paternal effect resulting in embryonic failure to achieve implantation, pregnancy loss, and/or developmental abnormalities resulting from "carried over" sublethal effects may be associated with sperm nuclear/chromatin defects, including the presence of aneuploidy, genetic anomalies, DNA damage, and possibly other causes (Tesarik et al., 2004; Seli and Sakkas, 2005; Tesarik, 2005; Aitken and De Iuliis, 2007a, 2007b; Barroso and Oehninger, 2007; Henkel, 2007; Barroso et al, 2009).

A simple strategy to overcome these above-mentioned effects would be to excise caution and develop more sophisticated methods for sperm selection. However, our current sperm selection parameters for use in ART are still based on morphology and motility, which may not be relevant to chromatin integrity (Razavi et al., 2010). In infertile men with moderate and severe teratozoospermia, DNA damage has been reported in spermatozoa with apparently normal morphology present in the motile fractions after sperm preparation by swim-up (Avendano et al, 2009). The negative impact of DNA fragmentation in morphologically normal sperm on embryo quality and probability of pregnancy in ICSI cycles has also been reported (Avendaño et al, 2010).

The results of studies highlighting the influence of sperm DNA damage to reduced fertility in humans and poor outcomes after IVF and/or ICSI have traditionally been made using estimates of damage in previous samples or on an aliquot from the sample used for the insemination. All of the existing methods for the detection of DNA damage in sperm lead to destruction of the same,
rendering them unusable for therapeutic approach (Stanger et al., 2010). The dilemma regarding the identification of a viable spermatozoon with low apparent DNA damage from at risk samples still eludes the scientific community.

Today, we have advanced protocols that allow sperm selection (Paasch et al., 2007) based on their ultra structural morphology (Antinori et al., 2008) or surface charges by electrophoresis (Kaneko et al., 1984; Engelmann et al., 1988; Ainsworth et al., 2005; Ainsworth et al., 2007). New insights into the molecular biology of spermatozoa have even prompted the development of molecular selection strategies such as hyaluronic acid mediated sperm selection (Jakab et al., 2005), the annexin V magnetic activated cell separation (MACS) (Said et al., 2006), and annexin V molecular glass wool filtration (Grunewald et al., 2007). Selection of sperm based on combined density gradient and Zeta method have been suggested to improve the outcome of ICSI (Kheirollahi-Kouhestani et al., 2009; Razavi et al., 2010). However, these strategies are expensive, which limits use of the same, particularly in a developing country like India. Due to the same reason, there is also an urgent need to develop a simpler and cost-effective sperm selection technique for routine use in ART.

First described for use with human sperm (Jeyendran et al., 1984), the hypo-osmotic swelling test (HOST) enables the identification of sperm with functionally intact membranes and is routinely employed in the determination of sperm viability (WHO, 1999). The assay is based on the fact that fluid transport occurs across an intact cell membrane under hypoosmotic conditions until equilibrium is reached. Due to the influx of fluid, the cell will expand and bulge, especially in the tail, and this change can be readily observed with a phase contrast microscope (Jeyendran
et al., 1992) The osmotic stress caused by the chosen hypo-osmotic medium must be sufficient to effect an influx of water into the cell to enable curling of the tail, but to prevent lysis of the sperm membrane (Matson et al., 2008).

It has been suggested that the sperm HOS test may be helpful to screen for any paternal factor associated with repeated embryonic or early fetal loss and in a resource-poor setting (Bhattacharya, 2010). The test has previously been reported to aid in identification of individual spermatozoa with minimal DNA fragmentation (Stanger et al., 2010). The study further reported 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 SCSA and TUNEL assay.

In the present study, use of alkaline comet assay in assessment of the relationship between tail swelling by HOST and the extent of DNA damage in individual HOST positive and negative spermatozoa has been reported. The assay assesses actual DNA strand breaks, alkali labile sites and can detect DNA damage equivalent to as few as 50 single-strand breaks per cell and varying number of double strand breaks (Tice and Strauss, 1995; Olive et al., 1998; Singh et al., 1988). The assay attracts adherents by its speed, simplicity, reproducibility, and sensitivity and has been considered to be suitable for the assessment of DNA damage in sperm (Collins, 2002). Relatively few numbers of cells ~50, has been found to be sufficient for the reproducibility of the comet assay (Hughes et al., 1997).
HOST positive spermatozoa have been found to have higher DNA integrity as revealed by lowest percent of tail DNA, Olive tail moment (OTM) and tail length by the alkaline comet assay compared to those that were exposed to hydrogen peroxide and HOST negative spermatozoa. Although the finding is statistically insignificant, which could be attributed to the relatively small sample size, the results of the present study agree with the previous finding that sperm selected by HOST have minimal DNA damage (Stanger et al., 2010). The HOS test has the distinct advantage of being quick, simple, requiring minimal equipment. Most importantly, it is non-destructive and cost effective compared to other advanced sperm selection methods.

It has been reported that sperm selected using the HOS test from samples with only non-motile spermatozoa can result in pregnancy and live births (Peeraer et al., 2004; Sallam et al., 2005), making it an ideal sperm selection tool for ICSI. By employing the comet assay, it has been demonstrated that DNA damage in individual spermatozoa can be related to the degree of tail swelling and suggest that viable spermatozoa identified by HOST - induced tail swelling may have negligible DNA damage. Although the present findings warrant validation using samples of larger size, at this juncture, at least in at risk samples, it seems reasonable to suggest the use of HOST for non-destructive, selective identification of viable spermatozoa with minimal DNA fragmentation for use in ART.