The outcome of intracytoplasmic injection of fresh and cryopreserved epididymal spermatozoa from patients with obstructive azoospermia—a comparative study

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Introduction
Azoospermia due to obstruction of the male genital tubal system, secondary to agenesis (i.e. congenital bilateral absence of the vas deferens - CBAVD), aplasia, infections and scarring following trauma, is no longer an untreatable condition requiring the use of donor spermatozoa for fertilization. The efficiency of intracytoplasmic sperm injection (ICSI) (Palermo et al., 1992), using non-ejaculated spermatozoa surgically retrieved from the epididymis or testis to achieve fertilization and pregnancies, has revolutionized the potential of treating patients suffering from azoospermia (Tournaye et al., 1994; Silber et al., 1994, 1995). Micro-epididymal sperm aspiration (MESA) (Temple-Smith et al., 1985; Silber et al., 1994; Schlegel et al., 1994; Tournaye et al., 1994; Tournaye et al., 1995; Tucker et al., 1995) was the first surgical sperm retrieval method introduced involving the epididymis. It involves open surgery. A less invasive method of sperm retrieval was introduced recently, percutaneous aspiration by fine needle, which also involves the epididymis (percutaneous epididymal sperm aspiration – PESA) (Craft and Tsirigotis, 1994; Shrivastav, 1994, 1995; Shrivastav et al., 1994; Craft et al., 1995 a,b). If epididymal sperm aspiration fails, then testicular sperm aspiration (TESA) (Craft and Tsirigotis, 1995; Tsirigotis and Craft, 1995) has also been shown to be efficient. In patients suffering from obstructive azoospermia, a high rate of retrieval success is possible due to the abundance of spermatozoa in the epididymal or testicular tubuli. After completion of the ICSI procedure, which requires the use of only a few spermatozoa, cryopreservation of excess spermatozoa is feasible in most patients, avoiding further surgical procedures for sperm retrieval in the future. The best epididymal sperm aspiration method is still in debate; the reports in the literature concerning use of frozen–thawed epididymal spermatozoa, especially those obtained by PESA, are scarce and no consensus exists concerning the most efficient cryopreservation protocol to be used.

Our aim was to compare the outcome of ICSI with fresh or cryopreserved–thawed epididymal sperm retrieved by MESA or PESA. To evaluate the benefits of epididymal sperm cryopreservation, the cumulative pregnancy rate achieved per surgical sperm aspiration procedure in this group of patients with obstructive azoospermia was also assessed.

Materials and methods
Study population
During the period of October 1995 to April 1997, 24 patients with obstructive azoospermia underwent 24 cycles of surgical sperm aspiration in conjunction with ICSI at Assaf Harofeh Medical Center’s IVF Unit. Aetiology for the obstruction included CB-AVD (18 patients, 75%), orchiepididymitis (four patients, 17%), trauma and vas deferens laceration (one case, 4%), and vasectomy reversal failure (one case, 4%). The methodology of surgical sperm retrieval included MESA (7 cycles) and PESA by fine needle (17 cycles). In all patients non-ejaculated spermatozoa were successfully retrieved, and in 23 of the 24 aspirations performed (96%) excessive spermatozoa were cryopreserved (in one case cryopreservation was not performed due to technical failure). These patients formed our study group. Following

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transferred to a 6-ml conical tube (Falcon, Becton Dickinson Labware, Corp., Tokyo, Japan) at 20–49 years). The mean age of the female partners was 31.8 ± 6.7 years (range 23–42 years). The male partners were previously investigated at the Assaf Harofeh Medical Center’s Male Infertility Clinic. Familial and personal history was taken, with emphasis on familial male infertility, undescended testicles in childhood, orchiepididymitis or mumps orchitis in the past. The patients underwent physical examination of their genitalia as well as testicular and transrectal sonography to determine the existence of a normal anatomy for seminal vesicles, prostate, distal vasa deferentia and ejaculatory ducts. Their hormonal profile was also assessed. Cytological analysis revealed a normal karyotype in all patients. The group was selected by rigorous criteria. All patients underwent extensive work-up of several ejaculates (at least three) prior to their surgical sperm retrieval. No sperm could be seen in any ejaculate provided, even after examination by the extended sperm preparation method (Ron-El et al., 1997). Those presenting with CBA VD were also screened for cystic fibrosis mutations and underwent genetic counselling. The male patients’ mean serum follicle stimulating hormone (FSH) level was 4.4 ± 2.6 IU/l (range 1.8–10.2 IU/l). Their testicular volume was within normal limits (i.e. >15 ml). Diagnosis of obstructive azoospermia was based upon a histological report taken previously and showing normal spermatogenesis.

**Sperm retrieval and preparation**

On the day of the oocyte retrieval, the male partners were asked to produce fresh ejaculates and, following analysis by the extended sperm preparation method (Ron-El et al., 1997), it was confirmed that no spermatozoa were found in their specimens.

**Methodology of microsurgical epididymal sperm aspiration (MESA)**

MESA was performed under general anaesthesia. The methodology of MESA was according to the technique published by others (Schlegel et al., 1994; Silber et al., 1994; Tournaye et al., 1994). However, after the first seven procedures, open surgery was abandoned and currently all epididymal aspirations are performed by PESA because, based upon the authors previous personal experience, it seems to be a much easier procedure for the patients, requiring less operating time and anaesthesia.

**Methodology of percutaneous epididymal sperm aspiration (PESA)**

PESA was performed using 23-gauge butterfly needles attached to a 20-ml plastic syringe serving as an aspiration device. While holding the testicle between the index finger and the thumb, the epididymis was palpated and the butterfly needle was directly passed through the scrotal skin into the epididymis. As much epididymal fluid as possible was aspirated and, before retrieving the needle from the epididymis, a small artery forceps was used to clamp the butterfly’s microtubing. If aspiration was not successful on one side, the contralateral epididymis was aspirated. The aspirates were immediately examined under an inverted microscope (Diaphot 300; Nikon Corp., Tokyo, Japan) at ×200 and ×400 magnification to detect the presence of any spermatozoa. The aspirate was then collected and transferred to a 6-ml conical tube (Falcon, Becton Dickinson Labware, New Jersey, USA) and centrifuged at 300 g for 10 min. Cell separation by discontinuous Percoll gradient was rarely performed as was recently suggested by Fujii et al. (1997). However, occasionally, when a lot of cell debris was seen, the resuspended pellet was layered on a mini-Percoll gradient (90–45%) (Sigma Chemical Co., St. Louis, MO, USA) prepared with Earle’s Balanced Salt Solution (EBSS) medium (Gibco BRL, Life Technologies, Paisley, Scotland) supplemented with pyruvic acid (Sigma) and centrifuged at 300 g for 20 min. The original pellet after red cell lysis (Sterile Water, Teva Medical, Petah-Tikva, Israel), or the spermatozoa-containing fraction following Percoll separation, was washed twice by addition of 6 ml of human tubal fluid medium supplemented with 7.5% synthetic serum (Irvine Scientific, Santa Ana, CA, USA) and centrifuged at 250 g for 5 min. The final pellet was incubated until the sperm injection (between 3 and 6 h). Prior to the injection, the final pellet was examined under the inverted microscope for the presence of spermatozoa using an injection dish (Falcon) containing multiple droplets (up to 50) of 10 μl each. If spermatozoa were identified, they were transferred to drops of 10% polyvinylpyrrolidone (PVP; Irvine Scientific) covered by pre-equilibrated embryo-tested light paraffin oil (Sigma).

**Epididymal sperm cryopreservation**

Following ICSI using fresh epididymal spermatozoa, the remaining spermatozoa were cryopreserved using a freezing protocol with Test Yolk Buffer freezing medium (Irvine Scientific). The spermatozoa-containing extract was diluted dropwise 1:1 with the freezing medium and sealed in freezing straws (0.5 ml, Instruments de Médecine Vétérinaire, IMV,l’Aigle, France). A simple two-step cryopreservation protocol was used. The straws were dropped in a nitrogen vapour chamber stabilized at ~80°C for 20 min (cooling rate of ~10°C/min) prior to immersion into liquid nitrogen for sperm storage at ~196°C. Following removal of the straws from the liquid nitrogen, rapid thawing occurred at room temperature. Then the frozen–thawed sperm mixture containing cryoprotectant was diluted with insemination medium, centrifuged at 300 g for 7 min and the pellet processed for examination in multiple droplets, as described above.

**Ovarian stimulation and oocyte retrieval**

Ovulation induction and oocyte retrieval were performed as described elsewhere (Ron-El et al., 1991), using a protocol of GnRH agonist (DTRP6, Decapeptyl 3.75 mg i.m.; Ferring, Malmo, Sweden) suppression, with human menopausal gonadotrophin (HMG, Pergonal, Teva, Petah Tikva, Israel) for ovarian stimulation. Oocytes were retrieved 36 h after administration of 10 000 IU of human chorionic gonadotrophin (HCG) (Chorigon, Teva) by vaginal ultrasound-guided follicular puncture.

**Sperm collection and ICSI procedure**

After cumulus and corona radiata cells were removed from oocytes following exposure to 80 IU/ml hyaluronidase (type IV-S; Sigma) in human tubal fluid medium (Irvine Scientific) for 30–60 s, their stages of nuclear maturation were determined by microscopic examination, so that only oocytes at metaphase II could be selected for injection. When spermatozoa could be identified and isolated, ICSI was performed as described by Van Steirteghem et al. (1993). Preferably motile sperm were used and, indeed in all cases, at least a few spermatozoa with some degree of motility were found for ICSI. Oocytes injected with immotile spermatozoa were not included in this study. Fertilization was assessed on the following day, 16–18 h post-sperm injection. If two distinct pronuclei were observed, then fertilization was judged to have occurred.

**Embryo transfer, luteal support and pregnancy evaluation**

After assessment of fertilization, embryonic cleavage and morphological quality approximately 24 h later, embryo transfer was performed using a Wallace catheter. Our policy is to limit the number of embryos transferred to three, except in older women (age >38 years) or in
Statistical evaluation was performed using Student’s t-test, χ² test, and Fisher’s exact test, where appropriate. Difference was considered significant at $P < 0.05$.

### Results

During the study period, non-ejaculated spermatozoa were successfully retrieved from all patients with obstructive azoospermia undergoing surgical sperm retrieval. In 24 cycles, spermatozoa were retrieved from the epididymis either by MESA (7 cycles) or PESA (17 cycles). Following all sperm retrievals but one (96%), excessive spermatozoa were cryopreserved for further use in subsequent cycles. No significant difference was found in the mean number of vials of spermatozoa cryopreserved following sperm retrieval by MESA or TESA (12.8/patient versus 10.6/patient, respectively, $P > 0.05$). With regard to possible complications following surgical epididymal sperm retrieval in this group of patients, no infections, severe haematomas or prolonged post-operative complications were noted following MESA or PESA. However, obviously PESA is clinically a more tolerable procedure, requiring a shorter time spent in the operation theatre, than MESA, with quicker convalescence.

In all thawed samples, enough motile spermatozoa were found to allow ICSI. Sperm parameters before and after cryopreservation are shown in Table I. Two patients (one in each group) failed to achieve embryo transfer following a trial with fresh spermatozoa: one had only one mature oocyte for sperm injection which did not fertilize, and the other patient had seven mature oocytes that failed to fertilize. The first patient achieved embryo transfer using cryopreserved–thawed sperm in a consecutive cycle, and the other did not repeat a treatment cycle using thawed spermatozoa. Six spouses of the 22 patients having embryo transfer (27%) achieved clinical pregnancy after ICSI using fresh epididymal spermatozoa.

Regarding the outcome of ICSI by sperm surgical retrieval procedures, no significant differences were found between the fertilization, embryo cleavage and implantation rates with fresh or cryopreserved sperm retrieved by PESA or MESA (Table II). Although ongoing pregnancy rates with fresh epididymal spermatozoa retrieved by PESA were higher than with those retrieved by MESA, and the opposite was observed for cryopreserved–thawed spermatozoa, the differences did not reach statistical significance, possibly due to the small group size.

Of the 23 patients with cryopreserved epididymal spermatozoa, ICSI was performed in 13 patients using cryopreserved–thawed spermatozoa. We compared the outcome of ICSI, using fresh or cryopreserved–thawed epididymal spermatozoa from in the same 13 patients with non-obstructive azoospermia, thus each patient served as his own control (Table III). In this group, spermatozoa were retrieved by MESA (three cycles) or PESA (17 cycles). No statistically significant differences were noted after comparing all parameters examined.

Comparing the outcome of 24 ICSI cycles with fresh non-ejaculated spermatozoa with the 21 ICSI cycles using cryopreserved spermatozoa, no significant differences were noted in the mean number of oocytes injected per cycle, their two-pronucleated fertilization and cleavage rates, mean number of embryos transferred and the relative distribution of the quality of the embryos transferred (Table IV). As shown in this table, the implantation rate and clinical and ongoing pregnancy rates per embryo transfer using frozen–thawed testicular spermatozoa were not inferior to those achieved following the use of fresh testicular spermatozoa ($P > 0.5$, χ² test and Fisher’s exact test). In fact, during the consecutive treatment cycles performing ICSI with cryopreserved–thawed non-ejaculated spermatozoa in 13 patients who underwent cryopreservation, a further seven clinical pregnancies were achieved. Another 10 patients have cryopreserved epididymal spermatozoa awaiting use in the future. Also five patients from both treatment groups have altogether 35 cryopreserved embryos for later transfer (Table IV).

In the group of patients whose fresh spermatozoa were used, all pregnancies but one were singleton pregnancies. One pregnancy resulted in twins. One pregnancy ended in a spontaneous first trimester miscarriage. The twin pregnancy ended in term delivery with one healthy newborn. The other died shortly after delivery due to a hypoplastic left heart. The patient objected to any prenatal diagnosis, including ultrasound examination, on religious grounds. Among the seven pregnancies achieved after using cryopreserved spermatozoa, one was twin and the other singletons. Two of the singleton pregnancies ended in first trimester spontaneous abortion. In total, following 24 surgical sperm aspirations in 24 patients with obstructive azoospermia, 14 clinical pregnancies were achieved of which 11 are ongoing or delivered. The delivery/ongoing pregnancy rate of 26% per embryo transfer (11% and 41%, using fresh or frozen testicular spermatozoa, respectively), gives a cumulative ongoing pregnancy rate per sperm retrieval procedure of 46% (11/24) in this group of patients.
Table II. Outcome of intracytoplasmic sperm injection (ICSI) with fresh and cryopreserved–thawed epididymal sperm in patients with obstructive azoospermia, according to the method of sperm retrieval

<table>
<thead>
<tr>
<th>Spermatozoa used for ICSI</th>
<th>Fresh</th>
<th>Cryopreserved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method of retrieval</td>
<td>MESA</td>
<td>PESA</td>
</tr>
<tr>
<td>No. of patients</td>
<td>7</td>
<td>17</td>
</tr>
<tr>
<td>No. of ICSI cycles</td>
<td>7</td>
<td>17</td>
</tr>
<tr>
<td>No. of injected oocytes</td>
<td>60</td>
<td>134</td>
</tr>
<tr>
<td>Fertilization rate (%)</td>
<td>55</td>
<td>56</td>
</tr>
<tr>
<td>Cleavage rate (%)</td>
<td>94</td>
<td>88</td>
</tr>
<tr>
<td>Mean no. of embryos per</td>
<td>4.5 ± 1.8</td>
<td>3.1 ± 1.0</td>
</tr>
<tr>
<td>embryo transfer ± SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Implantation rate (%)</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Clinical pregnancy rate</td>
<td>2/6(33)</td>
<td>5/16(31)</td>
</tr>
<tr>
<td>per embryo transfer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Delivery or ongoing</td>
<td>1/6(17)</td>
<td>5/16(31)</td>
</tr>
<tr>
<td>pregnancy rate per</td>
<td></td>
<td></td>
</tr>
<tr>
<td>embryo transfer</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a Student’s t-test.
*b χ² test.
*c Fisher’s exact test.

Values in parentheses are percentages.

Table III. The comparison of intracytoplasmic sperm injection (ICSI) outcome using fresh or cryopreserved–thawed epididymal spermatozoa in the same 13 patients with obstructive azoospermia

<table>
<thead>
<tr>
<th>Spermatozoa used for ICSI</th>
<th>Fresh</th>
<th>Cryopreserved–thawed</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>No. of ICSI cycles</td>
<td>13</td>
<td>21</td>
</tr>
<tr>
<td>No. of injected oocytes</td>
<td>115</td>
<td>205</td>
</tr>
<tr>
<td>Mean injected oocytes</td>
<td>8.8± 8.3</td>
<td>9.8± 5.5</td>
</tr>
<tr>
<td>per cycle ± SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of fertilized oocytes</td>
<td>69b  (60)</td>
<td>106b (52)</td>
</tr>
<tr>
<td>No. of cleaved embryos</td>
<td>64b  (93)</td>
<td>89b (84)</td>
</tr>
<tr>
<td>No. of embryo transfers</td>
<td>9</td>
<td>15</td>
</tr>
<tr>
<td>Mean no. of embryos</td>
<td>3.2± 1.8</td>
<td>2.5± 1.7</td>
</tr>
<tr>
<td>per embryo transfer ± SD</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Embryo quality:
- Excellent (%) 10 20
- Good (%) 28 44
- Fair (%) 62 36
+ Cryopreserved embryos 15 13

*a Student’s t-test.
*b χ² test.
Values in parentheses are percentages.

Discussion

The optimal surgical procedure to obtain epididymal spermatozoa in patients with obstructive azoospermia is in debate (Craft and Shrivastav, 1995; Schlegel et al., 1995; Tsirigotis et al., 1995; Tsirigotis and Craft, 1995, 1996; Khalifa and Grudzinskas, 1996; Silber, 1996). Advocating open surgery (MESA), Schlegel et al. (1994, 1995) argued that blood cells may contaminate percutaneous fine needle aspiration specimens and affect fertilization rates and that a cleaner specimen may be retrieved by microsurgical retrieval. Silber (1996) stressed the possibility to cryopreserve enough spermatozoa for 20 future ICSI cycles following MESA. One might argue that the freezability of epididymal sperm specimens retrieved by MESA could be different from those retrieved by PESA. Although PESA in patients with obstructive azoospermia was shown to be efficient (Craft and Shrivastav, 1995; Craft et al., 1995a,b; Tsirigotis et al., 1995; Meniru et al., 1997a,b), reports concerning cryopreservation of spermatozoa retrieved by PESA are scarce. No comparisons of the outcome of these two procedures were found in the literature. Our results show that not only is the fertilization rate of ICSI with fresh or cryopreserved–thawed epididymal spermatozoa retrieved by...

Table IV. The outcome of intracytoplasmic sperm injection (ICSI) using fresh or cryopreserved–thawed epididymal spermatozoa in 24 patients with obstructive azoospermia

<table>
<thead>
<tr>
<th>Spermatozoa used for ICSI</th>
<th>Fresh</th>
<th>Cryopreserved–thawed</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of ICSI cycles</td>
<td>24</td>
<td>21</td>
</tr>
<tr>
<td>No. of injected oocytes</td>
<td>194</td>
<td>212</td>
</tr>
<tr>
<td>Mean injected oocytes</td>
<td>8.0± 6.2</td>
<td>9.6± 5.6</td>
</tr>
<tr>
<td>per cycle ± SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of fertilized oocytes</td>
<td>108b (56)</td>
<td>113b (53)</td>
</tr>
<tr>
<td>No. of cleaved embryos</td>
<td>97b (90)</td>
<td>97b (86)</td>
</tr>
<tr>
<td>No. of embryo transfers</td>
<td>22</td>
<td>19</td>
</tr>
<tr>
<td>Mean no. of embryos</td>
<td>3.5± 1.4</td>
<td>3.1± 1.3</td>
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<tr>
<td>per embryo transfer ± SD</td>
<td></td>
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Embryo quality:
- Excellent (%) 14 20
- Good (%) 34 44
- Fair (%) 52 36
+ Cryopreserved embryos 20 15

Implantation rate | 8/77b (10) | 8/59b (14) | ns |
Clinical pregnancy rate per embryo transfer | 7/22b (32) | 7/19b (37) | ns |
Delivery or ongoing pregnancy rate per embryo transfer | 6/22c (27) | 5/19c (26) | ns |

*a Student’s t-test.
*b χ² test.
*c Fisher’s exact test.

Values in parentheses are percentages.
PESA equal to that retrieved by MESA, but also the clinical outcome does not differ significantly between the groups. Therefore, as PESA has definitive clinical advantages compared with the open surgery required in MESA, it may be offered as the treatment of choice for patients with obstructive azoospermia. Epididymal sperm cryopreservation is feasible and efficient in patients with obstructive azoospermia using a simple freezing protocol. Most studies reporting outcome of ICSI with frozen–thawed epididymal spermatozoa used a comparable cohort group of patients treated by ICSI with fresh epididymal spermatozoa as control. However, the optimal control group for comparing the efficacy of ICSI with cryopreserved–thawed epididymal spermatozoa is the one of ICSI with fresh epididymal spermatozoa from the same patients. Our results show that epididymal sperm cryopreservation is efficient, allowing the achievement of fertilization, cleavage and implantation rates comparable to those achieved when using fresh non-ejaculated sperm in the same patient group. These findings corroborate previous observations by Devroye et al. (1995), who reported comparable pregnancy and implantation rates following ICSI with fresh or cryopreserved epididymal sperm in a group of seven couples with obstructive azoospermia, using a similar sperm cryopreservation protocol. The same group reported similar results in a group of 43 fresh-MESA cycles compared to 9 cryopreserved–thawed cycles (Nagy et al., 1997). The outcome does not differ significantly between the groups.

References


Sperm freezing in obstructive azoospermia


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