Detection of chromosomal abnormalities by fluorescent in-situ hybridization in immotile viable spermatozoa determined by hypo-osmotic sperm swelling test*

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If randomly selected immotile spermatozoa are used for intracytoplasmic sperm injection (ICSI), pregnancy rates are significantly decreased. The hypo-osmotic swelling test (HOST) is the only method available to detect the viable, but immotile spermatozoa for ICSI. However, evidence is still lacking for the chromosomal abnormalities for the normal-looking, but immotile spermatozoa positive for HOST. Sperm samples from 20 infertile men with normal chromosomal constitution were obtained. After Percoll separation, morphologically normal but immotile spermatozoa were transported individually into HOST solution for 1 min using micropipettes. Cells that showed tail curling with swelling in HOST were then transferred back into human tubal fluid solution to allow reversal of swelling. These sperm cells were fixed and processed for the multicolour fluorescence in-situ hybridization (FISH) for chromosomes X, Y and 18. The same FISH procedure was applied for the motile spermatozoa from the same cohort, which formed the control group. The average aneuploidy rates were 1.70 and 1.54% in 1000 HOST positive immotile and motile spermatozoa respectively detected by FISH for each patient. Our results indicate that morphologically normal, immotile but viable spermatozoa have an aneuploidy rate similar to that of normal motile spermatozoa.

Key words: chromosome abnormality/hypo-osmotic swelling test/male infertility/oligozoospermia/spermatozoa

Introduction

Intracytoplasmic sperm injection (ICSI) is the assisted reproductive technology procedure of choice in cases of severe male-factor infertility and unexplained fertilization failure after conventional in-vitro fertilization (IVF) (Palermo et al., 1992). The advantages of ICSI are (i) the need of only one spermatozoon per oocyte and (ii) the ability to use not only the ejaculated spermatozoon, but also spermatozoa from testis and immature forms of sperm cells from the testis may be obtained with varying success.

Poor or absent fertilization rates are obtained in cases of ICSI using immotile ejaculated spermatozoa of unknown viability (Nagy et al., 1995). Total immotility may be caused by necrozooospermia, ultrastructural defects or metabolic immaturity of the spermatozoon.

In cases of total necrozooospermia in the ejaculate, motile spermatozoa can sometimes be recovered from a testicular biopsy (Tournaye et al., 1996). However, when selecting spermatozoa to inject into an oocyte, it is always preferable to use the one that has shown some sign of motility and with best morphology available.

The hypo-osmotic swelling test (HOST) or cell staining methods are often used to identify sperm viability. The mechanisms of HOST and eosin-Y staining are mainly related to the functional integrity of sperm membrane and normal functional activity of human spermatozoon (Jeyendran et al., 1984). Since there is no available cell-staining technique that does not impair the viability of the cell for future clinical use, HOST is the most useful method of selection for the viable spermatozoon in an asthenozoospermic population. Successful use of immotile spermatozoa has been reported in ICSI (Casper et al., 1996). Although such cells have been successfully used in ICSI, controversies exist over whether sperm phenotype abnormalities are indicative of genotype abnormalities (Engel et al., 1996). The multicolour fluorescence in-situ hybridization (FISH) may be one approach to give an insight into phenotype-genotype interaction of sperm cells, although not the most suitable technique.

This study aims to show the chromosomal status of the immotile spermatozoon which show HOST positivity compared to motile spermatozoon.

Materials and methods

Sperm samples from 20 infertile men who were referred to Baskent University IVF Unit were obtained by masturbation. All patients had sperm analysis showing oligozoospermia according to WHO criteria (WHO, 1999). <4% normal morphology according to Tygerberg strict criteria (Kruger et al., 1988) and normal chromosomal constitution in the peripheral lymphocyte metaphases. All patients had total motile sperm ratios that were <50% (according to WHO criteria: type A + type B <50%).

The semen was washed using two-gradient (95 and 47.5%) Percoll (Pharmacia AB, Uppsala, Sweden) separation, the pellet was diluted in 500 µl human tubal fluid solution (HTF) (IVF 50, Scandinavian Scientific, Gothenburg, Sweden). The morphologically normal but immotile spermatozoa were transported individually into HOST solution (Hypo-10, Scandinavian Scientific, Gothenburg, Sweden).
using micropipettes (Hunter Scientific, Saffron Walden, Essex UK) of a microinjection device (Nikon, Narishige, Japan); if the final washout did not produce sufficient immotile spermatozoa, then the spermatozoa from the interface between the gradients of Percoll were used. The spermatozoa were incubated in hypo-osmotic solution for 10 min. The cells which showed tail curling with swelling in HOST (viable cells) (Figure 1) were then transferred back into HTF solution to allow reversal of swelling. Then the cells were transferred to a glass microscope slide with a small amount of HTF solution. The cells were allowed to dry in the drop of HTF solution on the slide with constant viewing under the inverted microscope. After complete drying of the HTF solution, 1 drop (5 µl) of the Carnoy’s fixative (1 ml acetic acid, 3 ml methanol) was added to the spermatozoa. The cells were constantly observed under the inverted microscope and another drop of fixative was added before complete drying and this step repeated 5 times.

Decondensations of the spermatozoa were performed in 25 nmol/l dithiothreitol (DTT) in 0.1% trypsin (Biochrom KG, D-1000 Berlin, Germany). After incubation with DTT for 15 min, slides were rinsed twice in 2×SSC (sodium chloride and sodium citrate) and dehydrated through ethanol series (70, 85, 90%) and air dried (Coonen et al., 1991). Dehydrated samples were then denatured at 73°C in 70% formamide/2×SSC and hybridized overnight at 37°C, using probes for the chromosomes 18, X and Y in a three-colour hybridization protocol (Bischoff et al., 1994; Blanco et al., 1996). All probes were directly labelled with fluorophores and obtained commercially (Vysis Inc., Downer’s Grove, IL, USA). The post-hybridization wash was performed in 0.4×SSC/0.3% NP40 at 73°C for 2 min. The same fixation and FISH procedure were applied for the motile sperm from the same cohort, which served as the control group. Slides were examined using a DAPI/FITC/Texas Red triple-band pass filterblock (Nikon, Japan) allowing the simultaneous visualization of orange, green and blue fluorophores, and photographed with a camera (Nikon F-601 QD) (Figure 2). Hybridization efficiency was 95% in both motile and immotile sperm groups. An autosomal probe (chromosome 18 probe) served as internal control. Only intact cells and cells which did not overlap were scored. Slides were scored blindly by two independent investigators. The statistical analysis included χ²-analysis, and t-test. The mean values were given with their standard deviations (±SD) in addition to median values and the range.

Table I. Comparison of chromosomal status of motile and immotile sperm cells from 20 infertile men after FISH analysis

<table>
<thead>
<tr>
<th>Immotile HOST</th>
<th>Motile HOST</th>
</tr>
</thead>
<tbody>
<tr>
<td>X chromosome aneuploidy</td>
<td>0.46</td>
</tr>
<tr>
<td>Y chromosome aneuploidy</td>
<td>0.33</td>
</tr>
<tr>
<td>18 chromosome aneuploidy</td>
<td>0.23</td>
</tr>
<tr>
<td>Any combinations</td>
<td>0.81</td>
</tr>
<tr>
<td>Average incidence of spermatozoa with abnormal chromosomal status</td>
<td>1.70 ± 4.48</td>
</tr>
</tbody>
</table>

The two types of sperm cell were not significantly different.

Results

The mean sperm concentration of the patients was 7.3 ± 2.7×10⁶/ml and median was 7.5×10⁶/ml (range 2.3–12). Mean percentage total motile (type A + type B + type C) spermatozoa was 18.8 ± 7.6% (median: 19%; range: 7–35). The mean percentage normal morphology according to Tygerberg strict criteria was 1.8 ± 1.0% (median: 2.0; range: 0–3). Average X chromosome, Y chromosome and 18 chromosome anomaly rates individually were 0.46, 0.33 and 0.27% respectively. Overall, the average incidence of spermatozoa with abnormal chromosomal status was 1.70 ± 4.48% (0.9–2.6%) in immotile HOST positive sperm cells detected by FISH for each patient. These data are summarized in Table I.

On the other hand, FISH analysis of motile spermatozoa yielded the X chromosome, Y chromosome and 18 chromosome anomaly rates as 0.43, 0.25 and 0.23% respectively (Table I). Overall, the average incidence of spermatozoa with abnormal chromosomal status in motile HOST positive sperm cells detected by FISH for each patient was 1.54 ± 4.13% (0.8–2.5%). The statistical analysis showed that the difference in aneuploidy rate between the motile and immotile spermatozoa was not significant (P = 0.23).
Discussion

There is no described test with non-invasive properties to select a live spermatozoon for microinjection purposes, since the spermatozoon identified by supravital stains such as eosin-Y or acridine orange cannot be used for further clinical study. Desmet et al. reported that HOST might be useful in distinguishing viable from non-viable immotile spermatozoon for ICSI. A 30% fertilization rate was obtained with the injection of selected spermatozoa into 1-day-old oocytes, which had failed to fertilize after conventional IVF (Desmet et al., 1994). Casper et al. reported three pregnancies in eight clinical ICSI cycles after a fertilization rate of 43% of the oocytes injected with spermatozoa selected using HOST, compared to the fertilization rate 26% using random sperm injection; i.e. the fertilization rate was almost doubled (Casper et al., 1996). Vandervorst et al. used immotile spermatozoon intracytoplasmic injection in 11 patients without HOST and they had a 34% fertilization rate, but no pregnancies ensued and they postulated that they were able to find motile spermatozoon and succeeded in four pregnancies in the subsequent cycles (Vandervorst et al., 1997).

Verheyen et al. compared three hypo-osmotic solutions, namely, Jeyendran solution (Jeyendran et al., 1984) containing sodium citrate and fructose; milli-Q water; and a mixture of 50% culture medium and 50% milli-Q water (Verheyen et al., 1997). They found that the solution composed of 50% culture medium and 50% water was the most appropriate for selection of viable immotile spermatozoon for ICSI. We used a commercial HOST solution in this study which is mainly based on Jeyendran solution and we did not detect any toxicity, since a number of the spermatozoon lived up to 24 h after injection in 11 patients without HOST and they had a 34% fertilization rate; i.e. the fertilization rate was almost doubled (Casper et al., 1996). Vandervorst et al. used immotile spermatozoon intracytoplasmic injection in 11 patients without HOST and they had a 34% fertilization rate, but no pregnancies ensued and they postulated that they were able to find motile spermatozoon and succeeded in four pregnancies in the subsequent cycles (Vandervorst et al., 1997).

Chromosomal abnormalities are detected in ~15% of azoospermic subjects and 6–7% of subjects with <10×10⁶ spermatozoa/ml, either alone or in combination with other abnormalities of the semen (Bourrouillou et al., 1992). Even in subfertile patients with normozoospermia, an increased rate of chromosomal aberrations was detected (Matsuda et al., 1989). Sex and autosomal chromosomes were found to be abnormal in 4.2 and 1.5% of infertile men respectively. While sex chromosome anomalies were predominant in non-obstructive azoospermic patients, autosomal chromosomal anomalies were more common in patients with obstructive azoospermia (Van Assche et al., 1996). The most common abnormality involving sex chromosomes is XXY and the most common molecular defect is microdeletions in the AZF region of the long arm of the Y chromosome (Reijo et al., 1995). Bernardini investigated the cytogenetic constitution of infertile and normal men and found higher rates of sperm aneuploidy for autosomes 1 and 17 with decreasing semen quality (<5×10⁶/ml) compared to controls (1.43 versus 0.8%) (Bernardini et al., 1998). Guttenbach found a 0.31–0.41% disomy rate in individual sperm cells from 20 healthy fertile men using FISH with six different chromosome-specific DNA probes, and they found similar disomy rates as indicated by two distinct hybridization signals for all chromosomes, ranging from 0.31 to 0.34% (Guttenbach et al., 1994a,b). In addition, McInnes et al. investigated sperm samples from infertile men using FISH with probes for chromosomes 13 and 21 and found a highly significant increase in the frequency of spermatozoa disomic for chromosome 13 in infertile patients (0.28%) compared to control donors (0.13%) and for chromosome 21 (0.48% in infertile men versus 0.37% in controls) (McInnes et al., 1998).

It has been shown (Bischoff et al., 1994) that using single colour FISH was not sufficient to distinguish failed hybridization from nullisomy, or disomy from diploidy, but a multicolour FISH strategy provides an internal control. Three colour probes (sex chromosomes plus one autosomal chromosome) are sufficient to provide an internal control. For this reason, one of the probes for chromosomes 13, 18 or 21 is frequently used for the demonstration of autosomal chromosomal status (Bischoff et al., 1994).

Immotility or non-progressive motility in spermatozoa may result from centriolar or tubular abnormalities or absence of centrioles or microtubules such as supernumerary microtubules, imperfect organization of the microtubules (Sauville et al., 1983), absence of the central pair of microtubules or the complete anoxeme (Neugebauer et al., 1990) or ultrastructural abnormalities such as the absence of dynein arms (Gopalkrishnan et al., 1995). These abnormalities were commonly detected in many variants of immotile cilia syndromes. Despite these abnormalities, pregnancies were reported after intracytoplasmic injections of immotile spermatozoa from ejaculate or testicular extract of men with immotile cilia syndrome (Kahraman et al., 1997; Olmedo et al., 1997). However, anti-sperm antibodies, infection of the genital tract and prolonged periods of anejaculation are most common causes of total immotility (Vandervorst et al., 1997). In addition, the low energy production in the spermatozoa or exposure to radical oxygen species may cause inadequate use of the energy sources in the spermatozoa. Coenzyme Q10, being an antioxidant and energy promoter, is located in the mitochondria of the midpiece. Men treated with oral CoQ-10 did better in the next ICSI cycle after a failed cycle (Lewin and Lavon, 1997).

It is a common procedure to use morphologically normal spermatozoa for ICSI, if available, and it has recently been confirmed in an ESHRE abstract that decreased fertilization rates result with the injection of spermatozoa with abnormal morphology (De Vos et al., 1999). In addition, our collaborators have already shown a relationship between diminished sperm maturity, increased cytoplasmic retention/head size and increased rate of chromosomal abnormalities as detected by multicolour FISH (Moretti et al., 1997). A case report has recently been presented of a male with Y:16 chromosome translocation and FISH analysis of morphologically normal spermatozoa showing 51% chromosomally normal or balanced sperm cells (Giltay et al., 1999). However, if morphologically abnormal cells were also included in the FISH analysis nearly 90% of all the spermatozoa were unbalanced.

Based on the above evidence, we chose to assess the aneuploidy status of the morphologically normal but immotile spermatozoon in this study. Our data showed that normal but immotile spermatozoon might not differ from the chromosomal
status of the motile spermatozoa. The limited reports in the current literature support the use of immotile, alive and normal spermatozoa yielded to achieve normal pregnancy.

We suggest that any men with severe oligoasthenozoospermia should have genetic consultation to rule out chromosomal abnormalities or Y chromosome microdeletions. In such cases, preimplantation diagnosis to the embryos, or prenatal diagnosis should be offered to the couple. We also call for further studies to further the evaluation of the men with peripheral chromosomal abnormality. Our results show that the normal karyotype of the immotile but morphologically normal spermatozoa is likely to decrease the risk of genetic transmission of the chromosomal abnormalities. In conclusion, immotile spermatozoa from men with normal karyotype in the absence of microdeletions of the Y chromosome may be safely used for ICSI purposes.

References


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