

High fertilization and pregnancy rate after intracytoplasmic sperm injection with spermatozoa obtained from testicle biopsy

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In cases requiring microsurgical epididymal sperm aspiration (MESA) for congenital absence of the vas deferens (CAVD) or irreparable obstructive azoospermia, often no spermatozoa can be retrieved from the epididymis, or there may even be no epididymis present. We wished to see whether testicular biopsy with testicular sperm extraction (TESE) in such cases could yield spermatozoa that would result in successful fertilization and pregnancy (despite the absence of epididymal spermatozoa) using intracytoplasmic sperm injection (ICSI). In the same setting during the same 2-week period, 28 patients with CAVD or irreparable obstruction were treated; 16 consecutive fresh MESA–ICSI cycles and 12 cycles which required testicular biopsy with testicular sperm extraction (TESE–ICSI) were performed. Normal two-pronuclear fertilization rates were similar in both groups: 45% for epididymal spermatozoa and 46% for testicular biopsy-extracted spermatozoa. Cleavage rates were also similar (68% for epididymal and 65% for testicular spermatozoa). The ongoing pregnancy rates in this series were 50 and 43% respectively. We conclude that epididymal spermatozoa and testicular spermatozoa yield similar fertilization, cleavage and ongoing pregnancy rates using ICSI. When epididymal spermatozoa cannot be retrieved, a testicular biopsy can be performed and the few barely motile spermatozoa thus obtained can be used for ICSI. It appears that all cases of obstructive azoospermia can now be successfully treated.

Key words: intracytoplasmic sperm injection/microsurgical sperm aspiration/pregnancy/testicle biopsy

Introduction

It has been demonstrated that intracytoplasmic sperm injection (ICSI) can achieve normal fertilization and ongoing pregnancy rates in severe male factor infertility where traditional in-vitro fertilization (IVF) and embryo transfer has failed (Van Steirteghem *et al.*, 1993a,b). In cases of congenital absence of the vas deferens (CAVD), or otherwise irreparable obstructive azoospermia, microsurgical epididymal sperm aspiration (MESA)

with IVF has been shown to yield fertilization and pregnancy resulting in live births (Silber *et al.*, 1990). However, the results are poor and unpredictable, with pregnancy and fertilization rates of <10% (Silber *et al.*, 1994; Belker, 1994). A MESA series comparing ICSI to conventional IVF has now demonstrated completely normal fertilization and pregnancy rates with epididymal spermatozoa utilizing ICSI (Silber, 1994; Silber *et al.*, 1994). The purpose of the present study was to compare the use of ICSI using testicular biopsy spermatozoa to ICSI with epididymal spermatozoa in the same time period (December 1993). If there were spermatozoa in the epididymis MESA was performed, and if there were no spermatozoa in the epididymis, or if the latter was absent or destroyed, then testicular sperm extraction (TESE) was performed.

Materials and methods

A series of 28 consecutive MESA/TESE cycles was performed during a 10-day period in December 1993. If spermatozoa were present in the epididymis, MESA was performed. If there were no epididymal spermatozoa, TESE was used. Epididymal spermatozoa could be retrieved from 16 of these 28 cases, of which 21 cases were due to CAVD and seven to irreparable epididymal obstruction.

In 12 of the 28 cases no epididymal spermatozoa were retrievable. In these cases there were either no spermatozoa at all in the epididymis or there was actually no epididymis. All of these patients with no epididymal spermatozoa had undergone multiple previous scrotal surgeries and were massively scarred. In these cases, spermatozoa were retrieved by performing testicle biopsy, morselizing the tissue and spinning down the supernatant at 300 g for 5 min. This led to the recovery of very small numbers of non-motile or barely twitching testicular spermatozoa for ICSI.

Details of the surgical technique for epididymal and testicular sperm retrieval

Epididymal sperm aspiration has already been described (Silber, 1988; Silber *et al.*, 1990). A longitudinal microsurgical slit was made under $\times 25$ –40 magnification with a Zeiss operation microscope in the proximal epididymal tubule, and fluid, devoid of red blood cells, was aspirated with a plastic pipette (Medicuts 22 gauge, 0.7 mm/22 mm) (Argyle, Sherwood Medical/Argyle, St Louis, MO, USA). The distal epididymis was avoided because of the preponderance of senescent spermatozoa in that region. Some motility is crucial as a test for 'vitality' of the spermatozoa. Therefore, all epididymal spermatozoa were obtained from proximal epididymal tubules.

When there was no epididymis, or when the entire scrotum

was massively scarred, or when there were simply no spermatozoa in the epididymis, we then resorted to testicular biopsy for sperm retrieval. The surgical technique for testicular biopsy was extremely simple. A small 1 cm horizontal incision was made in the scrotal skin and carried through the peritoneal tunica vaginalis. Then a 0.5 cm incision was made in the tunica albuginea, and a small piece of extruding testicular tissue was excised and placed in a small Petri dish with 3 ml of HEPES-buffered Earle's medium. The tunica albuginea was closed with several 3–0 vicryl intracuticular stitches. When the retrieval of testicular tissue for sperm extraction was performed as a last resort because no epididymal spermatozoa were retrievable, then of course, the incision was more extensive than when it was known at the outset that testicular spermatozoa were all that was available.

In all of these 28 cases, our first choice was to obtain epididymal spermatozoa because we could freeze and save the majority of the retrieved epididymal spermatozoa for subsequent MESA-ICSI cycles without the need ever to re-operate on the husband. With TESE, however, the freezing of extra spermatozoa was not considered to be an easy option because of the tiny numbers of spermatozoa and because there was barely any apparent motility in most of the very few free spermatozoa. Epididymal sperm preparation was the same as has already been described (Silber *et al.*, 1994).

Details of testicular sperm preparation and the ICSI technique

The piece of testicular tissue was finely minced in HEPES-buffered media, and this effluent was then placed in a 5 ml Falcon tube and centrifuged for 5 min at 1800 g. The supernatant was removed with a Pasteur pipette; after adding 0.2 ml of Earle's medium the pellet was gently resuspended. Percoll separation would have been pointless to attempt. The number of spermatozoa in the droplet was so few and the motility so weak, that one had no choice but to pick the individual spermatozoon up out of the field of debris, red blood cells and Sertoli cells for ICSI. The sperm suspension was then kept at 37°C in the incubator (5% O₂, 5% CO₂, 90% N₂) until the moment for the ICSI procedure on the oocytes.

Oocyte collection and preparation for ICSI

Oocyte retrieval was carried out by vaginal ultrasound-guided puncture 36 h after human chorionic gonadotrophin (HCG) was administered. After oocyte retrieval, the cumulus–corona cell complexes were scored under the inverted microscope at $\times 40$ or $\times 100$ magnification as mature (type 1.0), slightly hypermature (type 1.2), slightly immature (type 1.1) or immature (type 2). Up to eight cumuli of a similar type were transferred into a Falcon tube with Earle's medium; these tubes were gassed prior to tight closure and were transported in a thermobox kept at 37°C to the micro-injection laboratory, which was located elsewhere on the Medical Campus at a distance of about 500 m.

The cells of the cumulus and corona radiata were removed by incubation for ~ 30 s in HEPES-buffered Earle's medium with 80 IU hyaluronidase/ml (type VIII, specific activity 320 IU/mg; Sigma Chemical Co, St Louis, MO, USA). The removal of the cumulus and corona cells was enhanced by aspiration of the complexes in and out of hand-drawn glass pipettes with an

opening of ~ 200 μ m. Afterwards, the oocytes were rinsed several times in droplets of HEPES-buffered Earle's and B₂ medium and then carefully observed under the inverted microscope at $\times 200$ magnification. This included an assessment of the oocyte and the zona pellucida as well as noting the presence or absence of a germinal vesicle or the first polar body. Besides the assessment of nuclear maturity, the cytoplasm of the oocyte was examined for the presence of vacuoles or other abnormalities in the texture of the ooplasm. The oocytes were then incubated in 25 μ l microdrops of B₂ medium covered by lightweight paraffin oil (British Drug House, Pasture, Brussels, Belgium) at 37°C in an atmosphere of 5% O₂, 5% CO₂ and 90% N₂. About 3–4 h later the oocytes were observed again to see whether more oocytes had extruded the first polar body. ICSI was carried out on all morphologically intact oocytes that had extruded the first polar body.

The ICSI procedure

The holding and injection pipettes were made from 30 μ l borosilicate glass capillary tubes (Drummond Scientific Company, Broomall, PA, USA) of 78 mm length and an inner and outer diameter of 0.69 and 0.97 mm respectively. These glass capillaries were cleaned by (i) sonication for 30 min in pure water (Milli-RO and Milli-Q; Millipore, Brussels, Belgium) with 2% (v/v) detergent (7X-PF O-MATIC; Flow Laboratories, Irvine, Scotland) and (ii) rinsing in running Milli-Q water for 30 min. Both cleaning steps were repeated before drying and sterilizing the pipettes in a hot-air oven (100°C for 6 h) (Memmert type ULE 500; Schwabach, Germany). The second sonication was done in water without detergent. The glass pipettes were obtained by drawing thin-walled glass capillary tubes (Drummond Scientific Company) using a horizontal microelectrode puller (type 753; Campden Instruments Ltd, Loughborough, Leics., 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 60 μ m and an inner diameter of 20 μ m. To prepare the injection pipette, the pulled capillary was opened on a microgrinder (EG-4 Micro-Grinder; Narishige) to an outer diameter of 7 μ m and an inner diameter of 5 μ m; the bevel angle was 50°. This grinding step required about 3 min and the wetstone of the grinder was humidified by slow water drip during the procedure. The microforge was 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 $\sim 45^\circ$ in order to facilitate the injection procedure in the Petri dish.

A 3–5 μ l sperm droplet was placed in the centre of a Petri dish (Falcon type 1006) and was surrounded by eight 5 μ l droplets of HEPES-buffered Earle's medium with 0.5% crystalline bovine serum albumin. These droplets were covered by ~ 3.5 ml of lightweight paraffin oil. The ICSI procedure was carried out on the heated stage (37°C; THN-60/16 and MS100 Controller; Linkam Scientific Instruments Ltd, London, UK) of an inverted microscope (Diaphot; Nikon Corporation, Tokyo, Japan) at $\times 400$ magnification using a Hoffman Modulation Contrast System (Modulation Optics Inc., Greenvale, New York, USA). The microscope was equipped with a Nikon F-601M camera for still pictures and a video camera (DXC-755 P; Sony Corporation, Brussels, Belgium) that allowed the procedure to be followed

on a Trinitron® colour video monitor (PVM-1443MD; Sony). The microscope was equipped with two coarse positioning manipulators (3D Motor Driven Coarse Control Manipulator MM-188; Narishige) and with two three-dimensional hydraulic remote-control micromanipulators (Joystick Hydraulic Micromanipulator MO-188; Narishige). The holding and injection pipettes were fitted to a tool holder and connected by Teflon tubing (CT-1; Narishige) to a micrometer-type microinjector (IM-6; Narishige). Solution delivery was controlled via 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 by slight negative pressure exerted on the holding pipette. The polar body was held at 12 or 6 o'clock and the micropipette pushed through the zona pellucida and the oolemma into the ooplasm at 3 o'clock. A single spermatozoon was injected into the ooplasm with ~1–2 pl of medium. The injection pipette was withdrawn gently and the injected oocyte released from the holding pipette. The aspiration of a single spermatozoon and injection into the ooplasm were repeated until all metaphase II oocytes were injected. The injected oocytes were then washed in B₂ medium and transferred into 25 µl droplets of B₂ medium covered by lightweight paraffin oil. The Petri dishes with the oocytes were incubated in an incubator (Heraeus, B5060 EK/O₂; Van der Heyden, Brussels, Belgium; 37°C, 5% CO₂ and 90% N₂).

Assessment of fertilization and embryo cleavage

The further handling of the injected oocytes was similar to our standard IVF procedure. About 16–18 h after the micro-injection, the oocytes were observed under the inverted microscope (×200 or ×400 magnification) for any sign of damage which may have been due to the micro-injection and for the presence of pronuclei and polar bodies. Fertilization was considered normal when two clearly distinct pronuclei containing nucleoli were present. The eventual presence of one pronucleus or three pronuclei was noted, together with the presence of one or two or fragmented polar bodies. If a single pronucleus was observed, a second evaluation was carried out ~4 h later in order to see whether the pronuclear status had changed. The embryo cleavage of the two-pronuclear oocytes was evaluated after a further 24 h of in-vitro culture. The embryos were scored according to the equality of size of the blastomeres and the number of anucleate fragments. Cleaved embryos with <50% of their volume filled with anucleate fragments were eligible for

transfer. Up to three embryos were loaded in a few microlitres of Earle's medium into a Frydman catheter (LG 4.5; Prodimed, Neuilly-en-Thelle, France) and transferred into the uterine cavity. Embryo replacement was usually done ~48 h after the micro-injection procedure.

If supernumerary embryos with <20% anucleate fragments were available, they were cryopreserved on day 2 or day 3 by the slow-freezing protocol with dimethyl sulphoxide.

Establishment and follow-up of pregnancy

Pregnancy was confirmed by detecting 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 echographic screening at 7 weeks of pregnancy. Prenatal diagnosis was carried out by chorionic villous sampling at 9–10 weeks of gestation or by amniocentesis at 16 weeks of gestation. Genetic counselling was done in view of the prenatal diagnosis and for the planning of a prospective follow-up study of the children born after ICSI. The referring gynaecologist and the patients were asked to provide detailed information of the evolution of the pregnancy and the outcome of the delivery.

Results

The comparison of results using ICSI with MESA versus TESE are summarized in Table I. In 16 of the 28 patients epididymal spermatozoa could be retrieved (MESA). In 12 of the 28 patients, only testicular biopsy spermatozoa (TESE) could be retrieved because of absence of the epididymis or absence of spermatozoa in the epididymis. In no case were both epididymal and testicular spermatozoa used. The fertilization rates were similar with testicular biopsy spermatozoa and epididymal spermatozoa. With MESA, 45% of injected eggs had two-pronuclear fertilization, and with TESE the corresponding proportion was 46%. The cleavage rate per fertilized oocyte was 68% with epididymal spermatozoa and 65% with testicular biopsy spermatozoa.

More important than the cumulative fertilization rate was the consistency of fertilization in 25 of the 28 patients. In all 16 MESA patients (100%), normal cleaved embryos were obtained and transferred. In nine of the 12 TESE patients (75%), normal cleaved embryos were obtained and transferred. There was consistently enough fertilization in every MESA case to allow embryo transfer. In the TESE cases, nine out of 12 had good embryos for transfer, but in three cases there was no fertilization. Interestingly, the cumulative fertilization and cleavage rates were the same with both testicular and epididymal spermatozoa.

Table I. Fertilization and cleavage rate after intracytoplasmic sperm injection with epididymal and testicular spermatozoa

Source of spermatozoa	No. of cycles	No. of metaphase II eggs injected	No. of 2PN oocytes (%)	No. of cleaved embryos (%)
Epididymal (MESA)	16	235	105 (45)	71 (68)
Testicular biopsy (TESE)	12	185	86 (46)	56 (65)

MESA = microsurgical epididymal sperm aspiration; TESE = testicular sperm extraction; 2PN = two pronuclei.

The total, clinical and ongoing pregnancy rates per transfer were similar in both groups. The ongoing pregnancy rate per transfer was almost identical for epididymal and testicular spermatozoa (50 versus 55%) (Table II). However, the pregnancy rate per stimulated cycle was moderately lower with testicular spermatozoa (43%) than with epididymal spermatozoa (50%) because of the lower transfer rate (75 versus 100%). Nonetheless, from this initial comparison series, the most obvious and startling observation was just how high the fertilization and pregnancy rates were with testicular biopsy-derived, relatively non-motile, and completely immature spermatozoa utilizing ICSI.

The ongoing implantation rate per replaced embryo was 25.5% for MESA cases and 23.3% for TESE cases (Table III). Testicular biopsy never yielded enough spermatozoa to seriously attempt freezing. Therefore, in patients where no epididymal spermatozoa were obtainable, the spectre of repeated operations for each future ICSI procedure was apparent. It is intriguing how few spermatozoa were obtainable by mincing of testicular tissue.

Discussion

There were two main objectives to this study, one clinical and the other physiological. The clinical objective was to determine whether ICSI could salvage the most severe MESA cases (either CAVD or irreparable obstructive azoospermia), where there were absolutely no epididymal spermatozoa retrievable at all, by resorting to testicle biopsy. The physiological objective was to see whether completely immature spermatozoa, which had only recently been released from the Sertoli cells, could fertilize and result in a normal pregnancy if freed from the need to penetrate the zona pellucida and to fuse with the oolemma membrane.

Our previous reports have verified the superiority of ICSI over conventional IVF using epididymal spermatozoa and have

indicated that testicular biopsy-extracted spermatozoa were capable of fertilization (Silber *et al.*, 1994; Devroey *et al.*, 1994; Schoysman *et al.*, 1993). In this paper we have reported a completely new series in which we compared the results of ICSI using epididymal spermatozoa with ICSI using totally immature spermatozoa derived from a testicle biopsy specimen, and also reported the first series of pregnancies with testicular biopsy spermatozoa.

Because of the consistently improved results with ICSI over conventional IVF, and indeed the good results with testicular spermatozoa (if epididymal spermatozoa are not obtainable), we advise the mandatory use of ICSI for all MESA patients. The consistently good fertilization rates with epididymal and testicular biopsy spermatozoa using ICSI seem to highlight the possible role of the epididymis in normal fertilization as having nothing to do with the intrinsic ability of the spermatozoa to fertilize and result in a normal embryo once penetration is completed. Most likely, the function of the epididymis is to give the already genetically completed spermatozoa the ability to fuse with the oolemma. Zona penetration and, perhaps more importantly, membrane fusion capacity may be what the spermatozoon develops as it is processed through the epididymis.

An additional benefit of this MESA/ICSI technology is that epididymal spermatozoa can be frozen and stored so that the husband will not require more than one operation for sperm retrieval. Yet his wife can undergo multiple cycles of IVF/ICSI with thawed epididymal spermatozoa until she finally gets pregnant. However, if testicular biopsy spermatozoa are required because of absence of the epididymis or absence of epididymal spermatozoa, then the numbers and motility may be too low to allow this additional benefit of freezing. Since the numbers and motility of testicular spermatozoa are so low, thus far cryopreservation has not been feasible. Therefore, the husband

Table II. Ongoing pregnancy rate after intracytoplasmic sperm injection with epididymal and testicular spermatozoa

Source of spermatozoa	No. of cycles	No. of transfers	No. of pregnancies/ transfer: positive HCG	No. of pregnancies	
				Clinical	Ongoing
Epididymal (MESA)	16	16	12	10	8 (50%)
Testicular biopsy (TESE)	12	9	6	5	5 (43%)

HCG = human chorionic gonadotrophin. For other abbreviations, see Table I.

Table III. Ongoing implantation rates after intracytoplasmic sperm injection with epididymal and testicular spermatozoa

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

^aIncluding two sets of triplets.

^bIncluding two sets of twins.

For abbreviations, see Table I.

has to undergo multiple biopsy procedures if subsequent cycles are needed.

It has to be noted that the implantation rate of embryos after ICSI with testicular biopsy (TESE-ICSI) is 23%, indicating the high capacity for implantation and the great similarity to results obtained using epididymal or ejaculated spermatozoa. These results indicate that in cases of obstructive azoospermia, epididymal spermatozoa give extraordinarily reliable fertilization with ICSI and have the additional benefit of being retrievable in relatively large quantities, which allows multiple aliquots to be frozen and used in subsequent cycles without the male partner needing any further invasive procedures. If epididymal spermatozoa are not retrievable, testicle biopsy would be a final resort that is also very effective, with most patients obtaining embryos for transfer and with high implantation rates in both cases. The potential disadvantage of testicular spermatozoa is that the numbers and the motility are so low that freezing for subsequent ICSI cycles is more problematic than with epididymal spermatozoa.

A blind percutaneous needle aspiration of epididymal spermatozoa is certainly an alternative to microsurgical approaches, but a blind stick would retrieve much smaller amounts of spermatozoa for freezing for subsequent ICSI cycles, is definitely not painless, would have to be repeated on multiple occasions and, therefore, may not have any benefit over a single microsurgical epididymal aspiration. Furthermore, if epididymal spermatozoa are not retrievable by a blind needle stick, it would not be clear whether the cause was truly absence of spermatozoa in the epididymis or simply a technical failure. We view this issue as relatively trivial compared to the overwhelming conclusions which we have drawn from this study. To summarize, the apparent efficacy of fertilization of testicular spermatozoa using ICSI would imply that there may be no case of obstructive azoospermia that cannot now be treated successfully.

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References

- Belker, A. (1994) The sperm microaspiration retrieval techniques study group. Results in the United States with sperm microaspiration retrieval techniques and assisted reproductive technologies. *J. Urol.*, **151**, 1255–1259.
- Devroey, P., Liu, J., Nagy, Z., Tournaye, H., Silber, S.J., and Van Steirteghem, A.C. (1994) Normal fertilization of human oocytes after testicular sperm extraction and intracytoplasmic sperm injection (TESE and ICSI). *Fertil. Steril.*, **62**, 639–641.
- Schoysman, R., Vanderzwalmen, P., Nijs, M., Segal, L., Segal-Bertin, G., Geerts, L., van Roosendaal, E. and Schoysman, D. (1993) Pregnancy after fertilisation with human testicular spermatozoa. *Lancet*, **342**, 1237.
- Silber, S.J. (1988) Pregnancy caused by sperm from vasa efferentia. *Fertil. Steril.*, **49**, 373–375.
- Silber, S.J. (1994) A modern view of male infertility. In Flaherty, S.P. and Matthews, C.D. (eds), *The Infertile Male*. CSIRO Australia (ISBN: 0-643-05584-3), pp. 93–104.
- Silber, S.J., Ord, T., Balmaceda, J., Patrizio, P. and Asch, R.H. (1990) Congenital absence of the vas deferens: the fertilizing capacity of human epididymal sperm. *N. Engl. J. Med.*, **323**, 1788–1792.
- Silber, S.J., Nagy, Z.P., Liu, J., Godoy, H., Devroey, P. and Van Steirteghem, A.C. (1994) Conventional IVF versus intracytoplasmic sperm injection for patients requiring microsurgical sperm aspiration. *Hum. Reprod.*, **9**, 1705–1709.
- Tournaye, H., Devroey, P., Liu, J., Nagy, Z., Lissens, W. and Van Steirteghem, A.C. (1994) Microsurgical epididymal sperm aspiration and intracytoplasmic sperm injection: a new effective approach to infertility as a result of congenital bilateral absence of the vas deferens. *Fertil. Steril.*, **61**, 1045–1051.
- Van Steirteghem, A.C., Liu, J., Joris, H., Nagy, Z., Janssenswillen, C., Tournaye, H., Derde, M., Van Assche, E. and Devroey, P. (1993a) Higher success rate by intracytoplasmic sperm injection than by subzonal insemination. Report of a second series of 300 consecutive treatment cycles. *Hum. Reprod.*, **8**, 1055–1060.
- Van Steirteghem, A.C., Nagy, Z., Joris, H., Liu, J., Staessen, C., Smits, J., Wisanto, A. and Devroey, P. (1993b) High fertilization and implantation rates after intracytoplasmic sperm injection. *Hum. Reprod.*, **8**, 1061–1066.

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