Human Reproduction vol.8 no.7 pp.1061–1066, 1993

High fertilization and implantation rates after intracytoplasmic sperm injection

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Previously reported better fertilization rate after intracytoplasmic single sperm injection (ICSI) than after subzonal insemination of several spermatozoa was confirmed in a controlled comparison of the two procedures in 11 patients. Intracytoplasmic sperm injection was carried out in 150 consecutive treatment cycles of 150 infertile couples, who had failed to have fertilized oocytes after standard in-vitro fertilization (IVF) procedures or who were not accepted for IVF because not enough motile spermatozoa were present in the ejaculate. A single spermatozoon was injected into the ooplasm of 1409 metaphase II oocytes. Only 117 oocytes (8.3%) were damaged by the procedure and 830 oocytes (64.2% of the successfully injected oocytes) had two distinct pronuclei the morning after the injection procedure. The fertilization rate was not influenced by semen characteristics. After 24 h of further in-vitro culture, 71.2% of these oocytes developed into embryos, which were transferred or cryopreserved. Only 15 patients did not have embryos replaced. Three-quarters of the transfers were triple-embryo transfers. High pregnancy rates were noticed since 67 pregnancies were achieved, of which 53 were clinical, i.e. a total and clinical pregnancy rate of 44.7% and 35.3% per started cycle and 49.6% and 39.2% per embryo transfer. A total of 237 supernumerary embryos were cryopreserved in 71 treatment cycles.

Key words: intracytoplasmic injection/in-vitro fertilization/male-factor infertility/oocyte/spermatozoon

Introduction

Intracytoplasmic sperm injection (ICSI) has recently been described as beneficial in alleviating infertility in couples who could not be helped by standard in-vitro fertilization (IVF) treatment or by subzonal insemination (SUZI) of the oocytes (Palermo *et al.*, 1992, 1993; Van Steirteghem *et al.*, 1993). Most of these infertile couples suffered from severe male-factor infertility and the number of motile spermatozoa in the ejaculate was sometimes too low for the couples to be accepted in an IVF programme.

This report describes the results of a controlled comparison of the SUZI and ICSI procedures on sibling oocytes in 11 treatment cycles as well as the outcome of 150 consecutive treatment cycles of assisted fertilization by ICSI in couples who failed to fertilize after IVF or after SUZI or who could not be accepted for IVF treatment because of extremely impaired semen characteristics.

Materials and methods

Patients

The controlled comparison between the SUZI and ICSI procedures was carried out between 3 August 1992 and 4 September 1992 on 11 treatment cycles, which were included in the recently submitted report on 300 consecutive treatment cycles (Van Steirteghem *et al.*, 1993). The 150 consecutive treatment cycles by ICSI in 150 couples were carried out between 20 October 1992 and 12 January 1993. The inclusion criteria for ICSI were (i) total absence or $<5\%$ of normal fertilization after standard IVF, (ii) $<500\,000$ progressively motile spermatozoa in the whole ejaculate, or (iii) failed or sporadic fertilization after SUZI. Previous treatments were carried out in our centre or in the centres which referred the couples to our centre specifically to have ICSI.

Patient counselling included information about the novelty of this new procedure of assisted fertilization. The patients signed a consent form which included prenatal diagnosis by chorionic villus sampling or amniocentesis as well as a prospective follow-up of the children born after the ICSI procedure.

The protocol was reviewed and approved by the ethical committee of the Medical Campus of the Dutch-speaking Brussels Free University.

Ovarian stimulation

Ovarian stimulation was carried out by a desensitizing protocol of the intranasally administered gonadotrophin-releasing hormone agonist (GnRHa) buserelin (Suprefact; Hoechst, Brussels, Belgium) in association with human menopausal gonadotrophins (HMG; Humegon; Organon, Oss, The Netherlands; or Pergonal; Serono, Brussels, Belgium) and human chorionic gonadotrophins (HCG; Pregnyl, Organon; Profasi, Serono). The details of the stimulation protocols have been described previously (Smitz *et al.*, 1988). The supplementation of the luteal phase was started on the day after HCG administration and consisted of natural micronized progesterone 600 mg per day intravaginally in three divided doses (Utrogestan, Piette, Brussels; Smitz *et al.*, 1992, 1993).

Semen evaluation and preparation

The evaluation of semen density and motility was carried out according to the recommendations of the World Health Organization (WHO, 1992). Strict criteria were used to evaluate sperm morphology (Kruger *et al.*, 1986). A semen sample was considered to be normal when the following criteria were fulfilled: (i) sperm density 20 × 10^6/ml, (ii) progressive motility of 40%, and (iii) at least 14% of spermatozoa with normal morphology. Semen evaluation and preparation was done at least once prior to the treatment cycle in order to evaluate whether enough spermatozoa were present in the ejaculate to perform ICSI. The semen preparation consisted of treatment on a Percoll discontinuous gradient and sometimes of a further treatment with electroporation or metabolic stimulants pentoxifylline and 2-deoxyadenosine.

Oocyte preparation for micro-injection

Oocyte retrieval was done by vaginal ultrasound-guided puncture of the ovarian follicles, 36 h after HCG administration. At the end of the oocyte retrieval, the cumulus–corona cell complexes were inspected under the inverted microscope at ×40 or ×100 magnification and classified as mature, slightly immature, slightly hypermature or immature (Khan *et al.*, 1989). The cumulus–corona cell complexes were transferred into 5 ml Falcon tubes with Earle's medium; these tubes were gassed (5% O_2, 5% CO_2, 90% N_2) prior to being closed tightly and then transported in a thermobox at 37°C to the micro-injection laboratory, which is located elsewhere on the Medical Campus at a distance of ~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, sp. act. 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. 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 ×200 magnification. This included an assessment of the oocyte and the zona pellucida as well as 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 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. 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.

Intracytoplasmic sperm injection procedure

The holding and injection pipettes were made from 30 μl 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 sonication for 30 min in high-quality water (Milli-RO and Milli-Q, Millipore, Brussels, Belgium) with 2% (v/v) detergent (7X-PF, Flow Laboratories, Irvine, UK) and 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) using a horizontal microelectrode puller (Type 753 from Campden Instruments Ltd, Loughborough, UK). The holding pipette was cut and fire-polished on a microforge (MF-9 Microforge from Narishige Co. Ltd, Tokyo, Japan) to obtain an outer diameter of 60 μm and an inner diameter of 20 μm. To prepare the injection pipette, the pulled capillary was opened on a microgrinder (EG-4 Micro-Grinder from Narishige) to an outer diameter of 7 μm and an inner diameter of 5 μm; the bevel angle was 50°. This grinding step required ~3 min and the wetstone of the grinder was humidified by having a 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 ~45° in order to facilitate the injection procedure in the Petri dish.

Just prior to the injection procedure, the sperm fraction was washed in a 1.5 ml Eppendorf tube containing T6 medium with a concentration of 5 mM $CaCl_2 \cdot 2H_2O$; the T6 medium used during the selection procedure contains 1.78 mM $CaCl_2 \cdot 2H_2O$. This tube is centrifuged at 1800 *g* for 5 min. The supernatant is removed and the pellet is re-suspended in 20–50 μl of medium. One μl of the selected sperm fraction was added to 2–4 μl of a 1 g/10 ml polyvinylpyrrolidone (PVP) solution in HEPES-buffered Earle's medium containing 0.5% (w/v) BSA (fraction V, Sigma). One gramme of PVP-K90 (MW 360 000, ICN Biochemicals, Asse-Relegem, Belgium) was weighed out and dissolved in 10 ml pure water. This 10% PVP solution was dialysed and lyophilized. The dialysis tubing (Visking size 9-36/32", Medicell International Ltd, London, UK) was boiled for 2 h in 1 l of a solution with 0.2 M Na_2Co_3 and 1 mM EDTA. The dialysis membranes were then cooled and rinsed extensively in Milli-Q water prior to being used. About 10 ml of the 10% PVP solution was transferred into the tubing and was dialysed in the cold room for 2 days in Milli-Q water by changing the water seven times a day. The dialysate was then lyophilized and stored at room temperature until further use. The PVP solution was filtered through an 0.8 μm filter (Millipore) and stored at 4°C for a maximum of 3 weeks.

The 3–5 μl sperm–PVP droplet was placed in the centre of a Petri dish (Falcon type 1006) and was surrounded by eight 5 μl droplets of HEPES-buffered Earle's medium with 0.5% crystalline BSA. These droplets were covered by ~3.5 ml of lightweight paraffin oil.

The ICSI procedures were carried out on the heated stage (37°C; THN-60/16 and MS100 Controller from Linkam Scientific Instruments Ltd, London, UK) of an inverted microscope (Diaphot, Nikon Corporation, Tokyo, Japan) at ×400 magnification using the Hoffman Modulation Contrast System (Modulation Optics Inc., Greenvale, NY, USA). The microscope was equipped with a Nikon F-601M camera for still pictures and a video camera (DXC-755 P from Sony Corporation, Brussels, Belgium) that allowed the procedure

to be followed on a Trinitron® colour video monitor (PVM-1443MD from 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 were connected by Teflon tubing (CT-1, Narishige) to a micrometer-type micro-injector (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 was 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 was pushed through the zona pellucida and the oolemma into the ooplasm at 3 o'clock. A single spermatozoon was injected into the ooplasm with ~1–2 pl of medium. The injection pipette was withdrawn gently and the injected oocyte was released from the holding pipette. The aspiration of a single spermatozoon and the injection into the ooplasm was 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/O_2, Van der Heyden, Brussels, Belgium; 37°C; 5% O_2, 5% CO_2 and 90% N_2).

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, two or fragmented polar bodies. If a single pronucleus is observed, a second evaluation is carried out about 4 h later in order to see whether the pronuclear status has changed (Staessen *et al.*, 1993). The embryo cleavage of the two pronuclear (2PN) 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 (Deschacht *et al.*, 1988; Staessen *et al.*, 1989). Cleaved embryos with less than 50% of their volume filled with anucleate fragments were eligible for transfer. Up to three embryos were loaded into a few μl of Earle's medium and 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 (Van Steirteghem *et al.*, 1987).

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 villus 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 the 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.

Statistical methods

All statistical tests were performed two-sided at the 5% level of significance. The calculations were performed using the StatWorks package (Cricket Software, Inc., Philadelphia, PA, USA) on a Macintosh personal computer. In the controlled study, the fertilization rates after SUZI or ICSI were compared by χ^2 and Fisher's exact test. A χ^2 goodness-of-fit test was applied in the 150 consecutive ICSI cycles for the normal fertilization rates and cleavage rates in the four categories of semen characteristics.

Results

The 11 treatment cycles included in the controlled comparison of SUZI and ICSI yielded 169 cumulus–corona cell complexes, which all had a mature and dispersed appearance under the inverted microscope. After enzymatic and mechanical removal of the cumulus and corona cells the following observations were

Table I. Controlled comparison of subzonal insemination (SUZI) and intracytoplasmic spermatozoon injection (ICSI) procedures on sibling oocytes

	SUZI No.	(%)	ICSI No.	(%)
Oocytes injected	71		73	
Oocytes intact (%)	69	(97)	66	(90)
2-Pronuclear oocytes (% of intact)	3	(4)[a]	48	(73)[a]
1-Pronuclear oocytes	1		1	
3-Pronuclear oocytes	0		4	
Cleaved embryos (% of 2-pronuclear)	2		38	(79)
Transferred or frozen embryos	2		36	

[a]$P < 0.0001$ by χ^2 or Fisher's exact test.

Table II. Transfer procedures in controlled comparison of subzonal insemination (SUZI) and intracytoplasmic spermatozoon injection (ICSI)

	1 embryo		2 embryos		3 embryos	
	No. ET	No. preg.	No. ET	No. preg.	No. ET	No. preg.
SUZI	0	0	0	0	0	0
ICSI	0	0	3	0	6	3[a]
SUZI and ICSI	0	0	1	1[b]	0	0

[a]One singleton and two twin pregnancies.
[b]Singleton pregnancy.
ET = embryo transfers; preg. = pregnancies.

made of the oocytes: 147 metaphase II, five metaphase I, and 10 germinal vesicles. In seven complexes an empty zona pellucida without an oocyte or a cracked zona pellucida was found. The metaphase II oocytes of each patient were allocated alternatively to SUZI or ICSI treatment. After ICSI had been attempted in three of these 147 oocytes, it was noted that the spermatozoon had probably not been injected into the cytoplasm; these three oocytes were considered to have undergone SUZI and ICSI. The results of this controlled comparison are summarized in Table I. Slightly fewer oocytes were damaged by SUZI than by ICSI. The fertilization rate, however, was substantially higher after ICSI ($P < 0.0001$ by χ^2 or Fisher's exact test). The outcome of the transfers is summarized in Table II. Ten out of 11 patients had embryos replaced and four of them became pregnant. The amniocenteses of three patients revealed four normal karyotypes, two 46,XX and two 46,XY. The evolution of these four pregnancies has so far been uneventful.

Table III. Nature of intact, fertilized oocytes after intracytoplasmic spermatozoon injection in relation to semen characteristics

Semen	No. of cycles	No. of oocytes injected	Intact oocytes		2PN oocytes	
			No.	%	No.	%
Normal	11	126	111	88.1	80	72.1
Single defect	21	203	186	91.6	130	69.9
Double defect	45	374	335	89.6	212	63.3
Triple defect	73	704	646	91.8	408	63.2
Totals	150	1407	1280	90.8	830	64.9

Differences non-significant by χ^2 goodness-of-fit test.

Table IV. Embryo cleavage of 2-pronuclear oocytes after intracytoplasmic spermatozoon injection

Semen	No. of 2-pronuclear oocytes	Embryos with <50% fragments		Embryos transferred or frozen	
		n	%	*n*	%
Normal	80	57	71.3	51	63.8
Single defect	130	111	85.4	104	80.0
Double defect	212	157	74.1	145	68.4
Triple defect	408	316	77.5	296	72.5
Totals	830	641	77.2	596	71.2

Differences non-significant by χ^2 goodness-of-fit test.

The semen characteristics of the 150 consecutive ICSI cycles which were carried out over a slightly less than 3-month period indicated: 73 cycles (48.7%) with oligoasthenoteratozoospermia, 45 cycles (30.0%) with two semen abnormalities, 21 cycles (14.0%) with a single semen defect, and 11 cycles (7.3%) with sperm density, motility and morphology within the limits of the reference ranges for semen characteristics. The (mostly referred) patients had had total or almost total fertilization failure in one, two or sometimes more standard IVF cycles, or the semen characteristics were so deficient that the patients could not be accepted for IVF. Some patients had also been unsuccessful when SUZI had been done on the oocytes. The mean age of the female patients was 32.9 years (range 25–46 years) and the mean age of their partner was 35.4 years (range 26–52 years).

After ovarian stimulation by an association of buserelin and HMG–HCG, 1717 cumulus–oocyte complexes (mean 11.4 per cycle) were retrieved by echographically guided vaginal puncture. The microscopic observation of these complexes revealed that only five had a frankly immature aspect. After enzymatic and mechanical removal of the surrounding cumulus and corona cells, morphological observation revealed that an oocyte with intact zona pellucida was found in 1645 complexes (95.8%); a zona pellucida without an oocyte, only cumulus and corona cells, an oocyte without zona pellucida, a cracked zona pellucida or a degenerated oocyte was found in 72 complexes (4.2%). At the time of the ICSI procedure, the nuclear maturity of the intact oocytes revealed 1438 oocytes in metaphase II (83.7%), 53 oocytes in metaphase I (3.1%), and 154 oocytes at the germinal vesicle stage. Nineteen metaphase II oocytes were not injected because (i) no spermatozoa were found in two ejaculates of the partner of a patient with 13 oocytes; the couple agreed to donate these oocytes for research; (ii) only one spermatozoon was found in the semen of a couple with six oocytes; five oocytes were not injected; and (iii) one oocyte was damaged just prior to the sperm injection. In 10 oocytes the ICSI failed and the single spermatozoon was deposited in the perivitelline space; only one of these was fertilized and cleaved but no pregnancy occurred after this embryo was replaced. ICSI was carried out on the remaining 1409 oocytes. For the four groups of semen characteristics, the number of oocytes that were intact after the ICSI procedure and the number of normally fertilized oocytes are summarized in Table III. Less than 10% of the oocytes were damaged by the injection procedure. The mean percentage of intact oocytes with two clearly distinct pronuclei was 64.9% and ranged from 63.2

Table V. Embryo transfers (ET) and pregnancies (preg.) after intracytoplasmic spermatozoon injection

Semen	Embryo transfer of							
	One embryo		Two embryos		Three embryos		All transfers	
	No. ET	No. preg.[a]	No. ET	No. preg.[a]	No. ET	No. preg.[a]	No. ET	No. preg.[a]
Normal	0	0	3	1 (1)	6	2 (1)	9	3 (2)
Single defect	1	0	1	0	17	11 (10)	19	11 (10)
Double defect	6	1 (1)	5	1 (1)	28	12 (9)	89	14 (11)
Triple defect	4	0	14	6 (4)	50	33 (26)	68	39 (30)
Total	11 (8.1%)	1 (1)	23 (17.0%)	8 (6)	101 (74.8%)	58 (46)	135	67 (53)

[a]Clinical pregnancies in parentheses.

to 72.1% in the four different groups of sperm characteristics. The fertilization rate was not significantly different for the four categories of semen (χ^2 goodness-of-fit test). A single pronucleus was observed in 50 injected oocytes (3.6%) and three pronuclei in 71 oocytes (5.0%). The unexpected finding of three pronuclei occurred mostly in oocytes with only one polar body.

The characteristics of embryo cleavage after further 24 h culture of the 2PN oocytes are summarized in Table IV. More than three-quarters (641/830, or 77.2%) of the 2PN oocytes cleaved to embryos which fulfilled transfer criteria. Since the number of embryos to be transferred was limited to a maximum of three, supernumerary embryos were cryopreserved if they had cleaved and had <20% of their volume filled with anucleate fragments. The percentage of 2PN oocytes actually transferred or frozen was 71.2% (596/830). Freezing of 237 supernumerary embryos was done in 71 cycles (47.3% of total), i.e. a mean of 3.4 frozen embryos per cycle with freezing.

The outcomes of the single-, double- and triple-embryo transfers are summarized in Table V. Embryo transfer was possible in 135 of 150 cycles (90%). A triple-embryo transfer was possible in 75% (101 cycles) of the cycles. Pregnancy was established in 67 cycles, corresponding to a pregnancy rate of 44.7% per started cycle and 49.6% per embryo replacement. At the time of writing, the evolution of these 67 pregnancies is as follows: 7 preclinical abortions, 6 first-trimester miscarriages, one ectopic gestation and 53 clinical pregnancies. There have been 36 singleton, 12 twin and five triplet pregnancies. After transfer of two embryos in one patient, two gestational sacs were seen, one of which contained a monozygotic triplet and the other a single fetus. This quadruplet pregnancy was therapeutically reduced to a singleton pregnancy and has had a normal evolution so far. The total implantation rate per transferred embryo was 26.7% and the clinical ongoing implantation rate per transferred embryo was 20.8%.

Discussion

Many centres, including the Brussels Free University Centre for Reproductive Medicine, had accumulated over the years a number of couples with long-standing infertility who could not be helped by conventional IVF treatment. Such failures occurred especially in couples with severe male-factor infertility. After insemination with even a high number of progressive motile spermatozoa, oocytes failed to fertilize in one, two or even more IVF treatment cycles. Furthermore, there was a subpopulation of couples who could not be accepted for IVF because too few (e.g. <500 000) progressive motile spermatozoa could be harvested from the semen. In the last 5 years, several procedures for assisted fertilization have been introduced. A rather moderate success in terms of fertilization, embryo cleavage and pregnancy was achieved by partial zona dissection and especially SUZI. The normal fertilization rate never exceeded 20–25% of the injected oocytes and the implantation and take-home baby rates remained low (Cohen *et al.*, 1991; Ng *et al.*, 1991; Fishel *et al.*, 1992). In the Brussels Centre the fertilization rate after SUZI of 3008 successfully injected oocytes was 16.6% (Palermo *et al.*, 1993; Van Steirteghem *et al.*, 1993). The first clinical application of direct ICSI seemed to be more promising in terms of fertilization and (clinical) pregnancy rates (Palermo *et al.*, 1992, 1993; Van Steirteghem *et al.*, 1993). Before possibly abandoning SUZI for the treatment of these patients, a controlled comparison of SUZI and ICSI on sibling oocytes of 11 patients was carried out. A substantially higher normal fertilization rate (4 versus 72%) provided confirmation that ICSI was superior to SUZI. It has to be added that on this rather small cohort of 150 oocytes SUZI (4%) scored rather low and ICSI rather high (72%) in terms of fertilization. Such an extreme difference can be only partly explained by the limited number of treatment cycles. However, in these 11 patients, 10 did have an embryo transfer and four did become pregnant (two singleton and two twin pregnancies) and are currently in their third trimester. Our previous experience of applying SUZI and ICSI and the results of this controlled study led us to establish ICSI as the routine treatment for assisted fertilization as of the last quarter of 1992. In fact, SUZI has been abandoned in our Centre since September 1992.

This report describes the results of 150 treatment cycles of ICSI over a 3-month period since the ICSI procedure was adopted as the only procedure for assisted fertilization. Preclinical and very limited experience had been reported by the Norfolk and Singapore groups (Lanzendorf *et al.*, 1988; Ng *et al.*, 1991). The first clinical successes of ICSI in terms of high fertilization, embryo cleavage, transfer and pregnancy rates were reported previously in two large series of patients who received SUZI or ICSI treatment (Palermo *et al.*, 1993; Van Steirteghem *et al.*, 1993). This series has not only confirmed the previous results but has provided even better results in terms of several aspects of the ICSI procedure: (i) the damage caused by the micro-injection procedure was <10% of the injected oocytes; (ii) the normal fertilization rate of the successfully injected oocytes was 65%; (iii) the abnormal fertilization rate as evidenced by the presence of a single pronucleus or three pronuclei in the injected oocytes was, respectively, 4% and 6% of the injected oocytes; (iv) after 24 h of further in-vitro culture >70% of the 2PN oocytes showed one, two or even three mitotic divisions and had <20% of their volume filled with anucleate fragments; (v) 90% of patients with severe and on many occasions extreme male-factor infertility had embryos transferred; (vi) three-quarters of the transfers were triple-embryo transfers; (vii) supernumerary embryos were cryopreserved in 47% of the 150 cycles; (viii) the total and clinical pregnancy rates per started cycle were 39 and 35%, respectively.

These consistent results have been steady in a busy clinical programme of two to three ICSI cycles per day as well as three to four daily standard IVF procedures. It is in fact not exceptional that the ICSI results equal or even exceed the IVF results when such data are reviewed at our weekly clinical laboratory review meeting. These results have been achieved by a team of two physician/scientists, one senior technician and four medical technologists. It is our conviction that such results can be consistently obtained if great care is taken with optimal conditions for the many factors involved in the procedures. Specific attention is required for many aspects such as the equipment needed to perform the procedures, the preparation of the holding and especially the injection micropipettes, the video facilities which allow the procedure to be followed by other members of the micro-injection staff, and the temperature control throughout the

procedure. The micro-injection staff are involved full-time in the clinical routine and in the many research projects which are related to this area of specialized embryology.

ICSI is a new clinical procedure, and therefore its efficacy and safety need to be established. There may be some concern that the natural selection process of fertilization is being completely bypassed in patients with gross semen abnormalities in terms of sperm numbers, motility and morphological appearance. These conventionally used semen parameters do not, however, provide information on the quality of the DNA which is present in the sperm head.

Since clinical experience with ICSI is limited, the couples were counselled extensively by the medical and paramedical staff about the novelty of the procedure. The couples signed a consent form including agreement to a prenatal diagnosis by chorionic villus sampling or amniocentesis. As reported previously, no abnormalities have been detected so far in the 57 fetal karyotypes from 48 clinical pregnancies (Palermo *et al.*, 1993; Van Steirteghem *et al.*, 1993). It goes without saying that a careful clinical and echographic monitoring of the pregnancies by the obstetrician has to be done. The patients and the referring gynaecologist provide the Centre with all the information on the pregnancy and delivery. The couples also agree to a prospective follow-up of the children by a team of geneticists and paediatricians. The results of these follow-up studies will be reported shortly and may provide the data to allow a valid full-scale evaluation of the safety of the ICSI procedure.

Acknowledgements

We are indebted to many colleagues: Geertrui Bocken, Bart Desmet, An Vankelecom and Heidi Van Ranst of the micro-injection team; Professor Inge Liebaers, Dr Maryse Bonduelle, Elvire Van Assche and Andrea Buysse from the Department of Medical Genetics; the clinical, paramedical and laboratory staff of the Centre for Reproductive Medicine. Frank Winter of the Language Education Centre edited, and Viviane De Wolf and Nadia Fenners typed, the manuscript. This work is supported by grants from the Belgian Fund for Medical Research.

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Received on March 17, 1993; accepted on April 15, 1993