Epididymal and Testicular Spermatozoa and Intracytoplasmic Sperm Injection

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Clinical interest in the fertilizing capacity of epididymal and testicular sperm originally developed from cases of obstructive azoospermia that could not be corrected surgically. Congenital absence of the vas deferens (CAV), failed vasoepididymostomy (V-E), and all irreparable obstructions were once frustrating conditions, because although patients with these conditions have normal spermatogenesis, they were untreatable.[1] Microsurgical epididymal sperm aspiration (MESA) along with in vitro fertilization (IVF) was introduced in 1985, and in 1988 was used to treat these cases, but only modest success was achieved.[1-3]

Research was undertaken in 1993 to determine whether intracytoplasmic sperm injection (ICSI) could improve the low fertilization and pregnancy rates in patients with microsurgically retrieved epididymal or testicular sperm.[4,5] In cases where there were no motile epididymal sperm retrievable, researchers were particularly interested in whether sperm obtained from testicular biopsy could be successfully employed. It was found that ICSI with retrieved epididymal or testicular sperm in men with CAV, or any other cause of obstructive azoospermia, gives reliable, 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 sperm with ICSI not only for obstructive, but also for nonobstructive, azoospermia.[6,7]

The purpose of this article is (1) to review the first use of ICSI with epididymal and testicular sperm to treat obstructive azoospermia of all causes (CAV, failed V-E, or irreparable obstruction); (2) to review the first use of testicular sperm and ICSI for the treatment of nonobstructive azoospermia, including Sertoli cell only, maturation arrest, cryptorchidism, and postchemotherapy-induced azoospermia, and even Klinefelter syndrome; and (3) to suggest what direction male infertility treatment will be taking in the future, and explore briefly its genetic implications.

NATURE OF EPIDIDYMAL AND TESTICULAR SPERM

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 nonobstructed epididymis.[8-12] Sperm with the greatest motility were always found most proximally in the obstructed epididymis.[1] The percentage of motility is usually low (from 1 to 30%), but the greatest motility is always found, ironically, in the most proximal region.

Sperm in the distal regions of an obstructed epididymis have poor to no motility because of senescence. Electron microscopic (EM) studies indicate that the distal sperm are aging and the more recently produced proximal sperm have not yet degenerated.[13,14] Therefore, the problem with fertilization using these epididymal sperm may be aging as well as immaturity.

Something other than motility related to epididymal sperm transit is required for maximum fertilization rate with conventional IVF. Proximally retrieved epididymal sperm often exhibit excellent motility but, nevertheless, a poor fertilization rate. After successful V-E, ejaculated sperm usually can fertilize, but retrieved epididymal sperm usually cannot. No IVF program has consistently produced greater than 5 to 10% success with retrieved epididymal sperm without the use of ICSI.[15] Testicular sperm usually exhibit poor motility and, often, no motility. Yet with ICSI, the fertilization and pregnancy rate from testicular sperm is high. Presumably, testicular sperm, despite poor motility, unlike epididymal or ejaculated sperm, have a high percentage of vitality because of minimal senescence.

MICROSURGICAL EPIDIDYMAL SPERM ASPIRATION

The term, MESA, an acronym for microsurgical epididymal sperm aspiration, was coined in 1988. In the early days of conventional IVF, MESA was an elaborately invasive procedure. The technique now can be performed elegantly with minimal invasion in an outpatient setting. Microsurgical technique is advocated to get the cleanest specimen, cause the least damage, and afford the greatest number of motile sperm for freezing. Epididymal sperm, in contrast to testicular sperm, must be motile to obtain good fertilization. The surgical technique in the male is as follows. The scrotum is entered through a small incision, the tunica vaginalis is opened, and the epididymis is exposed. Under ×10 to ×40 magnification with an operating microscope, a tiny incision is made with microscissors in the epididymal tunic to expose the tubules in the proximal portion of the obstructed epididymis. Sperm are aspirated directly from an opening in the epididymis tubule with a micropipette. The epididymal fluid is immediately diluted in HEPES-buffered Earle's medium and examined for motility and quality of progression. If there is no motility, another aspiration is made more proximally. Motile sperm usually are not obtained until the proximal-most portion

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of the caput epididymis or even the vasa efferentia is reached.[1,3] Currently, this procedure, though delicate, is brief and almost painless postoperatively.

The rationale for trying to find the most motile sperm, even with ICSI, is based on the following presumptions: ICSI with ejaculated sperm works best when there is some motility, no matter how poor, as a verification of vitality. In the obstructed epididymis, nonmotile sperm most often are senescent and degenerate. The most proximal sperm are least likely to have undergone senescent degenerative changes in the sperm head and are most likely to exhibit some motility.

TESTICULAR SPERM EXTRACTION

When no motile sperm are retrievable, a testicular biopsy is performed, and sperm are retrieved by morselizing the testicular specimen. Although many millions of motile sperm are retrievable from the obstructed epididymis, the number of sperm obtainable from a tiny piece of testicular tissue is less than 500,000; these sperm usually have only weak, slowly twitching motility, and sometimes exhibit no motility at all. Incubation of testicular sperm in culture medium for an hour or so often improves this poor motility somewhat.

Also, when there is no epididymis, or when the entire scrotum is massively scarred, or when there are simply no sperm in the epididymis, there is no choice but to resort to testicular biopsy for sperm retrieval. The surgical technique for testicular biopsy, when there is normal spermatogenesis, is extremely simple. A 4-mm incision is made in the scrotal skin and carried through the peritoneal tunica vaginalis. A tiny incision is then made in the tunica albuginea, and a small piece of extruding testicular tissue is excised and placed in a small Petri dish with 3 mL of HEPES-buffered Earle's medium. The tunica albuginea is closed with a 3-0 Vicryl suture.

PREPARATION OF EPIDIDYMAL AND TESTICULAR SPERM

There is nothing particularly unusual about the treatment of epididymal or testicular sperm for cases of obstructive azoospermia and normal spermatogenesis. Epididymal sperm are usually prepared with a routine mini-Percoll gradient, just as for conventional ICSI. Testicular sperm are simply concentrated by centrifugation because of the very small numbers and extremely poor motility.

As has been published by Liu et al,[16] the present investigation found that the method of sperm preparation has no effect on results, but only on the technical ease of performing the ICSI. Testicular-tissue sperm are retrieved in such small numbers, and with such minimum motility, that the only method of preparation is to morselize the tissue, centrifuge the effluent at 300 × *g* for 5 minutes, and resuspend in 100 μL. At the present time, testicular tissue sperm are not frozen and saved as is routinely done for epididymal sperm, because of the small numbers of barely undulating sperm and the large numbers of Sertoli cells and cytoplasmic debris.

When epididymal sperm are retrieved (MESA), the majority of sperm are frozen for subsequent cycles. Because for ICSI only a minor degree of motility is required (for vitality assessment only), freezing these fragile epididymal spermatozoa poses no serious difficulty. All of the epididymal sperm that are not used fresh for ICSI are diluted first in media and then 50:50 by volume with sperm-freezing media, drawn into 250-μL straws, suspended over liquid nitrogen vapor for several hours, and then plunged into liquid nitrogen in a standard fashion.[17,18]

With testicular sperm extraction (TESE), the small piece of testicular tissue is finely minced in HEPES-buffered media, and this effluent is then placed in a 5-mL Falcon tube and centrifuged for 5 minutes at 300 × *g*.[18–21] The supernatant is removed and the pellet is resuspended in medium. Percoll separation would be pointless to attempt. The sperm in the droplet are so few and the motility so weak that one has no choice but to pick the individual sperm up out of the field of debris, red blood cells, and Sertoli cells for ICSI. Sometimes the supernatant can be recentrifuged at 1800 × *g*; this produces a cleaner preparation for ICSI. The sperm suspension is then kept at 37°C in the incubator.

The motility of the few testicular biopsy sperm is so weak that the sperm droplet is not placed into the polyvinylpyrrolidone (PVP) droplet as is done with most ICSI cases. Rather, the individual barely motile sperm is 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 sperm in all cases is broken, because these sperm could exhibit increased motility later.

OOCYTE PREPARATION FOR THE INTRACYTOPLASMIC SPERM INJECTION PROCEDURE

Oocyte retrieval is carried out by vaginal ultrasound-guided puncture 36 hours after human chorionic gonadotropin (hCG) administration. After the oocyte retrieval, the cells of the cumulus and corona radiata are removed by incubation for about 30 seconds 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, Missouri). The removal of the cumulus and corona cells is enhanced by aspiration of the complexes in and out of hand-drawn glass pipettes with serially smaller openings of approximately 200 to 150 μm. Afterward, the oocytes are 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 are then placed in Ménézo B2 medium and carefully observed under the inverted microscope at ×200 magnification. This includes 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 are then incubated in 25-μL microdrops of B2 medium covered by lightweight paraffin oil (British Drug House, Pasture, Brussels, Belgium) at 37°C in an atmosphere of 5% O_2, 5% CO_2, and 90% N_2. Approximately 3 to 4 hours later, the oocytes are observed again to see whether more oocytes have extruded the first polar body. Intracytoplasmic sperm

injection is carried out on all morphologically intact oocytes that have extruded the first polar body.

INTRACYTOPLASMIC SPERM INJECTION PROCEDURE

The holding and injection pipettes are made from 30-μL borosilicate glass capillary tubes (Drummond Scientific Co., Broomall, Pennsylvania) 78 mm long and with an inner and outer diameter of 0.69 mm and 0.97 mm, respectively. The glass pipettes are obtained by drawing thin-walled glass capillary tubes (Drummond Scientific Co.) using a horizontal microelectrode puller (Type 753, Campden Instruments Ltd., Loughborough, Leicestershire, UK). The holding pipette is 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 is opened on a microgrinder (EG-4 Micro-Grinder, Narishige Co. Ltd.) to an outer diameter of 7 μm and an inner diameter of 5 μm; the bevel angle is 45 degrees. The grinding step requires approximately 3 minutes. The wetstone of the grinder is humidified by slow water drip during the procedure. The microforge is then used to make a sharp spike on the injection pipette and to bend the edge of the holding and injection pipettes to an angle of approximately 35 degrees to facilitate the injection procedure in the Petri dish.

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

The ICSI procedure is carried out on the heated stage (37°C; THN-60/16 and MS100 Controller, Linkam Scientific Instruments Ltd., London, UK) of an inverted microscope (Diaphot, Nikon Corp., Tokyo, Japan) at ×400 magnification using Hoffman Modulation Contrast System (Modulation Optics Inc., Greenvale, New York). The microscope is equipped with a Nikon F-601M camera for still pictures and a video camera (DXC-755 P, Sony Corp., Brussels, Belgium) that allows the procedure to be followed on a Trinitron color video monitor (PVM-1443MD, Sony). The microscope is equipped with two coarse positioning manipulators (3-D Motor Driven Coarse Control Manipulator MM-188, Narishige Co. Ltd.) and with two three-dimensional hydraulic remote-control micromanipulators (Joystick Hydraulic Micromanipulator MO-188, Narishige Co. Ltd.). The holding and injection pipettes are fitted to a tool holder and are connected by Teflon tubing (CT-1, Narishige Co. Ltd.) to a micrometer-type microinjector (IM-6, Narishige Co. Ltd.). Solution delivery is controlled via a 1-μL–resolution vernier micrometer.

For testicular sperm, a single weakly motile spermatozoon is selected from the central sperm droplet and is aspirated tail-first into the tip of the injection pipette. The spermatozoon is then placed in the separate 5-μL droplet of 10% PVP in HEPES-buffered Earle's medium and washed. The tail is broken with the tip of the injection pipette to immobilize it, and then it is picked up again into the injection pipette. The Petri dish is then moved to visualize an oocyte in one of the droplets surrounding the sperm suspension. The oocyte is immobilized by slight negative pressure exerted on the holding pipette. The polar body is held at 12 or 6 o'clock, and the micropipette is pushed through the zona pellucida and the oolemma into the ooplasm at 3 o'clock. A single spermatozoon is injected into the ooplasm with approximately 1 to 2 pL of medium. The injection pipette is withdrawn gently, and the injected oocyte is released from the holding pipette. The aspiration of a single spermatozoon and injection into the ooplasm are repeated until all Metaphase II oocytes are injected. The injected oocytes are then washed in B2 medium and transferred into 25-μL droplets of B2 medium covered by lightweight paraffin oil. The Petri dishes with the oocytes are then incubated (Heraeus, B5060 EK/O_2, Van der Heyden, Brussels, Belgium) at 37°C, 5% CO_2, and 90% N_2.

ASSESSMENT OF FERTILIZATION, EMBRYO CLEAVAGE, AND PREGNANCY

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

Pregnancy is confirmed by detecting increasing serum hCG concentration on at least two occasions at least 12 days after

Table 1. Comparison between Microsurgical Epididymal Sperm Aspiration with Conventional In Vitro Fertilization and with Intracytoplasmic Sperm Injection

Cycles	Mature Eggs	2PN Oocytes	Fertilization Rate (%)	Transfers n (%)	Delivered Pregnancies n (%)
IVF-MESA					
67	1427	98	7	13/67 (19)	3/67 (4.5)
ICSI-MESA					
33	431	201	47	31/33 (94)	12/33 (36.3)

embryo replacement. Clinical pregnancy is determined by observing a gestational sac by means of echographic screening at 7 weeks of pregnancy. Patients are asked to provide detailed information of the evolution of the pregnancy and the outcome of the delivery.

RESULTS WITH OBSTRUCTIVE AZOOSPERMIA

This is a cumulative report on the the first 131 consecutive cycles of ICSI for obstructive azoospermia using fresh epididymal, frozen-thawed epididymal, and testicular spermatozoa. Sixty-nine cycles were performed for CAV, and 62 were for failed V-E or irreparable blockage. Fifty-six of the 131 cycles utilized fresh epididymal sperm (MESA), 13 utilized frozen epididymal sperm, and 62 used testicular biopsy sperm (TESE). Results were equivalent in CAV, failed V-E cases, and irreparable blockage. Results were similar for MESA (epididymal sperm) and for TESE (testicular sperm). However, the results were greatly affected by the age of the woman and the number of retrievable oocytes. The TESE cases happened to have older wives. Therefore, although fertilization and cleavage was similar with TESE, pregnancy rate was lower, due to the age of the woman. Nonetheless, even balancing the effect of age, the embryo tranfer rate for epididymal sperm was better (98%) than that for testicular sperm (90%).

Table 1 compares the overall results of the first consecutive 33 MESA-ICSI cycles with the previous 67 consecutive MESA-conventional IVF cycles.[15] The fertilization rate with conventional IVF using epididymal sperm was 7%, and only 19% of these patients had an embryo transfer. With ICSI, using epididymal or testicular sperm, the fertilization rate was 47%, and 94% of patients had an embryo transfer. The delivered pregnancy rate with epididymal sperm 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.

Tables 2 and 3 summarize the fertilization and cleavage rates using fresh epididymal sperm, frozen-thawed epididymal sperm, and testicular-biopsy–extracted sperm. The standard MESA procedure, which yielded epididymal sperm, was the preferred approach, because the large numbers of sperm obtained from the epididymis could easily be frozen and used in subsequent cycles with no need for any further surgery on the man. However, in many of the most difficult cases with multiple previous surgeries, motile epididymal sperm simply were not retrievable. Only in these cases was it necessary to 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. When fresh epididymal sperm were used, no eggs from the patient were injected with previously frozen sperm or testicular biopsy sperm. When testicular biopsy sperm were used no eggs from that patient were injected with epididymal sperm. All 131 consecutive cases represented pure groups in this respect, for proper comparison.

The 2PN fertilization rates were remarkably similar with fresh epididymal (55%), frozen epididymal (48%), and testicular biopsy (49%) sperm. The cleavage rates in all three groups also were similar: 63% for fresh epididymal sperm, 79% for frozen epididymal sperm, and 61% for testicular spermatozoa.

Tables 4 and 5 summarize the embryo transfer and pregnancy rates using fresh epididymal, frozen epididymal, and testicular sperm. One hundred percent of patients with frozen-thawed epididymal sperm had embryos to transfer after ICSI. Fresh epididymal sperm similarly yielded a 98% transfer rate. Testicular sperm produced a still remarkable 90% transfer rate.

The delivered pregnancy rate per cycle was 34% for fresh MESA, 46% for frozen-thawed (MESA) sperm, and 18% for TESE. The delivered pregnancy rate per transfer and per cycle was obviously similar with fresh MESA and frozen-thawed MESA. For TESE (testicular biopsy sperm), the pregnancy rate appeared to be much lower, but this can be readily explained by the higher age of those women. Otherwise, the pregnancy

Table 2. Fertilization and Cleavage Rate after Intracytoplasmic Sperm Injection with Epididymal and with Testicular Biopsy Sperm*

Source of Sperm	Patient Cycles (n = 72)	M-II Eggs Injected (n = 962)	2PN Oocytes (n = 443) (46%)	Cleaved Embryos (n = 302) (68%)
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)

*First 72 cases

Table 3. Fertilization and Cleavage Rate after Intracytoplasmic Sperm Injection with Epididymal and with Testicular Biopsy Sperm (July 1993–May 1995)

Source of Sperm	Patient Cycles (n = 131)	M-II Eggs Injected (n = 1727)	2PN Oocytes (n = 890) (52%)	Normal Cleaved Embryos (n = 573) (64%)
Fresh epididymal (MESA)	56	751	413 (55)	261 (63)
Frozen epididymal	13	228	110 (48)	87 (79)
Testicular biopsy (TESE)	62	748	367 (49)	225 (61)

rate per transfer would be essentially identical in all three groups regardless of the sperm origin.

CRITICAL IMPACT OF WOMAN'S AGE ON RESULTS

The only factor that affected success in couples underoing MESA-ICSI or TESE-ICSI for obstructive azoospermia was the woman. Clearly, whether sperm were from the 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 (CF) genotype, the sperm morphology, and the quality of motility had no impact. The only factors in the woman that mattered were her age and the number of oocytes retrievable from her.

Table 6 demonstrates this relation. The only factor that dramatically affected the pregnancy rate was the age of the woman. When the woman was under 30 years of age, 71% of the MESA-ICSI cycles resulted in pregnancy, and 48% delivered. When the woman was 30 to 36 years of age, 48% became pregnant, and 30% delivered. When the woman was over 36 years of age, but under 40 years, 6% became pregnant, and when the woman was 40 years of age or older, although 20% became pregnant, only 5% delivered. The age of the woman affected only implantation and pregnancy rate. The 2PN fertilization rate was remarkably constant for all age groups (51%, 53%, 48%, and 51%). The cleavage rate was also unrelated to age of the woman. In fact, the fertilization rate was remarkably constant (about 52%) no matter how these patients were grouped: by the cause of obstruction, the quality of the sperm, whether from testicle or from epididymis, the number of eggs retrieved, or even the age of the woman. For women under 30 years of age, the overall delivery rate was 48%. For women between 30 and 36 years of age the delivery rate was 30%, and for women over 36 years of age, it was only 6%. Thus, the age of the woman dramatically affected the pregnancy and delivery rate, but had no effect at all on fertilization or cleavage.

Table 7 shows that women who yield more eggs (> 8) have 2PN fertilization and cleavage rates similar to those for women who yield very few eggs. However, the pregnancy rate is dramatically higher in women who produce more eggs despite transfer of similar numbers of embryos.

CYSTIC FIBROSIS (CF) AND CONGENITAL ABSENCE OF VAS (CAV): PREIMPLANTATION EMBRYO DIAGNOSIS

Virtually all patients with CF (one of the most common autosomal recessive genetic disorders in humans) also have CAV. Until recent years, most patients with CF did not survive sufficiently long even to consider trying to become a father. Therefore, none of the CAV patients initially treated with MESA (over 200) had clinical CF. A possible genetic link between CF and CAV was suggested more than 20 years ago.[22] However, it was not until mutation analysis in the CF gene became possible that this link was confirmed at the molecular level. In fact, infertile men with CAV probably have a mild reproductive (genital) form of CF that is inherited through mutations in the CF gene.[6,23–29]

Today, over 500 mutations have been identified in the cystic fibrosis transmembrane conductance regulator (CFTR) gene of patients with CF. With the exception of the ΔF508 mutation (deletion of the amino acid phenylalanine at position 508 of the CFTR), and a few others specific to certain ethnic groups like W1282 in Ashkenazy Jews, most of these mutations are rare and are present in only one or a few patients with CF. Routine screening methods have been developed for carrier detection in the general population. These methods usually take into account the frequency of mutations in CF proteins in the particular population being studied. Up to 20% of CAV males are carriers of two different mutations (compound heterozygotes). However, in these instances, at least one of the CF mutations is a weak one in reference to the clinical phenotype in patients with CF. In the remaining patients, no CF mutations can be found,

Table 4. Pregnancy and Delivery Rate after Intracytoplasmic Sperm Injection with Epididymal and with Testicular Biopsy Sperm*

Source of Sperm	Patient Cycles (n = 72)	Transfers (n = 65) (90%)	Clinical Pregnancies per Transfer (n = 38) (58%)	Delivered Pregnancies per Transfer (n = 24) (37%)	Delivered Pregnancies per Cycle (33%)
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

*First 72 cases

Table 5. Fertilization and Delivery Rate after Intracytoplasmic Sperm Injection with Epididymal and with Testicular Biopsy Sperm (July 1993–May 1995)

Source of Sperm	Patient Cycles (n = 131)	Transfers (n = 124) (95%)	Clinical Pregnancies per Transfer (n = 57) (46%)	Ongoing/Delivered Pregnancies per Transfer (n = 36) (29%)	Ongoing/Delivered Pregnancies per Cycle (27%)
Fresh epididymal (MESA)	56	55 (98)	32 (58)	19 (35)	34
Frozen epididymal	13	13 (100)	7 (54)	6 (46)	46
Testicular biopsy (TESE)	62	56 (90)	18 (32)	11 (20)	18

but recent work has shown noncoding mutations in the T5-allele of the CF gene in such patients.[30]

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

Thus, CF carrier status alone certainly does not cause CAV. The 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 other genes are involved. The latter idea is supported by a study of Mercier et al[31] of two brothers, one with CAV and one normally fertile, who have the same genotype at the CFTR gene. This confusion was finally cleared up by the study of Chillon et al,[30] demonstrating a defect in the noncoding T-5 allele of the CF gene in such patients.

It is clear that it is crucial to screen both the husband with CAV and his wife for CF mutations. If the woman turns out to be a CF 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. In 1994 the first case of successful preimplantation embryo diagnosis in a CAV-MESA case in which both partners were carriers of the ΔF508 mutation was published.[32] Embryo blastomere biopsy and preimplantation diagnosis are required as a routine approach whenever the female is discovered, upon screening, to be a CF carrier.

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

TESTICULAR SPERM EXTRACTION-INTRACYTOPLASMIC SPERM INJECTION FOR NONOBSTRUCTIVE AZOOSPERMIA

Sertoli Cell Only, Maturation Arrest, Cryptorchid Atrophy, Postchemotherapy, and Klinefelter Syndrome

The major effort with MESA, TESE, and ICSI until recently was concentrated on irreparable obstructive azoospermia. These patients generally have normal spermatogenesis. Although microsurgery is very successful and is the preferred treatment for most cases of obstructive azoospermia, it seems that there

Table 6. Fertilization and Pregnancy Rate after Intracytoplasmic Sperm Injection with Epididymal and with Testicular Sperm in Relation to Age of Woman (July 1993–May 1995)

Age of Woman (yr)	Patient Cycles (n = 131)	M-II Eggs Injected (n = 1727)	2PN Oocytes (n = 890) (52%)	Normal Cleaved Embryos (n = 573) (64%)	Clinical Pregnancies per Cycle (n = 57) (44%)	Ongoing/Delivered Pregnancies per Cycle* (n = 36) (27%)
< 30	31	486	247 (51)	155 (63)	22 (71)	15 (48)
30–36	63	870	457 (53)	293 (64)	30 (48)	19 (30)
37–39	17	141	68 (48)	51 (75)	1 (6)	1 (6)
40+	20	230	118 (51)	74 (63)	4 (20)	1 (5)

*Ongoing and delivered rates per cycle for women under age 37 yr = 36%

Table 7. Obstructive Azoospermia: Fertilization and Pregnancy Rate after Intracytoplasmic Sperm Injection with Epididymal and with Testicular Sperm in Relation to Number of Eggs (July 1993–May 1995)

Number of Eggs	Patient Cycles (n = 131)	M-II Eggs Injected (n = 1727)	2PN Oocytes (n = 890) (52%)	Normal Cleaved Embryos (n = 573) (64%)	Clinical Pregnancies per Cycle (n = 57) (44%)	Ongoing/Delivered Pregnancies per Cycle* (n = 36) (27%)
1–3	7	19	11 (58)	9 (82)	1 (14)	1 (14)
4–8	25	141	79 (56)	65 (82)	6 (24)	4 (16)
9–16	44	450	230 (51)	158 (69)	23 (52)	15 (34)
17–24	35	618	326 (53)	212 (65)	16 (46)	10 (29)
25 or more	20	499	244 (49)	129 (53)	11 (55)	6 (30)

are no cases of irreparable obstructive azoospermia that cannot be treated successfully with the approach of TESE or MESA plus ICSI. The next step in using ICSI to treat the azoospermic couple was for cases of germinal failure; that is nonobstructive azoospermia.

Original studies on spermatogenesis demonstrated that most patients with nonobstructive azoospermia had a miniscule amount of sperm production in the testes, despite no sperm ever reaching the ejaculate.[33,34] In 70% of azoospermic patients who are casually thought to be making zero sperm, there are nonetheless a few sperm being produced somewhere in the testicle.[35–37] Testicular sperm retrieval may afford a method to ensure getting nonsenescent sperm for ICSI when the ejaculate exhibits absolutely no sperm, or sperm with no motility. Although sperm motility is always markedly reduced in the testicle, the percentage of fresh, live "vital" sperm is extremely high. Therefore, if a careful search of the entire ejaculate reveals no sperm or no sperm with motility, it is wise to do a testicle biopsy and use that sperm for TESE and ICSI.

This concept of using TESE for nonobstructive male infertility has now taken a great leap. Most patients with nonobstructive azoospermia, who appear to have no sperm production, in truth have a few rare foci of sperm somewhere in their testicles. Review of the slides of many azoospermic patients with "Sertoli cell only" and "maturation arrest," revealed very small numbers of mature spermatids in most of these nonobstructive azoospermic patients. Thus far, in 70% of such patients with zero sperm in the spun-down ejaculate, enough sperm were found in an extensive testicle biopsy to perform ICSI and get normal embryos and a normal pregnancy rate. Even in patients whose routine diagnostic testicle biopsy failed to reveal any sperm, a more thorough 2-hour search of testicular tissue usually does reveal a few sperm that can be used for ICSI, resulting in fertilization and pregnancy rates no different from those of patients with obstructive azoospermia and normal spermatogenesis.[38,39] Patients with postchemotherapy and postcryptorchid atrophy almost always have sperm in the testes sufficient for ICSI, despite extremely high follicle-stimulating hormone levels and extremely small testes; whereas in the "genetic" cases of Sertoli cell only and maturation arrest only about 60% have sperm retrievable from the testes.

The evolution of TESE and ICSI is fascinating: ICSI provides a remarkably effective solution to severe male-factor infertility. Initial efforts were directed at severe oligospermia, or oligoasthenospermia. The severity of the oligospermia, even with 100% abnormal morphology, had no adverse effect on fertilization, cleavage, or pregnancy rate.[18] Next, ICSI with MESA or TESE was used for obstructive azoospermia when microsurgery had failed. Now, a sample of motile epididymal sperm is always frozen at the time of vasectomy reversal, V-E, or redoing a previously failed vasectomy reversal, in the unusual event that the repair should fail. Thus, in a sense, every case of obstructive azoospermia is microsurgically correctable. Now, even when sperm production is so extremely low as to make the ejaculate absolutely devoid of sperm, the testicle can still usually be found to contain enough sperm for ICSI. This means that there are now very few cases of azoospermia that are not treatable so long as the woman is young enough and has sufficient ovarian reserve.

GENETIC ETIOLOGY AND TRANSMISSION OF MALE INFERTILITY

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

The important factor that ICSI brings into this controversy is simple. If male infertility has nothing to do with all these etiologies, 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, as with CAV, most cases of nonobstructive male infertility are indeed of genetic origin.

Comparative biology of exotic species interestingly provides insight into the powerful genetic control of spermatogenesis. Roger Short has beautifully demonstrated that species with a promiscuous mating pattern have a much higher sperm count and larger testicles, owing to "sperm competition."[40–42] 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 sperm 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 poor semen quality compared with that of most other animals, with the exception of the gorilla, which also has a monogamous mating system.

O'Brien's studies on the inbreeding of cheetahs, and of certain population "bottlenecks" of lions, also points to the strong genetic control over spermatogenesis.[43] Inbreeding, and loss of genetic diversity, clearly leads to severe defects in spermatogenesis.[43–45] 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 to be no better than psychological counseling.[46] Most reproductive physicians today are not surprised by these results.

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 disorders, then all of the conventional therapies for male infertility could be permanently laid to rest, and ICSI alone relied upon. However, then in future decades an even higher percentage of severe male-factor infertility in the population could be expected.

Obstructive azoospermia most commonly is acquired (via vasectomy, infection, or hernia-type surgery). However, about 10% of obstructive azoospermia is congenital and inherited on the CF gene. It is caused either by compound heterozygosity with at least one "weak" deletion (10%), occasionally by weak deletion homozygosity, or most commonly by CF heterozygous carrier status combined with a noncoding dosage deletion on the T-5 exon of the opposite allele.[30] For this condition, particularly if both the man and the woman are carriers, genetic counseling is required.

Nonobstructive azoospermia and severe oligospermia, most commonly are caused by Sertoli cell only, or maturation arrest. These problems are usually genetic and appear to be inherited on the long arm of the Y chromosome.[47] There appear to be no phenotypic consequences of this deletion other than infertility, and with ICSI, this infertility can be treated successfully. Preimplanation embryo biopsy and diagnosis and genetic counseling are not necessary.

The major use of ICSI with testicular biopsy tissue will be for those who have a miniscule amount of spermatogenesis, with no sperm in the ejaculate. In fact, it is clear that even with nonobstructive azoospermia (e.g., Sertoli cell only or maturation arrest) there are usually some tiny foci of spermatogenesis that allow TESE with ICSI to be performed despite the man's being completely azoospermic from "germinal failure."

It is likely that severe male-factor infertility is genetically transmitted, possibly on the long arm of the Y chromosome. Therefore, although ICSI offspring have been shown to be completely normal, it is possible that the male children of these infertile couples will also require ICSI when they grow up and wish to have a family.

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