Pregnancies achieved with testicular and ejaculated spermatozoa in combination with intracytoplasmic sperm injection in men with totally or initially immotile spermatozoa in the ejaculate

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Introduction

Total asthenozoospermia is a severe problem because only the technique of micromanipulation can assist couples, and even then fertilization and pregnancy rates are very low. Total asthenozoospermia may be caused by ultrastructural abnormalities in the sperm tail, including immotile cilia syndrome associated with bronchiectases and situs inversus (Kartagener's syndrome; Yokota et al., 1993; Wolf et al., 1994). While the immotility of the spermatozoa is caused by ultrastructural abnormalities in the tail, most of the sperm population has been proved to be viable (Ukada et al., 1993; Hendry and Cole, 1994). For these patients, micromanipulation of the spermatozoa is recommended as a treatment mode. Although subzonal sperm injection has been shown to be successful for severe asthenozoospermic patients (Terrion et al., 1993; Ukada et al., 1996), intracytoplasmic sperm injection (ICSI) is now the treatment predominantly used. The achievement of high fertilization and pregnancy rates with ICSI has encouraged assisted conception units to prefer ICSI as a treatment mode for couples affected by severe male factor infertility (Palermo et al., 1992).

With the first publication of Schoysman et al. (1993) on fertility and pregnancy using testicular spermatozoa, a new era of male infertility treatment commenced. High fertilization and pregnancy rates after ICSI with spermatozoa obtained from testicular biopsy were reported by Silber et al. (1995). Our results have suggested that higher implantation and pregnancy rates can be achieved by using testicular spermatozoa in both non-obstructive and obstructive groups (Kahraman, et al., 1996).

In this study, the efficacy of ICSI using testicular and ejaculated spermatozoa in patients with totally or initially immotile spermatozoa was evaluated.

Materials and methods

Patients

A total of 24 couples with total asthenozoospermia who were referred to our infertility clinic were included in the study. In 10 patients ejaculated spermatozoa were used, whereas in 14 patients the spermatozoa were recovered from the testis. Of the 24 male partners, 14 also had total teratozoospermia. The sperm findings of total asthenozoospermia and total asthenoteratozoospermia were confirmed after Percoll separation.

The efficacy of intracytoplasmic sperm injection (ICSI) employing testicular and ejaculated spermatozoa was assessed in 24 couples with totally or initially immotile spermatozoa. No criteria were employed in selecting which patients would be treated with testicular or ejaculated spermatozoa. The men were chosen at random. Testicular spermatozoa obtained by testicular sperm extraction were used in 14 and ejaculated spermatozoa were used in 10 of these couples. In all cases, asthenozoospermia was total in their basal semen sample. In 12 male partners, spermatozoa were totally immotile before and after Percoll gradient fractionation (totally immotile). In the remaining 12 men, spermatozoa initially showed a total absence of motility; however, some of the spermatozoa had showed very poor motility (0.1%) after Percoll gradient fractionation and a 1.5–2.0 h incubation period (initially immotile). Of these 24 total asthenozoospermic males, 14 also had total teratozoospermia. The fertilization and cleavage rates in the testicular and ejaculated sperm groups were 53.5 and 96.3 and 54.5 and 94.4% respectively. One cycle resulted in complete fertilization failure, and in 23 embryo transfer cycles a total of 10 pregnancies were obtained (41.6%). Eight pregnancies were achieved in the testicular sperm group, while only two pregnancies were obtained in the ejaculated sperm group. Four pregnancies, two from the ejaculated sperm group and two from the testicular sperm group, resulted in clinical abortions in the first trimester. Of the remaining six pregnancies, two have already resulted in healthy births and four pregnancies are now in the second or third trimester in the testicular sperm group.

Using testicular spermatozoa in combination with ICSI can be an alternative mode of treatment in cases with totally or initially immotile spermatozoa in the ejaculate. Very low pregnancy rates have been obtained and no ongoing pregnancy has been achieved using ejaculated spermatozoa in these cases.
Sperm sampling
Where possible, semen samples were collected by masturbation after 3–5 days of sexual abstinence. Samples were allowed to liquefy for at least 30 min at 37°C before analysis. Sperm density and motility were evaluated according to the recommendations of the World Health Organization (1992). Sperm morphology was evaluated by Kruger’s strict criteria (Kruger et al., 1986). After liquefaction, the semen samples were washed in Earle’s medium by centrifuging for 5 min at 1800 g. The pellet was applied to a three-layer Percoll gradient (90/70/50%) and then centrifuged at 300 g for 20 min. The 90% Percoll fraction was again washed with Earle’s medium for 5 min at 1800 g and the pellet was centrifuged in Earle’s medium just before microinjection.

Testicular sperm sampling
Testicular tissue samples were obtained by open biopsy on the same day that the oocytes were retrieved. The testicular tissue obtained was placed in a Falcon tube containing 1 ml HEPES-buffered Earle’s medium. The testicular tissue was progressively divided into small segments. It was gently crushed between microneedles in a Petri dish containing HEPES-buffered medium to obtain a suspension of spermatozoa. This suspension was then transferred into a Falcon tube and rotated for 5–60 s with a vortex. It was then examined at ×200 magnification in a Petri dish containing 5 ml HEPES-buffered Earle’s medium. If sperm cells were revealed to be present, the suspension was centrifuged on a two-layer Percoll gradient (70/90%) and freed from all debris and eventually from red blood cells. The testicular tissue solution was kept in an incubator (5% CO₂ in air) at 37°C until the ICSI procedure.

Ovarian stimulation
Gonadotrophin-releasing hormone analogue (Suprefact; Hoechst, Frankfurt, Germany) was given as a nasal spray, starting in the luteal phase, and was continued for 14 days until sufficient pituitary down-regulation was achieved. Follicular development was then stimulated with an injection of follicle stimulating hormone (FSH; Metrodin; Serono, Rome, Italy) and human menopausal gonadotrophin (Humegon; Organon, Oss, The Netherlands). Ovulation was induced by a 10 000 IU human chorionic gonadotrophin (HCG; Pregnyl; Organon, Istanbul, Turkey) injection, and oocyte aspiration was performed 36 h later under guidance using vaginal ultrasound.

Oocyte preparation
After oocyte retrieval, the cumulus cells and the corona radiata were removed by a brief exposure to HEPES-buffered Earle’s medium containing 80 IU/ml hyaluronidase (type VIII; specific activity 320 IU/mg; Sigma Chemical Co., St Louis, MO, USA). The oocytes were completely denuded by aspiration of the cell complex in to and out of a hand-drawn glass pipette. The oocytes were rinsed several times in HEPES-buffered Earle’s medium and were assessed under an inverted microscope to determine their stage of maturity. Oocytes were incubated in Earle’s balanced salt solution medium at 37°C in an atmosphere of 5% CO₂ in air and covered by paraffin oil. Only metaphase II oocytes were microinjected.

ICSI procedure
The pipettes were made from 30 µl borosilicate glass capillary tubes (Drummond Scientific Company, Broomall, PA, USA) with a 0.97 mm external diameter, a 0.69 mm internal diameter and a length of 78 mm. After washing several times, the pipettes were pulled on a horizontal microelectrode puller (PB-7; Narishige Co. Ltd, Tokyo, Japan). The pulled capillary was opened on a microgrinder (EG-4; Narishige Co. Ltd). The inner diameter of the microinjecting pipette was 7 µm and the bevel angle was 50°, which was bent by a microforge (MF-9; Narishige Co. Ltd). The holding pipette was also cut and fire-polished on a microforge which had a 20 µm inner and a 80 µm outer diameter.

Oocytes were placed one by one in HEPES-buffered Earle’s medium droplets. The spermatozoa were aspirated one by one with a 15 µm (tip diameter) pipette and transferred into the injection dish containing a droplet of 5 µl HEPES-buffered Earle’s medium, before being transferred into a droplet of 10% polyvinylpyrrolidone (PVP; P5288; Sigma Chemical Co.). The ICSI procedure was performed on the heated stage of an inverted microscope (IMT-2; Olympus Corporation, Tokyo, Japan). The microscope was equipped with two coarse positioning manipulators (MM-188 and MO-109; Narishige Co. Ltd). A single living spermatozoon, immobilized by touching the tail, was aspirated tail first into the injection pipette. Touching of the tail was performed even in the presence of a totally immotile sperm sample. The oocyte was held by gentle suction on the holding pipette. The first polar body was placed at the 12 or 6 o’clock position. The injecting pipette containing the spermatozoon was introduced across the zona pellucida and into the oocyte’s cytoplasm. Gentle suction was applied carefully to break the oolemmal membrane and to aspirate a minimal amount of ooplasm until the visualization of cytoplasmatic breakage. The immobilized spermatozoa were inserted, together with the withdrawn ooplasm and the smallest volume of PVP solution possible, and the injecting pipette was withdrawn gently. The injected oocytes were placed in a four-well Nunc dish for incubation.

Further evaluation of injected oocytes and the establishment of pregnancy
Fertilization checks took place at 16–18 h after injection. Fertilization was assessed as normal when two clearly distinct pronuclei containing nuclei were present. The states of embryo cleavage and quality were assessed after a further 24 h of in-vitro culture. The embryos were evaluated according to the blastomere size equality and the relative proportion of anucleate fragments. A maximum of four embryos were transferred in exceptional cases when all available embryos had >50% fragmentation. All patients underwent an initial test for serum β-HCG at 12–14 days after embryo transfer. The existence of a pregnancy was confirmed by the detection of increasing serum β-HCG concentrations 3 days later. A clinical pregnancy was diagnosed by ultrasonography at 7 weeks of gestation. All couples were counselled and agreed to have a prenatal diagnosis. This was performed by amniocentesis at 16 weeks of pregnancy.

Results
The mean ± SD ages of the female and male patients were 28.8 ± 5.4 and 34.4 ± 6.9 years respectively, and the mean ± SD duration of infertility was 7.5 ± 4.9 years. The median value of sperm concentration was 1.1×10⁹/ml, with a range of 100×10³ to 100×10⁶. All sperm samples were totally or severely asthenozoospermic after Percoll gradient fractionation. The results are shown in Table I. Of the 207 oocytes retrieved, 167 were metaphase II and were subjected to injection. In all, 54 oocytes in the testicular sperm and 36 oocytes in the ejaculated sperm groups fertilized normally with two distinct pronuclei, of which 52 and 34 achieved cleavage in these two groups respectively. Fertilization and cleavage rates in the testicular and ejaculated sperm groups were 53.5 and 96.3 and 54.5 and 94.4% respectively. A total of 23 patients underwent embryo transfer; 52 embryos in the
Table I. Outcome of intracytoplasmic sperm injection in testicular and ejaculated sperm groups

<table>
<thead>
<tr>
<th>Sperm groups</th>
<th>Testicular</th>
<th>Ejaculated</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>10</td>
<td>14</td>
<td>24</td>
</tr>
<tr>
<td>No. of oocytes retrieved</td>
<td>92 (71.7)</td>
<td>115 (87.8)</td>
<td>207</td>
</tr>
<tr>
<td>No. of metaphase II oocytes</td>
<td>66 (101.0)</td>
<td>87 (101.0)</td>
<td>153</td>
</tr>
<tr>
<td>No. of oocytes fertilized</td>
<td>34 (54.5)</td>
<td>54 (53.5)</td>
<td>88</td>
</tr>
<tr>
<td>No. of embryos cleaved</td>
<td>34 (94.4)</td>
<td>52 (96.3)</td>
<td>86</td>
</tr>
<tr>
<td>No. of embryos transferred</td>
<td>34 (94.4)</td>
<td>52 (96.3)</td>
<td>86</td>
</tr>
<tr>
<td>No. of embryos transferred</td>
<td>10 (100)</td>
<td>13 (92.9)</td>
<td>23</td>
</tr>
<tr>
<td>No. of pregnancies</td>
<td>2 (20.0)</td>
<td>8 (57.1)</td>
<td>10</td>
</tr>
<tr>
<td>No. of abortions</td>
<td>2 (100.0)</td>
<td>2 (25.0)</td>
<td>4</td>
</tr>
<tr>
<td>No. of ongoing pregnancies</td>
<td>–</td>
<td>52 (62.9)</td>
<td>6</td>
</tr>
</tbody>
</table>

Values in parentheses are percentages.

testicular sperm group and 34 embryos in the ejaculated sperm group were transferred. A total of 10 (41.7%) pregnancies were achieved. Eight pregnancies were achieved in the testicular sperm group, both belonging to the initially immotile sperm group, but they resulted in a clinical abortion. Only two pregnancies were achieved in the ejaculated sperm group, both belonging to the initially immotile sperm group, but they resulted in a clinical abortion. The ongoing pregnancy rate per started cycle was 25.0%. Six pregnancies are now in the second or third trimester in the testicular sperm group, while no ongoing pregnancy was achieved in the ejaculated sperm group.

Discussion

With the improvement of microinsemination techniques, the range of treatments for male infertility has been enlarged. The first human pregnancies after ICSI were obtained by using motile spermatozoa (Palermo et al., 1992, 1993; Van Steirteghem et al., 1993a,b). The use of motile spermatozoa in humans has been recommended, and when immobilized spermatozoa are used they are believed to be necessary to provide positive activation of the oocyte's cytoplasm to achieve a successful fertilization (Hoshi et al., 1995). However, the treatment protocols for immotile spermatozoa have yet to be established. Immotile spermatozoa may be capable of fertilizing an oocyte after ICSI. As was suggested by Silber (1995), in certain cases, excluding Kartagener's syndrome, sperm senescent degeneration and perhaps delayed epididymal transport may be responsible for sperm immotility. However, in these cases, testicular spermatozoa may not be senescent and the lack of motility may not be a serious problem. It was also suggested by Silber that although no pregnancies have yet been achieved using ICSI with non-motile ejaculated or epididymal spermatozoa, one could speculate that pregnancies in such cases may be possible using testicular spermatozoa.

To evaluate the use of testicular spermatozoa when the ejaculate contains totally immotile spermatozoa, we attempted ICSI using both ejaculated and testicular spermatozoa and compared the results in terms of the fertilization, cleavage and pregnancy rates.

In our study, no significant differences were observed between the fertilizing capacities of testicular and ejaculated spermatozoa. The problem associated with using immotile spermatozoa is that the viability of the single spermatozoon used for injection is not determined, although most of these spermatozoa have been proved to be viable (Hendry and Cole, 1994). In this study, the efficacy of ICSI as a treatment mode for cases with totally immotile spermatozoa was assessed. The semen analysis of cases included in this study revealed no motility in their basal sample and 0.0 or <0.1% motility after Percoll gradient fractionation. In selecting the vital spermatozoa, sperm vitality tests were employed. However, during the ICSI procedure an immotile spermatozoon can cause difficulties and uncertainty as to whether or not it is viable.

When testicular sperm extraction is used, we prefer to fractionate the suspension on a Percoll gradient (47.5/95%) if a microscopic examination reveals the presence of sperm cells. Verheyen et al. (1995) compared four mechanical methods to retrieve spermatozoa from testicular tissue, and they concluded that the treatment of testicular tissue by fine mincing followed by discontinuous Percoll centrifugation was the most effective. Nagy et al. (1995) showed that there was no important influence from either the type or the extent of sperm impairment on the outcome of ICSI; even in the most severe form of male infertility, high fertilization and pregnancy rates were obtained in this way.

Only one condition appeared to have a strongly negative influence on the result of ICSI. This concerned injection into the oocyte of immotile spermatozoa (Nagy et al., 1995), and no pregnancy was achieved from this group in this study. The males who had totally immotile spermatozoa both before and after Percoll gradient fractionation were questioned to ascertain if they had ever had any signs of Kartagener's syndrome. They were all shown to be negative as no such signs were discovered. However, an electron microscopic evaluation of spermatozoa could not be performed to rule out this pathology. Ejaculated spermatozoa were obtained in seven cases, while spermatozoa were recovered from the testis in 12 cases. Morphologically no difference was found between ejaculated or testicular spermatozoa. In 14 cases, severe morphological defects were present, coupled with totally immotile sperm characteristics.

In three previous studies where totally immotile or initially immotile spermatozoa were used, no ongoing pregnancy was achieved. Immotile spermatozoa may be responsible for sperm immotility. However, in these cases, testicular spermatozoa may not be senescent and the lack of motility may not be a serious problem. It was also suggested by Silber that although no pregnancies have yet been achieved using ICSI with non-motile ejaculated or epididymal spermatozoa, one could speculate that pregnancies in such cases may be possible using testicular spermatozoa.

Table II. Outcome of intracytoplasmic sperm injection according to sperm motility after Percoll gradient fractionation

<table>
<thead>
<tr>
<th></th>
<th>Totally immotile</th>
<th>Initially immotile</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>12</td>
<td>12</td>
<td>24</td>
</tr>
<tr>
<td>No. of oocytes retrieved</td>
<td>103</td>
<td>104</td>
<td>207</td>
</tr>
<tr>
<td>No. of metaphase II oocytes</td>
<td>92 (89.3)</td>
<td>74 (71.2)</td>
<td>167</td>
</tr>
<tr>
<td>No. of oocytes fertilized</td>
<td>48 (52.2)</td>
<td>42 (56.8)</td>
<td>90</td>
</tr>
<tr>
<td>No. of embryos cleaved</td>
<td>46 (95.8)</td>
<td>40 (95.2)</td>
<td>86</td>
</tr>
<tr>
<td>No. of embryos transferred</td>
<td>46</td>
<td>40</td>
<td>86</td>
</tr>
<tr>
<td>No. of pregnancies</td>
<td>7 (58.3)</td>
<td>3 (25.0)</td>
<td>10</td>
</tr>
<tr>
<td>No. of abortions</td>
<td>3 (42.9)</td>
<td>1 (33.3)</td>
<td>4</td>
</tr>
<tr>
<td>No. of ongoing pregnancies</td>
<td>4 (33.3)</td>
<td>2 (16.7)</td>
<td>6</td>
</tr>
</tbody>
</table>

Values in parentheses are percentages.
obtained with ICSI (Nagy et al., 1995; Nijs et al., 1995; Van Steirteghem et al., 1995).

In our study the fertilization rate was the same in both the ejaculated and testicular sperm groups. The only ongoing pregnancies were achieved in the testicular sperm group. In the ejaculated sperm group only two pregnancies were obtained, which both resulted in a clinical abortion (Table I). The ongoing pregnancy rate was higher in the totally immotile sperm group, although no difference was observed between fertilization and cleavage rates (Table II).

The high number of totally immotile spermatozoa in the ejaculate is mostly thought to be degenerative, while totally immotile testicular spermatozoa are probably immature and can still gain their motility during maturation. The totally immotile spermatozoa in the testis may therefore be immature and viable. In non-obstructive azoospermia, it has been shown that fertilization and pregnancy can be achieved even if the spermatozoa are totally immotile (Devroy et al., 1995). In cases of obstructive azoospermia, where micro-epididymal sperm aspiration is impossible because of totally destroyed epididymis, or in non-obstructive azoospermia with severe spermatogenic defects, a higher pregnancy rate was achieved. Furthermore, in our unpublished data higher implantation and pregnancy rates were achieved. These results suggest that higher pregnancy rates can be obtained when testicular spermatozoa are used. Thus, testicular sperm extraction was performed in 14 couples and the results were compared with the ejaculated sperm group (n = 10).

Although the number of patients was low, the study suggests for the first time that a higher pregnancy rate can be achieved using testicular spermatozoa in cases of totally immotile spermatozoa. Thus, testicular spermatozoa may be preferential to ejaculated spermatozoa in obtaining pregnancies from men with totally immotile spermatozoa. It is possible that the role of the ductus epididymis should be questioned.

References


