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Fertilizing Capacity of Epididymal and Testicular Sperm using Intracytoplasmic Sperm Injection (ICSI)

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Abstract. For men with uncorrectable obstructive azoospermia, their only hope of fathering a child is microsurgical epididymal sperm aspiration (MESA) combined with *in vitro* fertilization (IVF). In 1988, proximal epididymal sperm were demonstrated to have better motility than senescent sperm in the distal epididymis, and it was thought that retrieval of motile sperm from the proximal epididymis would yield reliable fertilization and pregnancy rates after conventional IVF. However, the results to date have been poor, and although a minority of patients achieved good fertilization rates with IVF, the vast majority (81%) had consistently poor or no fertilization and the pregnancy rate averaged only 9%. Recently, intracytoplasmic sperm injection (ICSI) has been successfully used to achieve fertilization and pregnancies for patients with extreme oligoasthenozoospermia. ICSI has therefore been applied to cases of obstructive azoospermia and, in this report, 67 MESA–IVF cases are compared with 72 MESA–ICSI cases. The principle that motile sperm from the proximal segments of the epididymis should be used for ICSI was followed, although in the most severe cases in which there was an absence of the epididymis (or absence of sperm in the epididymis), testicular sperm were obtained from macerated testicular biopsies. These sperm only exhibited a weak, twitching motion. In 72 consecutive MESA cases, ICSI resulted in fertilization and normal embryos for transfer in 90% of the cases, with an overall fertilization rate of 46%, a cleavage rate of 68%, and ongoing or delivered pregnancy rates of 46% per transfer and 42% per cycle. The pregnancy and take-home baby rates increased from 9% and 4·5% with IVF to 53% and 42% with ICSI. There were no differences between the results for fresh epididymal, frozen epididymal or testicular sperm, and the number of eggs collected did not affect the outcome. The results were also unaffected by the aetiology of the obstruction such as congenital absence of the vas deferens or failed vasoepididymostomy. The only significant factor which affected the pregnancy rate was female age. It is concluded that although complex mechanisms involving epididymal transport may be beneficial for conventional fertilization of human oocytes (*in vivo* or *in vitro*), none of these mechanisms are required for fertilization after ICSI. Given the excellent results with epididymal and testicular sperm, ICSI is obligatory for all future MESA patients. Finally, the use of ICSI with testicular sperm from men with non-obstructive azoospermia is also discussed.

Extra keywords: IVF, congenital absence of the vas, MESA, TESE, cystic fibrosis.

Introduction

Congenital absence of the vas deferens (CAV) and irreparable obstructive azoospermia due to failed vaso-epididymostomy (V-E), are the cause of male infertility in a large and frustrating group of patients who have normal spermatogenesis, and from whom large numbers of sperm are surgically retrievable for *in vitro* fertilization (IVF) (Temple-Smith *et al.* 1985; Silber *et al.* 1990). A treatment protocol consisting of microsurgical epididymal sperm aspiration (MESA) from the proximal region of the epididymis and IVF was developed to solve this problem. The first successful fertilization and pregnancies using this approach were achieved in CAV patients (Silber *et al.* 1988, 1990) and this technique was applied to all cases of obstructive azoospermia including failed vasectomy reversal and epididymal blockage. In some men, successful V-E was also performed at the time of the MESA. The two major achievements from

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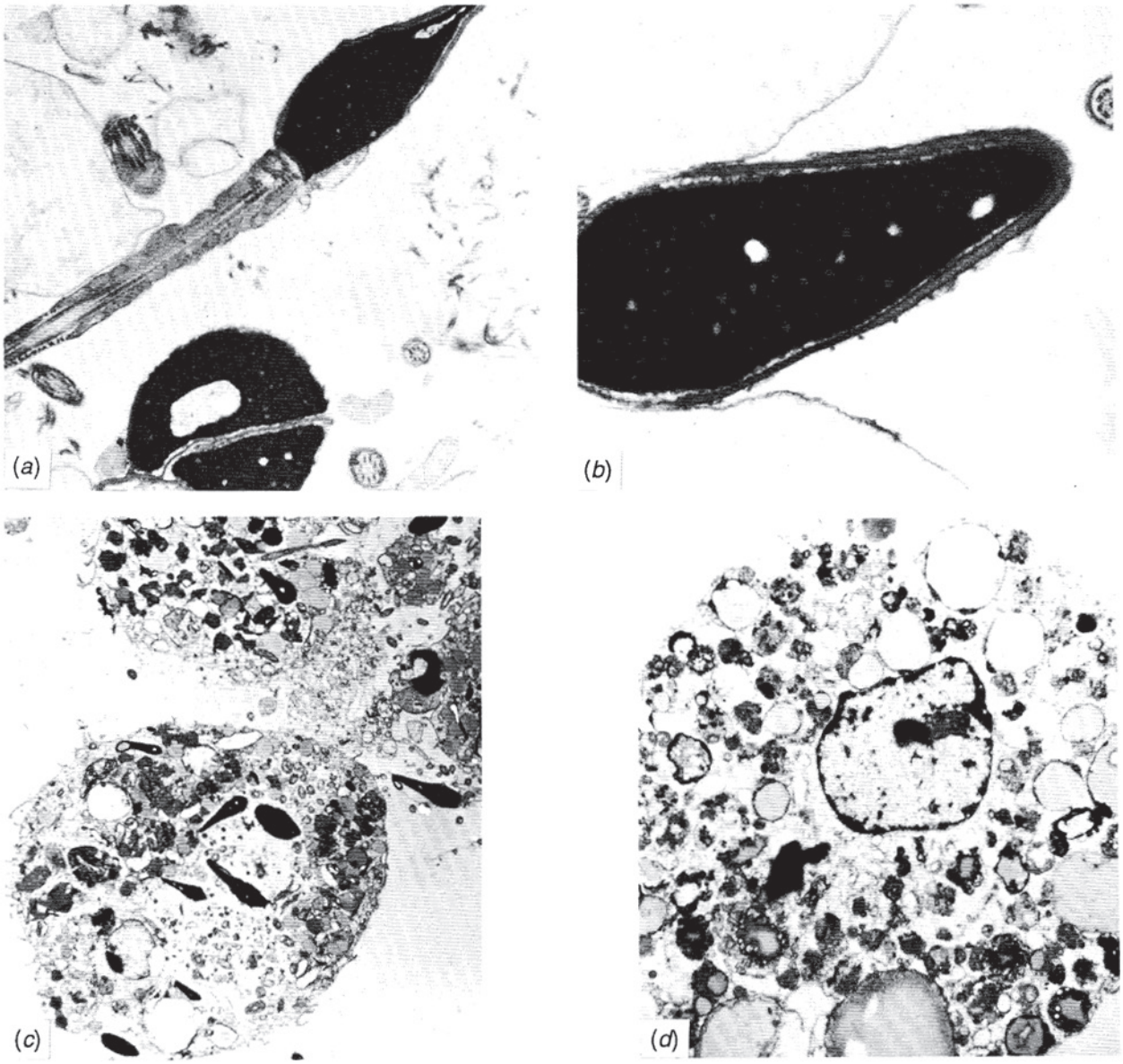

Fig. 1. *(a)* Ultrastructure of sperm obtained from the rete testis of a man with congenital absence of the vas deferens (CAV). Note the normal organization of the nucleus, acrosome and flagella. *(b)* Ultrastructure of a spermatozoon obtained from the vasa efferentia of a man with CAV. The organization of the nucleus, acrosome and flagella is similar to that of normal ejaculated sperm. *(c)* Ultrastructure of macrophages obtained from the corpus epididymis in a man with CAV. The cytoplasm of the macrophages contains large amounts of sperm remnants at different stages of degradation and digestion. *(d)* Ultrastructure of a macrophage obtained from the cauda epididymis of a man with CAV. Note the presence of prominent whorls of membranes and numerous lipid droplets indicating advanced stages of sperm degradation.

this work were: *(1)* the establishment of a successful treatment for male infertility caused by a condition that had previously been untreatable; and *(2)* a demonstration that in the human, sperm passage through the epididymis was not always required for fertilization and the live birth of normal children.

However, subsequent to the initial enthusiasm for using IVF with aspirated epididymal sperm, it soon became apparent that these sperm often failed to fertilize. The reason for this was not readily apparent and there were no recognizable differences between the quality of epididymal sperm that did or did not fertilize after IVF (Silber 1994). Most centres have obtained very low ($\leq$10%) fertilization rates and pregnancy rates of $\leq$9% (Anon. 1994).

Epididymal Sperm Motility and Conventional IVF

Sperm with the greatest motility are always found in the most proximal portion of the obstructed epididymis (Silber *et al.* 1990). Although the percent motility is

usually low (1–30%), the greatest motility is always found, paradoxically, in the most proximal region. The most distal site from which progressively motile sperm are usually recovered is the proximal corpus epididymis, and this only occurs in about 10% of cases. Incubation of proximal epididymal sperm usually results in a dramatic improvement in progressive motility, whereas incubation of distal epididymal sperm does not improve their motility.

The fertilizing capacity of sperm which have not traversed the entire epididymis can ideally be studied with the human clinical model. In every mammal that has been studied, spermatozoa from the caput epididymis are only capable of weak circular motion at most and are not able to fertilize (Orgebin-Crist 1969), whereas sperm from the corpus epididymis can occasionally fertilize but the pregnancy rate is low. However, few of these animal studies allowed the sperm time to mature *in vivo* and thereby potentially develop the capacity for fertilization. In our clinical procedures, sperm are aspirated from specific regions of the obstructed epididymis, which allows them time to mature in the epididymis despite the absence of epididymal transit. In animal studies in which the epididymis was experimentally ligated to determine if time alone could mature sperm, the obstructed environment was so pathological that no firm conclusion about fertility could be reached, and the initial increase in motility was followed subsequently by sperm senescence and poor motility associated with obstruction (Gaddum and Glover 1965; Bedford 1966; Gaddum 1969; Glover 1969).

Thus, the most striking finding in the retrieval of sperm from the chronically-obstructed epididymis is the inversion of the usual pattern of motility one would expect in a non-obstructed epididymis. Sperm in the distal regions of an obstructed epididymis have poor or no motility because of senescence, and sperm in the proximal regions have the best motility. Therefore, the poor fertilization rate obtained using distal epididymal sperm is probably due to aging as well as immaturity (Krylov and Borovikov 1984). There is experimental support for this concept. Young (1931) ligated the guinea-pig epididymis at various levels and examined the proximal sperm that had been trapped for varying periods of time. Contrary to expectation, the more distal sperm had the poorest motility and the proximal sperm had the best. Young (1931) concluded that in the obstructed epididymis the more distal sperm are senescent, whereas the more proximal sperm have had time to mature despite having not passed through the epididymis. Thus, sperm maturation (development of progressive motility) appears to be intrinsic in nature, and may not require epididymal transit.

Clinical studies of V-E in humans have demonstrated equivalent pregnancy rates irrespective of whether the sperm had passed through a long or short length of the corpus epididymis, and even when sperm had only passed through a portion of the caput, there were reasonable pregnancy rates (Silber 1989*a*, 1989*b*). Two pregnancies have been documented (with proven paternity) after end-to-end anastomosis of the vas deferens to the vasa efferentia, with normal motile sperm found in the post-operative ejaculate. Thus sperm do not require transit through the epididymis in order to fertilize oocytes (Silber 1980, 1988; Weiske 1994). However, despite good motility, sperm collected from the proximal regions of the obstructed epididymis by MESA often do not fertilize oocytes by conventional IVF (Silber 1994).

We demonstrated with electron microscopy (EM) that after vasectomy, sperm which are proximal to the site of occlusion undergo senescence and degeneration into what appears by light microscopy to be debris and dead sperm, but is in fact globules of broken-down sperm heads and tails (Friend *et al.* 1976). The debris and dead sperm are initially seen in the ejaculate after vasovasostomy, however, if there is no secondary epididymal occlusion, the ejaculate eventually contains normal sperm. More recent studies have demonstrated that the reason for poor sperm motility in the distal epididymis of MESA patients is exactly the same senescent phenomenon. The quality and integrity of sperm aspirated from the rete testis, vasa efferentia, and caput is always markedly superior to the sperm aspirated from the corpus and cauda of the obstructed epididymis. The distal epididymis contains mostly degenerating and necrotic sperm along with large numbers of giant sperm-engulfing macrophages. This is similar to the senescence changes in sperm proximal to a vas occlusion. Sperm aspirated from the vasa efferentia, rete testis, and caput epididymis are usually similar to ejaculated sperm in normospermic subjects (Fig. 1). The only difference is that some of the proximal epididymal sperm possess a cytoplasmic droplet around the posterior portion of the head and the initial segment of the flagellum. With that exception and regardless of IVF success or failure, all of the proximal epididymal sperm we examined had a normal ultrastructure.

Nevertheless, it is still difficult to predict the fertilizing capacity of epididymal sperm from the sperm characteristics. Some samples with good motility yield no fertilization, whereas a small number of samples with poor motility yield good fertilization rates.

Intracytoplasmic Sperm Injection and MESA

Intracytoplasmic sperm injection (ICSI) has been successfully used to treat extreme oligoasthenozoospermia (Palermo *et al.* 1992, 1993; Van Steirteghem *et al.* 1993*a*, 1993*b*). We have therefore examined the proposal that ICSI could be used to improve the poor fertilization and

pregnancy rates obtained with MESA–IVF. The present collaborative studies confirm that ICSI using epididymal or testicular sperm in men with CAV or any other cause of obstructive azoospermia gives reliable, and much higher, fertilization and pregnancy rates than conventional IVF with MESA (Tournaye *et al.* 1994; Silber *et al.* 1994, 1995).

The purpose of this paper is to: *(1)* document the first large-scale, systematic use of ICSI to treat obstructive azoospermia due to CAV, failed V-E or irreparable obstruction; *(2)* document the first systematic use of ICSI with testicular sperm; *(3)* predict the use of testicular sperm and ICSI for the treatment of non-obstructive azoospermia due to Sertoli cell-only syndrome and maturation arrest; and *(4)* review what the ICSI technique reveals about epididymal and testicular physiology.

Materials and Methods

Induction of Follicular Development and Oocyte Retrieval

The female partners underwent routine induction of multiple follicular development. Briefly, the protocol involved the daily subcutaneous administration (1 mg) of leuprolide acetate (Lupron; TAP Pharmaceuticals, North Chicago, IL, USA) until the day of follicular aspiration. After desensitization, patients received human follicle-stimulating hormone (FSH) (Metrodin; Serono, Randolph, MA, USA) and/or human menopausal gonadotrophin (hMG) (Pergonal; Serono) until multiple follicles 2·0 cm in diameter were noted by ultrasound. At that time, 10 000 I.U. of human chorionic gonadotrophin (hCG) (Profasi; Serono) was administered intramuscularly and, 35 h later, patients underwent transvaginal follicle aspiration.

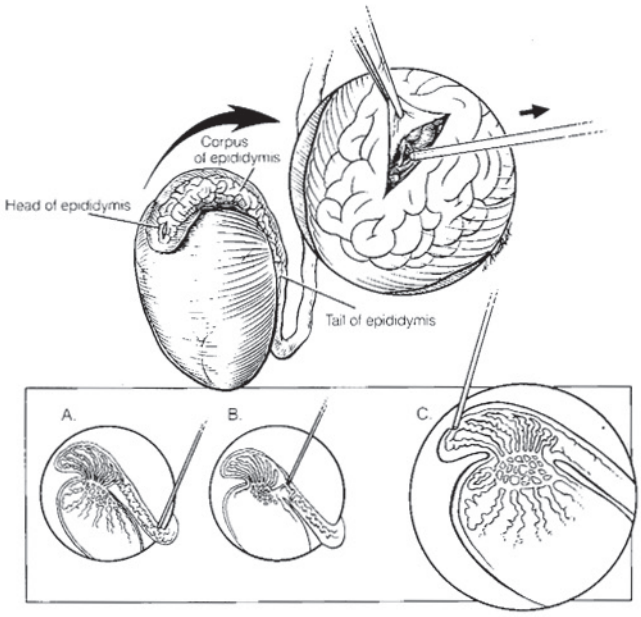

Fig. 2. Sperm were microsurgically aspirated from distal and proximal regions of the epididymis. The samples with the best motility were used for IVF. From Silber (1994).

Microsurgical Epididymal Sperm Aspiration

The male partners underwent scrotal exploration and MESA to obtain sufficient numbers of motile sperm for IVF or ICSI. The surgical technique 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 micro-scissors in the epididymal tunic to expose the tubules in the proximal portion of the obstructed epididymis. Sperm were aspirated directly from the opening in the tubule with a plastic micropipette (Medicuts 22G, 0·7 mm/22 mm; Cook Urological, Spencer, IN, USA). The epididymal fluid was immediately diluted in HEPES-buffered Earle's medium and an aliquot was examined for motility and quality of progression. If sperm exhibited poor motility or no motility, another aspiration was made more proximally (Figs 2 and 3). Motile sperm were usually not obtained until the proximal caput epididymis or vasa efferentia were reached (Silber *et al.* 1988, 1990). The rationale for proceeding proximally to find the most motile sperm for ICSI was based on the following presumptions: *(1)* ICSI with ejaculated sperm is most efficient when there is some motility, no matter how poor, as a verification of 'vitality'; and *(2)* the most proximal sperm are least likely to have undergone degenerative changes in the sperm head due to senescence.

Testicular Sperm Extraction

When there was no epididymis, the entire scrotum was massively scarred, or there simply were no sperm in the epididymis, testicular sperm extraction (TESE) was performed to recover sperm for ICSI. 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 extruded 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. When the retrieval of testicular tissue was performed as a last resort after unsuccessful MESA, the incision was more extensive because it was known that only testicular sperm would be available.

The testicular tissue was finely minced in HEPES-buffered Earle's medium and the suspension was placed in a 5-mL tube and centrifuged for 5 min at 300*g*. The supernatant was removed with a Pasteur pipette, and after adding 0·1–0·2 mL of Earle's medium, the pellet was gently resuspended. The sperm suspension was kept at 37°C in an incubator gassed with 5% O_2, 5% CO_2 and 90% N_2 until the injection procedure was performed. The number of sperm in the resulting droplet was few, so individual sperm had to be selected for ICSI from among the debris, red blood cells, and spermatid-laden Sertoli cells. The barely motile testicular sperm were placed in a PVP droplet which resulted in immediate and complete immotility.

In the 72 cases reported here, our first choice was to obtain epididymal sperm. The reason was that we could freeze and save the majority of the epididymal sperm for subsequent ICSI cycles without the need for further surgery. Freezing of testicular sperm was not considered because of the small numbers of sperm and their extremely poor motility.

MESA and Conventional IVF

Epididymal sperm were first diluted and examined in a 5-mL volume and then concentrated into a volume of 0·3 mL, layered onto a discontinuous mini-Percoll gradient (Ord *et al.* 1990), and centrifuged for 30 min. The entire 95% Percoll fraction was then washed twice and added to the entire cohort of eggs in a tube in 1 mL of HTF culture media (Quinn *et al.* 1985). After incubation at

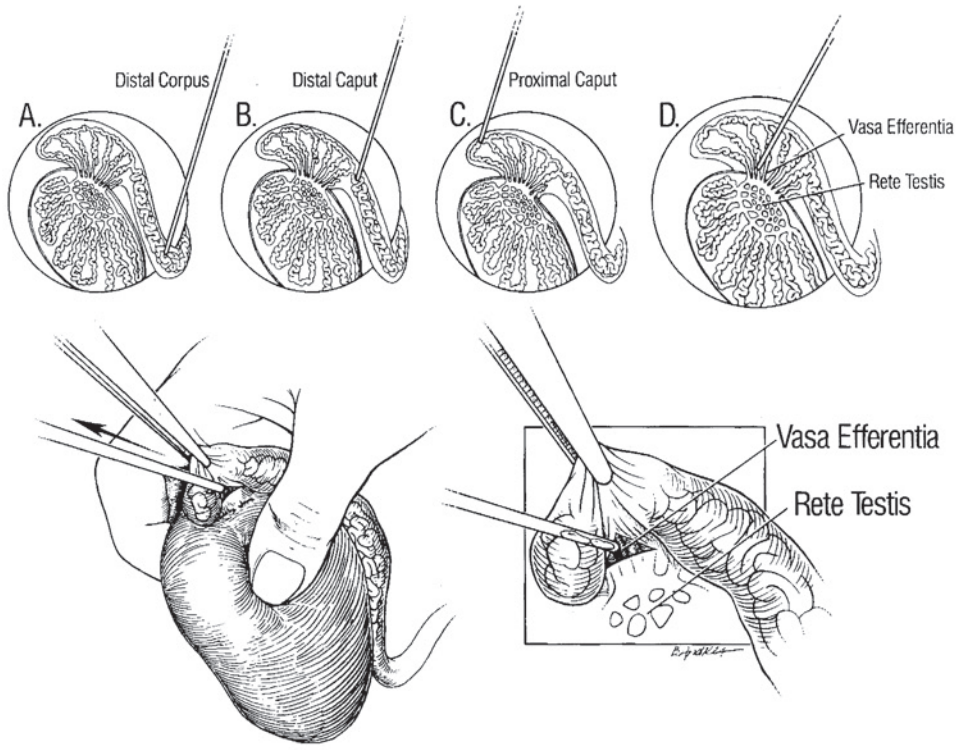

Fig. 3. Sperm in the distal and sometimes proximal epididymis were often immotile or poorly motile, and in these cases, vasa efferentia fluid was collected and usually contained the most motile sperm. From Silber (1994).

37°C and 5% CO_2 in air for two days, the embryos were transferred to the Fallopian tubes or to the uterus. The female partners received 50 mg day^{-1} progesterone in oil (intramuscularly), beginning on the day of oocyte collection.

MESA and ICSI

The procedures for sperm and oocyte preparation, microinjection and culture were essentially as described by Van Steirteghem *et al.* (1993*a*, 1993*b*). Details are provided below. A slightly different approach was used for MESA–ICSI. Since only very small numbers of sperm with weak motility (vitality) were required for ICSI, the unused epididymal fluid was diluted 1:1 with sperm freezing medium, drawn into 0·25-mL straws, frozen in liquid nitrogen vapour then plunged into liquid nitrogen.

Oocyte Preparation for ICSI

After oocyte retrieval, up to 8 cumulus–oocyte complexes were transferred into a tube containing Earle's medium. The tubes were gassed, tightly closed and then transported in a thermobox (37°C) to the microinjection laboratory which was about 500 m away. The cells of the cumulus and corona radiata were removed by incubation for about 30 s in HEPES-buffered Earle's medium containing 80 I.U. mL^{-1} of hyaluronidase (Type VIII; specific activity 320 I.U. mg^{-1}; Sigma, St Louis, MO, USA). Removal of the cumulus and coronal cells was enhanced by aspiration of the complexes in and out of hand-drawn glass pipettes with an opening of about 200 μm. The oocytes were rinsed several times in HEPES-buffered Earle's and B_2 media and then assessed at 200× magnification for nuclear maturity (germinal vesicle, polar body) and cytoplasmic abnormalities. Oocytes were then incubated in 25-μL microdrops of B_2 medium covered by lightweight paraffin oil (BDH, Brussels, Belgium) at 37°C in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2. About 3–4 h later, the immature oocytes were checked to determine if any of them had extruded the first polar body. Intracytoplasmic sperm injection was carried out on all morphologically intact oocytes that had extruded the first polar body (metaphase II stage).

Preparation of ICSI Pipettes

The holding and injection pipettes were made from 30-μL borosilicate glass capillary tubes which were 78 mm long and had an inner diameter (ID) of 0·69 mm and an outer diameter (OD) of 0·97 mm (Drummond Scientific Company, Broomall, PA, USA). The capillaries were sonicated for 30 min in Milli RO or Milli Q water (Millipore, Brussels, Belgium) containing 2% (v/v) detergent (7X-PF O-MATIC; Flow Laboratories, Irvine, Scotland), then rinsed in running Milli Q water for 30 min. This cleaning procedure was repeated before the capillary tubes were finally dried and sterilized at 100°C for 6 h in a ULE 500 oven (Memmert, Schwabach, Germany). The second sonication was done in water without detergent.

The pipettes were made using a Type 753 horizontal micro-electrode puller (Campden Instruments, Loughborough, Leicestershire, UK). The holding pipettes were cut and fire-polished on a MF-9 microforge (Narishige, Tokyo, Japan) to obtain an OD of 50 μm and an ID of 20 μm. To prepare the injection pipettes, the pulled capillaries were opened on an EG-4 microgrinder (Narishige) to an OD of 7 μm, an ID of 5 μm and a bevel angle of 50°. The microforge was used to make a sharp spike on each injection pipette and to bend the

edge of the holding and injection pipettes to an angle of about 45° in order to facilitate injection in a Petri dish.

ICSI Procedure

A 3–5-μL sperm droplet was placed in the centre of a Petri dish (Falcon 1006; Becton Dickinson, Lincoln Park, NJ, USA), surrounded by eight 5-μL droplets of HEPES-buffered Earle's medium which contained 0·5% crystalline bovine serum albumin (BSA). The droplets were covered with about 3·5 mL of lightweight paraffin oil. The ICSI procedure was carried out at 37°C on a Diaphot inverted microscope (Nikon, Tokyo, Japan) at 400× magnification using Hoffman modulation contrast optics (Modulation Optics, Greenvale, NY, USA) and a THN-60/16 heated stage (Linkam Scientific Instruments, London, UK). The microscope was equipped with a F-601M camera (Nikon) for still pictures and a DXC-755P video camera (Sony, Brussels, Belgium) that allowed the procedure to be followed on a PVM-1443MD Trinitron colour video monitor (Sony). The microscope was equipped with two coarse positioning manipulators (3D Motor Driven Coarse Control Manipulator MM-188; Narishige) and two three-dimensional hydraulic remote-control micromanipulators (Joystick Hydraulic Micromanipulator MO-188; Narishige). The holding and injection pipettes were fitted to tool holders and were connected by Teflon tubing (CT-1; Narishige) to IM-6 microinjectors (Narishige). Fluid delivery was controlled by a 1-μL resolution vernier micrometer.

A single, almost immotile spermatozoon was selected from the central droplet and aspirated tail-first into the tip of the injection pipette. The Petri dish was then moved in order to visualize an oocyte in one of the droplets surrounding the sperm suspension. The oocyte was immobilized against the holding pipette by slight negative pressure. The oocyte was positioned so that the polar body was at 12 o'clock or 6 o'clock and the injection pipette was pushed through the zona pellucida and the oolemma into the ooplasm at 3 o'clock. The spermatozoon was injected into the ooplasm with about 1–2 pL of medium. The injection pipette was withdrawn gently and the injected oocyte was released from the holding pipette. This was repeated until all the metaphase II oocytes had been injected. The injected oocytes were then washed in B_2 medium, transferred to 25-μL droplets of B_2 medium in Petri dishes covered by lightweight paraffin oil and incubated in a B5060 EK/O_2 incubator (Heraeus, Brussels, Belgium) at 37°C in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2.

Assessment of Fertilization and Embryo Cleavage

Further handling of the injected oocytes followed our standard IVF procedures. About 16–18 h after microinjection, the oocytes were observed under an inverted microscope at 200× or 400× magnification for any sign of damage and for the presence of pronuclei (PN) and polar bodies. Fertilization was considered to be normal when two distinct PN containing nucleoli were observed. The presence of one PN or three PN was noted together with the presence of one, two or fragmented polar bodies. If a single PN was observed, a second evaluation was carried out about 4 h later to see if another PN appeared. Cleavage of the 2 PN oocytes was evaluated after a further 24 h in culture and 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 embryos, and occasionally more (depending on female age and embryo quality), were loaded into a Frydman catheter (LG 4·5; Prodimed, Neuilly-en-Thelle, France) in a small volume of Earle's medium and transferred into the uterine cavity. Embryo replacement was usually done about 48 h after microinjection. If supernumerary embryos with < 20% anucleate fragments were available, they were cryopreserved on Day 2 or Day 3 by a slow-freezing protocol using dimethylsulfoxide (DMSO).

Establishment and Follow-up of Pregnancies

Pregnancy was confirmed by detection of increasing serum hCG concentrations on at least two occasions, at least 10 days after embryo replacement. Clinical pregnancy was determined by observing a gestational sac by means of ultrasound screening at 7 weeks of pregnancy. Prenatal diagnosis was carried out by chorionic villus sampling at 9–10 weeks of gestation or by amniocentesis at 16 weeks of gestation. Genetic counselling was given in view of the prenatal diagnosis and for planning a prospective follow-up study of the children born after ICSI. The referring gynaecologist and the patients were asked to provide detailed information on the outcome of the pregnancy and the delivery.

Results and Discussion

Comparison of MESA–ICSI and MESA–IVF

The objective of our first study of 17 patients with CAV or irreparable obstructive azoospermia who had consistently failed to achieve fertilization in previous cycles with MESA–IVF was to determine whether ICSI could produce better results than MESA–IVF (Silber *et al.* 1994). When ICSI was used with epididymal or testicular sperm, we achieved good fertilization rates and normal embryos in 82% of the cases, compared with only 19% with conventional IVF. Table 1 summarizes the results and also demonstrates that 11 of the 15 patients (70%) with CAV had common cystic fibrosis (CF) carrier genotypes and 8 had the ΔF508 mutation. The CF carrier status had no adverse effect on the fertilization or pregnancy rates after ICSI. The overall ICSI fertilization rate was 45% (85% cleaved normally), compared with a 7% fertilization rate for conventional IVF. The pregnancy rate with MESA–ICSI was 47% per cycle (normal delivery rate of 30%) compared with 4·5% for MESA–IVF.

We concluded from this first series of 17 MESA–ICSI cycles that complex mechanisms facilitated by epididymal passage are required by sperm for conventional fertilization of human oocytes (whether *in vivo* or *in vitro*), but these mechanisms are not required for fertilization after ICSI. Because of the consistently good results using epididymal sperm with ICSI in comparison to conventional IVF, and also the good results in extreme cases requiring testicular tissue sperm, we concluded that ICSI would be essential for all future MESA patients with CAV or irreparable obstructive azoospermia.

This has been fully validated by the results of an additional 72 consecutive cycles of MESA–ICSI which were compared with 67 previous consecutive cycles of MESA–IVF. Fifty-three of the MESA–ICSI cycles were performed for CAV, 18 were for failed V-E, and one was for irreparable blockage. Since the results were equivalent for CAV and failed V-E, all the data were analysed as one group of 72 patients. Table 2 compares

Table 1. Results for the 17 patients in the first MESA–ICSI series based on cystic fibrosis genotype

CAV, congenital absence of the vas deferens; FR, fertilization rate; ICSI, intracytoplasmic sperm injection; MESA, microsurgical epididymal sperm aspiration; M II, metaphase II; N, normal genotype; PN, pronuclei. From Silber *et al.* (1994)

No.	Genotype if CAV	No. of M II eggs	No. of 2 PN eggs	No. embryos transferred	FR(%)	Female: age	Pregnant
1	ΔF508, N	9	3	3	33	42	No
2	N, N	14	5	5	36	39	No
3	ΔF508, N	22	11	6	50	31	No
4	ΔF508, N	10	5	5	50	33	No
5	R117H, R117H	14	6	4	43	31	Yes
6	ΔF508, N	18	6	4	33	28	Yes
7	W1282X, N	16	5	3	31	36	Yes
8	ΔF508, N	3	2	2	67	25	No
9	N, N	3	0	0	0	40	No
10	N, N	7	3	3	43	28	Yes
11	N, N	10	8	3	80	38	No
12	ΔF508, N	8	1	1	13	35	Yes
13	ΔF508, N	12	5	3	42	36	Yes
14	ΔF508, N	10	0	0	0	35	No
15	R117H, N	11	5	3	45	29	Yes
16	N, N	22	10	3	45	32	No
17	N, N	8	5	2	63	42	Yes
Totals		197	89	50	41%		8/17(47%)

Table 2. Comparison of MESA–ICSI and MESA–IVF in a similar patient population

ICSI, intracytoplasmic sperm injection; IVF, *in vitro* fertilization; MESA, microsurgical epididymal sperm aspiration; PN, pronuclei; PR, pregnancy rate. Adapted from Silber *et al.* (1994)

	MESA–IVF	MESA–ICSI
No. of cycles	67	72
No. of mature eggs	1427	962
No. of 2 PN eggs	98	443
Fertilization rate	7%	46%
No. (%) of embryo transfers	13 (19%)	65 (90%)
PR (ongoing or delivered PR)	9% (4·5%)	53% (42%)

Table 3. Fertilization, cleavage and pregnancy rates after ICSI with fresh epididymal sperm, frozen epididymal sperm and testicular sperm

ET, embryo transfer; ICSI, intracytoplasmic sperm injection; MESA, microsurgical epididymal sperm aspiration; M II, metaphase II; PN, pronuclei; PR, pregnancy rate; TESE, testicular sperm extraction

	Fresh epididymal sperm (MESA)	Frozen epididymal sperm	Testicular sperm (TESE)	Combined totals
No. of cycles	33	7	32	72
No. of M II eggs injected	431	95	436	962
No. of 2 PN eggs	201	27	215	443
Fertilization rate	47%	28%	49%	46%
No. (%) of cleaved embryos	127 (63%)	20 (74%)	155 (72%)	302 (68%)
No. (%) of ETs	31 (94%)	7 (100%)	27 (84%)	65 (90%)
No. of clinical pregnancies	20	4	14	38
Clinical PR per ET	65%	57%	52%	58%
No. of ongoing[A] pregnancies	15	3	12	30
Ongoing[A] PR per ET	48%	43%	44%	46%
Ongoing[A] PR per cycle	45%	43%	38%	42%

[A] Ongoing or delivered pregnancies.

the results of these 72 MESA–ICSI cycles with the 67 MESA–IVF cycles. The overall fertilization rate with MESA–IVF was 7% and only 19% of the patients had an embryo transfer. With ICSI, using epididymal or testicular sperm, the fertilization rate was 46% and 90% of the patients had an embryo transfer. The ongoing or delivered pregnancy rate per stimulated cycle was 4·5% after MESA–IVF compared with 42% after MESA–ICSI. This represents a 7–10-fold improvement in the results with ICSI, and is all the more impressive because the 72 ICSI cycles included 32 patients who had no epididymal sperm and thus required the use of testicular sperm.

Overall Results with TESE–ICSI and MESA–ICSI

After establishing that ICSI was preferable to conventional IVF for MESA, we compared the results among the different groups of patients (Table 3). The standard MESA procedure was the preferred approach because large numbers of sperm could be obtained from the epididymis and frozen for use in subsequent cycles without the need for further surgery. However, in many of the men who had undergone MESA on several earlier occasions, epididymal sperm could not be retrieved, and in these cases we often used testicular sperm. The patients in each of the three groups did not represent mixtures of clinical conditions or techniques. For example, if fresh epididymal sperm were used, then no eggs in that patient were injected with frozen epididymal sperm or testicular sperm. Similarly, if testicular sperm were used, then none of the eggs were injected with epididymal sperm.

The 2 PN fertilization rates were remarkably similar with fresh epididymal (MESA) sperm (47%) and testicular sperm (49%), whereas frozen epididymal sperm yielded a lower fertilization rate (28%). The cleavage rates were similar in all three groups: 63% for MESA, 74% for frozen epididymal sperm and 72% for testicular sperm (Table 3). Despite the lower 2PN fertilization rate with frozen–thawed epididymal sperm, all of these patients had embryo transfers after ICSI. Fresh MESA yielded a 94% transfer rate, whereas the transfer rate was 84% with testicular sperm. The ongoing or delivered pregnancy rates per transfer were 48% (MESA), 43% (frozen–thawed epididymal sperm) and 44% (TESE). The ongoing or delivered pregnancy rates per cycle were 45% (MESA) and 43% (frozen–thawed epididymal sperm). The pregnancy rate per cycle was lower (38%) for testicular sperm because of the lower transfer rate. A significant number (16%) of TESE–ICSI cases resulted in no fertilization, whereas there were very few fresh or frozen epididymal sperm cases with no fertilization. Nevertheless, the pregnancy rate per transfer was similar in all three groups.

MESA–ICSI for Irreparable Obstructive Azoospermia

Table 4 summarizes the results in 18 of the 72 patients whose indication for MESA was irreparable, failed V-E. The results for these patients were not different from those with CAV or from the combined results for the 72 patients. The 2PN fertilization rate was 50%, the cleavage rate was 79%, and 94% of the patients had embryos for transfer. Forty-four percent of the couples achieved a clinical pregnancy, which indicates that the aetiology of obstructive azoospermia had no influence on the results.

Table 4. The use of MESA–ICSI for failed vasoepididymostomy

ET, embryo transfer; ICSI, intracytoplasmic sperm injection; MESA, microsurgical epididymal sperm aspiration; M II, metaphase II; PN, pronuclei; PR, pregnancy rate

No. of patients	18
No. of M II eggs	249
No. of 2 PN eggs	124
Fertilization rate	50%
No. (%) of cleaved embryos	98 (79%)
No. (%) of patients with ET	17 (94%)
No. of clinical pregnancies	8
Clinical PR per cycle	44%

Virtually all the couples preferred to have a concurrent V-E or vasectomy reversal because we could assure them of an 88% chance of surgical success (Silber 1989*a*, 1989*b*, 1989*c*). As such, the couple could consider having more children in the future without the need for further ICSI. Since our normal policy was not to perform MESA–ICSI on reconstructable cases, this means that the 18 patients in Table 4 were a selected group who had no chance for pregnancy without MESA–ICSI. The results in this seemingly dismal group were still quite acceptable.

Results of MESA–ICSI or TESE–ICSI in Relation to Female Factors

Clearly, it was unimportant whether the sperm were derived from the epididymis (frozen or fresh) or the testis, or whether the male had CAV or irreparable obstruction. The CF genotype, sperm morphology and quality of motility also had no impact. The only factor in the 72 couples which affected success was the female age. The only female selection criteria were a normal uterus based on a hysterosalpingogram and a normal menstrual cycle. Several patients had tubal disease, polycystic ovary disease (PCO), some degree of endometriosis, or non-intraluminal uterine fibroids.

Table 5 summarizes the fertilization and pregnancy rates for MESA–ICSI in relation to the number of metaphase II oocytes that were retrieved. The 2 PN fertilization rate was not related to the number of mature

Table 5. Fertilization and pregnancy rates after MESA–ICSI in relation to the number of mature eggs

ET, embryo transfer; ICSI, intracytoplasmic sperm injection; MESA, microsurgical epididymal sperm aspiration; PN, pronuclei; PR, pregnancy rate

	No. of metaphase II eggs:			Totals
	< 10	10–19	≥20	
No. of patients	24	30	18	72
2 PN fertilization rate	48%	48%	43%	46%
Cleavage rate	83%	77%	65%	68%
Average no. of embryos per ET	2·3	3·5	3·5	2·9
No. of pregnancies	8	20	10	38
PR per cycle	33%	67%	56%	53%

Table 6. Fertilization and pregnancy rates after MESA–ICSI in relation to female age

ET, embryo transfer; ICSI, intracytoplasmic sperm injection; MESA, microsurgical epididymal sperm aspiration; M II, metaphase II; PN, pronuclei; PR, pregnancy rate

	Female age (years):			Combined totals
	< 30	30–37	> 37	
No. of patients	20	35	17	72
No. of M II eggs	293	479	190	962
No. of 2 PN eggs	138	220	85	443
Fertilization rate	47%	46%	45%	46%
No. (%) of cleaved embryos	91 (66%)	160 (73%)	51 (60%)	302 (68%)
Total no. of embryos transferred	54	104	51	209
Average no. of embryos per ET	2·7	3·0	3·0	2·9
No. of pregnancies	15	19	4	38
PR per cycle	75%	54%	24%	53%

Table 7. Ongoing implantation rates per embryo after ICSI with epididymal and testicular sperm

ICSI, intracytoplasmic sperm injection; MESA, microsurgical epididymal sperm aspiration; TESE, testicular sperm extraction

	Epididymal (MESA)	Testicular (TESE)
No. of patients	16	12
No. of embryo transfers	16	9
No. of embryos transferred	47	30
No. of foetal hearts	12[A]	7[B]
Ongoing implantation rate	25·5%	23·3%

[A] Includes 2 sets of triplets. [B] Includes 2 sets of twins.

eggs but the cleavage rate was higher when fewer eggs were retrieved. Fewer embryos were transferred when fewer eggs were obtained, however, the implantation rates per embryo were similar (16–20%) regardless of whether there was a high or low number of mature eggs. The pregnancy rate was reduced (33% *v.* 53%) in women with < 10 eggs, but this was due to the lower number of embryos transferred (2·3 *v.* 3·5). In the 72 cycles, there were 962 metaphase II eggs and 302 cleaved embryos (Table 3), which is an average of 4·2 embryos per patient. An average of only 2·9 embryos were transferred per patient because only the best quality embryos were selected for fresh transfer.

Table 6 demonstrates the effect of female age. The 2 PN fertilization rate (45–47%) and the cleavage rate was unrelated to female age. However, increased female age dramatically reduced the implantation and pregnancy rates. When the female partner was < 30 years of age, 75% of the MESA–ICSI cycles resulted in pregnancy. When the female was 30–37 years of age, 54% became pregnant, but when the woman was > 37 years of age, only 24% became pregnant. Thus, of all the factors which might have been predicted to affect the treatment of obstructive azoospermia by ICSI, only the age of the female had an impact, and this was not on the fertilization rate but on the implantation and pregnancy rates.

Implantation Rates after MESA–ICSI and TESE–ICSI

Although embryo transfer rates were high with both MESA–ICSI and TESE–ICSI, a significant percentage (16%) of the TESE–ICSI cycles resulted in no fertilization. This was rare with MESA–ICSI. Hence, we performed a prospective study in a series of 16 MESA cases and 12 TESE cases to determine if the implantation rate was different for embryos derived from ICSI using epididymal (MESA) and testicular sperm (TESE). The results are summarized in Table 7. There was complete failure of fertilization in 3 (25%) of the TESE cycles, whereas fertilization and an embryo transfer occurred in all of the MESA cycles. However, the ongoing implantation rates per embryo were not different (25·5% *v.* 23·3%) for epididymal and testicular sperm. Thus, for reasons

we do not yet understand, testicular sperm do not always fertilize eggs after ICSI, but if fertilization does occur, the cleavage and implantation rates are not different from epididymal sperm.

Clinical Approach to Managing the CF Issue in CAV Patients Undergoing MESA

Virtually all patients with clinical CF, the most common genetic disorder in humans, also have CAV. Until recently, most CF patients did not survive long enough to consider fatherhood. None of the CAV patients we have treated thus far have had clinical CF. Until recently (Silber *et al.* 1991; Anguiano *et al* 1992; Patrizio *et al.* 1993), it was not recognized that infertile males with CAV had an isolated genital form of CF that was inherited via a mutation of the CF gene.

We routinely screen all CAV patients for the 36 most common CF mutations. About 70% of otherwise normal CAV patients have one of these CF mutations, despite a normal chloride sweat test and no clinical symptoms of CF. Our studies of the parents of these CAV patients, as well as their offspring, have revealed that: *(1)* one of the parents of the CF-positive, CAV male always has the same heterozygous mutation as their son; *(2)* male siblings of a CAV male have a 1 in 4 chance of also having CAV; *(3)* offspring of CAV males (after successful MESA) have in theory a 50% chance of having the same CF heterozygous mutation as the CAV father; *(4)* the CF heterozygous male offspring do not themselves have CAV, and indeed have normal bilateral vasa deferentia; *(5)* the presumption is that CF carrier status alone does not cause CAV; the child must acquire a mutation (perhaps undetectable with current methods) from both parents to develop CAV (Mercier *et al.* 1995); and *(6)* 10% of CAV patients have two different CF mutations, either two weak ones, or a weak and a strong one. These compound heterozygous carriers do not have clinical CF nor a positive sweat chloride test, yet compound heterozygosity can cause CF.

It is crucial to screen for CF in men with CAV and in their partners. If the female partner is negative for the 36 common CF mutations, we feel it is quite safe to perform MESA–ICSI since the chances of any male offspring having CAV are remote, and the chances of the child having CF is probably less than in a normal, unscreened population. However, if she turns out to be a CF carrier (4% incidence in the general population), the couple can still undergo MESA–ICSI, but pre-implantation embryo diagnosis or prenatal diagnosis should be performed.

We have published the first report of successful pre-implantation embryo diagnosis after MESA–ICSI in a couple in which the man had CAV and both partners were carriers of the ΔF508 CF mutation (Liu *et al.* 1994). Both were 35 years of age and they had given up hope many years earlier of ever having a child because of the low success rate of MESA–IVF. With the introduction of ICSI, they finally decided to attempt MESA, but the woman was also found to have the ΔF508 mutation on routine pre-MESA screening. This couple thus reflected a major question for those who are CF carriers, as both were heterozygous for ΔF508. Twelve metaphase II oocytes were retrieved and microinjected. One degenerated, 5 fertilized and cleaved to 4–8-cells by Day 3 post-oocyte recovery. One or 2 blastomeres were then removed with a micropipette, and underwent PCR and genetic analysis. Two of the 5 embryos were found to be homozygous for ΔF508 and therefore were not transferred. The other three embryos were all heterozygous-positive for ΔF508. The couple accepted the possibility of a child who was a CF carrier since they were both CF carriers themselves and 3 heterozygous embryos were replaced. The dilemma was that if the ΔF508 mutation in the embryo originated from the mother, then some unknown CF mutation from the father might be transmitted to the child along with the mother's ΔF508 mutation. Thus, any male child would not be a true heterozygote but would be similar to his father and would therefore most likely have CAV. Since the father had no phenotypic expression of CF other than CAV, it was felt there would be little or no risk of the child having CF. If the ΔF508 mutation in the embryo originated from the father, then there would be no undue concern. The woman became pregnant and delivered a normal baby boy who was confirmed to be a heterozygous ΔF508 carrier. The child's chloride sweat test was normal and both vasa deferentia were present and normal, indicating that the ΔF508 deletion probably originated from the father.

A more serious problem arises when the woman is discovered to have a CF mutation different from the man. In that case, unless both mutations can be tested for in the embryo, 50% of embryos (i.e. all embryos presenting with just one of the detectable mutations) would have to be discarded, even though only 25% would be expected to be potentially dangerous compound heterozygotes.

In summary, the issue of CF in the offspring from CAV males must be addressed seriously in any MESA–ICSI programme. Nonetheless, with routine, inexpensive CF genetic screening of the prospective parents and the availability of pre-implantation diagnosis, we are not aware of any children born with a CF phenotype or CAV despite transmission to the offspring of the father's CF gene mutation. But only with proper attention to these details can this potential risk to the offspring remain remote.

TESE–ICSI for Non-obstructive Azoospermia and Other Conditions

To date, our major effort with MESA–ICSI and TESE–ICSI has been concentrated on obstructive azoospermia. The results presented here indicate that all cases of obstructive azoospermia have the potential to be treated successfully and the only limitations appear to be the age and egg quality of the female partner. For ICSI, it is the vitality of the sperm and not their motility which is important, therefore retrieval of testicular sperm for ICSI enables injection of non-senescent, viable sperm when the ejaculated sperm are completely immotile.

No matter what the cause of total asthenozoospermia, the key to success with ICSI is to inject viable sperm. Selection of viable sperm is difficult when they are all immotile, so one solution to this problem is to retrieve sperm from the testis for ICSI. Although the motility of testicular sperm is always poor, the percentage of viable sperm should be high. This approach has been successful in a case of maturation arrest and in a case of Kartagener's syndrome. In addition, we have reviewed the slides of many of our azoospermic patients with diagnoses of Sertoli cell only syndrome or maturation arrest, and we found very small numbers of mature spermatids in these samples, indicating that TESE–ICSI could be used successfully in many of these non-obstructive azoospermic cases. Therefore, whenever the ejaculate fails to contain the few motile sperm that are required for ICSI, testicular sperm extraction provides a simple back-up solution.

Conclusions

Three years ago, ICSI provided a major breakthrough in the treatment of severe male factor infertility. Initial efforts were directed at severe oligozoospermia or oligoasthenozoospermia, and the only serious limitation was if there were no sperm in the ejaculate or if they were all immotile. This review documents the solution to the problem of obstructive azoospermia. If microsurgery is impractical or unsuccessful, MESA–ICSI or TESE–ICSI provide the most successful treatment. In fact, when we perform vasectomy reversals or V-E, we now always freeze a sample of motile epididymal sperm in case the microsurgery is unsuccessful. Thus, almost every case of obstructive azoospermia is now amenable to microsurgery and ICSI. The next step is to utilize TESE–ICSI to treat cases of non-obstructive azoospermia, and this will be the direction of our future research.

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Open Discussion

Simon Fishel (Nottingham):

Have you done antibody assessments on these MESA patients?

Silber:

We did antibody studies (immunobead tests) when we were doing conventional IVF on these patients and we didn't find any correlation between antibodies and fertilization. We also did computerized motion analysis with Russell Davis and Jim Overstreet, but no matter how carefully we studied these patients, we found nothing that we could measure that was predictive of fertilization after conventional IVF. We've lost interest in the antibodies now that we use ICSI and we haven't studied them with the patients currently undergoing ICSI.

Harold Bourne (Melbourne):

The cleavage rate in one of your slides was only 79%. Does that mean a lot of the embryos arrested at the pronuclear stage?

Silber:

Yes. On average, we achieved 46% two pronuclear fertilization instead of 60–65% as you would expect with ejaculated sperm, but this was very predictable with either testicular or epididymal sperm. The average cleavage rate was only 60–70%.

Bourne:

Any ideas why?

Silber:

No, I don't even know why we should have such consistently lower two pronuclear fertilization rates with epididymal and testicular sperm compared with ejaculated sperm.

Herman Tournaye (Brussels):

Your definition of Sertoli cell only syndrome seems to be exactly the same as hypospermatogenesis. As soon as you find some gonadal cells, then by definition it's not Sertoli cell only syndrome.

Silber:

Hypospermatogenesis is defined by most pathologists as a condition in which you see many tubules with only a small amount of spermatogenesis in them. We traditionally think of Sertoli cell only as a condition in

which you look all over the slide and all you see is Sertoli cells, but then you might see one isolated tubule making sperm. David Page found that deletions in all these cases occur at the same location regardless of whether they're complete Sertoli cell only syndrome or there are occasional tubules which appear to be making sperm. This is an example of what geneticists call a 'leaky gene'. So I think it's probably fair to call it Sertoli cell only as long as I define carefully what I mean.

In most cases of hypospermatogenesis, most of the tubules are producing small numbers of sperm and sperm can be found in the ejaculate after it has been centrifuged, and these cases don't even come into consideration for testicular sperm extraction (TESE). But in many cases of Sertoli cell only in which there is an occasional tubule producing sperm, there are no sperm in the ejaculate even after centrifugation at 1800*g*. We have gone back over Sertoli cell only cases carefully and it's amazing how many of them have one or two tubules that are making a few spermatids.

David de Kretser (Melbourne):

It shouldn't be called Sertoli cell only syndrome. It really is a question of what the aetiology of the material is and we don't fully understand that yet.

Silber:

We can debate terminology, but the interesting thing is that, if we see that picture rather than classical hypospermatogenesis, we consistently find the same gene defect on Yq (78 patients). They have isolated the gene and they're about to start sequencing it.

Alan Trounson (Melbourne):

Are you saying that ΔF508 is genomically imprinted for congenital absence of the vas (CAV)?

Silber:

Every patient with CAV is presumed by geneticists to have a CF deletion, but in 30% of cases you don't pick it up since we routinely screen for only 36 mutations and there are certainly over 300 mutations that we don't screen for.

Whenever we found a ΔF508 heterozygote, for example in a male with CAV, there was a ΔF508 either in his mother or in his father. If it was in his father it was obvious his father had bilateral vas and was fertile. Furthermore, if the male with CAV had ΔF508, then there was a 50% chance that the offspring from MESA would have ΔF508, and if it were a male offspring with ΔF508, he would have the vas deferens present bilaterally. So therefore the mere fact of carrier status for CF does not cause congenital absence of the vas. We presume it requires either compound heterozygosity, homozygosity or something from both parents.

Trounson:

Two years ago, you said that IVF fertilization rates with MESA were about 30%. Now it's only 7%. Is this radical difference due to the patients or techniques?

Silber:

In the first 100 MESA cases that we did with conventional IVF, we reported 16 pregnancies with live births. We were pretty excited about that. But in the next 67 cases we had only 9 pregnancies and half of those miscarried (4·5% live birth rate per cycle). The fertilization rate was always low though, 17% in the first 100 cases and only 8% in the next 67 cases. I don't know whether it was a different group or whether we did repeat cycles on people that were less likely to succeed. I really can't explain why it got worse, but frankly it was never very high.