Fertilizing Capacity of Epididymal and Testicular Sperm with ICSI

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INTRODUCTION

Congenital absence of the vas deferens (CAV) and irreparable obstructive azoospermia are the cause of male infertility in a very large group of patients who have normal spermatogenesis, but who previously were nonetheless frustratingly untreated (25; 19). Microsurgical sperm aspiration (MESA) from the epididymis and along with IVF was developed in 1988 to solve this problem for CAV patients but was also tried on patients with failed vasoepididymostomy (18; 19). We thus demonstrated that epididymal sperm transit is not always mandatory for fertilization and pregnancy.

However, subsequent to the initial enthusiasm for using IVF with aspirated sperm, it soon became apparent that epididymal sperm often did not fertilize, and the reason was not readily apparent. There appeared to be no easily recognizable difference between the quality of epididymal sperm that fertilized with conventional IVF, versus epididymal sperm that did not fertilize (22). Most centers attempting this procedure have obtained no greater than 9% pregnancy and fertilization rates (3).

We wished to see whether intracytoplasmic sperm injection (ICSI), could improve the poor fertilization rate and pregnancy rate in patients
with microsurgically retrieved epididymal or testicular sperm. Moreover, we wanted to see whether in the terribly difficult cases where there are no motile epididymal sperm retrievable, if testicular biopsy-derived sperm could fertilize as well (and, of course, produce babies) using ICSI. Our collaboration demonstrated clearly that ICSI using epididymal or testicular sperm in men with CAV, or any other cause of obstructive azospermia, does give reliable, and much higher, fertilization and pregnancy rates than conventional IVF.

METHODOLOGY FOR MESA-ICSI AND MESA-IVF

Egg and Sperm Retrieval

The female partners undergo a fairly routine induction of multiple follicular development with the following protocol: leuprolide acetate (Lupron, TAP Pharmaceuticals, North Chicago, IL) 1 mg subcutaneously is administered daily until the day of follicular aspiration. After desensitization, patients receive human follicle stimulating hormone (FSH) (Metrodin, Serono Laboratories, Inc., Randolph, MA) and/or human menopausal gonadotropins (hMG) (Pergonal, Serono) until many follicles of 2.0 cm are noted on ultrasound. Then human chorionic gonadotropin (hCG) (Profasi, Serono) 10,000 IU is administered intramuscularly. Thirty-six hours after hCG administration, the patients undergo transvaginal follicular aspiration.

The husband undergoes microsurgical scrotal exploration with the intention of aspirating sufficient numbers of motile spermatozoa to utilize for IVF with the wife’s eggs. The surgical technique in the male is as follows: scrotal contents are extruded through a small incision, the tunica vaginalis is opened and the epididymis is exposed. Under 10-40x magnification with an operating microscope, a tiny incision is made with microscissors in the epididymal tunic to expose the tubules in the proximal portion of the obstructed epididymis. Sperm are aspirated directly from the opening in the epididymal tubule with a micropipette. The epididymal fluid is immediately diluted in HEPES-buffered Earle’s medium and examined for motility and quality of progression. If there is no motility or poor motility, another aspiration is made more proximally. Motile sperm are usually not obtained until we reach the proximal-most portion of the caput epididymis or even the vasa
efferentia (18; 19). The rationale for trying to find the most motile sperm even with ICSI is based on the following presumptions: ICSI with ejaculated sperm works best when there is some motility, no matter how poor, as a verification of "vitality." When distal epididymal sperm is used, the pregnancy rate even with ICSI is low and the abortion rate is high, because the distal epididymal sperm are senescent. The most proximal sperm are least likely to have undergone senescent degenerative changes in the sperm head (26).

When no motile sperm are retrievable from the epididymis on either side, or when the epididymis is absent, a testicular biopsy is performed, and sperm are retrieved by morselizing the testicular specimen. Invariably only occasional small numbers of sperm are obtainable from minced testicular tissue, along with large numbers of spermatid-laden Sertoli cells. The few free spermatozoa have only weak, slowly twitching motility. This has never been successful for conventional IVF, but is extraordinarily effective for ICSI. It has been observed by everyone in the field that incubation of epididymal sperm in culture medium for an hour or so dramatically improves the motility. Even 100% non-motile testicular tissue sperm will develop some very weak motility with incubation in culture medium. There is nothing particularly unusual about the treatment of epididymal or testicular sperm. Epididymal sperm are usually prepared with a routine mini-Percoll gradient, whether for conventional IVF, or for ICSI. Testicular sperm are simply concentrated by centrifugation because of the very small numbers and extremely poor motility.

Oocyte Preparation for the Intracytoplasmic Injection Procedure

Oocyte retrieval is carried out by vaginal ultrasound-guided puncture 35 h after hCG. After the oocyte retrieval, the cumulus-corona-cell complexes are transferred directly into a Falcon tube with Earle's medium; these tubes are gassed prior to tight closure and are transported in a thermobox that is kept at 37°C, to the microinjection laboratory which is located elsewhere on the Medical Campus at a distance of about 500 m.

The cells of the cumulus and corona radiata are removed by incubation for about 30 seconds in HEPES-buffered Earle's medium with 80 IU hyaluronidase/ml (Type VIII, specific activity 320 IU/mg, Sigma Chemical Co, St. Louis, MO, USA). The removal of the cumulus and corona cells is enhanced by aspiration of the complexes in and out of hand-drawn glass pipettes with serially smaller openings of about
200 to 150 micrometers. Afterwards, the oocytes are rinsed several times in droplets of HEPES-buffered Earle's and B2 medium and then carefully observed under the inverted microscope at X200 magnification. This includes an assessment of the oocyte and the zona pellucida as well as noting the presence or absence of a germinal vesicle or the first polar body. Besides the assessment of nuclear maturity, the cytoplasm of the oocyte is examined for the presence of vacuoles or other abnormalities in the texture of the ooplasm. The oocytes are then incubated in 25 µl microdrops of B2 medium covered by lightweight paraffin oil (British Drug House, Pasture, Brussels, Belgium) at 37°C in an atmosphere of 5% O₂, 5% CO₂ and 90% N₂. About 3 to 4 h later the oocytes are observed again to see whether more oocytes have extruded the first polar body. Intracytoplasmic sperm injection is carried out on all morphologically intact oocytes that have extruded the first polar body.

**Intracytoplasmic Sperm Injection (ICSI) Procedure**

The holding and injection pipettes are made from 30 µl borosilicate glass capillary tubes (Drummond Scientific Company, Broomall, PA, USA) of 78 mm length and an inner and outer diameter of 0.69 mm and 0.97 mm. These glass capillaries are cleaned by (1) sonication for 30 min in pure water (Milli-RO and Milli-Q, Millipore, Brussels, Belgium) with 2% (vol/vol) detergent (7X-PF O-MATIC, Flow Laboratories, Irvine, Scotland) and (2) rinsing in running Milli-Q water for 30 min. Both cleaning steps are repeated before drying and sterilizing the pipettes in hot air oven (100°C for 6h) (Memmert type ULE 500, Schwabach, Germany). The second sonication is done in water without detergent. The glass pipettes are obtained by drawing thin-walled glass capillary tubes (Drummond Scientific Company) using a horizontal microelectrode puller (Type 753 from Campden Instruments Ltd, Loughborough, Leics., United Kingdom). The holding pipette is cut and fire-polished on a microforge (MF-9 Microforge from Narishige CO. Ltd., Tokyo, Japan) to obtain an outer diameter of 50 um and an inner diameter of 20 um. To prepare the injection pipette, the pulled capillary is opened on a microgrinder (EG-4 Micro-Grinder from Narishige) to an outer diameter of 7 um and an inner diameter of 5 um; the bevel angle is 35°. This grinding step required about 3 min and the wetstone of the grinder is humidified by slow water drip during the procedure. The microforge is then used to make a sharp spike on the injection pipette and to bend the edge of the holding and injection pipettes to an angle of about 30° in order to facilitate the injection procedure in the Petri dish.
A 3 to 5 µl sperm droplet is placed in the center of a Petri dish (Falcon type 1006) and is surrounded by eight 5 µl droplets of HEPES-buffered Earle's medium with 0.5% crystalline BSA. These droplets are covered by about 3.5 ml of lightweight paraffin oil. The intracytoplasmic sperm injection procedure is carried out on the heated stage (37°C; THN-60/16 and MS100 Controller from Linkam Scientific Instruments Ltd, London, United Kingdom) of an inverted microscope (Diaphot, Nikon Corporation, Tokyo, Japan) at X400 magnification using Hoffman Modulation Contrast System (Modulation Optics Inc., Greenvale, New York, United States of America). The microscope is equipped with a Nikon F-601M camera for still pictures and a video camera (DXC-755 P from Sony Corporation, Brussels, Belgium) that allows the procedure to be followed on a Trinitron color video monitor (PVM-1443MD from Sony). The microscope is equipped with two coarse positioning manipulators (3D Motor Driven Coarse Control Manipulator MM-188, Narishige) and with two three-dimensional hydraulic remote-control micromanipulators (Joystick Hydraulic Micromanipulator MO-188, Narishige). The holding and injection pipettes are fitted to a tool holder and are connected by Teflon tubing (CT-1, Narishige) to a micrometer-type microinjector (IM-6, Narishige). Solution delivery is controlled via a one-µl-resolution vernier micrometer.

A single almost immotile spermatozoan is selected from the central droplet and is aspirated tail-first into the tip of the injection pipette. The spermatozoan is then placed in a separate 5 µl droplet of 10% PVP in Hapes-buffered Earle's medium, washed, and then picked up again into the injection pipette. The Petri dish is then moved in order to visualize an oocyte in one of the droplets surrounding the sperm suspension. The oocyte is immobilized by slight negative pressure exerted on the holding pipette. The polar body is held at 12 or 6 o'clock and the micropipette is pushed through the zona pellucida and the oolemma into the ooplasm at 3 o'clock. A single spermatozoan is injected into the ooplasm with around one to two picolitre of medium. The injection pipette is withdrawn gently and the injected oocyte is released from the holding pipette. The aspiration of a single spermatozoan and injection into the ooplasm are repeated until all metaphase II oocytes are injected. The injected oocytes are then washed in B2 medium and transferred into 25 µl droplets of B2 medium covered by lightweight paraffin oil. The Petri dishes with the oocytes are then incubated ( Heraeus, B5060 EK/O2, Van der Heyden, Brussels, Belgium) at 37°C, 5% CO2 and 90% N2).
Table 1 compares the overall results of these 72 MESA-ICSI cycles to the previous 67 consecutive MESA-IVF cycles (23). The fertilization rate with IVF using epididymal sperm was 7%, and only 19% of these patients had an embryo transfer. With ICSI, using epididymal or testicular sperm, the fertilization rate was 46%, and 90% of patients had an embryo transfer. The delivered pregnancy rate with epididymal sperm using IVF was 4.5%. With ICSI the ongoing or delivered pregnancy rate was 42% per stimulated cycle. There was clearly a seven-to-tenfold improvement in results with ICSI. This improvement is all the more dramatic when it is realized that the 72 consecutive ICSI cases included 32 otherwise rejected MESA patients who had no epididymal sperm, and thus required testicular biopsy for sperm retrieval.

**TABLE 1. Comparison of MESA-ICSI results to conventional MESA-IVF results in a similar patient population**

<table>
<thead>
<tr>
<th>Cycles</th>
<th>Mature Eggs</th>
<th>2 PN</th>
<th>Fertilization Rate</th>
<th>Transfers</th>
<th>Pregnancy Rate (Ongoing)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVF-MESA 67</td>
<td>1427</td>
<td>98</td>
<td>7%</td>
<td>13/67 (19%)</td>
<td>9% (4.5%)</td>
</tr>
<tr>
<td>ICSI-MESA 72</td>
<td>962</td>
<td>443</td>
<td>46%</td>
<td>65/72 (90%)</td>
<td>53% (42%)</td>
</tr>
</tbody>
</table>

We viewed the standard MESA procedure which yielded epididymal sperm to be the preferred approach because the large numbers of sperm obtained from the epididymis could easily be frozen and used in subsequent cycles with no need for any further surgery on the husband. However, in many of the most difficult referrals, who had had multiple previous surgeries, motile epididymal sperm were simply not retrievable. Only in these cases did we resort to testicular biopsy for sperm retrieval.

The patients in each of these three groups (fresh epididymal, frozen epididymal and testicular) represented no mixture whatsoever. If we used fresh epididymal sperm, then no eggs from that patient were injected with previously frozen sperm, or testicular biopsy sperm. If testicular biopsy sperm was used, then no eggs from that patient were injected with epididymal sperm. All 72 consecutive cases represented pure groups in this respect for proper comparison.
Table 2 summarizes the embryo transfer and pregnancy rates using fresh epididymal (MESA), frozen epididymal, and testicular biopsy extracted (TESE) sperm. Despite the somewhat lower 2PN fertilization rate already noted with frozen-thawed epididymal sperm, 100% of these patients had embryos to transfer after ICSI. Fresh MESA yielded a 94% transfer rate, and TESE (testicular sperm) a remarkable 84% transfer rate.

**TABLE 2. Pregnancy rate after ICSI with epididymal and with testicular biopsy sperm**

<table>
<thead>
<tr>
<th>Source of Sperm</th>
<th>Patients Cycles</th>
<th>Number Transfers</th>
<th>Number Clinical Pregnancies per Transfers</th>
<th>Number Ongoing or Delivered Per Transfer</th>
<th>Ongoing or Delivered Per Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh Epididymal (MESA)</td>
<td>33</td>
<td>31 (94%)</td>
<td>20 (65%)</td>
<td>15 (48%)</td>
<td>45%</td>
</tr>
<tr>
<td>Frozen Epididymal</td>
<td>7</td>
<td>7 (100%)</td>
<td>4 (57%)</td>
<td>3 (43%)</td>
<td>43%</td>
</tr>
<tr>
<td>Testicular Biopsy (TESE)</td>
<td>32</td>
<td>27 (84%)</td>
<td>14 (52%)</td>
<td>12 (44%)</td>
<td>38%</td>
</tr>
<tr>
<td>Totals</td>
<td>72</td>
<td>65 (90%)</td>
<td>38 (58%)</td>
<td>30 (46%)</td>
<td>42%</td>
</tr>
</tbody>
</table>

The delivered or ongoing pregnancy rate per transfer was 48% for fresh MESA, 43% for frozen-thawed (MESA) sperm, and 44% for TESE. The ongoing or delivered pregnancy rate per cycle was obviously similar with fresh MESA (45%) and frozen-thawed MESA (43%). The pregnancy rate per cycle was obviously somewhat lower (38%) for TESE (testicular biopsy sperm) because of the lower (84%) transfer rate. Stated more simply, 16% of TESE cases had no fertilization at all, and only 5% of MESA cases exhibited no fertilization. Yet the pregnancy rate per transfer was essentially identical in all three groups regardless of the sperm origin.

The only factor which affected success in couples undergoing MESA-ICSI or TESE-ICSI for obstructive azospermia was the wife. Furthermore, the only factor in the wife that mattered was her age.

Table 3 demonstrates this relationship. The age of the wife only affected implantation and pregnancy rate. The age of the wife had no impact on fertilization rate. When the wife was under 30 years of age,
75% of the MESA-ICSI cycles resulted in pregnancy. When the wife was 30 to 37 years of age, 54% became pregnant. When the wife was over 37 years of age, 24% became pregnant. The age of the wife only affected implantation and pregnancy rate.

TABLE 3. Fertilization and pregnancy rate after MESA-ICSI in relation to age of wife

<table>
<thead>
<tr>
<th>Age of Wife</th>
<th>Number of Patients</th>
<th>Number of Eggs M-II</th>
<th>2 PN (%)</th>
<th>Number Cleaved</th>
<th>Number Transferred Per Patient</th>
<th>Number Pregnant</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;30</td>
<td>20</td>
<td>293</td>
<td>138 (47%)</td>
<td>91/138 (66%)</td>
<td>54 (2.7)</td>
<td>15 (75%)</td>
</tr>
<tr>
<td>30-37</td>
<td>35</td>
<td>479</td>
<td>220 (46%)</td>
<td>160/220 (73%)</td>
<td>104 (3.0)</td>
<td>19 (54%)</td>
</tr>
<tr>
<td>&gt;37</td>
<td>17</td>
<td>190</td>
<td>85 (45%)</td>
<td>51/85 (60%)</td>
<td>51 (3.0)</td>
<td>4 (24%)</td>
</tr>
<tr>
<td>Totals</td>
<td>72</td>
<td>962</td>
<td>443 (46%)</td>
<td>302/443 (68%)</td>
<td>209 (2.9)</td>
<td>38 (53%)</td>
</tr>
</tbody>
</table>

CYSTIC FIBROSIS AND CONGENITAL ABSENCE OF VAS (CAV): PRE-IMPLANTATION EMBRYO DIAGNOSIS

Virtually all patients with cystic fibrosis (the most common genetic disorder in humans) also have congenital absence of the vas (CAV). Until recent years, most cystic fibrosis (CF) patients did not survive sufficiently long to even consider trying to become a father. Therefore, none of the CAV patients initially treated (over 200) had clinical cystic fibrosis. In fact, until the papers of Anguiano et al (1) and our papers in 1991 and 1993 (1; 21; 15), there was no awareness that infertile males with CAV in fact had an isolated genital form of cystic fibrosis that was inherited via a mutation on the CF gene.

All CAV patients and their wives about to undergo MESA must be screened for at least the 36 most common mutations on the CFTR (cystic fibrosis transmembrane protein) gene. In fact, there are over 300 CF mutations but most of them are rare and usually not found on routine screening. But they are important in CAV. About 70% of otherwise
normal CAV patients have one of these CF mutations, despite not having clinical CF.

The inheritance of CAV on the CF gene follows simple Mendelian rules. The male child must have inherited a CF mutation from each parent and at least one of these mutations is usually rare and not detectable on routine screening. That is why the CAV patient usually appears to be a heterozygous CF carrier. Most male heterozygous CF carriers do not have CAV. To have CAV, the patient had to receive two CF alleles, one from each of his parents. One of the two parents of CF positive CAV males always has the same heterozygous mutation as their son. Siblings of the CAV male obviously have a one-in-four chance of also having CAV. Offspring of CAV males (after successful MESA) have a 50% chance of having the same CF heterozygous mutation as the CAV father. Yet the CF positive heterozygous male offspring of CAV males do not themselves have CAV, and indeed have normal vasa bilaterally. In like manner, the CAV patient’s father in 50% of cases is a carrier for the same mutation as his son, but obviously did not have CAV, and clearly was fertile.

Thus, CF carrier status alone certainly does not cause CAV. The child must receive not just one CF mutated gene from one parent to have CAV. He must get some mutation (perhaps undetectable with current methods) from both parents (10). Ten percent (10%) of CAV patients have two obviously different CF mutations, (either two weak ones, or a weak and a strong one, or sometimes two different strong CF mutations) detectable with common CF screening. These “compound” heterozygous CF carriers do not usually have clinical CF if one of the mutations is a weak one. They do not have a positive sweat chloride test. Yet this obvious compound heterozygosity does cause CAV.

It is crucial to screen both the husband with CAV, and his wife, for CF mutations. If the wife is negative for any of the 36 common CF mutations, we feel it is quite safe to perform MESA-ICSI on the couple. The chances of a male offspring having CAV are very remote, and the chances of the child’s having cystic fibrosis is probably less than in a normal, unscreened population.

However, if the wife turns out to be a CF carrier herself (4% incidence in the general population), the couple can still undergo MESA-ICSI, but preimplantation embryo diagnosis would then be mandatory. In fact, CAV-MESA patients may represent the largest single group who will need to use this technique. We have published the first case of successful pre-implantation embryo diagnosis in a CAV-
MESA case in which both partners were carriers of the ΔF508 mutation (9). We require embryo blastomere biopsy and pre-implantation diagnosis as a routine approach whenever the female is discovered, upon screening, to be a CF carrier.

An important ancillary finding is that for the purpose of ICSI, it is the "vitality" of the sperm and, of course, not the motility that matters. Presumably, the more proximal the sperm retrieval, the greater the percent of "viable" sperm. Therefore, testicular sperm retrieval (TESE) may afford a method to assure getting non-senescent, so-to-speak "fresh," sperm for ICSI when the ejaculate exhibits no motility. We first used TESE-ICSI under this rationale for a case of Kartagener's syndrome with successful fertilization and ongoing pregnancy. But no matter what the cause of total asthenospermia, the key to success with ICSI is to inject "live" sperm. But total lack of motility would make selection of "live" sperm difficult. The solution to this problem is to retrieve sperm from testicular tissue obtained via biopsy. Although the sperm motility is always terrible in the testicle, the percentage of fresh, live "vital" sperm should be high.

O'Brien's studies on the inbreeding of cheetahs, and of certain population "bottlenecks" of lions, also points to the strong genetic control over spermatogenesis. Inbreeding, and loss of genetic diversity, clearly leads to severe defects in spermatogenesis (30, 31). If male infertility is for the most part genetic, it would explain the dismal results achieved by every method of treatment attempted thus far, except of course for ICSI. Varicocelectomy, the last bastion of conventional treatment for male infertility, has been shown in a beautiful controlled study by Nieschlag to be no better than psychological counseling. Most reproductive physicians today are not surprised by these results (32).

**SUMMARY**

The purpose of this paper was to review the results and rationale for using testicular and epididymal sperm with ICSI for severe cases of male infertility. Seventy-two consecutive MESA cases were performed for congenital absence of vas (CAV) and for irreparable obstructive azospermia, using direct intracytoplasmic injection (ICSI) of an individual sperm into metaphase II oocytes of the wife. Fertilization and normal embryos were obtained for transfer in 90% of cases. There was
an overall fertilization rate of 46%, and a normal cleavage rate of 68%. The ongoing and delivered pregnancy rates per transfer were 46% and 42% per cycle.

In many cases, there was no epididymal sperm available, and testicular biopsy, i.e., testicular sperm extraction, (TESE) was resorted to for sperm retrieval. This approach had only a minor negative effect on results. The transfer rate was lower with TESE (84% versus 96%), and the sperm could not be frozen and saved for future cycles. But there was no dramatic difference in pregnancy rates with epididymal or testicular tissue sperm.

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