Fertilizing ability of immotile spermatozoa after intracytoplasmic sperm injection

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Introduction

After Palermo performed the first intracytoplasmic sperm injection (ICSI) in 1991 (Palermo et al., 1992), the ICSI procedure was applied routinely all over the world. ICSI indications are also evolving with time. Initial indications were low sperm count, poor morphology and low motility. Other types of patient have been identified as benefiting from this technique: failure of fertilization with classic in-vitro fertilization (IVF) or subzonal insemination (SUZI) (Van Steirteghem et al., 1993; Fishel et al., 1994), globozoospermia (Trokoudes et al., 1995) and azoospermia with spermatozoa extracted from the epididymis or testis (Schoysman et al., 1993a,b).

In our laboratory, we have been confronted with samples from ejaculate, epididymis or testis showing no motility at all at the time of collection, and even after preparation and purification on a Percoll gradient. For some patients a few spermatozoa with very poor motility were recovered after several hours of incubation. The motility of these spermatozoa may be impaired by a number of factors, including disorders of the sperm energy metabolism, a wide variety of ultrastructural abnormalities in the sperm tail (Ryder et al., 1990) or defects in the pathways of cellular signalling. The occurrence of antisperm antibodies or genital infection can also be a cause of sperm immotility (Ansbacher et al., 1971; Del Porto et al., 1975). Because these patients have no chance of obtaining fertilization with classic IVF methods, it was decided to submit their samples for ICSI. The aim of our study was to analyse the possible fertilizing capacity of totally immotile spermatozoa and initially immotile spermatozoa from various origins, i.e. ejaculate, epididymis or testis, after injection into the cytoplasm of human oocytes.

One of our patients was identified as suffering from Kartagener syndrome. The ultrastructurally defective immotile spermatozoa were used for both ICSI and SUZI because fertilization has been obtained by the latter technique (Wolf et al., 1993). Fertilization rates, embryo quality, pregnancy rates and ongoing pregnancy rates were analysed for all types of sperm origin.

Materials and methods

In our IVF laboratory, patients are treated with ICSI when the total motile sperm count is <600 000, the number of abnormal forms in the ejaculate is >85%, when total motility is <20% or when patients have a history of fertilization failure with classic IVF. In this study, 103 patients with severe sperm motility disorders underwent ICSI (azoospermia).

Ovarian stimulation

As described previously by Lejeune et al. (1990), ovarian stimulation was carried out by administering gonadotrophin-releasing hormone (GnRH; buserelin; Suprefact, SP; Hoechst, Brussels, Belgium) in...
Association with human menopausal gonadotrophin (HMG; Pergonal; Serono Laboratories Inc., Brussels, Belgium) Human chorionic gonadotrophin (HCG; Pregnyl; Organon, Oss, The Netherlands; Profasi, Serono Laboratories Inc.) was given when the cohort of follicles reached a diameter of ~20 mm. Luteal phase support consisted of administering 5000 IU HCG on days 4 and 8 after transfer.

**Sperm preparation**

Ejaculates were received as split samples Micro-epidydymal sperm aspirations (MESA) and testicular sperm extraction (TESE) biopsies were prepared as described previously (Schoysman et al., 1993a, Vanderzwalmen et al., 1994). For 103 patients, the absence of motility and sperm concentration were assessed on samples from ejaculate, epididymis or tests biopsy (Table I). Samples were centrifuged at a higher speed than normal (500 g) and/or placed on a discontinuous Percoll gradient (Vanderzwalmen et al., 1991) with two or three layers for 15-40 min (depending on the concentration). After a washing step in Earle’s medium (Sigma, Bornem, Belgium) supplemented with 0.5% human serum albumin (Irvine Scientific, Zelhik, Belgium), a drop of the sample was placed under oil (Sigma) in the cover of a Petri dish (Falcon, Vel, Leuven, Belgium) and incubated at 37°C in an atmosphere of 5% CO₂ for 2–3 h. When motile spermatozoa were present after preparation and incubation, they showed a poor head or tail displacement and sometimes moved out from the central part of the drop which contained dead spermatozoa, immotile spermatozoa and blood cells (‘swim-out’ technique). Few moved to the edge of the droplet. These sperm samples were defined as containing initially immotile spermatozoa. Other samples showed no motility at all, even after extended incubation, and were classified as containing totally immotile spermatozoa. After an electron microscopy investigation, one patient was identified as carrying Kartagener syndrome. Some of this partner’s oocytes were submitted for ICSI and others for SUZI.

**Oocyte preparation**

At 2–3 h after oocyte retrieval, the cumulus cells were removed by a very brief incubation in 80 IU hyaluronidase (Type VIII, Sigma) in IVF 50 culture medium (Scandinavian IVF Science, Göteborg, Sweden). The corona radiata was removed by aspirating the oocytes in and out of a glass pipette with a diameter ranging from 180 to 220 μm in IVF 50. The oocytes were washed four times and incubated in culture medium under oil until further processing. ICSI was performed on oocytes that had extruded their first polar body (metaphase II oocytes), 3–4 h after their retrieval.

**Sperm collection**

Prior to micromanipulation, a micromanipulation dish was prepared containing one central droplet of 10% polyvinylpyrrolidone (PVP, Sigma), five droplets of 10 μl culture medium–1% HEPES and one droplet of 3 μl culture medium–1% HEPES under oil.

Details of the preparation of the micropipettes have been described previously by Vanderzwalmen et al. (1996). Two types of micropipette were used. For the initially immotile spermatozoa a 10–13 μm pipette was used to retrieve those spermatozoa that showed some motility out of the swim-out droplet. These spermatozoa were released into the 3 μl droplet in the micromanipulation dish. For some patients with initially immotile spermatozoa, the final concentration in the swim-out droplet was sufficient to aspirate some of the sluggishly motile spermatozoa on the edge of the droplet with a mouth-controlled glass pipette of 30 μm diameter. These sperm sampling steps were all performed using an inverted microscope (Olympus IMT2; Omnilabo, Brussels, Belgium). Narashige manipulators and injectors were used to perform the ICSI procedure (Narashige; Omnilabo).

**Intracytoplasmic sperm injection**

No alterations were made to the ICSI procedure itself or to the media conditions when compared with our routine ICSI with motile spermatozoa. For the ICSI procedure itself, a 7.5 μm (outer diameter) micropipette was used to aspirate individual spermatozoa from the sperm collection droplet and to place them into the PVP. Although most of the spermatozoa were immotile, all tails were broken by gentle rubbing with the micropipette. The spermatozoon was aspirated (tail first) with some PVP into the micropipette and injected into the equatorial region of the oocyte after aspirating some ooplasm.

The injected oocytes were washed four times and incubated in culture medium at 37°C in an atmosphere of 5% CO₂.

**Subzonal sperm injection procedure**

In all, 10–15 spermatozoa were aspirated with a 10 μm (outer diameter) pipette from the sperm collection droplet and released underneath the zona pellucida of the oocytes.

**Assessment of fertilization and embryo cleavage**

At 18–20 h after the ICSI procedure the oocytes were checked for the presence of one, two or more pronuclei. The number of degenerated oocytes was recorded. Prior to transfer (i.e. 42 h after ICSI), the embryos were scored for their number of cells and overall quality aspect: the number and shape of the blastomeres, the percentage of fragments (Nijs et al., 1993a). In general, three grade A embryos were selected for transfer. If available, grade A supernumerary embryos were frozen using the slow propanediol–sucrose protocol (Nijs et al., 1993b).

The transfers were performed using an Edwards-Wallace embryo transfer catheter (International Medical, Brussels, Belgium) ~42–45 h after the ICSI procedure.

**Follow-up**

Luteal phase support consisted of 5000 IU HCG given on days 4 and 8 after transfer Pregnancy was diagnosed on the basis of an HCG concentration >30 mlU/ml in serum on day 15, with confirmation on days 20 and 25 after transfer Vaginal ultrasound sonography was carried out in week 6 after transfer. The presence of a yolk sac, the

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Table I. Motility and concentration of sperm samples of patients included in the study

<table>
<thead>
<tr>
<th>Origin</th>
<th>Ranges of sperm concentration (no/ml)</th>
<th>Initial motility (%)</th>
<th>Motility after preparation and incubation (%)</th>
<th>No of patients included (total = 103)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ejaculate</td>
<td>10–40 × 10⁶</td>
<td>0</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>100 000–60 × 10⁶</td>
<td>0</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Epididymis</td>
<td>50 000–10 × 10⁶</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Testes</td>
<td>100–10 000</td>
<td>0</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>1000–1 × 10⁶</td>
<td>0</td>
<td>0</td>
<td>26</td>
</tr>
</tbody>
</table>

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Table II. Subzonal insemination (SUZI) and intracytoplasmic sperm injection (ICSI) with totally immotile spermatozoa from a patient suffering from Kartagener syndrome

<table>
<thead>
<tr>
<th></th>
<th>SUZI</th>
<th>ICSI</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of oocytes manipulated</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td>No of two-pronuclear/manipulated oocytes</td>
<td>3/12</td>
<td>0/4</td>
</tr>
<tr>
<td>No of embryos transferred</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td>Pregnancy</td>
<td>1</td>
<td>–</td>
</tr>
</tbody>
</table>

Table III. The use of totally immotile spermatozoa from ejaculate, epididymis or testis in an intracytoplasmic sperm injection programme: fertilizing capacity, pregnancy rate, ongoing pregnancy rates, patients entering the freezing programme

<table>
<thead>
<tr>
<th></th>
<th>Ejaculate</th>
<th>Epididymis</th>
<th>Testis</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of patients</td>
<td>12</td>
<td>3</td>
<td>26</td>
</tr>
<tr>
<td>No of two-pronuclear/ injected oocytes</td>
<td>75/141</td>
<td>18/30</td>
<td>142/220</td>
</tr>
<tr>
<td>Fertilization rate (%)</td>
<td>55*</td>
<td>60</td>
<td>65*</td>
</tr>
<tr>
<td>No of transfers</td>
<td>12</td>
<td>3</td>
<td>26</td>
</tr>
<tr>
<td>Patients with transfers (%)</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Mean no of embryos/transfer</td>
<td>3 0</td>
<td>4 3</td>
<td>3 0</td>
</tr>
<tr>
<td>No of pregnancies/transfer</td>
<td>1/12b</td>
<td>0 3</td>
<td>8/26b</td>
</tr>
<tr>
<td>Pregnancy (%)</td>
<td>8</td>
<td>–</td>
<td>31</td>
</tr>
<tr>
<td>Miscarriages/biochemical pregnancy</td>
<td>1* (100)</td>
<td>–</td>
<td>3 (38)</td>
</tr>
<tr>
<td>No of ongoing pregnancies</td>
<td>0 (0)</td>
<td>–</td>
<td>5 (19)</td>
</tr>
<tr>
<td>Patients with freezing programme</td>
<td>–</td>
<td>–</td>
<td>4/26 (15)</td>
</tr>
</tbody>
</table>

Values in parentheses are percentages

*Values with the same superscripts were significantly different (P < 0.05)

Embryo quality and freezing programme

Figure 1 represents the quality scoring of the embryos 42 h after ICSI. When totally immotile spermatozoa were injected into oocytes, the overall embryo quality of the embryos was lower than when initially immotile spermatozoa were used. Fewer grade A embryos resulting from totally immotile spermatozoa (and subsequently more grade B embryos) were available for transfer. Hence, fewer embryos were available for freezing when totally immotile spermatozoa were used (Tables III–IV). Of the patients with ‘initially immotile’ spermatozoa, 15–20% had embryos that could enter the freezing programme. When totally immotile testicular spermatozoa were used, freezing was possible for the embryos from 15% of the patients.

Transfer and pregnancy rates

All 103 patients in this study had an embryo transfer (Tables III and IV). A mean number of 3.2 embryos was transferred.

Table IV. The use of initially immotile spermatozoa from ejaculate, epididymis or testis in an intracytoplasmic sperm injection programme: fertilizing capacity, pregnancy rate, ongoing pregnancy rates, patients entering the freezing programme

<table>
<thead>
<tr>
<th></th>
<th>Ejaculate</th>
<th>Epididymis</th>
<th>Testis</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of patients</td>
<td>20</td>
<td>15</td>
<td>27</td>
</tr>
<tr>
<td>No of two-pronuclear/ injected oocytes</td>
<td>105/162</td>
<td>100/160</td>
<td>143/238</td>
</tr>
<tr>
<td>Fertilization rate (%)</td>
<td>65</td>
<td>63</td>
<td>60</td>
</tr>
<tr>
<td>No of transfers</td>
<td>20</td>
<td>15</td>
<td>26</td>
</tr>
<tr>
<td>Patients with transfers (%)</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Mean no of embryos/transfer</td>
<td>2 6</td>
<td>3</td>
<td>3 3</td>
</tr>
<tr>
<td>No of pregnancies/transfer</td>
<td>6/20a</td>
<td>9/14b</td>
<td>8/27b</td>
</tr>
<tr>
<td>Pregnancy (%)</td>
<td>30</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>Miscarriages/biochemical pregnancy</td>
<td>3 (50)</td>
<td>1 (11)</td>
<td>1 (13)</td>
</tr>
<tr>
<td>No of ongoing pregnancies</td>
<td>3 (15)</td>
<td>8 (53)</td>
<td>7 (26)</td>
</tr>
<tr>
<td>Patients with freezing programme</td>
<td>3/20 (15)</td>
<td>3/15 (20)</td>
<td>4/26 (15)</td>
</tr>
</tbody>
</table>

Values in parentheses are percentages

*Values with the same superscripts were significantly different (P < 0.05)
A mammalian oocyte is not a novel observation: pronucleus formation has been described after the injection of sperm nuclei or intact dead spermatozoa from several species (human, hamster, mouse, rabbit and bull) into hamster oocytes (Uehara and Yanagimachi, 1976, 1977; Martun et al., 1988; Perreault et al., 1988). Gearon et al. (1995) injected non-viable human spermatozoa into aged human oocytes and observed fertilization and early cleavage. Ghunaim et al. (1995) injected totally immotile human spermatozoa stored for 2 days at 4°C and obtained fertilization and development. Other authors did not find any fertilization after ICSI with totally immotile spermatozoa (Li et al., 1995). The reason is probably because of a technical problem rather than the fertilizing potential of the spermatozoan. Because these spermatozoa are immotile, one might think that it is not necessary to immobilize them before injection. However, their tail must still be touched before injection (Vanderzwalmen et al., 1996) to induce a destabilization in the plasma membrane with the release of an activating factor ‘oscillin’ into the ooplasm (Dozortsev et al., 1995; Parrington et al., 1996). Another possibility is that depolymerization of the sperm membrane is necessary for ooplasmic enzymes to reach the sperm nucleus in order to start chromatin decondensation (Tesarik et al., 1994). Totally immotile spermatozoa from ejaculates fertilized significantly fewer oocytes than all the other sperm types. Perhaps the patient had a long period of abstinence and hence spermatozoa had a long transit time through the epididymis. This increases the presence of degenerating or dead spermatozoa in the sperm sample (Bedford, 1994). These aged spermatozoa contain fragile DNA, the protamine packaging has become unstable and pronucleus formation is subsequently hampered.

Overall, 95% of the two-pronuclei oocytes developed into 4- or 8-cell embryos, and all asthenozoospermic patients had embryos transferred.

The observation that totally immotile human spermatozoa can support the early embryo and fetal development confirms studies in the cow by Goto et al. (1990). They described the birth of a calf after ICSI with immobilized killed spermatozoa. In our study, a difference in embryo quality according to the type of immotility, however, was observed: fewer good quality embryos were obtained when totally immotile spermatozoa (of

**Figure 1.** Embryo quality at the moment of transfer (day 2) after intracytoplasmic sperm injection with initially and totally immotile spermatozoa from ejaculate (ejac), epididymus (epid) or testis. Y axis = percentage of embryos X axis = quality/grade of embryo, grade A, B, C.
any origin) were used compared with embryos derived from
initially immotile spermatozoa. Consequently, no embryos
could be cryopreserved for patients with totally immotile
spermatozoa from ejaculate or epididymis. As already
explained, ageing of spermatozoa can result in DNA strand
breaks and, although fertilization and first cleavage stages can
occur, the genome of the spermatozoon is not capable of
completing embryogenesis. Defective protamine packaging in
sperm DNA and their incorrect replacement by histones
during fertilization and cleavage could create problems like
asynchrony and delays during cell cycles just after fertilization.

Some of the anomalies arising in embryos after the use of
immotile spermatozoa could be due to abnormal spindle
formation because the centrioles were abnormal (Asch et al.,
1995). Because for these groups not enough supernumerary
embryos of good quality were available for further culture
in vitro up to the blastocyst stage, we could not confirm this
hypothesis of complete embryo blockage as yet. It confirms,
however, the studies of Janny and Ménéo (1994), who
identified a strong paternal effect on human embryo develop-
ment and blastocyst formation. The presence of only one
biochemical pregnancy (for the totally immotile ejaculated
sperm group), the absence of any pregnancy for totally
immotile epididymal spermatozoa and the relatively high
miscarriage rate (50%) in the initially immotile ejaculated
sperm group can again be explained by the low potential of
the embryonal genome.

Why do some samples show totally immotile spermatozoa
and others obtain some form of motility after a short period?
One of the causes of immotility is the presence of antisperm
antibodies. Their presence interferes considerably with preg-
nancy outcome because they cause a developmental block just
before genomic activation (Naz, 1992). Another type of defect
is structural abnormality. Kartagener syndrome patients suffer
from the absence of dynein arms. However, in the ejaculate
of our patient, dead spermatozoa could also be present. When
performing the micromanipulation, no distinction can be made
between these types of sperm cell. When SUZI was applied,
the theoretical probability that one mature spermatozoon was
present was >10 spermatozoa injected was obviously higher
than when only one spermatozoon is retrieved for ICSI. This
could explain the difference in fertilization rates. Although
only one patient was included in this study, SUZI seems to
be the method of choice for this specific condition (Wolf et al.,
1993).

Some initially immotile spermatozoa obtained some form
of motility after culture. Bedford (1994) described that during
transit through the testis and epididymis, spermatozoa undergo
several biophysical alterations: S–S bonds are stabilized in the
sperm nucleus, in the perinuclear matrix and in various tail
structures. Perhaps preparation, purification and incubation of
these immotile spermatozoa can circumvent these in-vivo
maturation processes (Schowsman et al., 1995). The initially
immotile spermatozoa (from ejaculate, epididymis or testis) have the capacity to recover sluggish motility because most
of this sperm population is probably immature. For the totally
immotile sperm cells, however, it is thought that for epididymal
and ejaculated cells the majority of the spermatozoa are
either degenerative (dead) or mature but structurally affected.
Testicular totally immotile spermatozoa may have delayed
maturation and have not yet switched on their energetic
pathways for motility. This latter maturation process is perhaps
time and/or culture dependent. We are currently evaluating
the influence of a long-term culture (up to 48 h) and different
culture conditions on totally immotile spermatozoa of any
origin. The use of the swelling test (Van der Ven et al., 1986)
was applied on some samples but did not prove to be efficient
because most of the samples contained few spermatozoa after
preparation. We are currently investigating in more detail,
however, if this test (in combination with ICSI) could be used
to identify those spermatozoa that are viable but have not yet
acquired any form of motility. These spermatozoa could then
be injected into oocytes and could prove to be better candidates
for ICSI: in this way, better fertilization rates, better embryo
quality and more ongoing pregnancies could be obtained. If
longer incubation times of totally immotile ejaculated or
epididymal spermatozoa still do not result in an improvement,
one could try to extract sperm cells from the testis of these
patients and determine whether these spermatozoa show the
same pathology.

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human fertilization arrests microtubule and chromosome configurations in
insenminated oocytes which failed to complete fertilization and development

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sperm injection are greater than subzonal insemination but are dependent

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