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Two essential steps for a successful intracytoplasmic sperm injection: injection of immobilized spermatozoa after rupture of the oolema

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A total of 740 cycles of intracytoplasmic sperm injection (ICSI) were performed: 625 cycles when $<6\times10^5$ total motile spermatozoa were harvestable from the ejaculate and 115 cycles in cases with a history of previous fertilization failure after classic in-vitro fertilization or subzonal sperm injection. An average of two pronuclei were observed in 63% of the injected oocytes, allowing 725 transfers of a maximum of three embryos (98%). Of 214 pregnancies initiated, 179 were established (25% of ICSI attempts). Because the fertilization rates from our initial 80 ICSI cycles were 2-fold less than those achieved previously, we changed the injection procedure and analysed, in 740 ICSI attempts, the importance of interfering technical factors and how to establish a successful ICSI programme. A remarkable change in the fertilization rate up to 68% (595 cycles) occurred when two steps in the injection procedure were performed well, i.e. immobilization of the spermatozoon and placement of the spermatozoon into the ooplasm after cytoplasmic aspiration into the pipette until oolema rupture. This immobilization, by touching the tail with the pipette, is mandatory for increasing the percentage of fertilization, even with totally non-motile spermatozoa (41%). Because aspiration of the cytoplasm is an invasive part of the ICSI procedure and influences the quality of the embryos, it is essential to reduce the amount of cytoplasm drawn into the pipette. This could be attained by using a spikeless injection pipette with the smallest possible internal diameter.

Key words: cytoplasmic aspiration/immobilization of the spermatozoon/immotile spermatozoa/ICSI/male factor infertility

Introduction

Classic in-vitro fertilization (IVF) techniques may fail in couples with severe male factor infertility. Moreover, some couples cannot be accepted for IVF if too few motile spermatozoa are observed in the ejaculate. Therefore, since 1990, to overcome such problems, micro-assisted fertilization procedures such as partial zona dissection (PZD; Malter and Cohen, 1989; Vanderzwalmen *et al.*, 1992) and subzonal sperm injection (SUZI; Ng *et al.*, 1991; Vanderzwalmen *et al.*, 1993) have been applied routinely in our centre. Encouraging results obtained by Palermo *et al.* (1992, 1993) and Van Steirteghem *et al.* (1993a,b) led us to offer intracytoplasmic sperm injection (ICSI) in our centre from April 1993 onwards. It quickly became apparent that the fertilization rates obtained were less than those expected when we started the ICSI programme.

The aim of this paper is to report the importance of some technical parameters which could influence the outcome of ICSI in 740 attempts with couples having very severe male factor infertility and using ejaculated spermatozoa. Parameters such as the quality of the injection pipette and the injection procedure, i.e. immobilization of the spermatozoon and aspiration of cytoplasm, were evaluated.

Materials and methods

Patient selection and semen evaluation

Between April 1993 and June 1995 a total of 516 couples underwent 740 cycles of ICSI using ejaculated semen as the only source of spermatozoa. The evaluation of sperm concentration and motility was carried out according to World Health Organization (WHO, 1993) criteria. When enough spermatozoa were present in the semen sample, sperm morphology was evaluated according to Tygerberg's strict criteria (Kruger *et al.*, 1986). We adopted the unit 'total number of motile spermatozoa in the ejaculate' (TMSE), as recommended by Fishel *et al.* (1993), to classify the semen samples.

The patients were classified into two groups. In the first group, 625 ICSI cycles were performed when the total number of motile spermatozoa in the ejaculate was $<6\times10^5$, i.e. when the TMSE in the ejaculate was insufficient to allow us to perform standard IVF with optimal results under our working conditions. When the TMSE was $<1\times10^5$ (299 ICSI attempts), too few motile spermatozoa could be harvested from the ejaculate; previously such patients were rarely accepted onto our SUZI programme. This group included seven patients with non-motile spermatozoa. An accurate morphological examination was not always possible because of very low sperm concentrations. However, when such an assessment could be performed, teratozoospermia was detected in 79% of the spermatozoa. In the second group, 115 ICSI attempts were performed on patients with a history of fertilization failure following two previous standard IVF programmes or one failed SUZI procedure.

Ovarian stimulation

Ovarian stimulation was carried out as described previously by Lejeune *et al.* (1990) by administrating nasal buserelin (Suprefact, SP; Hoechst, Belgium) in association with human menopausal gonadotrophin (HMG; Pergonal; Serono Laboratories Inc., Brussels, Belgium). Human chorionic gonadotrophin (HCG; Pregnyl; Organon, Oss, The Netherlands; Profasi, Serono) was administered when the cohort of follicles reached a diameter of ~20 mm. Luteal phase support consisted of administering 5000 IU HCG on days 4 and 8 after embryo transfer.

Sperm preparation

Split semen samples were obtained after 3 or 4 days of sexual abstinence on the morning of oocyte collection. In cases of extreme oligozoospermia, first a centrifugation step at 300 *g* for 5 min was performed prior to centrifugation at 300 *g* through a discontinuous Percoll gradient in two (70 and 95%) or three layers (50, 70 and 90%) (Ord *et al.*, 1990; Vanderzwalmen *et al.*, 1991). The centrifugation time ranged from 15 to 40 min, depending on the sperm concentration. The 90 or 95% Percoll fraction was washed with Earle's medium supplemented with 7.5% cord serum or 0.5% human serum albumin (CM; Irvine Scientific, Sentena, USA).

After washing and centrifugation for 5 min at 300 *g* of the 95% Percoll fraction, the sperm suspension was placed in a drop of CM containing little channels so that the motile spermatozoa could 'swim out' around the central part of the drop which comprised dead spermatozoa, immobile spermatozoa and other debris. The extension of the 'swim out' drop and channels depended on the semen concentration and final sperm motility. This 'swim out' drop was left at 37°C for 0.5–2.0 h until the ICSI procedure was performed.

Oocyte preparation

The cumulus and corona cells were removed from the oocytes 2–3 h after oocyte retrieval by incubation in 80 IU/ml hyaluronidase (Type VIII; Sigma Chemical Co., St Louis, MO, USA) for a maximum of 30 s. The removal of these cells was accomplished in another drop of CM free of hyaluronidase by aspiration of the complex cumulus corona into and out of a glass pipette with an inner diameter ranging from 180 to 220 µm. The oocytes were rinsed eight times in groups of five in 0.5 ml CM. Then they were incubated in CM covered with light paraffin oil at 37°C in an atmosphere of 5% CO_2 in air. ICSI was carried out on oocytes that had extruded their first polar body.

ICSI

Details of the preparation of the microtools for ICSI have been described previously by Van Steirteghem *et al.* (1993a,b). When the injection needles had a heat-formed spike pulled from the tip of the bevel using the microforge MF9 (Narashige; Tokyo, Japan), the outer diameter ranged from 7.0 to 10.0 µm and the corresponding inner diameter from 5.5 to 8.0 µm. For ICSI using spikeless injection pipettes, the inner diameter ranged from 4.0 to 4.5 µm. Before starting ICSI, a polyvinylpyrolidone (PVP; Sigma) droplet surrounded by five to seven drops of HEPES-buffered CM was placed in the centre of a Petri dish (Falcon type 3004). When the concentration of motile spermatozoa was judged to be sufficient, some motile spermatozoa were aspirated on the edge of the channels of the 'swim out' drop using a suction-controlled glass pipette of 30 µm diameter. This step was performed under an inverted microscope (Olympus IMT_2 or Olympus IX70). The spermatozoa were deposed at the 6 o'clock position on the edge of the PVP droplet (Figure 1A).

In cases of very severe oligozoospermia, non-motile spermatozoa or significant amounts of debris, an intermediate step was performed. In such situations, we first aspirated the spermatozoa from the 'swim-out' dishes using a microinjector with a 15 µm pipette. Then the spermatozoa were transferred to an additional droplet of CM situated next to the PVP droplet, before being transferred to the PVP drop (Figure 1B).

The ICSI procedure was performed under an inverted microscope (Olympus IMT_2 or Olympus IX 70) equipped with two coarse manipulators (Narashige) and two three-dimensional hydraulic micromanipulators (Mo-188; Narashige). The holding and injection systems (IM_6; Narashige) were filled with light mineral oil.

The sperm injection procedure used developed with time. Three different methods were used (Figure 2).

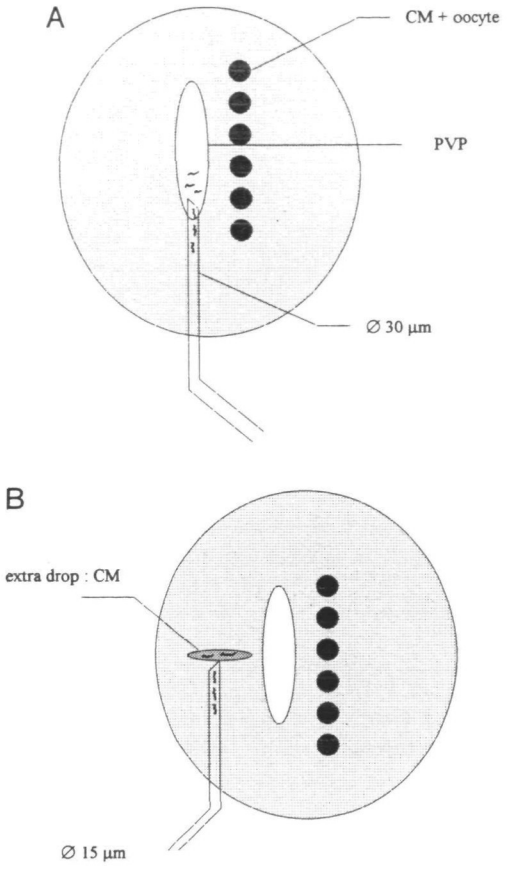

Figure 1. Preparation of the microdish for intracytoplasmic sperm injection: for severe oligoasthenoteratozoospermic samples, spermatozoa were placed immediately in the polyvinylpyrrolidone (PVP) drop (**A**); for extreme oligoasthenoteratozoospermic samples, spermatozoa were deposited first in a medium (CM) droplet (**B**).

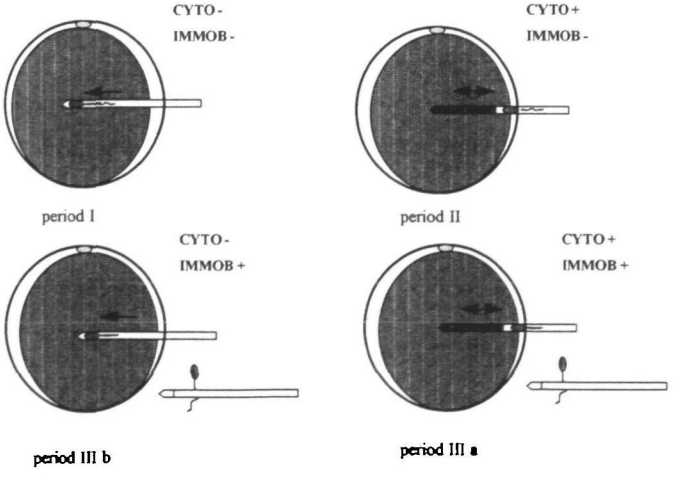

Figure 2. Diagram representing the four procedures for the injection of a spermatozoon performed during the different time periods. CYTO = cytoplasmic aspiration; IMMOB = sperm immobilization.

During period I (80 ICSI attempts), one motile spermatozoon which looked morphologically normal was aspirated out of the PVP drop. After holding the oocyte with the holding pipette (internal diameter 15 µm) and placing the polar body at the 12 or 6 o'clock position, the injection pipette was pushed through the zona pellucida

Table I. Effect of technical factors like sperm immobilization (immob) and aspiration of the cytoplasm (cyto) on the outcome of 740 intracytoplasmic sperm injection (ICSI) cycles

	cyto − immob − (period I)	cyto+ immob − (period II)	cyto − immob+ (period IIIb)	cyto+ immob+ (period IIIa)	Total
No. of ICSI cycles	80	50	15	595	740
No. of two pronuclei per injected oocyte (%)	210/580 (36)[a]	220/408 (54)[b]	50/120 (42)[c]	3163/4674 (68)[d]	3643/5782 (63)
No. of oocytes injected per puncture	7.3	8.2	8.0	7.7	7.8
No. of embryos obtained per ICSI cycle	2.7	4.4	3.3	5.0	4.8
No. of cleaving embryos (%)	205 (98)	211 (96)	48 (96)	3069 (97)	3533 (97)
No. of embryo transfers (%)	75 (94)	49 (98)	15 (100)	586 (98)	725 (98)
No. of pregnancies per ICSI cycle (%)	17 (21)	15 (30)	2 (13)	180 (30)	214 (29)
No. of miscarriages (%)	3 (18)	2 (13)	0 (0)	30 (17)	35 (16)
No. of ongoing pregnancies per cycle (%)	14 (18)[e]	13 (26)[e]	2 (13)[e]	150 (25)[e]	179 (25)

[a] versus [c], χ^2 not significant; [a] versus [b], $P < 0.001$; [b] versus [d], $P < 0.001$; [e] versus [e], not significant.

Table II. Fertilization rate as a function of the different injection procedures in a sibling oocyte study

Sibling oocyte study (21 ICSI attempts)	cyto − immob − (period I)	cyto − immob+ (period IIIb)	cyto+ immob − (period II)	cyto+ immob+ (period IIIa)
Fertilization (%)	21/70 (30)	13/35 (37)	27/52 (52)	43/54 (80)

cyto = cytoplasmic aspiration; ICSI = intracytoplasmic sperm injection; immob = sperm immobilization.

Table III. Effect of immobilization of the spermatozoa and cytoplasmic aspiration on fertilization rates after performing intracytoplasmic sperm injection (ICSI) according to the different severities of the semen sample or after fertilization failure

Total number of motile spermatozoa per ejaculate		cyto − immob − (period I)	cyto+ immob − (period II)	cyto − immob+ (period IIIb)	cyto+ immob+ (period IIIa)	Total
Non-motile	N ICSI		3[a]		4[a]	7
	2 PN (%)	4/24 (17)		19/46 (41)	23/70 (33)	
$<1 \times 10^5$	N ICSI	30[c]	21[d]	2[e]	239[f]	292
	2 PN (%)	83/252 (33)	110/189 (58)	4/10 (40)	1231/1785 (69)	1428/2236 (64)
$<6 \times 10^5$	N ICSI	27[g]	19[h]	6[i]	274[j]	326
	2 PN (%)	74/194 (38)	78/142 (55)	23/57 (40)	1510/2192 (69)	1685/2585 (65)
Fertilization failure						
	N ICSI	23[k]	7[l]	7[m]	78[n]	115
	2 PN (%)	53/134 (40)	28/53 (53)	23/53 (43)	403/651 (62)	507/891 (57)

2 PN = two pronuclei.
[a] versus [b], $P < 0.05$; [c] versus [d], $P < 0.001$; [c] versus [e], not significant; [d] versus [f], $P < 0.05$; [e] versus [f], $P < 0.05$; [g] versus [h], $P < 0.01$; [g] versus [i], not significant; [h] versus [j], $P < 0.001$; [i] versus [j], $P < 0.01$; [k] versus [l], not significant; [k] versus [m], not significant; [l] versus [n], not significant; [m] versus [n], $P < 0.001$.

at the equatorial level until the opposite side of the egg was reached. Injection of the motile spermatozoon was performed immediately.

During period II, 50 ICSI attempts were performed by first drawing back the plasma membrane into the injection pipette. Before expelling the motile spermatozoon, the cytoplasm was aspirated gently until a rapid outflow of cytoplasm was visualized in the injection pipette signalling rupture of the oolema. After re-injection of the cytoplasm and the motile spermatozoon, the injection pipette was withdrawn and the injected oocyte was released from the holding pipette.

For the last ICSI period (covering a 14 month period and 610 ICSI attempts) the spermatozoon was immobilized by touching the tail. The spermatozoon was positioned perpendicular to the tip of the pipette and was subsequently immobilized by gently rubbing its tail between the injection pipette and the bottom of the Petri dish. A combination of cytoplasmic aspiration and immobilization of the spermatozoon occurred in 595 ICSI (period IIIa) attempts; in another 15 ICSI attempts immobilization alone was performed before direct injection (period IIIb).

The injections were performed by touching the tail even in the presence of totally immotile spermatozoa. Independent of the injection procedure used, the selected spermatozoon was aspirated tail first into the tip of the injection pipette. The injected oocytes were then washed four times in fresh CM, placed in four-well Nunc dishes and incubated at 37°C in an atmosphere of 5% CO_2.

To analyse if fertilization was influenced by technique rather than only training and experience, a sibling oocyte study was performed during the last time period. When we retrieved enough oocytes, a comparative study of the different injection procedures was performed. Oocytes from the same patients were injected following the four different procedures.

We also analysed in a sibling oocyte study the usefulness of a spike on the injection pipette for its capacity to easily penetrate

through the oolema into the ooplasm. Oocytes from the same patient were injected without previous aspiration of the cytoplasm using spike or spikeless pipettes.

Assessment of fertilization and embryo development

Fertilization was checked 20 h after injection by the presence of two pronuclei. On certain occasions one or three pronuclei were detected.

The zygotes were cultured up to the 4-cell stage in Earle's medium supplemented with 15% cord serum. After 24 h of in-vitro culture, embryo quality was checked and the embryos were classified according to the number and form of the blastomeres and the percentage of anucleate fragments. A maximum of three embryos (2.8 embryos per transfer) were transferred to women with a mean age of 34 years (range 23–42).

Pregnancy was diagnosed on the basis of doubled endogenous HCG concentrations in serum. The analysis was confirmed on blood samples on days 15 and 22 after transfer. An ultrasound detection was performed between weeks 6 and 8 after transfer. The presence of a yolk sac, fetus and heart activity was evaluated and registered as an ongoing pregnancy.

Statistical methods

Comparisons between the groups of ICSI experiments were performed using Pearson's χ^2 test and Yates' correction when necessary.

Results

Out of 6785 oocytes collected (mean 9.2 oocytes per cycle), 5782 (85%) were injected. The remaining 15% consisted of metaphase I oocytes, germinal vesicle oocytes or oocytes with empty zonae pellucidae. Out of the 5782 oocytes injected, 404 (7%) were damaged during the injection procedure. By 18 h after the ICSI procedure, one and three pronuclei were observed respectively in 6.0 (347) and 0.9% (52) of the injected oocytes. The percentage of injected oocytes that fertilized normally reached 63% (3643/5782).

Cytoplasmic aspiration and immobilization of the spermatozoa

A comparison of the ICSI procedure following injection of motile or immobilized spermatozoa into the oolema with or without aspiration of cytoplasm is summarized in Table I. Different rates of fertilization were observed between the periods, each corresponding to a change in the ICSI procedure used.

The first increase in the percentage of two pronuclear eggs was observed between periods I (where no cytoplasmic aspiration was performed; 36%, 210/580) and II (where cytoplasmic aspiration was performed until there was a breach in the oolema; 54%, 220/408) when a motile spermatozoon was injected ($P < 0.001$).

A dramatic increase in the fertilization rate up to 68% (3163/4674) was noted (period IIIa) when we injected an immobilized spermatozoon into the ooplasm after rupture of the oolema. After this injection procedure, a statistically significant increase in the fertilization rate was observed compared with the injection procedure performed with a motile spermatozoon (period II, 220/408, 54%) ($P < 0.001$).

If no cytoplasmic aspiration was performed, then no difference in the rate of pronuclear egg formation was observed after the injection of a motile (36%) or immobilized (42%) spermatozoon.

The effect of the different injection procedures on the formation of two pronuclei in a sibling oocyte study showed the same evolution in the fertilization results compared with the large-scale study (Table II). Out of 21 ICSI attempts, fertilization rates of 30, 52, 80 and 37% were obtained respectively for periods I, II, IIIa and IIIb. These results show that not only training and experience but also improvements in the technique, i.e. aspiration of the cytoplasm and immobilization of the spermatozoon, resulted in an increase in the fertilization rate.

Independent of the semen categories, we observed that the fertilization rates corresponded to the changes in the ICSI procedure during the different time periods. The most remarkable increase was obtained when we combined aspiration and immobilization (Table III).

The beneficial effect of touching the sperm tail was also observed when we were confronted with totally immotile spermatozoa. A statistically significant difference in the fertilization rate was observed in favour of the 'touching tail' group. The proportion of fertilized eggs increased from 17 to 41% after breaking the tail with the pipette ($P < 0.05$).

Out of 3643 zygotes, 3533 (97%) cleaved, and no difference in the cleavage rate or the embryo transfer rate could be observed between the different time periods (Table I).

Nevertheless, the average number of oocytes injected was similar in each group, but the mean percentage of embryos obtained per ICSI was higher for period IIIa. As a consequence, more embryos were transferred and there were more embryos to select from before replacement for period IIIa. We obtained an average of 2.7 embryos per ICSI cycle for period I and 5.0 for period IIIa.

After 725 transfers (98%; 2.8 embryos per transfer, maximum of three embryos), a total of 214 pregnancies were established, corresponding to a pregnancy rate of 29% per ICSI cycle. Miscarriages occurred in 35 (16%) attempts, resulting in an overall ongoing pregnancy rate of 24% (179).

Compared with period I, although not statistically different, a higher ongoing pregnancy rate was obtained in periods II and IIIa when at least four embryos were obtained. We obtained ongoing pregnancy rates per started ICSI cycle of 26 and 25% for each period respectively.

Microinjection pipette and cytoplasmic aspiration

The percentages of two pronuclear embryos, grade A embryos and degenerated oocytes obtained during the first period, as a function of the external diameter of the injection needle, are shown in Table IV. Out of 140 oocytes injected using a pipette of 7 μm external diameter, the fertilization rate reached 26%. If we use pipettes of 8, 9 and 10 μm external diameter then the diameter of the needle did not influence the fertilization rate significantly.

When using pipettes of 7 and 8 μm external diameter, the percentages of good quality embryos (grade A) were 42 and 48% respectively; this percentage decreased to 14 and 17% when ICSI was performed with pipettes of 9 and 10 μm external diameter respectively. A significant increase in the

Table IV. Percentage of two pronuclei, grade A embryos and degenerated oocytes as a function of external diameter of the injection needle

	External diameter (μm)			
	7	8	9	10
No. of ICSI cycles	20	22	18	12
No. of oocytes injected	140	148	166	66
2 PN (%)	36 (26)[a]	60 (41)[b]	66 (40)[c]	24 (36)[d]
No. of cleaved embryos	36	58	64	23
No. of grade A embryos	15 (42)[e]	28 (48)	9 (14)[g]	4 (17)[h]
No. of degenerated oocytes	7 (5)[i]	15 (10)[f]	27 (16)[j]	19 (29)[k]

2 PN = two pronuclei; ICSI = intracytoplasmic sperm injection.
χ^2 values are: [a] versus [b–d], $P < 0.05$; [e] versus [h], $P < 0.05$; [e] versus [g], $P < 0.01$; [i] versus [j], $P < 0.01$; [i] versus [f], not significant.

Table V. Effect of the presence or absence of a spike on the fertilization rate and the percentage of degenerated oocytes after intracytoplasmic sperm injection (ICSI)

	No spike	Spike
No. of ICSI cycles	6	6
2 PN (%)	12/30 (40)	14/37 (38)
No. of degenerated oocytes (%)	1 (3)	2 (5)

2 PN = two pronuclei.

percentage of degenerated oocytes from 16 to 29% was observed when pipettes of 9 and 10 μm external diameter were used respectively.

Injection needles with and without spikes were employed to analyse the usefulness of the spike. We observe in Table V that no differences in the fertilization rate or the rate of degenerated oocytes were observed when an immobilized spermatozoon was injected directly. We obtained fertilization rates of 40 and 38% when pipettes with and without spikes were used respectively.

Discussion

Encouraged by the ICSI results published by Van Steirteghem *et al.* (1993a,b), we introduced this micro-assisted technique into our programme in April 1993. A total of 740 ICSI cycles using ejaculated semen were performed on 516 couples who had previously experienced failed IVF or who could not be accepted onto a standard IVF programme because of a low motile sperm concentration. As reported previously by different groups (Palermo *et al.*, 1993; Tucker *et al.*, 1993; Van Steirteghem *et al.*, 1993a,b; Payne *et al.*, 1994; Tsirigotis *et al.*, 1994; Alikani *et al.*, 1995; Svalander *et al.*, 1995), our overall results, covering a time period of 26 months, confirmed that ICSI increases fertilization and pregnancy rates even in the most severe forms of male factor infertility.

However, it was apparent that even after our 4 years of experience with PZD (Vanderzwalmen *et al.*, 1992) and SUZI (Vanderzwalmen *et al.*, 1993), the fertilization rates obtained were disappointing.

At the beginning of the ICSI programme some experience and training were necessary to help us to produce good quality injection needles and, as a consequence, low rates of degenerated oocytes.

Nevertheless, after 9 months (80 ICSI attempts) of the programme the resulting percentage of sperm decondensation and subsequent pronucleus formation was 2-fold less than the results presented by Van Steirteghem *et al.* (1993a,b).

When beginning our ICSI programme in April 1993 very few technical details were available. As a consequence, during the first period (period I) we performed ICSI in a simple and non-invasive way, i.e. by directly injecting a motile spermatozoon without aspiration of the cytoplasm. In such a situation, the oolema was not drawn back into the pipette until its rupture prior to injecting the motile spermatozoon. After this injection procedure, we frequently observed a channel in the ooplasm of the oocyte corresponding to the shape of the injection pipette when the latter was removed. The fertilization rate remained very poor because, during the vanishing of the invagination of the membrane into the egg, the motile spermatozoon was frequently rejected into the perivitelline space, a situation comparable with the SUZI technique (Payne *et al.*, 1994; Svalander *et al.*, 1995).

Therefore a series of experiments was designed to identify those technical factors in the sperm microinjection technique which could influence the fertilization rate. The problem was to determine if the low fertilization rate was caused by the impairment of sperm decondensation or by a technical problem concerned with injecting the spermatozoon into the cytoplasm.

Our data are in agreement with those obtained by Svalander *et al.* (1995), who stated that aspiration of the cytoplasm until a breach in the oolema is observed increases the rate of fertilization.

Gearon *et al.* (1995) and Tesarik and Sousa (1995b) have reported that the mechanical disruption of the ooplasm alone and the degree of cytoplasmic aspiration may increase the activation rates producing, as a consequence, an increase in the fertilization rate. Indeed, aspiration itself may provide a mechanical stimulus to induce an important release of intracytoplasmic calcium stores (Tesarik *et al.*, 1994). We also observed that even if the spermatozoon is injected correctly into the oocyte's cytoplasm, an increase in the fertilization rate from 69 to 83% is observed when vigorous aspiration of the cytoplasm is performed, with the degree of aspiration corresponding to at least the radius of the oocyte (P.Vanderzwalmen, unpublished data). However, even after ensuring the correct injection of the spermatozoon into the ooplasm, the appearance of two pronuclei on the following day does not necessarily occur.

It appears from cytoanalysis studies by Dozortsev *et al.* (1994) that most unfertilized oocytes after ICSI show evidence of intact spermatozoa in the cytoplasm. This confirms the previous studies of Selva *et al.* (1993) and Urner *et al.* (1993) which proposed that after SUZI, a relatively high number of spermatozoa penetrate the oocyte without attaining head decondensation.

To ensure normal sperm decondensation, partial sperm membrane damage prior to injection is required. This damage could be inflicted after immobilizing the spermatozoon by gently touching its tail with the pipette and leads to an

increase in the fertilization rate (Dozortsev *et al.*, 1994; Payne *et al.*, 1994).

How does touching the tail increase sperm decondensation and why do non-immobilized spermatozoa not progress to the pronucleus stage? Different studies (Dozortsev *et al.*, 1994; Tesarik *et al.*, 1994) have indicated that intracytoplasmic calcium wave oscillations following ICSI are correlated with oocyte activation. This was confirmed in a study by Homa and Swann (1994) who suggested that human oocytes are activated at fertilization by the diffusion of a protein from the spermatozoon into the ooplasm, with subsequent calcium oscillations and hyperpolarization of the oocyte. This activation factor appears to be present in the cytosolic fraction of the sperm cell (Dozortsev *et al.*, 1995).

In the case of injection of an intact non-demembranated spermatozoon, only a monotonic increase of calcium insufficient for activation was detected. In such a situation, the oocyte may fail to initiate the biochemical processes necessary for oocyte activation (Tesarik *et al.*, 1994).

Damaging the sperm tail probably induces a mechanical disorder in the plasma membrane as well as biochemical alterations. This destabilization permits the release of a substance into the cytoplasm of the oocyte which is able to initiate activation of the egg.

Another hypothesis is that the non-demembranated sperm nucleus remains poorly accessible to those factors required for chromatin decondensation and released by the activated oolema, and that there is a need for the sperm membrane to be breached so that cytoplasmic enzymes in the ooplasm can act on the sperm nucleus (Tesarik and Kopecny, 1989; Montag *et al.*, 1992). During oocyte activation, ooplasmic factors such as thiol-reducing agents (Perreaults *et al.*, 1984; Homa and Swann, 1994; Van Blerkom *et al.*, 1994) are involved directly in the process of sperm chromatin decondensation. Immobilization of the spermatozoon by gently touching its tail probably permits it to initiate oocyte activation, while sperm chromatin decondensation is activated by oocyte factors.

Alternatively, immobilizing the spermatozoon before injection may prevent the interference of the spermatozoon with the cytoskeleton and metaphase spindle of the oocyte. However, a rapid decrease in sperm motility is observed after injection into the cytoplasm. In addition, the process of aspiration of the cytoplasm may be more deleterious for the different organelles inside the cytoplasm. A further possibility may be that the immobilized spermatozoon is injected into the cytoplasm with a consistently lower volume of carrier medium than may be required for more motile spermatozoa (Catt *et al.*, 1995).

Nevertheless, even after ensuring the correct injection of the immobilized spermatozoon into the ooplasm, a significant proportion of the oocytes remained at metaphase II. Flaherty *et al.* (1995) and Tesarik and Sousa (1995a) determined that the failure of oocyte activation is the main cause of fertilization failure after ICSI. In this situation, Flaherty *et al.* (1995) showed that the oocytes contained a swollen sperm head which was arrested at various stages of decondensation, indicating that the oocyte had failed to activate and complete the second meiotic division. According to Flaherty *et al.* (1995), different hypothetical reasons can explain this absence of activation, for example an inability to undergo cytoplasmic maturation, a deficiency or absence of sperm cytosolic factors or an inability of the oocytes to exhibit multiple Ca^{2+} oscillations after microinjection.

Because ICSI is more invasive than the other micro-assisted techniques, an important factor is the design and quality of the microtools (Clarke and Johnson, 1988). When evaluating the effect of the shape and size of the pipette aperture on embryo quality and the rate of degenerated oocytes, it was clear that using larger diameter needles (9 and 10 μm) entailed a higher percentage of degenerated oocytes and poor quality embryos. Although the exact amount of fluid expelled from the injection needle was difficult to control, it appeared by visual examination that a larger volume of PVP–sperm suspension may have been injected when non-uniform and relatively larger pipettes were used.

Cytoplasmic aspiration is a delicate part of the procedure. A crucial factor appeared to be the amount of fluid aspirated and reinjected into the oocyte. The amount of cytoplasm and fluid aspirated into the pipette before reinjection was found to be related directly to the internal diameter of the injection pipette. A small volume of cytoplasm was aspirated if the internal diameter of the pipette was 3.5–4.0 μm. However, the aperture is easily closed when making the spike on the microforge using such pipettes, thus preventing entry of the spermatozoa. Therefore if a pipette with the smallest internal diameter (4.0–4.5 μm) was required, no spike was used.

If cytoplasmic aspiration is mandatory in the majority of injection procedures, it means that we are unable to cross the oolema directly. It may be influenced by oocyte quality and maturity, as well as the type of microtool used. The presence of a spike can facilitate passage through the zona pellucida but not through the oolema because no difference in the fertilization rate or the rate of degenerated oocytes could be observed when we used a pipette with or without a spike.

We observed that the minimal volume of ooplasm aspirated before reinjection generally occurred when we first used the spikeless pipettes or when the oocytes were not overmature, as judged by the appearance of the cumulus.

Apart from the reports of Bongso *et al.* (1989) and Wolf and Jouannet (1991), it has been found that the SUZI technique is not suitable for patients with totally immotile spermatozoa. In contrast to the ICSI technique, our data show that fertilization can be obtained with non-motile spermatozoa. Even in such a situation, it is necessary to damage the tail before injection so as to increase the fertilization rate. Data presented by Gearon *et al.* (1995) have shown that non-viable human spermatozoa injected into aged human oocytes are capable of fertilizing oocytes and undergoing cleavage if the medium contains calcium.

Gearon *et al.* (1995) have also suggested that activation occurs in the presence of paternal DNA, in the form of non-viable spermatozoa, and that male pronucleus formation appears to occur in the presence of normal concentrations of calcium in the different culture media available. In addition, Ng *et al.* (1993) have proposed that metabolic activity of the spermatozoon is only required for membrane mobilization

involved in fusion with the oolema. As long as the nuclear DNA of the spermatozoon is unaltered or proteolysis has not occurred, it may still be possible for the sperm genome to function once it is introduced into the oocyte. On the other hand, Liu *et al.* (1995) have shown that total fertilization failure after ICSI occurs if couples only have immotile spermatozoa available for injection.

Even though the efficiency is low, immotile ejaculated spermatozoa can produce viable young in the bovine model (Goto *et al.*, 1990; Ng *et al.*, 1993). However, for a limited number of cases we have observed (Nijs *et al.*, 1995) that no pregnancy is obtained with ejaculated compared with immotile testicular spermatozoa. It is probable that the higher pregnancy rate obtained with immotile testicular spermatozoa means that a high proportion of the spermatozoa are alive but immotile because of their immaturity. This may be related to the viability of the spermatozoa chosen for injection and reflect an impairment at the level of the centriole in non-motile ejaculated spermatozoa because of its involvement in the organization of mitotic spindles during early cleavage. The presence of a defective centriole may be responsible, in part, for irregular cleavage patterns or even the arrest of embryo development (Ng *et al.*, 1993).

Live immotile spermatozoa cannot be distinguished from dead spermatozoa. However, the distinction between a vital and a non-vital spermatozoon is important in relation to the results obtained for ICSI with immotile spermatozoa. Desmet *et al.* (1994) suggested that the hypo-osmotic swelling test could be useful in selecting immotile but vital spermatozoa.

Improvements and the levelling out of our results after some months of practice were the result of our growing technical expertise. Mechanical immobilization of the spermatozoon before an adequate injection played an important role in achieving consistently high fertilization rates. Exposure of the sperm nucleus or other internal components to the oocyte's cytoplasm after sperm immobilization promoted oocyte activation, decondensation of the spermatozoon and other events of normal fertilization.

Moreover, the outcome of the results depended mainly on technical factors such as the dimensions of the microinjection pipette which were adjusted to cause the minimum of trauma.

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