Fertilization and pregnancy outcome with intracytoplasmic sperm injection for azoospermic men

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The evident ability of the intracytoplasmic sperm injection (ICSI) procedure to achieve high fertilization and pregnancy rates regardless of semen characteristics has induced its application with spermatozoa surgically retrieved from azoospermic men. Here, ICSI outcome was analysed in 308 cases according to the cause of azoospermia; four additional cycles were with cases of necrozoospermia. All couples were genetically counselled and appropriately screened. Spermatozoa were retrieved by microsurgical epididymal aspiration or from testicular biopsies. Epididymal obstructions were considered congenital ($n = 138$) or acquired ($n = 103$), based on the aetiology. Testicular sperm cases were assessed according to the presence ($n = 14$) or absence ($n = 53$) of reproductive tract obstruction. The fertilization rate using fresh or cryopreserved epididymal spermatozoa was 72.4% of 911 eggs for acquired obstructions, and 73.1% of 1524 eggs for congenital cases; with clinical pregnancy rates of 48.5% ($50/103$) and 61.6% ($85/138$) respectively. Spermatozoa from testicular biopsies fertilized 57.0% of 533 eggs in non-obstructive cases compared to 80.5% of 1181 eggs in non-obstructive cases compared to 80.5% of 1181 eggs in non-obstructive cases compared to 80.5% of 1181 eggs in congenital cases ($P = 0.0001$) in obstructive azoospermia. The clinical pregnancy rate was 49.1% ($26/53$) for non-obstructive cases and 57.1% ($8/14$) for testicular spermatozoa obtained in obstructive azoospermia, including three established with frozen–thawed testicular spermatozoa. In cases of obstructive azoospermia, fertilization and pregnancy rates with epididymal spermatozoa were higher than those achieved using spermatozoa obtained from the testes of men with non-obstructive azoospermia.

Key words: azoospermia/intracytoplasmic sperm injection/microsurgical epididymal sperm aspiration/testicular sperm extraction

Introduction

Intracytoplasmic sperm injection (ICSI) has gained an increasing popularity due to its consistent fertilization rate and high pregnancy outcome (Palermo et al., 1992, 1993, 1995; Van Steirteghem et al., 1993). ICSI has been used largely to treat male factor infertility, with fertilization and pregnancy rates being comparable to those obtained in couples with good semen parameters undergoing standard in-vitro insemination. The evident ability of ICSI to achieve high fertilization and pregnancy rates regardless of ejaculated semen parameters has extended its application to azoospermic patients. Injected epididymal (Tournaye et al., 1994) and testicular spermatozoa (Schoysman et al., 1993) have been used to effect fertilization and pregnancies (Nagy et al., 1995). These fertilization rates were significantly lower than the rates obtained with freshly ejaculated spermatozoa, but increased fertilization and pregnancy rates were seen after a more aggressive permeabilization of the sperm membrane (Palermo et al., 1996b).

Original concerns about ICSI relating to its aggressiveness and arbitrary sperm selection have been eased by reports on the outcome and follow-up of the ICSI newborns (Bonduelle et al., 1994; Palermo et al., 1996a). The presence of a significant frequency of Y chromosome microdeletions and karyotypic alterations (de Kretser et al., 1972; Martin and Rademaker, 1988; Rucker et al., 1998) in men with non-obstructive azoospermia has raised concerns about the risks of treatment for these men.

The aetiology of azoospermia can be divided into obstructive and non-obstructive categories. The former is characterized by normal sperm production and is often caused by congenital maldevelopment of the vas deferens, a condition known to be associated with cystic fibrosis gene mutations. The treatment for obstructive azoospermia is microsurgical epididymal sperm aspiration (MESA), and when epididymal access is lacking is testicular sampling appropriate. Non-obstructive azoospermia is characterized by a varying degree of spermatogenic failure and at times is associated with an increased number of chromosomal abnormalities (Reijo et al., 1996; Girardi et al., 1997). The only method to retrieve spermatozoa from this form of azoospermia is by direct extraction of spermatozoa or germ cells from the testis.

The present study analyses the outcome of 308 consecutive ICSI cycles with spermatozoa surgically retrieved from azoospermic patients, and any possible relationship between the collection method, the aetiology of the azoospermia, and the ICSI outcome. The genetic profile of the patients presenting with obstructive azoospermia was recorded. To assess the safety of the ICSI procedure, the incidence of chromosomal abnormality in the embryos and fetuses and the occurrence of neonatal malformations were evaluated.

Materials and methods

Patients

From September 1993 through December 1997, assisted fertilization by ICSI was performed in 308 cycles using fresh or cryopreserved...
surgically retrieved spermatozoa. Prior to assisted reproduction, all men were evaluated to determine the aetiology of azoospermia. An extensive questionnaire, physical examination and hormonal analysis were performed to detect any treatable causes of azoospermia. For men with obstructive azoospermia, the option of microsurgical reproductive tract reconstruction was provided. For men with treatable abnormalities that may cause non-obstructive azoospermia, including hormonal anomalies, gonadotoxic exposures or varicoceles, treatment or removal of gonadotoxic influences was allowed. At least two centrifuged semen analyses were carefully examined. A delay of at least 6 months after prior diagnostic tests, testicular sperm extraction (TESE) or other scrotal procedures was provided to optimize the chances of sperm retrieval (Schlegel and Su, 1997). A repeat semen analysis was obtained on the day of sperm retrieval for all men, except those with bilateral congenital absence of the vas deferens. A total of 241 cycles was performed with epididymal spermatozoa and 67 cycles with testicular spermatozoa. Four additional testicular biopsy cycles were performed in patients with necrozoospermia. In 14 couples, no spermatozoa were retrieved and an attempt to inject immature germ cells was made. The mean age of the female partner was 34.5 ± 5 years. Patients undergoing ICSI agreed with the proceedings elucidated in the Clinical Informed Consent for Assisted Fertilization devised by The Center for Reproductive Medicine and Infertility. The form details all steps of the in vitro fertilization (IVF) procedure including the risk of carrying multiple fetuses after embryo transfer.

Genetic screening

All couples with non-obstructive azoospermia were offered genetic screening with molecular analysis for Y chromosome deletions as well as standard peripheral karyotype analysis. High resolution G-banding was performed on mitotically arrested lymphocytes isolated from peripheral blood (Seabright, 1971; Rucker et al., 1998). An assessment for Yq microdeletions was performed on DNA of peripheral leukocytes by multiplex PCR (Girardi et al., 1997).

Men with idiopathic or congenital obstructive azoospermia, including those with congenital bilateral absence of the vas deferens and their female partners were recommended to have cystic fibrosis gene mutation analysis prior to treatment (Schlegel et al., 1995a). Genetic counselling was offered to all couples. Cytophenetic analysis of amniotic fluid was performed by direct culture of cytrophoblastic cells overnight and harvest of chromosmes within 24 h, or after long-term culture of mesechnymal cells with a harvest of chromosmes within 7 days. Giemsa banding (trypsin) staining procedure was subsequently performed on both direct and long-term cultures (Wolf, 1974).

Semen collection and analysis

Epididymal sperm retrieval

Men with irreparable obstructive azoospermia were offered the choice of MESA (Schlegel et al., 1994; 1995b) or percutaneous testicular retrieval of spermatozoa (Sheynkin and Schlegel, 1997). For MESA procedures, 1–5 µl of fluid was aspirated from the lumen of an individual epididymal tubule in the midportion of the obstructed epididymis. Initial puncture with a 300–350 µm glass pipette was secured; this fluid was then diluted with 300 µl of human tubal fluid (HTF) medium. Additional proximal punctures of the epididymis were performed until enough spermatozoa of optimum quality were obtained. Because spermatozoa are highly concentrated (often over 1x10^6/µl) in epididymal fluid, only microlitre quantities are generally required. During 131 attempts at MESA simultaneously with ICSI for obstructive azoospermia, epididymal or vasal spermatozoa were retrieved in 129 procedures (98.5%). Testicular sperm retrieval was performed in one case because of prior removal of the epididymis from a scrotal surgical procedure, and testicular sperm retrieval was performed in a second case to allow microsurgical cross-over vasal reconstruction in the presence of a contralateral non-functioning testis with ipsilateral vasal obstruction. ICSI cases using fresh or cryopreserved epididymal spermatozoa were considered congenital (n = 138) or acquired (n = 103) according to the aetiology of the obstruction. Epididymal fluid was diluted, analysed, and processed by two- or three-layer density gradient centrifugation. After removal of the density gradient medium, a 1 µl aliquot of the final suspension at ~1x10^6/ml was placed in the injection dish (Palermo et al., 1993, 1995).

Cryopreservation of epididymal spermatozoa

Excess epididymal spermatozoa were cryopreserved (n = 112) for later use, thus avoiding the need for repeated microsurgery (Verheyen et al., 1993). An average of 5.5 vials of spermatozoa were obtained per MESA procedure (Brandell et al., 1998a). The sperm suspension (at a concentration of ~3x10^6/ml) was diluted with an equal amount of cryopreservation medium (freezing medium: test–yolk buffer with glycerol; Irvine Scientific, Irvine, CA, USA). Up to 1 ml aliquots of the final solution were placed in 1 ml cryogenic vials (Nalgene® Brand Products, Rochester, NY, USA). The vials were kept at –20°C for 35 min, exposed to liquid nitrogen vapour at –70°C for 10 min, and then plunged in liquid N₂ at –196°C. When required, vials were brought to room temperature to allow thawing. Epididymal samples were processed similarly to fresh semen and when necessary were exposed to a motility enhancer (3.5 mM pentoxifylline) to allow selection of viable spermatozoa (Palermo et al., 1996b).

Testicular sampling

For men with non-obstructive azoospermia, spermatozoa were retrieved directly from the testis because they do not collect in the epididymis. Typically, multiple biopsies are needed to find the rare spermatogenic foci that are present within the testicles of men with non-obstructive azoospermia (Figure 1). A single open biopsy was obtained from the testis with better histology on diagnostic biopsy, or from the larger testis when a diagnostic biopsy result was not available. Biopsies were performed under optical magnification to identify and preserve the sub tunical testicular blood supply, and biopsy incisions were placed in avascular regions. Each biopsy was dispersed and analysed as described below (Schlegel et al., 1997). If spermatozoa were not retrieved, subsequent biopsies were obtained until either sperm cells were found or no avascular regions of the testis were left. Up to 14 biopsies were required to first identify spermatozoa (Ostad et al., 1998).

Each biopsy specimen (Figure 2) of up to 500 mg was rinsed in culture medium to remove red blood cells, separated into individual tubules with sterile glass slides, and minced using scissors. The resulting suspension of seminiferous tubules was then sequentially passed through a 24 gauge angiocatheter to enhance disruption of the tubules. Individual testicular samples were distributed into 5.0 ml centrifuge tubes (Falcon; Becton Dickinson, Lincoln Park, NJ, USA) containing an excess of culture medium. To assess the presence of spermatozoa, a small amount of suspension medium was carefully observed under a phase-contrast microscope at ×200–400. In preparation for ICSI, after the shredded testicular tissue was removed, the suspension medium was subsequently centrifuged for 500–1800 g for 5 min, with the pellet being subjected to a single-layer density gradient centrifugation. In recent cases, it has been possible to identify individual, enlarged seminiferous tubules that presumably contain spermatogenesis in a background of thin, sclerotic tubules (Figure 3). In the five most recent cases only 3–10 mg of testicular tubules
ICSI in azoospermic patients

Figure 1. The typically small size of the testis from a man with non-obstructive azoospermia (Klinefelter’s syndrome) during a testicular biopsy attempt. This testis measures only 1×1.5 cm.

Figure 2. A standard testis biopsy is viewed under optical magnification during a testicular sperm extraction procedure. An ample portion of testicular parenchyma is removed.

Figure 3. Testicular biopsy specimen from a man with non-obstructive azoospermia. In one tubule Sertoli cell-only pattern can be observed while others are sclerotic. Extensive hyperplasia of interstitial (Leydig) cells is also present (original magnification ×100).

Figure 4. Testicular biopsy specimen obtained from a man with Klinefelter’s syndrome (haematoxylin and eosin). The stromal tissue is prevalent over the distribution of the seminiferous tubules (original magnification ×100).

were excised (Schlegel et al., 1998). Testicular cases were analysed according to the presence (n = 14) or absence (n = 53) of reproductive tract obstruction. Percutaneous sperm retrieval was performed under sterile conditions after a spermatic cord block was secured with 7–10 ml of 1% lignocaine. Local anaesthetic was also introduced into the scrotal skin at the percutaneous biopsy site. Testicular samples were obtained with a 14 gauge automatic biopsy gun. Biopsy samples were placed into 200–300 µl aliquots of culture medium and examined under a phase-contrast microscope to determine the adequacy of sperm retrieval and repeat biopsies were obtained through the same site until the presence of spermatozoa was documented. The specimen was then placed directly into a 40 µl drop under oil where the tips of two 25 gauge needles on 1 ml syringes were used to fray the testicular tissue. The tissue was removed from the droplet and 3 µl of the suspension was placed under oil directly onto the injection dish.

Cryopreservation of testicular spermatozoa

Testicular tissue was cryopreserved similarly to epididymal spermatozoa in five cases. Suspensions of testicular tissue were cryopreserved by addition of equal amounts v/v of cryopreservation medium (Irvine Scientific) and processed as described above.

Analysis of retrieved sperm specimens

Sperm concentration and motility were assessed in a sperm counting chamber. The morphology parameters were evaluated and classified according to strict criteria (Kruger et al., 1986).

Ovarian stimulation and oocyte preparation

Oocyte retrieval was performed after pituitary desensitization with gonadotrophin-releasing hormone agonist and ovulation induction with gonadotrophins (Palermo et al., 1995, 1996a,b) and the oocytes were exposed to hyaluronidase to remove cumulus–corona cells (Palermo et al., 1995).

Microinjection procedure and embryological evaluation

The micromanipulation setting, the characteristics of the microtools as well as the details of injection procedure have been previously described (Palermo et al., 1995, 1996b), as have the selection of the spermatozoon and its immobilization–permeabilization method (Palermo et al., 1996a). When no spermatozoa were available, an attempt to retrieve round spermatids was made according to the criteria that we have previously described (Colombero et al., 1997a). Oocytes were examined 12–17 h after the injection procedure for fertilization, defined by the presence of two distinct pronuclei and two clear polar bodies. The evaluation for early embryonic cleavage was performed after an additional 24 h. Morphologically good quality embryos were transferred into the uterine cavity on the third day after the microinjection procedure.
and whether samples used were fresh or cryopreserved. Spermatozoa were used according to the aetiology of the azoospermia used to compare the age in the patients where surgically retrieved partners with normal sperm concentration (Table II). Male mentions. The frequency of Yq microdeletions was analysed in azoospermia who were screened for presence of Yq microdeletions, 15 (20.0%) (Table I). Ninety-nine testicular biopsies were performed in 16 cases of obstructive azoospermia and in 83 of non-obstructive azoospermia. Spermatozoa were successfully retrieved in all (n = 14) of the obstructive azoospermia cycles and in 63.9% azoospermia analysed for chromosomal abnormalities and Yq deletions, 13 had Y chromosome abnormalities. These cases included 10 patients with Yq microdeletions, one case with mosaic 45,X/46,XY, and one case of 46,XX with sex determining gene present (SRY+). (Sinclair et al., 1990; Rucker et al., 1998). For men with a Y chromosome deletion involving the AZFb region, seven attempts of TESE were performed and no mature spermatozoa were obtained from the testis. In two of these cases, round spermatogonial cells were isolated and injected into oocytes without achieving any normal fertilization (Brandell et al., 1998b). For the 75 patients with non-obstructive azoospermia who underwent testicular biopsy for sperm retrieval for ICSI, karyotypic abnormalities were found in 15 (20.0%) (Table I). These patients represent a subset of the total 218 patients included 10 patients with Yq microdeletions, one case with mosaic 45,X/46,XY, and one case of 46,XX with sex determining gene present (SRY+) (Sinclair et al., 1990; Rucker et al., 1998). For men with a Y chromosome deletion involving the AZFb region, seven attempts of TESE were performed and no mature spermatozoa were obtained from the testis. In two of these cases, round spermatogonial cells were isolated and injected into oocytes without achieving any normal fertilization (Brandell et al., 1998b).

### Results

#### Cytogenetics

For the 75 patients with non-obstructive azoospermia who underwent testicular biopsy for sperm retrieval for ICSI, karyotypic abnormalities were found in 15 (20.0%) (Table I).

These patients represent a subset of the total 218 patients previously evaluated at our centre with oligozoospermia and azoospermia who were screened for presence of Yq microdeletions. The frequency of Yq microdeletions was analysed in relation to the concentration of spermatozoa (Table II). Male partners with normal sperm concentration (>20×10^6/ml) had no deletions. Some oligozoospermic patients had microdeletions when the semen concentration was ≤5×10^6/ml (3/38; 7.9%) and a similar rate was observed in the azoospermic group (14/155; 9.0%). Among 75 men with non-obstructive azoospermia, 30 (40.0%) had Yq deletions, 15 (20.0%) had Yq microdeletions, 10 (13.3%) had Yq mosaic, and 12 (16%) had no Yq deletions. The frequency of Yq deletions was analysed in non-obstructive azoospermia cycles and in 63.9% and frozen–thawed epididymal spermatozoa.

### Table I. Chromosomal abnormalities of male partners with non-obstructive azoospermia

<table>
<thead>
<tr>
<th>Chromosomal abnormalities</th>
<th>Autosomal</th>
<th>Gonosomal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Translocation</td>
<td>1</td>
<td>46,XY(delYq)</td>
</tr>
<tr>
<td>Inversions</td>
<td>2</td>
<td>mos45/X/46,XY</td>
</tr>
<tr>
<td></td>
<td></td>
<td>46,XX(SRY+)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Klinefelter's syndrome</td>
</tr>
<tr>
<td></td>
<td></td>
<td>47,XXY</td>
</tr>
<tr>
<td></td>
<td></td>
<td>46,XY/47,XXY/48,XXXY</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48,XXXY/49,XXXXY</td>
</tr>
<tr>
<td>Total</td>
<td>3</td>
<td>12</td>
</tr>
</tbody>
</table>

a(SRY+) = sex determining gene positive on Y chromosome.

### Table II. Incidence of Yq chromosome microdeletions

<table>
<thead>
<tr>
<th>Sperm concentration (×10^6/ml)</th>
<th>Male patients</th>
<th>With deletions (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>155</td>
<td>14 (9.0)</td>
</tr>
<tr>
<td>1</td>
<td>23</td>
<td>2 (8.7)</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
<td>1 (6.6)</td>
</tr>
<tr>
<td>20</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>&gt;20</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>218</td>
<td>17 (7.8)</td>
</tr>
</tbody>
</table>

**Pregnancy assessment and therapeutic implantation support**

Starting on the day of oocyte retrieval, methylprednisolone (16 mg/day) and tetracycline (250 mg every 6 h) were administered for 4 days to all patients. Progesterone administration (25–50 mg i.m./day) was started on day 3 after human chorionic gonadotrophin administration and was continued until the establishment of pregnancy.

**Statistical analysis**

Statistical comparison was carried out by χ² analysis, two-tailed at 5% level of significance using the Statistical Analysis System (SAS Institute, Cary, NC, USA) to evaluate all hypotheses. Where appropriate, Fisher-type adjustments were made to ensure no violations because of small cell counts in χ² procedures. Student’s t-test was used to compare the age in the patients where surgically retrieved spermatozoa were used according to the aetiology of the azoospermia and whether samples used were fresh or cryopreserved.

### Table III. Sperm parameters and intracytoplasmic sperm injection outcome with epididymal spermatozoa according to the origin of the obstruction

<table>
<thead>
<tr>
<th>Obstruction</th>
<th>Congenital</th>
<th>Acquired</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycles</td>
<td>138</td>
<td>103</td>
</tr>
<tr>
<td>Mean concentration (×10^6/ml ± SD)</td>
<td>24.8 ± 39</td>
<td>16.0 ± 21</td>
</tr>
<tr>
<td>Mean motility (% ± SD)</td>
<td>11.8 ± 15</td>
<td>10.6 ± 16</td>
</tr>
<tr>
<td>Mean morphology (% ± SD)</td>
<td>2.8 ± 3</td>
<td>1.9 ± 2</td>
</tr>
<tr>
<td>Fertilization (%)</td>
<td>111/1524 (73.1)</td>
<td>660/911 (72.4)</td>
</tr>
<tr>
<td>Clinical pregnancies (%)</td>
<td>85 (61.6)</td>
<td>50 (48.5)</td>
</tr>
</tbody>
</table>

### Table IV. Sperm parameters and intracytoplasmic sperm injection outcome with fresh or frozen–thawed epididymal spermatozoa

<table>
<thead>
<tr>
<th>Spermatozoa</th>
<th>Fresh</th>
<th>Frozen–thawed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycles</td>
<td>129</td>
<td>112</td>
</tr>
<tr>
<td>Mean concentration (×10^6/ml ± SD)</td>
<td>24.4 ± 37</td>
<td>17.2 ± 27</td>
</tr>
<tr>
<td>Mean motility (% ± SD)</td>
<td>18.5 ± 17ª</td>
<td>2.9 ± 7ª</td>
</tr>
<tr>
<td>Mean morphology (% ± SD)</td>
<td>2.7 ± 2</td>
<td>2.1 ± 2</td>
</tr>
<tr>
<td>Fertilization (%)</td>
<td>1027/1429 (71.9)</td>
<td>747/1006 (74.3)</td>
</tr>
<tr>
<td>Clinical pregnancies (%)</td>
<td>83 (64.3)ª</td>
<td>52 (46.4)ª</td>
</tr>
</tbody>
</table>

ªStudent’s t-test, two independent samples; effect of cryopreservation on the motility parameter, P < 0.0001. bχ², 2×2, 1 df; effect of cryopreservation on pregnancy rate, P = 0.01.

### Epididymal cycles

Among 241 ICSI cycles performed with epididymal spermatozoa there was no difference in sperm concentration, motility and morphology between congenital obstructive versus acquired azoospermia. Fertilization characteristics, pregnancy and delivery rates were grouped according to the origin of the obstruction (Table III). The fertilization and pregnancy rates were comparable in both groups. The same number of cycles cited in Table III was also analysed according to the use of fresh or frozen epididymal spermatozoa (Table IV). The freezing process clearly depressed motility parameter and the pregnancy outcome (P = 0.01), but had no influence on the fertilization rate after ICSI.

### Testicular cycles

Ninety-nine testicular biopsies were performed in 16 cases of obstructive azoospermia and in 83 of non-obstructive azoospermia. Spermatozoa were successfully retrieved in all (n = 14) of the obstructive azoospermia cycles and in 63.9%...
Clinical pregnancies (%) 31 (50.0) 3 (60.0)

Fertilization (%) 367/608 (60.4) 32/43 (74.4)

Fertilization (%) 95/118 (80.5) a 304/533 (57.0) a

Mean motility (%) 6

Mean concentration (×10^6/ml ± SD) 14 53
Mean motility (%) ± SD 1.6 ± 2 0.4 ± 1
Fertilization (%) 4.0 ± 7 6.7 ± 14
Clinical pregnancies (%) 95/118 (80.5) a 304/533 (57.0) a

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Clinical pregnancies (%) 95/118 (80.5) a 304/533 (57.0) a

CI{S} in azoospermic patients

Table V. Sperm parameters and intracytoplasmic sperm injection outcome with testicular spermatozoa according to the type of azoospermia

<table>
<thead>
<tr>
<th></th>
<th>Obstructive</th>
<th>Non-obstructive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycles</td>
<td>14</td>
<td>53</td>
</tr>
<tr>
<td>Mean concentration</td>
<td>1.6 ± 2</td>
<td>0.4 ± 1</td>
</tr>
<tr>
<td>Mean motility (%)</td>
<td>4.0 ± 7</td>
<td>6.7 ± 14</td>
</tr>
<tr>
<td>Fertilization (%)</td>
<td>95/118 (80.5) a</td>
<td>304/533 (57.0) a</td>
</tr>
<tr>
<td>Clinical pregnancies (%)</td>
<td>31 (50.0)</td>
<td>26 (49.1)</td>
</tr>
</tbody>
</table>

Z^2; 2×2; 1 d.f; effect of aetiology of azoospermia on fertilization rate \( P = 0.0001 \).

Table VI. Sperm parameters and intracytoplasmic sperm injection outcome with fresh or frozen–thawed testicular spermatozoa

<table>
<thead>
<tr>
<th></th>
<th>Fresh</th>
<th>Frozen–thawed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycles</td>
<td>62</td>
<td>5</td>
</tr>
<tr>
<td>Mean concentration</td>
<td>0.7 ± 2</td>
<td>0.2 ± 0.4</td>
</tr>
<tr>
<td>Mean motility (%)</td>
<td>6.2 ± 14</td>
<td>5.2 ± 11</td>
</tr>
<tr>
<td>Fertilization (%)</td>
<td>367/608 (60.4)</td>
<td>324/43 (74.4)</td>
</tr>
<tr>
<td>Clinical pregnancies (%)</td>
<td>31 (50.0)</td>
<td>3 (60.0)</td>
</tr>
</tbody>
</table>

(n = 53) of the non-obstructive cases undergoing testicular biopsy. Semen parameters, fertilization and pregnancy rates for both obstructive and non-obstructive groups were compared (Table V). There were no normal forms in the testicular samples analysed. The fertilization and pregnancy rates did not differ whether the testicular group was separated according to the origin of the azoospermia (Table V) or whether the samples were used fresh or cryopreserved (Table VI).

For men with obstructive azoospermia, a clinical pregnancy rate of 56.1% (143/255), and ongoing pregnancies and deliveries were achieved for 50.6% (129/255) of attempts. In the 53 cases of testicular sperm extraction (TESE) in men with non-obstructive azoospermia, the clinical pregnancy rate was 49.1% (26/53). These cycles included one pregnancy from a cryopreserved sample.

Evolution of ICSI pregnancies with surgically retrieved spermatozoa

No differences in the pregnancy loss nor in the ongoing pregnancy and delivery rates were observed as a function of the origin of the sample and the aetiology of the azoospermia when the use of fresh and frozen spermatozoa was considered together (Tables VII and VIII). The incidence of miscarriages with surgically retrieved spermatozoa was similar to that noted with the use of ejaculated spermatozoa during a concurrent period of ICSI treatments. None of the fetuses conceived with surgically retrieved spermatozoa was available for cytogenetic analysis, and when this was performed on the amniotic fluid, in 12% (18/150) of such cases, only one abnormal karyotype was observed. Similarly, the incidence of congenital malformation did not vary in relation to the sample origin or the cause of azoospermia (Table VII). A comparison of the influence of cryopreservation in the epididymal and testicular groups showed no significant difference in terms of pregnancy losses and ongoing pregnancies (Table VIII). The difference in clinical pregnancy rate observed in the epididymal group was lost when deliveries and ongoing pregnancies were considered (Table VIII). In both epididymal and testicular spermatozoa, neonatal abnormalities were observed when the fresh sample was used (Table VIII). In the couples where round cells were injected (n = 14), no normal fertilization was observed. In the four cycles with necrozoospermia, the fertilization rate was 55.2% (16/29). Supravital staining performed on spermatozoa present in the ejaculates of these patients indicated 0% viability. Two clinical pregnancies were established and three healthy newborns were delivered from the two pregnancies.

ICSI outcome in Klinefelter’s patients

Among men with non-obstructive azoospermia that had a sex chromosomal abnormality, five had a 47,XXY karyotype (Klinefelter’s) syndrome, with one mosaic Klinefelter’s. Spermatozoa were obtained in four out of seven TESE procedures (Figure 4) on these six men with non-mosaic Klinefelter’s syndrome (Palermo et al., 1998). After 50 mature oocytes were injected with spermatozoa harvested from the testis, 34 (68%) were fertilized, 11 embryos were transferred, and five fetal heartbeats were obtained (45.4%). Five clinical pregnancies were maintained, one singleton and two twin pregnancies — all karyotypically normal. Five healthy newborns were delivered.

Discussion

Pre-operative evaluation of azoospermic men is critical to their subsequent treatment. Classification of men as having either obstructive or non-obstructive azoospermia was performed for all patients treated at our centre. Although sperm can be retrieved from at least some men with both non-obstructive and obstructive azoospermia, the approach to each of these conditions is quite different. For men with obstructive azoospermia, reproductive tract reconstruction may often be an alternative that is frequently more successful and less involved than assisted reproduction (Pavlovich and Schlegel, 1997). In addition, men with idiopathic obstructive azoospermia or congenital bilateral absence of the vas deferens are likely to have their condition caused by mutations of the cystic fibrosis gene (Mak and Jarvi, 1996). If the aetiology is not recognized and female partners appropriately tested, a high risk of having offspring affected by cystic fibrosis will result.

Non-obstructive azoospermia is associated with a significant risk of failure to retrieve spermatozoa and a variety of genetic risks (Devroey et al., 1995, 1996; Kahraman et al., 1996; Friedler et al., 1997). In our experience, ~40% of men with carefully documented non-obstructive azoospermia were unable to have spermatozoa extracted from the testis despite extensive biopsies of testicular tissue. For these men, simultaneous attempts at sperm retrieval during an IVF cycle may result in retrieved oocytes without spermatozoa to inject. Couples with this risk must consider the options of donor insemination or adoption prior to entering an attempt of sperm retrieval with ICSI.

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Table VII. Effect of the aetiology of azoospermia on intracytoplasmic sperm injection outcome and incidence of neonatal malformations

<table>
<thead>
<tr>
<th>A Cycles</th>
<th>Obstructive</th>
<th>Congenital</th>
<th>Acquired</th>
<th>Testicular spermatozoa</th>
<th>Azoospermia</th>
<th>Obstructive</th>
<th>Non-obstructive</th>
</tr>
</thead>
<tbody>
<tr>
<td>138</td>
<td>14</td>
<td>33.0 ± 5a</td>
<td>36.4 ± 5a</td>
<td>32.6 ± 6</td>
<td>53</td>
<td>34.8 ± 5</td>
<td></td>
</tr>
<tr>
<td>B Pregnancy losses (% of A)</td>
<td>11 (12.9)</td>
<td>2 (4.0)</td>
<td>1 (12.5)</td>
<td>4 (15.4)</td>
<td>7 (50.0)</td>
<td>21 (39.6)</td>
<td></td>
</tr>
<tr>
<td>74 (53.6)</td>
<td>48 (46.6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C Newborns</td>
<td>104</td>
<td>85 (61.6)</td>
<td>50 (48.5)</td>
<td>8 (57.1)</td>
<td>53</td>
<td>26 (49.1)</td>
<td></td>
</tr>
<tr>
<td>Newborns with malformations (% of C)</td>
<td>1 (0.9)</td>
<td>1 (2.1)</td>
<td>0</td>
<td>1 (4.5)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Student’s t-test, two independent samples; difference in maternal age when epididymal spermatozoa were used, P < 0.0001.

Table VIII. Effect of cryopreservation on intracytoplasmic sperm injection outcome and incidence of neonatal malformations

<table>
<thead>
<tr>
<th>A Cycles</th>
<th>Fresh (%)</th>
<th>Frozen (%)</th>
<th>Fresh (%)</th>
<th>Frozen (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>129</td>
<td>122</td>
<td>62</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>B Clinical pregnancies (% of A)</td>
<td>33.7 ± 5a</td>
<td>35.4 ± 5a</td>
<td>34.2 ± 5</td>
<td>36.2 ± 8</td>
</tr>
<tr>
<td>83 (64.3)b</td>
<td>52 (46.4)b</td>
<td>31 (50.0)</td>
<td>3 (60.0)</td>
<td></td>
</tr>
<tr>
<td>Pregnancy losses (% of B)</td>
<td>11 (13.3)</td>
<td>2 (3.8)</td>
<td>5 (16.1)</td>
<td>0</td>
</tr>
<tr>
<td>Ongoing pregnancies and deliveries (% of A)</td>
<td>73 (56.6)</td>
<td>50 (44.6)</td>
<td>25 (40.3)</td>
<td>3 (60.0)</td>
</tr>
<tr>
<td>C Newborns</td>
<td>52</td>
<td>25</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Newborns with malformations (%)</td>
<td>2 (2.0)</td>
<td>0</td>
<td>1 (4.0)</td>
<td>0</td>
</tr>
</tbody>
</table>

*Student’s t-test, two independent samples; difference in maternal age when epididymal spermatozoa were used, P < 0.1.

It has been clearly demonstrated that men with impaired sperm production have a higher prevalence of chromosomal abnormalities (de Kretser et al., 1972). Similarly in this study, we observed chromosomal abnormalities in 20.0% of treated men with non-obstructive azoospermia. The majority were sex chromosomal abnormalities. Sex chromosomal abnormalities were also detectable at a molecular level in the form of Yq microdeletions for 7.8% of men with non-obstructive azoospermia. Although these abnormalities were assessed in leukocytes, chromosomal defects have also been seen in the spermatozoa of infertile men (Martin and Rademaker, 1988; Colombero et al., 1997a,b); thus there is a demonstrated need for careful genetic screening. We have previously noted that the detection of genetic abnormalities has several important roles in the treatment of men with non-obstructive azoospermia (Rucker et al., 1998). First of all, detection of chromosomal abnormalities allows the performance of preimplantation genetic diagnosis. Secondly, identification of a genetic abnormality provides men with reassurance of the aetiology for low sperm production and eases concerns about personal culpability for the subfertile condition. In addition, 21% of couples where the man has an identifiable genetic abnormality will choose to proceed with a different approach to parenting, including donor insemination or adoption.

In order to most accurately reflect the chances of success for a couple with azoospermia, we have presented the results for initial simultaneous attempts at sperm retrieval with assisted reproduction, as well as the overall results of treatment. Couples were typically treated with simultaneous sperm retrieval and ICSI whether cryopreserved spermatozoa were generally retrieved after a previous ICSI failure. Therefore, results for simultaneous collection, i.e. spermatozoa and oocytes, cannot be fairly compared to cycles using cryopreserved spermatozoa, regardless of sperm source, as a bias toward worse results may occur for frozen cycles due to our treatment approach. Female age may also affect treatment results and can be a confounding factor for results of treatment of male factor infertility. For the series presented here, men with acquired obstructive azoospermia are older than men with congenital obstructive azoospermia at the time of treatment. Therefore, the female partners were older, as reflected by the difference in maternal ages noted for men with obstructive azoospermia (Tables VII and VIII).

Where epididymal spermatozoa were used for ICSI in
obstructive azoospermia, the fertilization and pregnancy rates were at least comparable to those obtained contemporarily with ejaculated spermatozoa. Moreover, the origin of the obstruction, congenital or acquired, did not influence ICSI outcome. Cryopreservation had a deleterious effect on sperm motility in this obstructive group, and although it did not affect the fertilization rate presumably it impaired pregnancy outcome.

Fertilization after ICSI with testicular spermatozoa was higher in the obstructive group without affecting the clinical pregnancy rate. Obstructive patients were biopsied because of the failure of epididymal aspiration or the absence of the epididymis. The complete absence of morphologically normal testicular spermatozoa is related to the particular characteristics of these ‘immature’ sperm cells that do not fit with the conventional criteria (Kruger et al., 1986). The good performance of the cryopreserved testicular spermatozoa, although presented here in a small series, may be related to the membrane characteristics of these cells which enable them to sustain the stress of the freezing process (Palermo et al., 1996b).

Pregnancy loss in couples with surgically retrieved spermatozoa ranged between 4.0 and 15.4%, comparable to that seen with ejaculated spermatozoa. The aetiology of azoospermia did not affect the live birth rate nor was there any relationship to neonatal malformations (Van Steirteghem et al., 1998). The cryopreservation of epididymal and testicular spermatozoa did not increase pregnancy loss nor the ongoing pregnancies and deliveries.

The similarly high pregnancy outcome with surgically retrieved spermatozoa, whether fresh or cryopreserved in men with obstructive azoospermia, suggests that such cryopreservation be used as a routine clinical practice for subsequent attempts. It has been clearly demonstrated that repeating a testicular biopsy within 6 months may decrease the chance of sperm retrieval and increase the risk of permanent testicular devascularization areas (Schlegel et al., 1997). When ejaculated spermatozoa are used, the origin of the sample as well as the semen parameters appeared irrelevant to the ICSI outcome, and this also seems to be true for azoospermic men.

Earlier concerns related to ICSI may now apply to the treatment of azoospermia where spermatozoa are obtained from men with severely abnormal sperm production. Nonetheless, in spite of being associated with a higher frequency of genetic abnormalities, the present results suggest that these conditions can be successfully treated with ICSI without a significant increase in adverse outcomes for progeny. The only factor that clearly depresses the pregnancy rate with ICSI independently of the origin of the spermatozoon is maternal age (Silber et al., 1996).

Even men with Klinefelter’s syndrome can produce some fully developed spermatozoa and contribute to successful pregnancies using testicular sperm extraction with ICSI (Palermo et al., 1998). On the other hand, the need for careful genetic screening of patients with severely compromised spermatozoa or azoospermia prior to ICSI treatment is still necessary, as is the monitoring of such pregnancies. In addition, newborns should be carefully evaluated at birth and in the early years of development. With a continued careful approach to follow-up of pregnancy results and offspring, we can thus far confirm the safety of ICSI.

Acknowledgements

We thank the clinical and scientific staff of The Center for Reproductive Medicine and Infertility, Peggy King for her careful follow-up of patients after treatment, Prof. J. Michael Bedford for his critical review of the manuscript, and Queenie Neri for editorial assistance.

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Received on May 5, 1998; accepted on November 24, 1998.