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The use of epididymal and testicular spermatozoa for intracytoplasmic sperm injection: the genetic implications for male infertility

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The results and rationale of using testicular and epididymal spermatozoa with intracytoplasmic sperm injection (ICSI) for severe cases of male infertility are reviewed. A total of 72 consecutive microsurgical epididymal sperm aspiration (MESA) cases were performed for congenital absence of the vas (CAV) and for irreparable obstructive azoospermia. ICSI was used to obtain normal embryos for transfer and fertilization in 90% of the cases. The overall fertilization rate was 46% with a normal cleavage rate of 68%. The pregnancy and delivery rates per transfer were 58 and 37% respectively. The delivery rate per cycle was 33%. In many cases, no epididymal spermatozoa were available and so testicular sperm extraction (TESE) was used for sperm retrieval. The transfer rate was lower with TESE (84 versus 96%) and the spermatozoa could not be frozen and saved for use in future cycles. However, there was little difference in pregnancy rates using epidiymal or testicular spermatozoa. The results were not affected by whether the obstruction was caused by CAV or failed vasoepididymostomy. Both fresh and frozen spermatozoa gave similar results; the only significant factor appeared to be the age of the female. Because of the consistently good results obtained using epididymal sperm with ICSI when compared with conventional IVF, and the similarly good results with testicular tissue spermatozoa, ICSI is mandatory for all future MESA patients. All CAV patients and their partners should be offered genetic screening for cystic fibrosis; hence pre-implantation embryo diagnosis should be available in any full service MESA programme. It is now clear that even with non-obstructive azoospermia, e.g. Sertoli-cell only, or maturation arrest, there are usually some small foci of spermatogenesis which allow TESE with ICSI to be carried out. This means that even in men with azoospermia due to absence of spermatogenesis or to a block in meiosis, there are usually a few spermatozoa available in the testes that are adequate for successful ICSI. Finally, it is likely that some forms of severe male factor infertility are genetically transmitted and although ICSI offspring have been shown to be completely normal, it is possible that the sons of these infertile couples will also require ICSI when they grow up and wish to have a family.

Key words: epididymal spermatozoa/ICSI/male factor infertility/testicular spermatozoa

Introduction

Clinical interest in the fertilizing capacity of epididymal and testicular spermatozoa developed originally from cases of obstructive azoospermia that could not be corrected surgically. Congenital absence of the vas deferens (CAV), failed vasoepididymostomy and all irreparable obstructions are very frustrating because these patients have normal spermatogenesis but nonetheless have been untreatable in the past (Silber *et al.*, 1990). Microsurgical epididymal sperm aspiration (MESA) and in-vitro fertilization (IVF) were introduced in 1985 and 1988 respectively to treat these cases, with modest success (Temple-Smith *et al.*, 1985; Silber *et al.*, 1988, 1990).

However, soon after the initial enthusiasm for using IVF with aspirated spermatozoa, it became apparent that fertilization failed more often with retrieved epididymal spermatozoa, and there was no readily apparent reason. There was no recognizable difference between the motility of epididymal spermatozoa that fertilized with conventional IVF versus epididymal spermatozoa that did not fertilize (Silber, 1994). Most centres attempting this procedure have obtained ≤9% fertilization and pregnancy rates (Belker, 1994).

Intracytoplasmic sperm injection (ICSI) has revolutionized the treatment of oligoasthenoteratozoospermia (Palermo *et al.*, 1992, 1993; Van Steirteghem *et al.*, 1993a,b). Therefore, we wanted to determine whether ICSI could improve the poor fertilization and pregnancy rates in patients with microsurgically retrieved epididymal or testicular spermatozoa. We were particularly interested in cases where there were no motile epididymal spermatozoa retrieved to see if the spermatozoa obtained from testicular biopsy could actually fertilize and develop into a fetus using ICSI. Our collaboration demonstrated that ICSI using epididymal or testicular spermatozoa in men with CAV or any other cause of obstructive azoospermia does give reliable, very high fertilization and pregnancy rates with normal deliveries. This discovery led to an intense interest in the use of fresh and frozen epididymal and testicular spermatozoa with ICSI, not only for obstructive but also for non-obstructive azoospermia. Our collaboration also stimulated a concerted study of the genetic aspects of male infertility.

The purposes of this paper are (i) to review the first use of ICSI to treat obstructive azoospermia of all causes (CAV, failed vasoepididymostomy or irreparable obstruction), (ii) to review the first systematic use of ICSI with testicular tissue spermatozoa, (iii) to discuss the use of testicular spermatozoa and ICSI for the treatment of non-obstructive (e.g. Sertoli cell only and maturation arrest) as well as obstructive azoospermia, and (iv) to suggest what direction male infertility treatment will be taking in the future, and to touch briefly on its genetic implications.

Epididymal sperm motility and conventional IVF

The most striking finding in the retrieval of spermatozoa from the chronically obstructed epididymis is the inversion of the usual pattern of motility one would have expected in a non-obstructed epididymis (Young, 1931; Gaddum and Glover, 1965; Bedford, 1966; Gaddum, 1969; Glover, 1969). Spermatozoa with the greatest motility have always been found most proximally in the obstructed epididymis (Silber *et al.*, 1990). The percentage motility is usually low (1–30%), but the greatest motility is always found, ironically, in the most proximal region.

Spermatozoa in the distal regions of an obstructed epididymis have poor to no motility because of senescence. Electron microscopic studies have indicated that the distal spermatozoa are ageing, and the more recently produced proximal spermatozoa have not yet degenerated (Friend *et al.*, 1976; Asch *et al.*, 1992). Therefore, the problems with fertilization using these epididymal spermatozoa may be ageing as well as immaturity (Figure 1).

Assuming that distal dead spermatozoa are avoided, the most baffling observation with epididymal sperm aspiration and conventional IVF is the apparent inability to predict fertilization from sperm motility. Something other than motility which is related to epididymal sperm transit is required to achieve a maximum fertilization rate with conventional IVF. After successful vasoepididymostomy, ejaculated spermatozoa can usually fertilize, but retrieved epididymal spermatozoa cannot. No IVF programme has consistently produced >5–10% success with retrieved epididymal spermatozoa without the use of micromanipulation.

Materials and methods

Methodology for MESA–ICSI and MESA–IVF

Egg and sperm retrieval

The female partners underwent a fairly routine induction of multiple follicular development using leuprolide acetate (Lupron; TAP Pharmaceuticals, North Chicago, IL, USA); 1 mg s.c. was administered daily until the day of follicular aspiration. After desensitization, patients received human follicle stimulating hormone (FSH, Metrodin; Serono Laboratories Inc., Randolph, MA, USA) and/or human menopausal gonadotrophin (HMG, Pergonal; Serono) until many follicles ~2.0 cm in diameter were noted on ultrasound. Then 10 000 IU human chorionic gonadotrophin (HCG, Profasi; Serono) were administered i.m. The patients underwent transvaginal follicular aspiration 36 h after HCG administration.

The husband underwent microsurgical scrotal exploration with the intention of aspirating motile spermatozoa to utilize for IVF or ICSI with the wife's eggs (Figure 2). The surgical technique in the male was as follows. The scrotal contents were extruded through a small incision, the tunica vaginalis was opened and the epididymis was exposed. Under ×10–40 magnification with an operating microscope, a tiny incision was made with microscissors in the epididymal tunic to expose the tubules in the proximal portion of the obstructed epididymis. Spermatozoa were aspirated directly from the opening in the epididymal tubule with a micropipette. The epididymal fluid was diluted immediately in HEPES-buffered Earle's medium and examined for motility and quality of progression. If there was no motility, another aspiration was made more proximally. Motile spermatozoa were not usually obtained until the most proximal portion of the caput epididymis or even the vasa efferentia was reached (Silber *et al.*, 1988, 1990).

The rationale for trying to find the most motile spermatozoa even with ICSI is based on the following presumptions. ICSI with ejaculated spermatozoa works best when there is some motility, no matter how poor, as a verification of 'vitality'. All previous experience with ICSI has demonstrated minimal success with absolutely non-motile spermatozoa. In the obstructed epididymis, non-motile spermatozoa are most often senescent and degenerate. The most proximal spermatozoa are least likely to have undergone senescent degenerative changes in the sperm head, and most likely to exhibit some motility. An early study of ICSI with MESA by Tournaye *et al.* (1994) suggested that such an approach would give better results.

When no motile spermatozoa were retrievable from the epididymis on either side, or when the epididymis was absent, a testicular biopsy was performed and spermatozoa were retrieved by morselizing the testicular specimen. Invariably, only occasional small numbers of spermatozoa were obtainable from minced testicular tissue, along with large numbers of spermatid-laden Sertoli cells. The few free spermatozoa had only weak, slowly twitching motility. In general, this method has not been successful for conventional IVF but is quite effective for ICSI (Craft *et al.*, 1993; Schoysman *et al.*, 1993; Devroey *et al.*, 1994; Silber *et al.*, 1994). The incubation of testicular spermatozoa in culture medium for ~1 h improves this motility dramatically. Even 100% non-motile testicular tissue spermatozoa will develop some very weak motility with incubation in culture medium.

There is nothing particularly unusual about the treatment of epididymal or testicular spermatozoa. Epididymal spermatozoa are usually prepared with a routine mini-Percoll gradient, whether for conventional IVF or ICSI. Testicular spermatozoa are simply concentrated by centrifugation because of the very small numbers and extremely poor motility.

Epididymal spermatozoa (MESA) and ICSI

The techniques of sperm and oocyte preparation, microinjection and culture were as described by Van Steirteghem *et al.* (1993a,b) for severe male factor cases. All embryos were transferred atraumatically to the uterus via a Frydman catheter. Stimulation protocol, egg retrieval, sperm retrieval and luteal support were similar to those in conventional IVF.

However, using the ICSI technique for MESA allowed several differences in approach from conventional IVF to be adopted. Since only very small numbers of spermatozoa are required for ICSI, the majority of the retrieved epididymal spermatozoa were frozen for subsequent cycles. Because for ICSI only a minor degree of motility is required (for vitality assessment only), freezing these fragile epididymal spermatozoa posed no serious problem. All of the epididymal fluid that was not used fresh for ICSI was diluted 50:50 by volume with sperm freezing medium, drawn into 250 µl straws, suspended over liquid nitrogen vapour for several hours and then

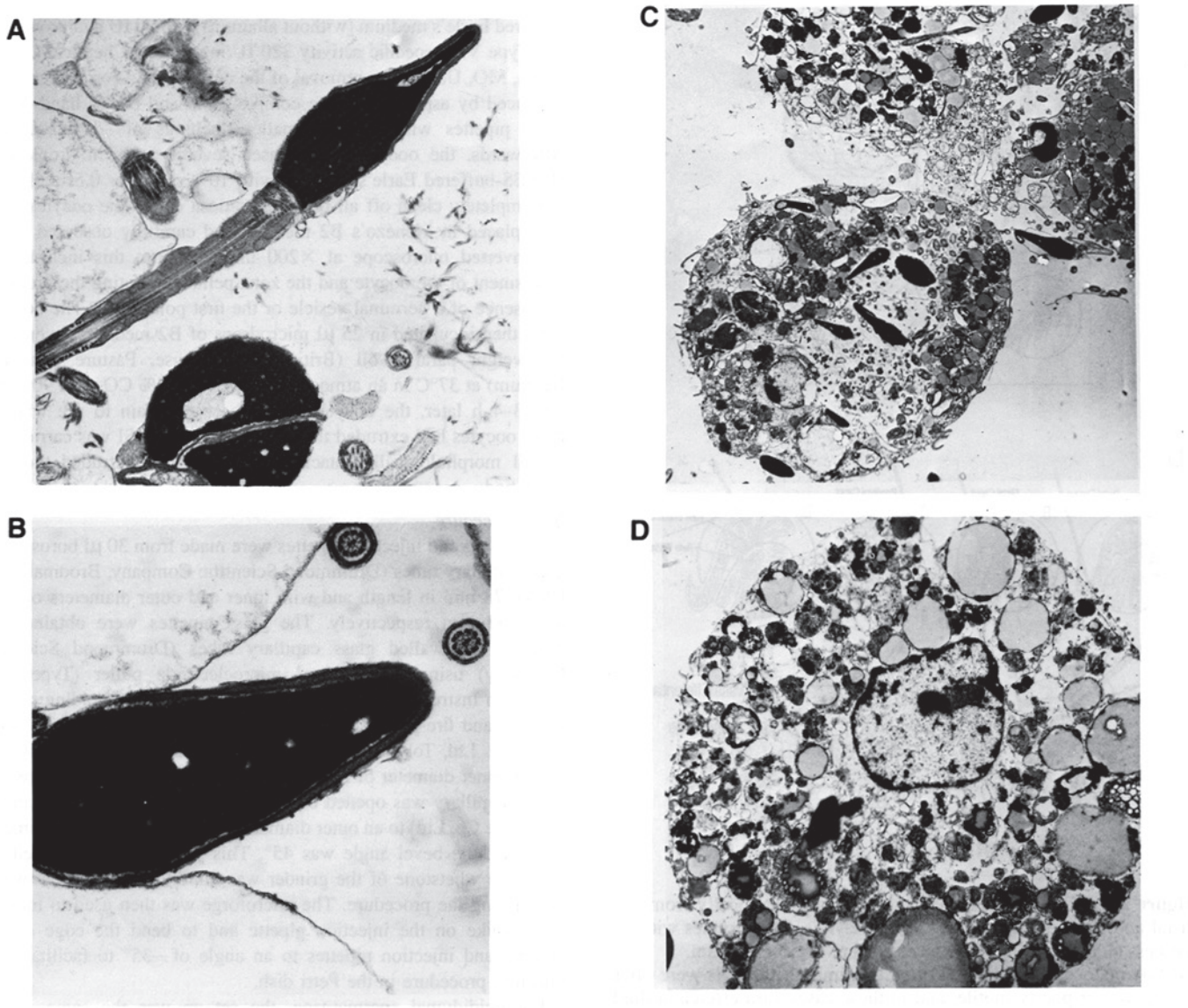

Figure 1. (**A**) Ultrastructure of spermatozoa obtained from the rete testis from a man with congenital absence of the vas deferens (CAV). Note the similarities in the organization of the nucleus acrosome and flagella to normal ejaculated spermatozoa. (**B**) Ultrastructure of spermatozoa obtained from the vasa efferentia from a man with CAV. Note the similarities in the organization of the nucleus acrosome and flagella to normal ejaculated spermatozoa. (**C**) Ultrastructure of cells obtained from the corpus epididymis in a man with CAV. Note the macrophages with their cytoplasm occupied by very large amounts of phagocytized sperm remnants in different degrees of degradation and digestion. (**D**) Ultrastructure of macrophage obtained from the cauda epididymis from a man with CAV. Note the presence of prominent whorls of membranes and numerous lipid droplets indicating advanced stages of sperm degradation.

plunged into liquid nitrogen in a standard fashion (Devroey *et al.*, 1995; Nagy *et al.*, 1995).

Epididymal spermatozoa were prepared for microinjection in the same manner as for conventional IVF using Percoll. As has been published by Liu *et al.* (1994), we found that the sperm preparation method has no effect on the results but does have an effect on the technical ease of performing ICSI. Testicular tissue spermatozoa were retrieved in such small numbers and with such minimum motility that the only preparation method was to morselize the tissue, centrifuge the effluent at 300 *g* for 5 min and resuspend in 100 µl. At present, testicular tissue spermatozoa are not frozen and saved, as is routinely carried out for epididymal spermatozoa, because of the small numbers of barely undulating spermatozoa and the large numbers of Sertoli cells, debris and red blood cells.

Testicular spermatozoa and ICSI

When there was no epididymis, when the entire scrotum was massively scarred, or when there were simply no spermatozoa in the epididymis, we resorted to testicular biopsy for sperm retrieval. The surgical technique for testicular biopsy was extremely simple. A small 1 cm horizontal incision was made in the scrotal skin and carried through the peritoneal tunica vaginalis. Then a 0.5 cm incision was made in the tunica albuginea, and a small piece of extruding testicular tissue was excised and placed in a small Petri dish with 3 ml of HEPES-buffered Earle's medium. The tunica albuginea was closed with several 3-0 vicryl intracuticular stitches.

The piece of testicular tissue was finely minced in HEPES-buffered medium, and this effluent was then placed in a 5 ml Falcon tube and centrifuged for 5 min at 300 *g* (Schoysman *et al.*, 1993; Devroey

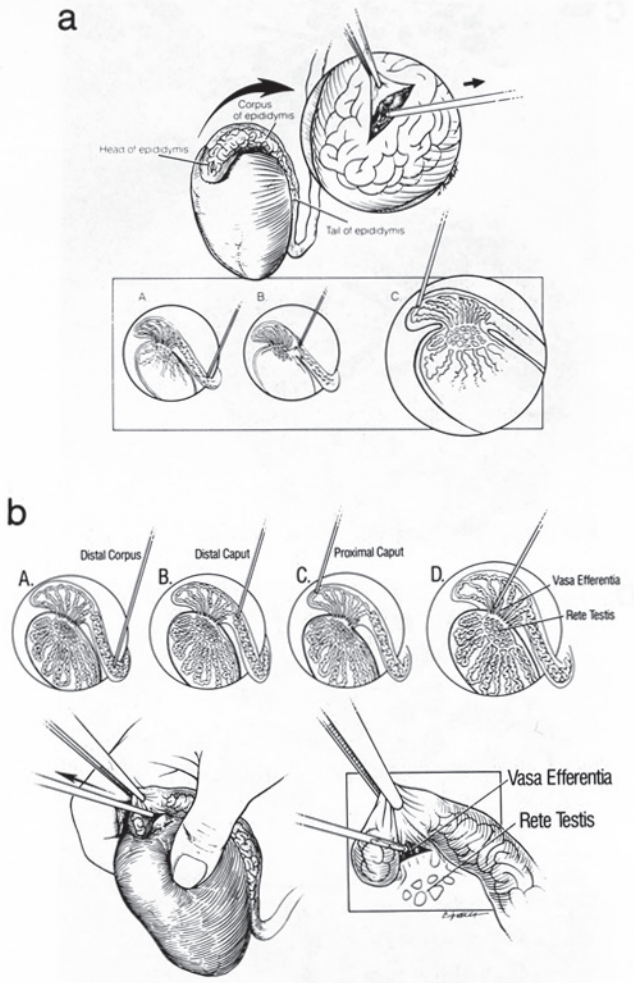

Figure 2. (**a**) Spermatozoa were aspirated microsurgically from distal and proximal regions of the epididymis. The samples with the best quality motility were used for in-vitro fertilization. (**b**) Spermatozoa in distal and often proximal epididymis were often non-motile or poorly motile, and in these cases vasa efferentia fluid usually had the most motile spermatozoa.

et al., 1994; Nagy *et al.*, 1995; Silber *et al.*, 1995). The supernatant was removed and the pellet was resuspended in medium. It would be pointless to attempt Percoll separation. The number of spermatozoa in the droplet was so few and the motility was so weak that there was no choice but to pick the individual spermatozoon up out of the field of debris, red blood cells and Sertoli cells for ICSI. The sperm suspension was then kept at 37°C in the incubator (5% O_2, 5% CO_2, 90% N_2) in Earle's medium until it was time for the ICSI procedure to be performed on the oocytes.

The motility of the few testicular biopsy spermatozoa was so weak (really barely apparent at all in many cases) that the sperm droplet was not placed into the polyvinylpyrrolidone (PVP) droplet, as is performed in most ICSI cases. Rather, the individual barely motile spermatozoon was picked up out of the droplet of testicular concentrate and then placed in the PVP droplet, which often results in complete immotility. The tail of the spermatozoon in all cases should still be broken because these spermatozoa often exhibited increased motility later on.

Oocyte preparation for the ICSI procedure

Oocyte retrieval was carried out by vaginal ultrasound-guided puncture 36 h after HCG. After oocyte retrieval, the cells of the cumulus and corona radiata were removed by incubation for ~30 s in HEPES-buffered Earle's medium (without albumin) 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 was enhanced by aspiration of the complexes in and out of hand-drawn glass pipettes with serially smaller openings of ~150–200 µm. Afterwards, the oocytes were rinsed several times in droplets of HEPES-buffered Earle's medium with 10% serum or 0.5% albumin to completely clean off all of the granulosa cells. The oocytes were then placed in Ménézo's B2 medium and carefully observed under the inverted microscope at ×200 magnification; this included an assessment of the oocyte and the zona pellucida, noting the presence or absence of a germinal vesicle or the first polar body. The oocytes were 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% O_2, 5% CO_2 and 90% N_2. At ~3–4 h later, the oocytes were observed again to see whether more oocytes had extruded the first polar body. ICSI was carried out on all morphologically intact oocytes that had extruded the first polar body.

ICSI procedure

The holding and injection pipettes were made from 30 µl borosilicate glass capillary tubes (Drummond Scientific Company, Broomall, PA, USA), 78 mm in length and with inner and outer diameters of 0.69 and 0.97 mm respectively. The glass pipettes were obtained by drawing thin-walled glass capillary tubes (Drummond Scientific Company) using a horizontal microelectrode puller (Type 753, Campden Instruments Ltd, Loughborough, UK). The holding pipette was cut and fire-polished on a microforge (MF-9 Microforge, Narishige Co. Ltd, Tokyo, Japan) to obtain an outer diameter of 50 µm and an inner diameter of 20 µm. To prepare the injection pipette, the pulled capillary was opened on a microgrinder (EG-4 Microgrinder, Narishige Co. Ltd) to an outer diameter of 7 µm and an inner diameter of 5 µm; the bevel angle was 45°. This grinding step required ~3 min. The whetstone of the grinder was humidified by a slow water drip during the procedure. The microforge was 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 ~35° to facilitate the injection procedure in the Petri dish.

For epididymal spermatozoa, the set up was the same as for ejaculated spermatozoa. A 5 µl droplet of 10% PVP (Sigma Chemical Co.) in HEPES-buffered Earle's medium with 0.5% bovine serum albumin (BSA) was placed in the centre of a Petri dish (Falcon type 1006), and a 1–2 µl sperm droplet was placed into the 10% PVP droplet. As the spermatozoa swam out into the PVP droplet, they were slowed down by the PVP and then picked up by the injection pipette. For testicular spermatozoa which were barely motile, a 3–5 µl sperm droplet was placed in the centre of the Petri dish (Falcon type 1006) right next to a 5 µl PVP droplet which was surrounded by eight 5 µl droplets of HEPES-buffered Earle's medium with 0.5% BSA. These droplets were all covered by ~3.5 ml of lightweight paraffin oil.

The ICSI procedure was carried out on the heated stage (37°C; THN-60/16 and MS100 controllers, Linkam Scientific Instruments Ltd, London, UK) of an inverted microscope (Diaphot, Nikon Corporation, Tokyo, Japan) at ×400 magnification using the Hoffman Modulation Contrast System (Modulation Optics Inc., Greenvale, New York, USA). The microscope was equipped with a Nikon F-601M camera for still pictures and a video camera (DXC-755 P, Sony Corporation, Brussels, Belgium) that allowed the procedure to be followed on a Trinitron® colour video monitor (PVM-1443MD, Sony Corporation). The microscope was equipped with two coarse positioning manipulators (3D Motor Driven Coarse Control Manipulator MM-188, Narishige Co. Ltd) and with two three-dimensional

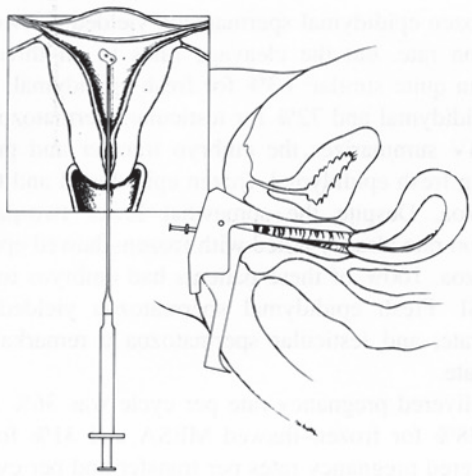

Figure 3. Pick-up of weakly motile spermatozoa from Petri dish microdroplet.

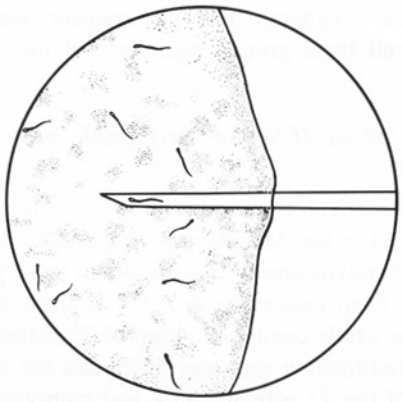

Figure 4. Preparing to inject spermatozoa into egg.

hydraulic remote-control micromanipulators (Joystick Hydraulic Micromanipulator MO-188, Narishige Co. Ltd). The holding and injection pipettes were fitted to a tool holder and were connected by Teflon tubing (CT-1, Narishige Co. Ltd) to a micrometer-type microinjector (IM-6, Narishige Co. Ltd). Solution delivery was controlled via a 1 µl resolution vernier micrometer.

For testicular spermatozoa, a single weakly motile spermatozoon was selected from the central sperm droplet and was aspirated tail-first into the tip of the injection pipette. The spermatozoon was then placed in the separate 5 µl droplet of 10% PVP in HEPES-buffered Earle's medium and washed. The tail was broken with the tip of the injection pipette to immobilize it, and was then picked up again into the injection pipette. The Petri dish was moved to visualize an oocyte in one of the droplets surrounding the sperm suspension. The oocyte was immobilized by applying slight negative pressure on the holding pipette. The polar body was held at 12 or 6 o'clock and the micropipette was pushed through the zona pellucida and the oolemma into the ooplasm at 3 o'clock. A single spermatozoon was injected into the ooplasm with ~1–2 pl of medium. The injection pipette was withdrawn gently and the injected oocyte was released from the holding pipette. The aspiration of a single spermatozoon and injection into the ooplasm were repeated until all metaphase II oocytes were injected. The injected oocytes were 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 were then incubated (Heraeus, B5060 EK/O_2, Van der Heyden, Brussels, Belgium) at 37°C, 5% CO_2 and 90% N_2 (Figures 3 and 4).

Table I. Comparison of micro-epididymal sperm aspiration (MESA)–intracytoplasmic sperm injection (ICSI) with conventional MESA–in-vitro fertilization (IVF) in a similar patient population

Method	Cycles	Mature eggs	2PN	Fertilization rate (%)	Transfers (%)	Pregnancy rate (% delivered)
IVF–MESA	67	1427	98	7	13/67 (19)	3/67 (4.5)
ICSI–MESA[a]	33	431	201	47	31/33 (94)	12/33 (36.3)

2PN = two pronuclei.
[a]This does not include results with frozen epididymal sperm cycles and testicular sperm cycles (see Tables III and IV).

Assessment of fertilization, embryo cleavage and pregnancy

Further handling of the injected oocytes was similar to our standard IVF procedure. At ~16–18 h after microinjection, the oocytes were observed under the inverted microscope (×200 or ×400 magnification) for any sign of damage which may have been incurred during microinjection and for the presence of pronuclei and polar bodies. Fertilization was considered normal when two clearly distinct pronuclei containing nucleoli were present. The presence of one pronucleus or three pronuclei was noted, together with the presence of one, two or fragmented polar bodies. If a single pronucleus was observed, a second evaluation was carried out ~4 h later to see whether the pronuclear status had changed. The embryo cleavage of the two-pronuclear oocytes was evaluated after a further 24 h of in-vitro culture. The embryos were scored according to the equality of size of the blastomeres and the number of anucleate fragments. Cleaved embryos with <50% of their volume filled with anucleate fragments were eligible for transfer. Up to three and occasionally more embryos (depending on the age of the wife and the embryo quality) were loaded in a few microlitres of Earle's medium into a Frydman catheter (LG 4.5, Prodimed, Neuilly-en-Thelle, France) and transferred into the uterine cavity. Embryo replacement was usually performed ~48 h after the microinjection procedure. If supernumerary embryos with <20% anucleate fragments were available, they were cryopreserved on day 2 or day 3 by a slow-freezing protocol with dimethylsulphoxide.

Pregnancy was confirmed by detecting increasing serum HCG concentrations on at least two occasions at least 12 days after embryo replacement. Clinical pregnancy was determined by observing a gestational sac by echographic screening at 7 weeks of pregnancy. Patients were asked to provide detailed information of the evolution of the pregnancy and the outcome of the delivery.

Results

This is a cumulative report on our first 72 consecutive cycles of ICSI for obstructive azoospermia using fresh epididymal, frozen–thawed epididymal and testicular spermatozoa. A total of 52 cycles were performed for CAV, and 20 were performed for failed vasoepididymostomy or irreparable blockage; 33 of the 72 cycles utilized fresh epididymal spermatozoa (MESA), seven used frozen epididymal spermatozoa and 32 employed testicular biopsy spermatozoa (testicular sperm extraction; TESE). Results were equivalent in CAV, failed vasoepididymostomy cases and irreparable blockage. Results were also equivalent for MESA (epididymal spermatozoa) and TESE (testicular spermatozoa).

Table I compares the overall results of our first consecutive

33 MESA–ICSI cycles with the previous 67 consecutive MESA–conventional IVF cycles (Silber *et al.*, 1994). The fertilization rate with conventional IVF using epididymal spermatozoa was 7%, and only 19% of these patients had an embryo transfer. With ICSI, using epididymal or testicular spermatozoa, the fertilization rate was 47%, and 94% of patients had an embryo transfer. The delivered pregnancy rate with epididymal spermatozoa using IVF was 4.5%. With ICSI, the delivered pregnancy rate was 36.3% per stimulated cycle. There was clearly a 7- to 10-fold improvement in results with ICSI. This improvement is all the more dramatic when it is realized that these 33 consecutive MESA–ICSI cases showed results similar to 32 otherwise rejected MESA patients who had no epididymal spermatozoa and thus required testicular biopsy for sperm retrieval (see Tables III and IV).

Table II extensively summarizes the results of our first 52 cases of MESA–ICSI for CAV. Of 52 patients, 37 (71%) had common cystic fibrosis carrier genotypes and 24 had the most common ΔF508 mutation. This table is an expansion of the original 14 cases in the study reported by us in Silber *et al.* (1994). The cystic fibrosis carrier status, including ΔF508, had no adverse effect on fertilization or pregnancy using ICSI. Among the 37 patients carrying at least one cystic fibrosis mutation, 23 (62%) became pregnant. Among the 16 cystic fibrosis-negative patients, six (38%) became pregnant (Table II). This is not a significant difference, and in any event it is clear that cystic fibrosis-positive carrier status does not negatively affect fertility results with ICSI. None of the 52 patients presenting with CAV had clinical signs of cystic fibrosis, and all had a normal chloride sweat test. Of the 52 CAV patients, 29 (56%) became pregnant; nine of the 20 failed vasoepididymostomy patients (45%) became pregnant. Again, this represents no significant difference (see Table V).

Results using epididymal versus testicular spermatozoa

Table III summarizes the fertilization and cleavage rates using fresh epididymal spermatozoa, frozen–thawed epididymal spermatozoa and testicular biopsy-extracted spermatozoa. We viewed the standard MESA procedure which yielded epididymal spermatozoa to be the preferred approach because the large numbers of spermatozoa obtained from the epididymis could be frozen easily 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 undergone multiple previous surgeries, motile epididymal spermatozoa 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 spermatozoa, then no eggs from that patient were injected with previously frozen spermatozoa or testicular biopsy spermatozoa. If testicular biopsy spermatozoa were used, then no eggs from that patient were injected with epididymal spermatozoa. All 72 consecutive cases represented pure groups in this respect for proper comparison.

The two-pronuclear fertilization rate was remarkably similar for fresh epididymal (47%) and testicular biopsy (49%) spermatozoa. Frozen epididymal spermatozoa yielded a lower (28%) fertilization rate, but the cleavage rates in all three groups were again quite similar: 63% for fresh epididymal, 74% for frozen epididymal and 72% for testicular spermatozoa.

Table IV summarizes the embryo transfer and pregnancy rates using fresh epididymal, frozen epididymal and testicular spermatozoa. Despite the somewhat lower two-pronuclear fertilization rate already noted with frozen–thawed epididymal spermatozoa, 100% of these patients had embryos to transfer after ICSI. Fresh epididymal spermatozoa yielded a 94% transfer rate, and testicular spermatozoa a remarkable 84% transfer rate.

The delivered pregnancy rate per cycle was 36% for fresh MESA, 28% for frozen–thawed MESA and 31% for TESE. The delivered pregnancy rates per transfer and per cycle were obviously similar for fresh MESA, frozen–thawed MESA and TESE. However, 16% of TESE cases had no fertilization at all, and only 4% of MESA cases exhibited no fertilization. However, the pregnancy rate per transfer was essentially identical in all three groups regardless of the origin of the spermatozoa.

Results of MESA–ICSI for irreparable obstructive azoospermia

Table V summarizes the results in 20 patients out of the 72 whose indication for MESA was irreparable obstruction or failed vasoepididymostomy. The results in these patients were no different from patients with CAV or from the aggregate results of the whole combined group of 72 patients. The two-pronuclear fertilization rate was 56%, and the cleavage rate was 80%. Of the 72 patients, 95% had embryos for transfer, 45% had a clinical pregnancy and 40% delivered. Therefore, the aetiology of obstructive azoospermia had no important or obvious influence on the results.

Virtually all couples preferred to have a successful vasoepididymostomy or vasectomy reversal because we could assure most patients a very high percentage (88%) of surgical success (Silber, 1989a,b,c). The wife would then not have to go through IVF. Furthermore, they could continue to have more than one child in the future without the need for any further medical or surgical procedures. Therefore, our usual policy was not to routinely perform MESA–ICSI on reconstructable cases. This means that these 20 obstructed patients who had MESA or TESE with ICSI were a very select group who had no chance of pregnancy without MESA–ICSI. Yet the results even in this seemingly poor prognosis group were quite acceptable. Therefore, we now freeze spermatozoa that are retrieved at the time of microsurgical vasoepididymostomy or vasectomy reversal as a back-up procedure just in case the reconstruction should fail. In that event, ICSI can be performed with this frozen epididymal sample which was retrieved at the time of attempted vasoepididymostomy.

Results of MESA or TESE with ICSI in relation to female factors, type of spermatozoa retrieved and implantation rates

The only factor which affected success in couples undergoing MESA–ICSI or TESE–ICSI for obstructive azoospermia was the woman. Clearly, whether spermatozoa were from the

Table II. Fertilization, pregnancy and delivery with ICSI in the first 52 patients with congenital absence of the vas deferens (CAV): relationship to cystic fibrosis genotype

Patient	Genotype if CAV	No. of mature eggs (MII)	No. of fertilized eggs (2PN)	No. of embryos transferred	Fertilization rate (%)	Age of wife	Pregnancy	Delivered
1	ΔF508/N	9	3	3	33	42	No	No
2	N/N	14	5	5	36	39	No	No
3	ΔF508/N	22	11	6	50	31	No	No
4	ΔF508/N	10	5	5	50	33	No	No
5	R117H/R117H	14	6	4	43	31	Yes	No
6	ΔF508/N	18	6	4	33	28	Yes	Yes
7	W1282X/N	16	5	3	31	36	Yes	Yes
8	ΔF508/N	3	2	2	67	25	No	No
9	N/N	3	0	0	0	40	No	No
10	N/N	7	3	3	43	28	Yes	Yes
11	ΔF508/N	8	1	1	13	35	Yes	No
12	ΔF508/N	12	5	3	42	36	Yes	Yes
13	ΔF508/N	10	0	0	0	35	No	No
14	R117H/N	11	5	3	45	29	Yes	No
15	N/N	8	4	3	50	32	No	No
16	N/N	12	3	2	25	32	Yes	Yes
17	ΔF508/N	9	0	0	0	42	No	No
18	R553X/N	3	2	2	67	33	Yes	Yes
19	N/N	7	4	3	57	39	No	No
20	N/N	24	12	6	50	40	Yes	No
21	G542X/N	11	8	3	73	34	Yes	Yes
22	N/N	23	16	4	70	26	Yes	Yes
23	ΔF508/R117H	16	11	3	69	26	Yes	Yes
24	W1282X/N	6	3	3	50	29	No	No
25	N/N	13	5	4	38	24	Yes	No
26	ΔF508/R117H	18	9	3	50	33	Yes	Yes
27	W1282X/N	34	17	3	50	32	Yes	No
28	N/N	3	1	1	33	42	No	No
29	N/N	21	1	1	5	40	No	No
30	ΔF508/R117H	17	8	4	47	34	Yes	No
31	ΔF508/N	11	8	3	73	31	Yes	No
32	ΔF508/N	22	7	3	32	23	Yes	No
33	N1303K/N	22	6	0	27	24	No	No
34	W1282X/N	20	3	3	15	23	Yes	Yes
35	ΔF508/N	19	4	2	21	32	No	No
36	R117H/R117H	15	5	3	33	32	Yes	Yes
37	W1282X/N	6	2	2	33	25	Yes	Yes
38	ΔF508/R117H	14	8	3	52	21	Yes	Yes
39	ΔF508/N	11	3	0	27	33	No	No
40	ΔF508/N	6	4	4	66	42	Yes	No
41	N/N	22	13	10	59	40	Yes	No
42	N/N	4	3	2	75	38	No	No
43	W1282X/N	23	10	4	43	36	Yes	Yes
44	N/N	2	2	2	100	43	No	No
45	ΔF508/N	15	6	3	40	29	Yes	Yes
46	ΔF508/N	11	7	5	64	36	No	No
47	ΔF508/N	15	14	4	93	32	No	No
48	ÆF508/N	8	4	3	50	36	No	No
49	N/N	25	4	2	16	25	No	No
50	R117H/N	13	6	3	46	30	Yes	No
51	ΔF508/N	5	4	4	80	31	Yes	No
52	ΔF508/I1139V	24	12	3	50	27	No	No
Totals	37/52 (70%) cystic fibrosis-positive	695	296	2.9 ± 1.3 (av.)	43%	32.7 ± 5.8 (av.)	29/52 (56%)	16/52 (31%)

There were 37 out of 52 cystic fibrosis-positive patients. Of the 52 patients with CAV partners, 29 became pregnant and 16 delivered. MII = metaphase II.

Table III. Fertilization and cleavage rate after ICSI with epididymal and testicular biopsy spermatozoa

Source of spermatozoa	No. of patient cycles	Metaphase II eggs injected	Two-pronuclear oocytes (%)	Cleaved embryos (%)
Fresh epididymal (MESA)	33	431	201 (47)	127 (63)
Frozen epididymal	7	95	27 (28)	20 (74)
Testicular biopsy (TESE)	32	436	215 (49)	155 (72)
Totals	72	962	443 (46)	302 (68)

Table IV. Pregnancy and delivery rates after ICSI with epididymal and testicular biopsy spermatozoa

Source of spermatozoa	No. patient cycles	No. transfers (%)	No. clinical pregnancies per transfer (%)	No. delivered per transfer (%)	No. delivered per cycle (%)
Fresh epididymal (MESA)	33	31 (94)	20 (65)	12 (39)	36
Frozen epididymal	7	7 (100)	4 (57)	2 (28)	28
Testicular biopsy (TESE)	32	27 (84)	14 (52)	10 (37)	31
Totals	72	65 (90)	38 (58)	24 (37)	33

TESE = testicular sperm extraction.

Table V. The use of ICSI with testicular or epididymal spermatozoa for failed vasoepididymostomy or non-congenital irreparable obstruction

No. of patients	No. of mature eggs	No. of 2PN oocytes (%)	No. of cleaved embryos (%)	No. of patients with embryo transfer (%)	No. of clinical pregnancies (%)	No. delivered (%)
20	267	147 (56)	117 (80)	19 (95)	9 (45)	8 (40)

2PN = two pronuclear.

Table VI. Fertilization and pregnancy rate after ICSI with epididymal and testicular spermatozoa in relation to age of wife

Age of wife	No. of patients	No. of eggs (MII)	2PN (%)	No. of cleaved embryos (%)	No. of embryos transferred per patient	No. pregnancies (%)	No. delivered (%)
<30	20	293	138 (47)	91/138 (66)	54 (2.7)	15 (75%)	12 (60)
30–38	35	479	220 (46)	160/220 (73)	104 (3.0)	19 (54%)	11 (31)
>38	17	190	85 (45)	51/85 (60)	51 (3.0)	4 (24%)	1 (6)
Totals	72	962	443 (46)	302/443 (68)	209 (2.9)	38 (53%)	24 (33)

2PN = two pronuclei. Delivery rate for wives <38 years = 23/55 (42%).

epididymis or the testis, frozen or fresh, or whether the male had CAV or irreparable obstruction from a variety of other causes, made no meaningful difference. The cystic fibrosis genotype, sperm morphology and the quality of motility also had no impact. The only factor in the woman that mattered was her age. Table VI demonstrates this relationship.

The only factor which dramatically affected pregnancy rate was the age of the woman. When the woman was <30 years of age, 75% of the MESA–ICSI cycles resulted in pregnancy; when 30–38 years of age, 54% of cycles resulted in pregnancy; and when >38 years of age, 24% of cycles resulted in pregnancy. The age of the woman affected only implantation and pregnancy rates. The two-pronuclear fertilization rate was remarkably constant for all age groups (47, 46 and 45% respectively). The cleavage rate was also unrelated to age of the woman. In fact, the fertilization rate was remarkably constant (~46%) no matter how these patients were grouped: by the cause of obstruction, the quality of the spermatozoa, whether from testicle or epididymis, the number of eggs retrieved, or even the age of the woman.

More dramatically, the overall delivery rate for all 72 patients was 33%, but for women <30 years it was 60%. For women between 30 and 38 years the delivery rate was 31%, and for women >38 years it was only 6%.

Although it is clear that embryo transfer rates are very high with both epididymal and testicular sperm ICSI, a significant percentage of TESE cases had no fertilization (16%) which was rare with MESA using ICSI. Therefore, we performed a prospective study in one single series of 16 MESA cases and 12 TESE cases to determine if the implantation rate was any different for embryos derived from epididymal spermatozoa versus testicular spermatozoa (Silber *et al.*, 1995). Table VII summarizes these results.

Of the TESE patients, 25% failed to fertilize or develop any embryos for transfer. All of the MESA patients fertilized well and had embryo transfers performed. However, the ongoing implantation rate per embryo transferred was not significantly different (25.5 versus 23.3%) whether epididymal or testicular spermatozoa were used (Silber, 1995). Thus, for reasons we do not yet understand, testicular spermatozoa usually fertilize quite well with ICSI, but not always. Nonetheless, if fertilization does occur, the cleavage and implantation rates are no different from epididymal spermatozoa, and appear to be no worse than for ejaculated spermatozoa (Nagy *et al.*, 1995). In fact, the pregnancy rate, if anything, is somewhat higher with testicular and epididymal spermatozoa (42.0% ongoing or delivered) compared with the first 1275 cycles of ejaculated sperm ICSI (which was 28.4% ongoing or delivered). The total pregnancy rate for ejaculated sperm ICSI was 36.1% per cycle, and for epididymal or testicular spermatozoa it was 53.0% (Van Steirteghem *et al.*, 1994).

Discussion

Cystic fibrosis and CAV: pre-implantation embryo diagnosis

Virtually all patients with cystic fibrosis (one of the most common autosomal recessive genetic disorders in humans)

Table VII. Ongoing implantation rates after ICSI with epididymal and testicular spermatozoa

Source of spermatozoa	No. of patients	No. of embryo transfers	No. of embryos replaced	No. of fetal hearts	Ongoing implantation rate (%)
Epididymal (MESA)	16	16	47	12[a]	25.5
Testicular biopsy (TESE)	12	9	30	7[b]	23.3

After Silber *et al.* (1995).
[a]Including two sets of triplets.
[b]Including two sets of twins.

also have CAV. Until recent years, most cystic fibrosis patients did not survive a sufficient length of time to even consider trying to become a father. Therefore, none of the CAV patients initially treated with MESA (>200) had clinical cystic fibrosis. A possible genetic link between cystic fibrosis and CAV was suggested >20 years ago (Holsclaw *et al.*, 1977). However, it was not until mutation analysis in the cystic fibrosis gene became possible that this link was confirmed at the molecular level. In fact, infertile males with CAV probably have a mild reproductive (genital) form of cystic fibrosis that is inherited through mutations in the cystic fibrosis gene (Dumur *et al.*, 1990; Rigot *et al.*, 1991; Silber *et al.*, 1991; Anguiano *et al.*, 1992; Gervais *et al.*, 1993; Osborne *et al.*, 1993; Patrizio *et al.*, 1993; Oates and Amos, 1994).

Today, >500 mutations have been identified in the cystic fibrosis transmembrane conductance regulator (CFTR) gene of cystic fibrosis patients. With the exception of the ΔF508 mutation (deletion of the amino acid phenylalanine at position 508 of the CFTR), most of these mutations are rare and are present in only one or a few cystic fibrosis patients. Routine screening methods have been developed for carrier detection in the general population. These methods usually take into account the frequency of mutations in cystic fibrosis proteins in the particular population being studied. This is probably the reason why only up to 60% of CAV males are found to be carriers of one cystic fibrosis mutation upon routine screening. Presumably, the second mutation is a rare one that is not included in routine screening. Up to 20% of CAV males are carriers of two different mutations (compound heterozygotes), but in these instances at least one of the cystic fibrosis mutations is weak in reference to the clinical phenotype in cystic fibrosis patients. In the remaining patients, no cystic fibrosis mutations can be found.

The inheritance of CAV on the cystic fibrosis gene seems to follow simple Mendelian rules, just as in cystic fibrosis. The male child must have inherited a cystic fibrosis mutation from each parent and at least one of these mutations is usually rare and not detectable on routine screening. However, most male cystic fibrosis carriers do not have CAV. To have CAV, the patient has to receive two cystic fibrosis alleles, one from each of his parents. One of the two parents of cystic fibrosis-positive CAV males always has the same heterozygous mutation as their son. Male 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 cystic fibrosis heterozygous mutation as the CAV father. Yet the cystic fibrosis-positive heterozygous male offspring of CAV males do not themselves have CAV, and indeed have normal vasa bilaterally. In a similar manner, the CAV patient's father in 50% of cases is a carrier of the same mutation as his son, but obviously did not have CAV and was clearly fertile (Patrizio *et al.*, 1993).

Thus, cystic fibrosis carrier status alone certainly does not cause CAV. The infertile patient must receive not just one mutated F gene from one parent to have CAV. The CFTR gene obviously plays a major role in the genesis of CAV, but the inability to identify a second mutation in carriers, or any mutation in other CAV males, could mean that, at least in some of the patients, another gene or genes are involved. The latter idea is supported by a study of Mercier *et al.* (1995) of two brothers, one with CAV and one normally fertile, who have the same genotype at the CFTR gene.

Anyway, it is clear that it is crucial to screen both the husband with CAV and his wife for cystic fibrosis mutations. If the wife is negative for cystic fibrosis mutations, then the couples' residual risk of having a cystic fibrosis or CAV (male) child will depend on the mutation detection rate. Assuming a one in 25 carrier frequency of cystic fibrosis, their residual risk will be about one in 163 at a 70% detection rate and one in 500 at a 90% detection rate. For the latter case, one might guess with what we know today a one in 1000 risk of cystic fibrosis and one in 1000 risk of CAV.

However, if the wife turns out to be a cystic fibrosis carrier herself, 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 preimplantation embryo diagnosis in a CAV–MESA case in which both partners were carriers of the ΔF508 mutation (Liu *et al.*, 1994). We require embryo blastomere biopsy and preimplantation diagnosis as a routine approach whenever the female is discovered upon screening to be a cystic fibrosis carrier.

In our first such case, both partners were 35 years of age. The husband was known to have CAV and they had given up hope many years earlier of ever having a child because of the low success rate of MESA with conventional IVF. With the introduction of ICSI, they finally decided to attempt MESA. The husband was a carrier of ΔF508, and the wife, on routine pre-MESA screening, was also found to carry ΔF508. Therefore, preimplantation diagnosis for cystic fibrosis was performed.

This first couple answered all of the major questions for couples who are cystic fibrosis carriers. A total of 12 oocytes

were retrieved, all were metaphase II and all were microinjected: one degenerated and five fertilized and cleaved to the 4- to 8-cell stage by day 3. One or two blastomeres were then removed with a micropipette from each of the five embryos and underwent genetic analysis. None of the five embryos were damaged. Two of the five embryos were found to be homozygous for ΔF508 and were not transferred because this genotype represents severe cystic fibrosis. The other three embryos were all ΔF508 carriers.

It would have been simple if there were embryos completely free of the ΔF508 mutation, but this would only occur in 25% of embryos; in this patient it did not occur at all. The couple had no problem with having a child who was a cystic fibrosis carrier so long as the child did not have clinical cystic fibrosis. It was obvious that these three heterozygous embryos should be replaced in the wife. However, the dilemma was that if the ΔF508 mutation in the embryo came from the mother, then some as yet unknown mutation in the CFTR gene from the father would have been transmitted to the embryo. Thus, the child would not be, purely speaking, a true heterozygote but would in fact have an identical CFTR genotype to his father. In the case of a male embryo, it would most likely have CAV itself. Since the father had no phenotypic expression of cystic fibrosis other than CAV, we felt there would be little risk of the child, either boy or girl, having cystic fibrosis. But, of course, caution demands a slight hedge on this question. If the ΔF508 mutation in the embryo came from the father, then there would be no concern whatsoever. The wife became pregnant and delivered a normal baby boy who was confirmed to be a heterozygous ΔF508 carrier. His chloride sweat test was normal, and both vasa deferentia were present and normal, indicating that the ΔF508 most probably came from his father.

Nevertheless, we think that it would be best to replace only embryos that are non-carriers of the ΔF508 mutation. The crucial clinical effort in such cases must be to recruit as many eggs and therefore as many embryos as possible, because only 25%, not 50%, will be transferable. The use of intragenic polymorphism to discriminate between the non-ΔF508 alleles of both parents could also be useful. So far, we have not found any informative polymorphism in the couple described above.

Another situation presents when the mother is discovered, on screening, to carry a cystic fibrosis mutation different from the father. In this case, it is possible to select for replacement all embryos that are non-carriers of the maternal mutation (50%). In this way, both compound carrier embryos (probably resulting in cystic fibrosis children) and embryos carrying the maternal mutation along with a probably unknown mutation of the father (that could eventually result in cystic fibrosis or CBAVD) are avoided. By using this strategy, 50% of embryos will be transferable.

In summary, the issue of cystic fibrosis in the offspring is to be addressed seriously in any MESA–ICSI programme for CAV. Nonetheless, with routine inexpensive cystic fibrosis genetic screening of the prospective parents and the more complex availability of good embryo preimplantation genetic testing, we have not seen any children born with a cystic fibrosis phenotype or with CAV, despite transmission to these offspring of their father's cystic fibrosis gene. Only with proper attention to this matter can this risk to offspring remain remote.

TESE–ICSI for non-obstructive azoospermia

Our major effort with MESA, TESE and ICSI until recently was concentrated on otherwise untreatable obstructive azoospermia. These patients generally have normal spermatogenesis. It seems that there are no such cases of obstructive azoospermia that cannot be treated successfully. The only limitation would be the age (i.e. the egg quality) of the female partner.

An important ancillary finding is that for the purpose of ICSI, it is the 'vitality' of the spermatozoa and not the motility that matters. Presumably, the more proximal (i.e. head of the epididymis or testicle) the sperm retrieval, the greater the percentage of 'viable' spermatozoa. Therefore, TESE may be a method of obtaining non-senescent, so-called 'fresh' spermatozoa for ICSI when the ejaculate exhibits absolutely no motility. We first used TESE–ICSI under this rationale for a case of Kartagener's syndrome, with successful fertilization and ongoing pregnancy. However, no matter what the cause of total asthenozoospermia, the key to success with ICSI is to inject 'live' spermatozoa. Total lack of motility would make the selection of 'live' spermatozoa difficult. In most cases of 0% motility there are, in truth, a few motile spermatozoa, which is not what we are referring to here. The solution to the problem of absolutely 100% zero motility in the ejaculate is to retrieve spermatozoa from testicular tissue obtained via biopsy. Although sperm motility is always markedly reduced in the testicle, the percentage of fresh live 'vital' spermatozoa is extremely high. Therefore, if a careful search of the entire ejaculate reveals no motility, it is wise to do a testicular biopsy and to use the spermatozoa for 'TESE' and ICSI.

This concept of using TESE for non-obstructive male infertility has now taken an even greater leap. Most patients with non-obstructive azoospermia, who appear to have no sperm production, in truth have a few rare foci of spermatozoa somewhere in their testicles. We have reviewed the slides of many azoospermic patients with 'Sertoli cell only' and 'maturation arrest', and have found very small numbers of mature spermatids in most of these non-obstructive azoospermic patients, indicating that TESE might also be successful in many of these cases (Silber *et al.*, 1995). Thus far, in 17 of 19 such patients with no spermatozoa in the spun-down ejaculate, enough spermatozoa were found in an extensive testicle biopsy (TESE) to perform ICSI, and to obtain normal embryos and a normal pregnancy rate. Even in patients whose routine diagnostic testicular biopsy failed to reveal any spermatozoa, a more thorough 2 h search of testicular tissue usually revealed a few spermatozoa which were then used for ICSI, resulting in fertilization and pregnancy rates no different from patients with obstructive azoospermia and normal spermatogenesis. In fact, whenever the patient's ejaculate fails to exhibit the very few motile spermatozoa that are required for ICSI, resorting to testicular biopsy for sperm extraction (TESE) will usually provide enough spermatozoa for ICSI (Figures 5 and 6).

The evolution of TESE and ICSI is fascinating. ICSI provides a remarkably effective solution to severe male factor

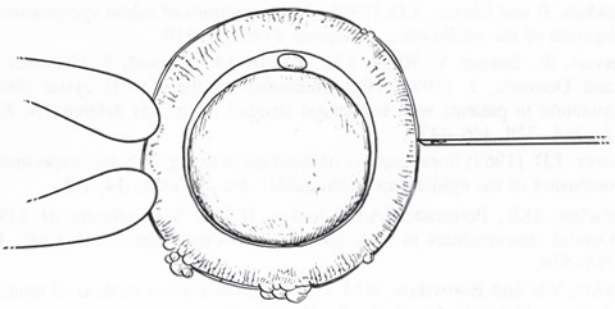

Figure 5. Testicular biopsy of a patient with obstructive azoospermia and normal spermatogenesis requiring testicular sperm extraction because of an absence of motile spermatozoa in the epididymis.

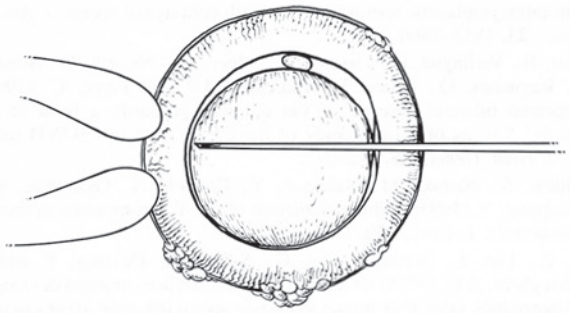

Figure 6. Patient with predominantly 'Sertoli cell only' has a single tubule showing normal spermatogenesis next to a typical 'Sertoli cell only'.

infertility. The initial efforts were directed at severe oligozoospermia or oligoasthenozoospermia. The severity of the oligozoospermia, even with 100% abnormal morphology, had no adverse effect on the fertilization, cleavage or pregnancy rates (Nagy *et al.*, 1995). Now ICSI with MESA or TESE can also be used for obstructive azoospermia when microsurgery fails or is not even worth attempting. In fact, at the time of all cases of vasectomy reversal, vasoepididymostomy or re-attempting a previously failed vasectomy reversal, we now always freeze a sample of motile epididymal spermatozoa just in case the repair should fail. Therefore, in a sense, every case of obstructive azoospermia is microsurgically correctable, but the technique now goes much further.

Our current research is focused on utilizing ICSI with TESE in cases of non-obstructive azoospermia. This includes 'Sertoli cell only' and 'maturation arrest'. The least senescent spermatozoa can be found in the testicle and, therefore, vitality is high despite poor to no motility. In many cases of deficient spermatogenesis, the production of spermatozoa is quantitatively so low that the ejaculate may contain nothing, even after centrifugation. Yet even when sperm production is so extremely low as to make the ejaculate absolutely devoid of spermatozoa, the testicle can still usually be found to contain enough spermatozoa for ICSI (Silber, 1995).

Genetic aetiology and transmission of male infertility

There have been many decades of debate over the causes and treatment of male infertility. There have been long lists of strongly advocated treatments such as varicocelectomy, clomiphene, HMG, HCG, HCG plus varicocelectomy, prednisone (for antibodies), cold, wet athletic supporters and vitamins. Many have argued that the aetiology of oligozoospermia was too warm a scrotal environment, which could be corrected by varicocelectomy and testicular cooling. Some argued that the problem was clandestine seminal infection, and advocated tetracycline for improving semen quality. The thousands of papers on the so-called aetiologies and treatments for male infertility are just too vast to cite.

The important question that ICSI brings into this controversy is simple. If male infertility has nothing to do with all these aetiologies and is simply genetically mediated, it is possible that future generations will have more male infertility than at present because ICSI will allow these severely infertile men to father more infertile males. In fact, it is beginning to appear that like CAV, most cases of non-obstructive male infertility are indeed of genetic origin.

Comparative biology of exotic species interestingly provides an insight into the powerful genetic control of spermatogenesis. Roger Short (1995) has demonstrated beautifully that species with a promiscuous mating pattern have a much higher sperm count and larger testicles owing to 'sperm competition' (Seuanez *et al.*, 1977; Silber, 1991). This means that when a number of different males inseminate the same female, as with chimpanzees, the most fertile male is most likely to have his spermatozoa result in the pregnancy. Thus, subsequent male offspring in this sort of mating system are likely to have higher sperm counts. In fact, on average, the human male has very very poor semen quality compared with most other animals, with the exception of the gorilla that also has a monogamous mating system.

O'Brien's studies on the inbreeding of cheetahs, and of certain population 'bottlenecks' of lions, also point to the strong genetic control over spermatogenesis. Inbreeding and loss of genetic diversity clearly lead to severe defects in spermatogenesis (O'Brien *et al.*, 1986, 1987; Wildt *et al.*, 1987). 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. Even varicocelectomy, the last bastion of conventional treatment for male infertility, has been shown in a controlled study by Nieschlag *et al.* (1994) to be no better than psychological counselling. Most reproductive physicians today are not surprised by these results.

A strong effort has been applied for several years by our clinic in St Louis and David Page's laboratory at MIT to study in intricate detail the Y chromosome of the most severe cases of male infertility, i.e. non-obstructive azoospermia (Foote *et al.*, 1992; Vollrath *et al.*, 1992; Behlke *et al.*, 1993; Nagafuchi *et al.*, 1993; Zinn *et al.*, 1993; Bogan and Page, 1994; Reijo *et al.*, 1995). Also, mutations of the androgen receptor gene have been clearly demonstrated to cause severe male factor infertility (Yong *et al.*, 1994). It seems more and more likely that most cases of spermatogenic defects are genetic in origin. If specific gene defects can continue to be isolated from most of these infertile male patients, and various mutations perhaps found for the different types of spermatogenic disorder, then we could permanently lay to rest all of the conventional

therapies for male infertility and rely solely on ICSI. However, we can then expect to see in future decades an even higher percentage of severe male factor infertility in the population.

Obstructive azoospermia is most commonly acquired (vasectomy, infection or hernia-type surgery). However, ~10% of obstructive azoospermia is congenital and inherited on the cystic fibrosis gene. It is caused by either compound heterozygosity with at least one 'weak' deletion (10%), very occasionally by weak deletion homozygosity or most commonly by cystic fibrosis heterozygous carrier status combined with a non-coding dosage deletion on the T-5 exon of the opposite allele (Chillon *et al.*, 1995). For this condition, particularly if husband and wife are carriers, genetic counselling is required.

Non-obstructive azoospermia and severe oligozoospermia are most commonly caused by 'Sertoli cell only' or 'maturation arrest'. These problems are usually genetic and they appear to be inherited on the long arm of the Y chromosome. There appear to be no phenotypic consequences of this deletion other than infertility, and with ICSI this infertility can be treated successfully. Preimplantation embryo biopsy and diagnosis, and genetic counselling, are probably not necessary.

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