

# Ongoing pregnancies and birth after intracytoplasmic sperm injection with frozen–thawed epididymal spermatozoa

Paul Devroey<sup>1</sup>, Sherman Silber<sup>2</sup>, Zsolt Nagy, Jiaen Liu, Herman Tournaye, Hubert Joris, Greta Verheyen and Andre Van Steirteghem

Centre for Reproductive Medicine, University Hospital, Dutch-speaking Brussels Free University, Laarbeeklaan 101, 1090 Brussels, Belgium and <sup>2</sup>St Luke's Hospital West, 224S Woods Mill Road, Suite 730, St Louis, MO 63017, USA

<sup>1</sup>To whom correspondence should be addressed

In seven patients who did not become pregnant following microsurgical epididymal sperm aspiration (MESA) and intracytoplasmic sperm injection (ICSI), a subsequent ICSI was performed using previously cryopreserved supernumerary epididymal spermatozoa without re-operating on the husband. During the original MESA procedure a mean sperm concentration of  $12.3 \times 10^6/\text{ml}$  was achieved. The supernumerary spermatozoa were cryopreserved for later use. After thawing frozen epididymal spermatozoa a mean concentration of  $1.9 \times 10^6$  spermatozoa/ml was obtained in straws containing a total volume of sperm suspension of 250  $\mu\text{l}$ . From 68 intact oocytes injected with frozen–thawed epididymal spermatozoa, a two pronuclear fertilization rate of 45% and a cleavage rate of 82% were obtained. A total of 17 embryos were replaced in the seven patients, resulting in two ongoing singleton pregnancies and one twin delivery. Six embryos were cryopreserved. In conclusion, it would appear mandatory to cryopreserve supernumerary spermatozoa during a MESA in order to avoid subsequent further scrotal surgery.

**Key words:** fertilization/frozen–thawed epididymal spermatozoa/intracytoplasmic sperm injection/pregnancy

## Introduction

Pregnancies have been obtained after failed vasectomy reversal or in the presence of bilateral absence of the vas deferens using a combination of microsurgical epididymal sperm aspiration (MESA) and classic in-vitro fertilization (IVF; Temple-Smith *et al.*, 1985; Silber *et al.*, 1988b, 1990). Aspiration of spermatozoa from the proximal region of the epididymis has yielded a fertilization rate of 6.9% per oocyte and pregnancy with live birth (4.5%; Silber *et al.*, 1994). The fertilization rate after MESA in conjunction with regular IVF, however, is low and unpredictable, depending on the motility and number of retrieved spermatozoa. Intracytoplasmic sperm

injection (ICSI), i.e. the injection of one spermatozoon into the cytoplasm of a metaphase-II oocyte, has solved this problem because morphology, number and progressive motility cease to be crucial (Palermo *et al.*, 1992, 1993; Van Steirteghem *et al.*, 1993a,b). Recently, high fertilization and pregnancy rates have been reported in association with MESA and ICSI (Silber *et al.*, 1994; Tournaye *et al.*, 1994). Because with ICSI only a few sperm cells are needed, the supernumerary spermatozoa obtained during a fresh MESA procedure are now cryopreserved and then thawed for a subsequent treatment cycle. In a collaborative project between the Centre for Reproductive Medicine of the Dutch-speaking Brussels Free University, Belgium, and St Luke's Hospital in St Louis, USA, the fertilizing capacity of frozen–thawed epididymal spermatozoa after ICSI and the outcome of such fertilization were compared with those of fresh epididymal spermatozoa.

## Materials and methods

### Patient population

From October 1992 until December 1993, seven couples, in whom the husband suffered from obstructive azoospermia, underwent MESA and ICSI and failed to become pregnant. The diagnoses were congenital bilateral absence of the vas deferens ( $n = 4$ ), failed vaso-epididymostomy ( $n = 2$ ) and failed vasovasostomy ( $n = 1$ ). The mean female age was 31.2 years (range 25–35) and the mean male age was 48.2 years (range 28–60). All had spermatozoa frozen and saved for a subsequent ICSI cycle.

### Microsurgical epididymal sperm aspiration

Spermatozoa were retrieved by the following technique. The scrotum was opened and after incision of the tunica vaginalis the epididymis was inspected. A tiny longitudinal incision was made in one dilated tubulus located in the head of the epididymis. Using the operating microscope under  $\times 40$  magnification, fluid was aspirated with a plastic pipette (0.7 mm OD  $\times$  22 mm, Medicut<sup>®</sup>; Sherwood, Tullamore, Ireland) on a tuberculin syringe (Becton Dickinson, Belgium). Haemostasis was obtained with microbipolar forceps. Epididymal fluid was diluted in HEPES-buffered Earle's medium and the presence of motile spermatozoa was checked in the adjacent laboratory. As much fluid as possible was aspirated. The tubule was then microsurgically closed with 10-0 nylon sutures. The tunica vaginalis space was then filled with heparinized saline and the scrotum closed with 000 Vicryl interrupted sutures.

From 1992 onwards, epididymal spermatozoa were cryopreserved after MESA. When motile spermatozoa were

obtained they were used fresh during the ICSI procedure. The remaining spermatozoa were cryopreserved. Sperm motility and concentration were assessed according to the World Health Organization (WHO, 1992) guidelines. The Tygerberg criteria were applied for morphological evaluation (Kruger *et al.*, 1986).

#### Cryopreservation of spermatozoa

After the Percoll gradient procedure and before freezing, the sperm suspension was diluted to  $2 \times 10^6$ /ml in order to be able to freeze a maximum number of straws. Semen was prepared for cryopreservation by dropwise dilution with sperm cryopreservation medium containing glycerol (concentration 7%), egg yolk, citrate, glycine, glucose and antibiotics (Richardson, 1976). After equilibration in a 37°C water bath for 10 min, the spermatozoa-medium mixture was drawn into 0.25 ml straws [Instruments de Médecine Vétérinaire (IMV), l'Aigle, France]. The straws were rapidly frozen in liquid nitrogen vapour. [Straws were placed horizontally ~10 cm above the liquid nitrogen surface for 10 min (room temperature to -80°C; rate -10°C/min), and then plunged into the liquid nitrogen (-196°C).] For thawing, the straws were removed from the liquid nitrogen and placed in a 37°C chamber for 10 min (Verheyen *et al.*, 1993). To remove the cryoprotectant, thawed specimens were put on a two-layer Percoll gradient (95.0-47.5%) and centrifuged for 20 min at 300 g. The 95% fraction was washed by centrifugation with Earle's medium at 1800 g for 5 min. Then the pellet was centrifuged in a 1.5 ml Eppendorf tube (1800 g, 5 min) and the spermatozoa from it used for microinjection. The Eppendorf tube is needed to concentrate the small number of spermatozoa into a small volume (20-50 µl).

#### Ovarian stimulation

The female partners were stimulated by a desensitizing protocol of busserelin (Suprefact; Hoechst, Brussels, Belgium; leuprolide acetate, Lupron; Tap Pharmaceuticals, Deerfield, IL, USA) associated with human menopausal gonadotrophin (HMG; Humegon, Organon, Oss, The Netherlands; Pergonal, Serono, Brussels, Belgium) and human chorionic gonadotrophin (HCG, Pregnyl, Organon; Profasi, Serono). This protocol has been described extensively elsewhere (Smitz *et al.*, 1988). Supplementation of the luteal phase was commenced on the day after HCG injection by administering 600 mg of natural micronized progesterone intravaginally (Utrogestan; Piette,

Brussels, Belgium) or by daily i.m. injections of 50 mg of progesterone (Progesteron; Sterop, SV, Brussels, Belgium; Federa, SV, Brussels, Belgium; Smitz *et al.*, 1992, 1993).

#### Intracytoplasmic sperm injection

The preparation of holding and injection pipettes have been described extensively elsewhere. The preparation of spermatozoa and the sperm injection procedure have also been reported in detail (Van Steirteghem *et al.*, 1993a,b).

#### Assessment of fertilization

At ~16 h after microinjection, oocytes were observed under the inverted microscope ( $\times 200$  or  $\times 400$ ) for the presence of two clearly distinct pronuclei containing nucleoli (Nagy *et al.*, 1994). Egg damage was evaluated and the presence of polar bodies was noted. Further embryo cleavage was observed after 24 h of in-vitro culture. The embryos were assessed according to equality of size of the blastomeres and the number of anucleate fragments (Deschacht *et al.*, 1988; Staessen *et al.*, 1989). Cleaved embryos with <50% of their volume filled with anucleate fragments were eligible for transfer. Up to three embryos were loaded in 200 µl of Earle's medium into a Frydman catheter (LG 4.5; Prodimed, Neuilly-en-Thulle, France). Embryo replacement was performed ~48 h after the microinjection procedure (Wisanto *et al.*, 1989).

If supernumerary embryos with <20% anucleate fragments were available, they were cryopreserved as reported earlier (Van Steirteghem *et al.*, 1987).

#### Establishment and follow-up of pregnancy

The establishment of pregnancy was confirmed by determining two increasing serum HCG values at least 10 days after embryo transfer. Clinical pregnancy was determined by visualization of a gestational sac by vaginal ultrasound examination at 7 weeks. Prenatal diagnosis was proposed, including chorionic villus sampling at 10 weeks or amniocentesis at 16 weeks of gestation. A continued follow-up of any children born was required (Bonduelle *et al.*, 1994).

#### Results

The semen parameters of the seven patients, including concentration, total motility and normal morphology of the fresh and frozen-thawed epididymal spermatozoa, are summarized in Table I. After thawing, the mean concentration and the total

**Table I.** Concentration, total motility and morphology of fresh and frozen-thawed epididymal spermatozoa

Epididymal spermatozoa	Mean concentration ( $\times 10^6$ /ml)	Total motility (%)	After Percoll gradient		
			Mean concentration ( $10^6$ )	Total motility (%)	Normal morphology (%)
Fresh	12.3 (2.6-27.5)	29.1 <sup>a</sup> (12-61)	7.4 (0-26)	38.7 (10-70)	17.7 (2-28)
Frozen-thawed	1.9 <sup>b</sup> (0.1-8.7)	0 <sup>c</sup>	0.46 (0-1.18)	0 <sup>c</sup>	ND <sup>d</sup>

Values in parentheses are ranges.

<sup>a</sup>No progressive motility type A.

<sup>b</sup>Mean concentration in straws containing 250 µl diluted cryopreservation medium.

<sup>c</sup>Some spermatozoa were found with extremely reduced motility which were chosen for the intracytoplasmic sperm injection.

<sup>d</sup>Not determined.

motility were extremely low because the suspension had had to be diluted to  $2 \times 10^6$  spermatozoa/ml before freezing.

Of the 69 intact metaphase-II eggs, 39 (56%) were fertilized normally after ICSI with fresh epididymal spermatozoa, and 28 out of 62 (45%) were fertilized normally after injection with frozen-thawed epididymal spermatozoa. The cleavage rates (79 and 82%) are comparable (Table II).

As shown in Table III, embryo quality was similar after injection with fresh or frozen-thawed epididymal spermatozoa. In all, 18 embryos were replaced after injection with fresh epididymal spermatozoa and 17 after injection with frozen epididymal spermatozoa. Out of these, seven and six embryos respectively were cryopreserved for later use. In the first procedure with fresh spermatozoa, a mean of 2.6 embryos were replaced; in the second procedure with frozen-thawed spermatozoa a mean of 2.4 embryos were replaced. Three ongoing pregnancies (two singleton and one twin) were obtained using frozen epididymal spermatozoa. The ongoing implantation rate was 23% (4/17). Amniocentesis of the twin pregnancy revealed two normal karyotypes (46XX and 46XY). This patient gave birth to a healthy boy (2670 g) and a healthy girl (1980 g) by Caesarean section prematurely at 36 weeks of gestation.

## Discussion

It has been observed that spermatozoa do not need an epididymal passage to initiate fertilization and subsequent pregnancy. This has been demonstrated after an anastomosis between the vas deferens and the vasa efferentia (Silber *et al.*, 1988a). Similarly, pregnancies after MESA in the caput and classic IVF or ICSI have been obtained. The method of choice now appears to be MESA with ICSI. An ongoing pregnancy rate with live birth of 30% (5/17) has been obtained with ICSI versus 4.5% (4/67) with conventional IVF (Silber *et al.*, 1994). If fresh spermatozoa are used, the husband requires multiple operations. By cryopreserving epididymal spermatozoa, only a limited number of aspirations are needed. The wife can undergo multiple oocyte retrievals to be injected with stored epididymal spermatozoa.

**Table II.** Two pronuclear (2PN) fertilization after intracytoplasmic sperm injection (ICSI) with fresh and frozen-thawed epididymal spermatozoa

Epididymal spermatozoa	No. of oocytes injected	No. of intact oocytes	2PN oocytes (%)	Cleaved embryos (%)	Transferable embryos
Fresh	77	69	39 (57)	31 (79)	26
Frozen-thawed	68	62	28 (45)	23 (82)	22

**Table III.** Distribution of embryos according to quality after ICSI with fresh and frozen-thawed epididymal spermatozoa

Epididymal spermatozoa	Embryo quality		
	Excellent	Good	Fair
Fresh	5	18	3
Frozen-thawed	1	14	7

The use of cryopreserved human spermatozoa has been well and widely accepted in artificial insemination. In the modern era, donor spermatozoa are stored and used only after safety with respect to sexually transmitted diseases has been assured. However, cryopreservation decreases sperm motility and introduces changes in the penetration of zona-free hamster eggs, in the acrosomal structure, and in the activity of acrosin (Pedersen and Lebech, 1971; Goodpasture *et al.*, 1981; Critser *et al.*, 1987; Keel and Webster, 1989). These observations have been made in initially normal sperm samples. Similar changes can be expected in impaired semen samples. It has been observed that cryopreservation of spermatozoa decreases the acrosin activity significantly by 24% and the incidence of intact acrosomes by 27%. It has been suggested that acrosomal loss during cryopreservation is associated with cell death (Cross and Hanks, 1991). The 45% fertilization rate after the use of frozen-thawed epididymal spermatozoa suggests that despite clearly diminished sperm quality after cryopreservation, enough thawed epididymal spermatozoa nevertheless retain their integrity for successful ICSI. Moreover, the fertilization rates of fresh and frozen-thawed spermatozoa were quite similar, 57 and 45% respectively.

Normally, spermatozoa with impaired motility are not frozen for all the above reasons. However, ICSI can be applied to spermatozoa with very poor motility. As demonstrated here, the initial motility of fresh epididymal spermatozoa was severely impaired and no initial progressive motility (type A) was observed, while after thawing the total motility in all samples was almost zero. The injection was carried out even with one spermatozoon with severely impaired motility. It has to be noted that before freezing, the sperm suspension was diluted to  $2 \times 10^6$ /ml to optimize the number of straws, and that after the Percoll gradient procedure the concentration was reduced to  $0.46 \times 10^6$ /ml. It is remarkable to observe that even such poor, frozen-thawed impaired spermatozoa yielded a 45% normal two pronuclear fertilization rate. All seven patients had an embryo transfer and three patients became pregnant, including two ongoing pregnancies and the delivery of healthy twins. No difference in two pronuclear fertilization rates and cleavage rates between the fresh and frozen samples was noticed. The ongoing implantation rate after the use of frozen-thawed epididymal spermatozoa was 23.5%.

In summary, it is mandatory to cryopreserve supernumerary spermatozoa during a MESA. After thawing, this can be used in a subsequent IVF cycle using ICSI, thus possibly avoiding future scrotal surgery.

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