

Intracytoplasmic sperm injection: laboratory set-up and injection procedure

H.Joris¹, Z.Nagy, H.Van de Velde, A.De Vos and A.Van Steirteghem

Centre for Reproductive Medicine, Dutch-speaking Brussels Free University (Vrije Universiteit Brussel),
Laarbeeklaan 101, 1090 Brussels, Belgium

¹To whom correspondence should be addressed

Intracytoplasmic sperm injection (ICSI) as treatment for male-factor infertility has been introduced worldwide in the past few years in many laboratories using assisted reproduction techniques. Some changes in the existing set-up are necessary before implementing this procedure. The equipment can be divided into two groups: that required for preparation of the microtools and that required for the microinjection procedure itself. A pipette puller, grinder and microforge are necessary for preparation of the microtools. The correct settings and use of these instruments are of crucial importance in preparing a good needle, which in turn is crucial to the injection procedure itself. The microscope has to be equipped with a heated stage, correct optics and manipulators and injectors. The correct settings and use of this equipment also influence the injection procedure and may influence the success rate. Retrospective analysis of the evolution of ICSI in our centre clearly shows a marked improvement following the introduction of some modifications into the procedure. These modifications were (i) reducing the concentration of hyaluronidase used for cumulus and corona radiata removal, (ii) selecting a motile spermatozoon that was immobilized prior to the injection and (iii) aspiration of cytoplasm to ensure rupture of the oocyte membrane. The injection procedure itself can also be influenced by oocyte characteristics. It has been reported that the reaction of the oocyte to the penetration by the pipette has an influence on the success rate. The ICSI procedure has about the same success rate as IVF in cases of non-male infertility. However, work can

still be done to improve the success rate of this procedure.

Key words: injection procedure/intracytoplasmic sperm injection/laboratory set-up/technique

Introduction

Intracytoplasmic sperm injection (ICSI) offers possibilities to couples who have a very limited chance or no chance at all of having children of their own using conventional in-vitro fertilization (IVF) or other assisted-fertilization procedures such as zona drilling (ZD), partial zona dissection (PZD) or subzonal insemination (SUZI).

The requirement for successful ICSI is the presence of one motile spermatozoon for each metaphase II oocyte to be injected. High fertilization and pregnancy rates can be achieved when a motile spermatozoon is injected (Van Steirteghem *et al.*, 1993). Rates fall when only immotile ejaculated spermatozoa or, probably, non-vital spermatozoa are injected (Nagy *et al.*, 1995a). Other semen parameters, such as concentration, morphology (except for globozoospermia; Liu *et al.*, 1995) and high titres of antisperm antibodies (Nagy *et al.*, 1995b), do not influence the success rates of ICSI.

Oocytes that have extruded the first polar body can be successfully injected. Other morphological aspects of oocytes, as regards the zona pellucida, the perivitelline space and the cytoplasm, do not influence the outcome of ICSI (De Sutter *et al.*, 1996).

A number of technical aspects can also influence the result of an ICSI cycle. An inverted microscope equipped with manipulators and injectors is neces-

sary to perform the injection procedure. Carefully prepared microneedles with the appropriate features and the correct positioning of these needles on the inverted microscope are required for an easy injection procedure. Such technical aspects are important but are in turn valueless if the operator does not have the skill necessary to perform a proper injection. Training in the injection procedure is of crucial importance before anyone can start to treat patients by ICSI.

The injection of an oocyte can be very difficult due to the characteristics of the oocyte. Penetration of the zona pellucida and the way in which the oolemma is ruptured can influence the result of the injection procedure. Such oocyte characteristics cannot usually be detected by a morphological evaluation and only become evident during the injection procedure.

The aim of this paper is (i) to describe the additional equipment needed by an IVF laboratory to ensure a good ICSI procedure and (ii) to discuss some technical aspects of the ICSI procedure. Details of the procedure as performed in our centre have been published previously (Van Steirteghem *et al.*, 1995). Results obtained over a long period using this protocol have also been published (Van Steirteghem *et al.*, 1996).

Equipment necessary for ICSI

The introduction of ICSI has revolutionized the treatment of male-factor infertility. Other assisted fertilization procedures such as ZD, PZD and SUZI (Gordon *et al.*, 1988; Cohen *et al.*, 1991; Palermo *et al.*, 1992a) have been used by only a limited number of centres. Over the past 3 years, many centres all over the world have initiated ICSI programmes. The equipment necessary for preparation of the microneedles and the manipulators and injectors had been developed earlier for use in research on living cells. Microinjection of oocytes and spermatozoa had been performed in various species since the early 1960s.

The set-up as used in our centre is described here in detail. There are two main aspects to be considered in setting up an ICSI laboratory: the equipment necessary for preparation of the microneedles and the inverted microscope equipped with manipulators and injectors.

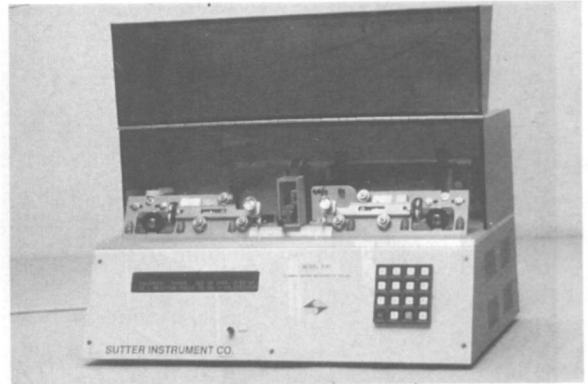


Figure 1. Horizontal pipette puller.

Preparation of microneedles

A general remark on the preparation of needles for ICSI is that this aspect should not be underestimated and that it takes a lot of training and patience to make good ICSI needles.

The type of capillary used influences the preparation of the microtools. The physical properties of the glass capillaries may influence the settings of the instruments. Borosilicate and Pyrex glass are mainly used. The inner and outer diameter and the wall thickness influence the characteristics of the needle that will be obtained. We use borosilicate capillaries (Drummond, Broomall, USA) with an inner and an outer diameter of 0.69 and 0.97 mm respectively and a length of 10 cm. The capillaries are washed and sterilized before use. Washing starts with sonication for 30 min in milli-Q water containing 2% detergent and rinsing in milli-Q water for at least 30 min. These steps are repeated but sonication is done without detergent. The capillaries are then dried in hot air and sterilized at 120°C for 6 h. Three instruments are necessary to prepare both holding and injection pipettes: a pipette puller, a beveller and a microforge.

A horizontal pipette puller (P-97, Sutter Instrument Company, Novato, USA) is used to pull the capillaries to form a needle with a long fine tip (Figure 1). Different parameters, including temperature, time, pulling force and velocity influence the shape of the needle. The parameters need no further modification once they have been established. The needles we use have a taper of approximately 11 mm in length. There is only a slight increase in the diameter (~1–2 µm for each

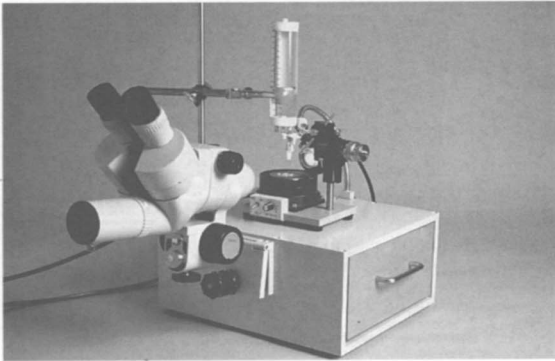


Figure 2. Grinder placed on a table carrying a stereo microscope that allows the grinding procedure to be monitored.

100 μm in length) in the first part. At this point it is difficult to measure the wall thickness. Whether the needle is stiff enough will become clear when injection of the oocyte is attempted. At our centre, both holding and injection pipettes are made from the same needles. Payne (1995) uses glass capillaries of different thickness to prepare holding and injection pipettes.

After pulling, the beveller and microforge are used to finalize the preparation of the needles. In order to make an injection pipette, the needle is ground. The whetstone of the grinder (EG-4; Narishige, Tokyo, Japan) rotates at a speed of 1500 rpm. There is a constant drip of milli-Q water during the grinding procedure. The needle is fixed in the holder, which is positioned at an angle of 50° and is lowered until contact with the whetstone is made. This procedure is monitored through a stereo microscope (Nikon, Tokyo, Japan) mounted on a table (Figure 2). In the past, pulled needles were ground until the correct opening was obtained (Van Steirteghem *et al.*, 1995). Currently, needles are cut on the microforge (MF-9; Narishige; see Figure 3) at an outer diameter of $\sim 6\text{--}7\ \mu\text{m}$. The grinding procedure then takes only a few seconds. After this, the needle is again mounted on the microforge. If necessary, excess water is removed by heating the filament (a platinum wire with a diameter of 100 μm on which a glass node is made) and moving the needle upwards alongside the filament until all the water has evaporated. The opening of the needle is turned to the front before the spike is made. The position of the needle

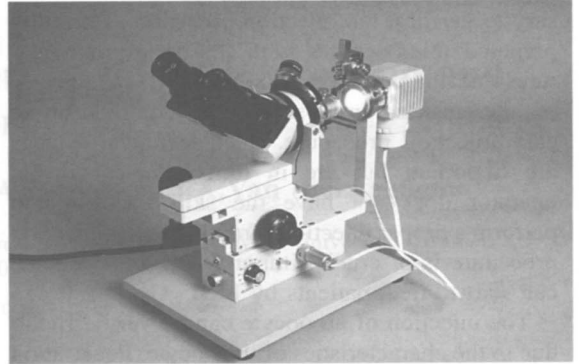


Figure 3. Microforge used for preparation of holding and injection pipettes.

against the glass node is checked before gentle heating is applied. The needle is lowered, brief contact with the glass node is made and the spike is formed by pulling back the needle. Finally, the needle is moved down next to the filament until the opening is out of the field. The needle is bent to an angle of $\sim 30^\circ$ by gentle heating. Too much ventilation may cause incorrect bending and should be avoided.

The preparation of a holding pipette is less complicated. A pulled needle is fixed on the microforge and placed horizontally. It is moved until the diameter at the level of the filament is 60–80 μm . The needle is then placed on the filament, heating starts and the temperature is increased until the glass of the needle starts to melt. Heating is stopped suddenly, the platinum wire returns to its original position and the needle breaks. The tip is moved away from the glass node and the opening is positioned in front of the glass node. The sharp edge is smoothed and this is continued until the opening has a diameter of $\sim 15\ \mu\text{m}$. The needle is now placed vertically and is bent similarly as for an injection pipette. A holding pipette can also be prepared by heating in a flame and pulling by hand. This procedure may be faster but is a cruder way of preparing holding pipettes.

Once prepared, the needles are stored in special containers. First they are stored in a closed plastic box (Plexilabo, Drongen, Belgium). The day before use needles are placed in another container with a metal foot and a glass cover (Research Instruments, Cornwall, UK; Figure 4) and are sterilized by dry heat at 120°C for 6 h.

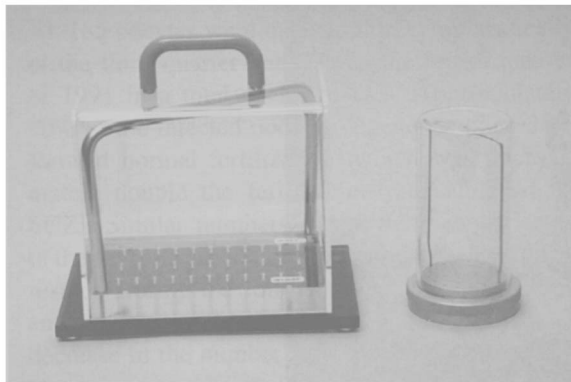


Figure 4. Pipette containers to store pipettes after preparation (left side) or for sterilization the day before the injection procedure (right side).

Currently there are a number of companies supplying holding and injection pipettes for ICSI. These may constitute an alternative for centres that do not want to invest in either the equipment or the time necessary for training in the preparation of pipettes.

The inverted microscope

A number of procedures in the IVF laboratory are performed using a stereo microscope. Even the presence of pronuclei and embryo quality can be checked in this way. However, a proper evaluation should be carried out on an inverted microscope using $\times 200$ or $\times 400$ magnification because many details are lost at a lower magnification. The inverted microscope can be modified so as to be able to perform the manipulation procedures.

The microscopes (Diaphot TMD, Diaphot 300; Nikon) in our centre (Figure 5) are equipped with a heated stage (Linkam, Surrey, UK) so that work can be carried out at 37°C . It has been shown that oocytes are sensitive to a fall in temperature. Transient cooling of oocytes to room temperature for only 10 min can be sufficient to cause irreversible damage to the spindle (Pickering *et al.*, 1990). The safety of performing the injection procedure at room temperature (Atlee *et al.*, 1995) may therefore be questioned.

We originally used a home-made heated stage. The heating element was a 1-cm-high horse shoe-shaped aluminium ring that was fixed on the table of the microscope. Calibration was carried out by

placing a small electrode in the injection dish on the microscope table. The central part of the microscope table is now equipped with commercial heated stages. Calibration has shown that it is necessary to increase the settings of the controller to more than 37°C . With these heated stages, the presence of a metal horse shoe-shaped ring still has a positive influence on the temperature in the dish, creating a kind of mini chamber in which there is reduced circulation of air. The presence of such a ring can make a difference of 1°C . Another possibility that we have not evaluated in our centre is the use of an environmental chamber on the inverted microscope (Payne, 1995). This allows work at 37°C in an environment of 5% CO_2 .

The optics used also facilitate the injection procedure. Phase-contrast optics provide a view in only one plane. Direct interference contrast (DIC) and Hoffman modulation contrast (HMC) offer greater differentiation. The choice of either of these two systems depends on the chamber used for the injection procedure. DIC is better when glass is used, while HMC is better for plastic dishes.

Two identical sets of two manipulators are mounted on the microscope. The one on the left-hand side is used for manipulation of the holding pipette, the one on the right-hand side for the injection pipette. Both manipulators can move in the x -, y - and z -axes. An electrical manipulator (MM-188; Narishige) permits coarse movements. This is used mainly for positioning the needles and to move the needles up or down when an injection dish is replaced. The second manipulator is a hydraulic remote-control manipulator (MO-188; Narishige) with a hanging joystick, which is used for fine movements. The surface to be reached with one rotation of the joystick can be adjusted and fixed in such a way that the whole field can be covered when working at $\times 400$ magnification. Each manipulation set is completed with a microinjector (IM-6; Narishige). These are used to either fix or release the oocyte or to aspirate and inject a spermatozoon. The injectors consist of an 800 μl syringe, Teflon tubing and a steel holder. The plunger is controlled by a micrometer. The syringe, the tubing and the steel holder are filled with the same oil as is used for embryo culture. Care is taken to avoid the presence of air bubbles in the system.

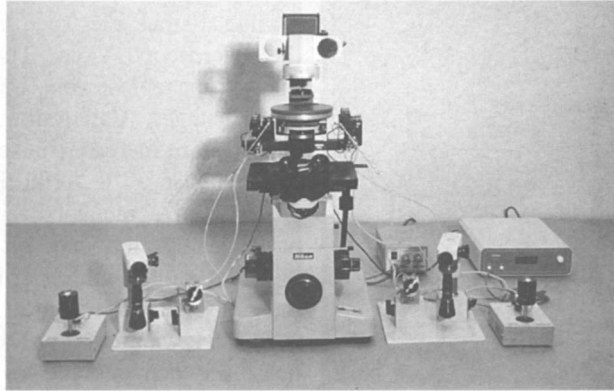


Figure 5. Set-up used for intracytoplasmic sperm injection. The inverted microscope is equipped with Hoffman modulation contrast optics, a heated stage and two identical sets of micromanipulators and injectors. The set on the left side is used for manipulating the holding pipette, the set on the right side for manipulating the injection pipette.

Both the hydraulic manipulator and the injector are fixed on a metal plate by means of a magnet. This ensures a fixed position and makes it possible to work while looking through the microscope.

The microscopes are also equipped with high quality cameras (DXC-755 P; Sony, Tokyo, Japan), and all manipulations can be followed on a monitor (Trinitron; Sony). This allows immediate discussion of new or unexpected aspects of the procedure among the colleagues present in the laboratory and is very important when new persons are being trained.

The whole set-up is placed on an anti-vibration table to prevent vibrations from interfering with the injection procedure.

The ICSI procedure

The use of sucrose to shrink oocytes when performing SUZI has never been considered in our centre, neither in mice nor in humans. When performing SUZI in mice, it was extremely seldom that the oolemma was ruptured following penetration of the zona pellucida, and rupture always led to immediate lysis. From the start of these procedures, the injection pipette was pushed through the zona pellucida at the three o'clock position, and this practise was continued when the clinical programme of SUZI started. The oolemma continued to be ruptured accidentally in some oocytes.

The first description of the ICSI procedure

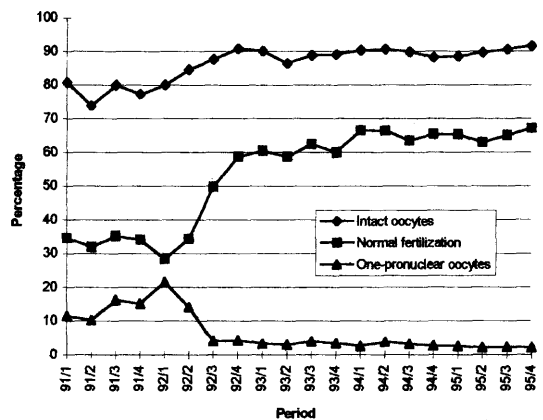


Figure 6. Percentages of intact, normally fertilized and activated oocytes after ICSI from January 1991 until December 1995. Each point represents the average results over a 3 month period.

originates from this period (Palermo *et al.*, 1992b). After the first promising results, more attempts were made to inject spermatozoa into the cytoplasm of the oocyte. It soon became evident that the oolemma did not always rupture immediately after penetration of the zona pellucida.

Figure 6 represents the results of ICSI from 1991 to 1995. Each point represents the mean results over a period of 3 months. All results are expressed as a percentage of the injected oocytes. The normal fertilization rate was ~35% during the first 18 months. A total of 114 oocytes were injected by ICSI in 123 cycles during the first 6 months. Many of these were accidental ICSI. In

all, 185 oocytes were injected intracytoplasmically in the third quarter and 370 in the fourth quarter of 1991 in a total of 141 cycles. Approximately 20% of the injected oocytes degenerated and 35% showed normal fertilization, which was approximately double the fertilization rate achieved by SUZI. Similar numbers of oocytes were injected in the first and second quarters of 1992 with similar results. During the following 6 months there was an increase in the number of intact oocytes, a decrease in the number of activated oocytes and a steep increase in the normal fertilization rate. In this period, a number of modifications were introduced. First the concentration of hyaluronidase used to remove the surrounding cumulus oophorus and corona radiata cells was reduced. Secondly, a motile spermatozoon was selected and immobilized for ICSI. Finally, if breakage had not occurred upon penetration of the zona pellucida, the oolemma was aspirated into the injection pipette until it ruptured. These three modifications will be discussed here in more detail.

Hyaluronidase is known to activate oocytes. This has been shown in the mouse (Kaufman, 1983) and in humans (Pickering *et al.*, 1988). Owing to the introduction of assisted-fertilization techniques such as ZD, PZD and SUZI, it became increasingly necessary to remove the cumulus and corona cells. Information about the concentration of hyaluronidase used and the time of exposure to the enzyme was often limited or even omitted. Different authors using PZD or SUZI reported different concentrations and different times of exposure to hyaluronidase. At the start of our assisted fertilization programme, we used hyaluronidase at a concentration of 760 IU/ml (Palermo *et al.*, 1993). This was reduced to 160 IU/ml when activation of oocytes was observed even before commencement of the injection procedure. At a later stage, a concentration of 80 IU/ml was used. The normal fertilization rate then increased and the number of activated oocytes decreased drastically.

Recently, we investigated whether a further reduction in the concentration of hyaluronidase could influence the exposure time to the enzyme and the intactness of the oocyte and its fertilization and embryo development after ICSI. We compared different concentrations of hyaluronidase on sibling

oocytes (Joris *et al.*, 1996). Cumulus removal was carried out in two steps, and a significantly longer incubation in hyaluronidase was recorded in medium containing only 10 IU hyaluronidase/ml. No significant differences in oocyte intactness, fertilization and activation rate and embryo development were found. However, a lower degeneration rate was observed at lower concentrations of the enzyme, which itself justifies the use of a lower concentration of hyaluronidase.

Although the majority of groups use a concentration of 80 IU hyaluronidase/ml, it has been suggested that use of a higher concentration leads to a comparable rate of formation of one-pronuclear oocytes (Payne, 1995; Payne and Matthews, 1995).

The second modification to the procedure was the immobilization of the spermatozoa that were selected for injection. For SUZI, only motile spermatozoa were selected for injection. For a short period, only immotile spermatozoa were selected when ICSI was attempted, to avoid degeneration of oocytes due to the injection of motile spermatozoa. This injection of immotile spermatozoa may partly explain the lower normal fertilization and higher activation rate during the first quarter of 1992, as a higher number of non-vital cells were probably selected for injection. Immobilization of motile spermatozoa is carried out as follows: a motile and if possible 'normal looking' spermatozoon is selected and aspirated into the injection pipette. It is gently expelled from the pipette and the sperm cell is placed perpendicular to the injection pipette, which is then moved over the tail of the spermatozoon. The pipette is lowered and the tail is fixed for a short time between the pipette and the bottom of the dish. For a proportion of spermatozoa, this action alone is sufficient to immobilize them, but other spermatozoa remain motile. The pipette is then moved over the tail in a short firm way until immobilization is achieved. It may be that the tail remains intact during this procedure, or it may show a breakage at one point. After immobilization, the spermatozoon is again aspirated, now tail-first, into the injection pipette.

The positioning of the injection pipette influences performance of immobilization. The optimal position for injection is with the bent part of the needle held completely horizontal. However, the

pipette may easily stick to the bottom of the dish, making manipulation of the spermatozoon rather difficult. It is therefore better to position the needle in such a way that only the tip touches the bottom of the dish first. The spermatozoon can then be immobilized using the tip of the needle and the injection procedure is not hampered.

The final modification in the procedure was the aspiration of cytoplasm to ensure the rupture of the cytoplasmic membrane. As more oocytes were injected by ICSI it became evident that the oolemma often did not rupture when the pipette was pushed through the zona pellucida deep into the oocyte. Attempts were then made to break the membrane by pushing the pipette in as far as possible. Where this also was insufficient, the injection needle was pulled back and pushed forward again in another position further away from the polar body. However, if penetration was attempted more than once, the shape of the oocyte sometimes became completely deformed.

In order to minimize damage to the oocyte, the next step was to try to inject the spermatozoon after breaking the membrane by aspiration of it into the injection pipette. This procedure proved to be successful.

Two of the changes introduced also influenced the success rates of other studies. A fertilization rate of 35.5% in 17 cycles increased to 59.9% in 24 cycles when the spermatozoon was immobilized (Gerris *et al.*, 1995). When a comparison was made of immobilizing spermatozoa by aspiration into the pipette until they stopped moving and squeezing the tail between the bottom of the dish and the injection pipette prior to injection into unfertilized oocytes from IVF, the second group showed a significantly higher fertilization rate (Van den Bergh *et al.*, 1995). Comparing results of ICSI with (i) sluggish motile spermatozoa, (ii) gentle immobilization, (iii) permanent immobilization or (iv) immotile spermatozoa, a higher rate of fertilization and also a better cleavage rate were achieved using permanent immobilization of the spermatozoa (Fishel *et al.*, 1995). A similar curve with a high increase in the fertilization rate (as shown in Figure 6) was found when the results of a 9-month period were analysed on a weekly basis (Svalander *et al.*, 1995). This increase in the

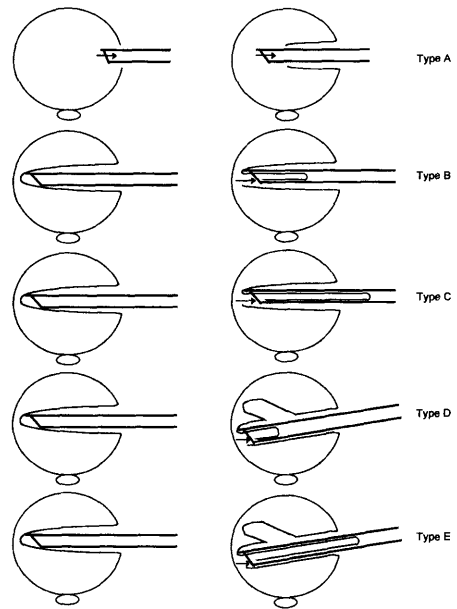


Figure 7. The five different types of membrane breakage. See text for more details.

success rate was due to better injection pipettes, injection of immobilized spermatozoa and aspiration of cytoplasm. Comparing the results of ICSI with or without immobilization of the spermatozoa and with or without aspiration of cytoplasm, the best results were obtained following immobilization and aspiration (Vanderzwalmen *et al.*, 1996). A 'vigorous' aspiration of cytoplasm also led to a higher fertilization rate than 'gentle' aspiration (Tesarik and Sousa, 1995). The volume aspirated was approximately 10× larger with vigorous aspiration than with gentle aspiration. However, a recent report (Mansour *et al.*, 1996) questions the necessity of cytoplasmic aspiration. Mansour *et al.* routinely injected without aspiration of cytoplasm and achieved high fertilization rates. A comparison of sibling oocytes injected with or without aspiration of cytoplasm showed no difference in fertilization.

Polyvinylpyrrolidone (PVP) has been used since the beginning of our assisted fertilization programme (Lanzendorf *et al.*, 1988). It facilitates manipulation of the spermatozoa and allows good control of the fluid in the injection pipette. Control of the volume injected into the oocyte is more difficult when PVP is not used (personal observa-

tion). However, similar results can be obtained without using PVP (Harari *et al.*, 1995).

Extensive training of staff before they treat patients is crucial. Even some centres which had had an assisted-fertilization programme and which switched from SUZI to ICSI did not immediately obtain good results (Gordts *et al.*, 1995; Vanderzwalmen *et al.*, 1996). Training in the injection procedure itself is stepwise. Initially, there are a few days of observation of the injections performed by experienced colleagues. The first active step is then to try to aspirate and immobilize spermatozoa. It is best to practise on a daily basis for not more than 1–2 h. One of the problems that have to be overcome is that the operator cannot see whether there is negative pressure, positive pressure or no pressure being applied by the injector. This can cause very strong aspiration or blowing of air. After some days of manipulating the spermatozoa, the injection of an oocyte can be attempted. At the start, 2-day-old unfertilized oocytes from IVF or ICSI are used. After injecting a number of these oocytes over a period of a few weeks, the trainee can start to inject 1-day-old unfertilized oocytes from IVF or in-vitro-matured oocytes. The results of these injections are evaluated and, when they are acceptable and constant over several weeks, the injection of fresh oocytes can commence. This starts with the injection of only two or three oocytes from patients who have at least 15 to 20 oocytes available for injection. When the results are constant, the number of oocytes injected can be increased gradually. All these steps are carefully monitored by experienced colleagues and all the results obtained are constantly evaluated to see whether the training curve shows the expected evolution. In our experience, it takes at least 3 months before new personnel feel comfortable performing the injection procedures independently. The presence of a camera on the microscope influences this training period because constant monitoring offers the possibility of receiving advice at any point in the procedure.

Certain aspects of the injection procedure related to the oocyte have also been studied. These included examining the position of the polar body at the time of injection and the reaction of the oocyte membrane to the introduction of the injection pipette.

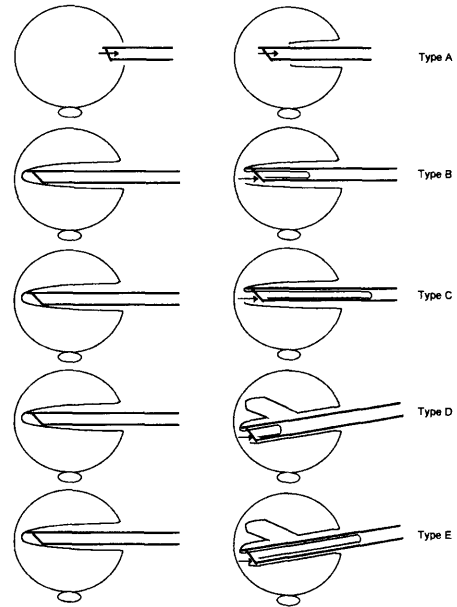


Figure 7. The five different types of membrane breakage. See text for more details.

To avoid damage to the meiotic spindle, oocytes were always injected with the polar body as far away from the injection site as possible. It must, however, be emphasized that the position of the polar body is not always a reliable guide to the location of the metaphase plate (Palermo *et al.*, 1995). For the injection procedure, the polar body was placed at 6 or 12 o'clock. To determine whether either of these two positions of the polar body influenced survival and fertilization of the oocyte and embryo development, they were compared in 100 ICSI cycles (Nagy *et al.*, 1995c). This study demonstrated that, although there was no difference in survival and fertilization relating to the position of the polar body, there was a difference in embryo development. Since it was shown that better cleavage occurred when the polar body was positioned at 6 o'clock, we always inject with the polar body fixed at the opening side of the injection pipette.

Different patterns of breakage of the oolemma following penetration by the injection pipette were found. The results of ICSI in 6860 oocytes were analysed after recording the mode of rupture of the oocyte membrane (Nagy *et al.*, 1995c). Five different types of membrane breakage were estab-

Table I. Summary of the results published by Palermo *et al.* (1996) and Nagy *et al.* (1995c) on the different patterns of breakage^a of the oolemma following penetration of the injection pipette. The results in **bold face** represent the results shown by the authors in their study. The results that are underlined are the results found to be significantly different by the respective group. The other data are our recalculations to make direct comparisons between the two studies

	Palermo <i>et al.</i> (1996)			Nagy <i>et al.</i> (1995c)		
	Sudden	Normal	Difficult	Sudden A	Normal B+C	Difficult D+E
Number of injected oocytes	297	2164	407	1401	5071	388
% of injected oocytes	11.8	73.9	14.3	20.4	73.9	5.7
% survival	<u>86.1</u>	95.7	97.1	<u>81.9</u>	92.3	88.2
2 PN						
% of injected oocytes	<u>57.4</u>	64.3	69.0	57.5	65.8	62.9
% of intact oocytes	<u>66.6</u>	67.1	71.0	70.1	71.3	71.7
1 PN						
% of injected oocytes	7.2	6.8	5.5	2.5	3.4	2.6
% of intact oocytes	8.4	7.1	5.7	3.8	3.1	3.3
3 PN						
% of injected oocytes	<u>6.1</u>	2.7	3.8	6.4	3.7	5.2
% of intact oocytes	<u>7.0</u>	2.9	3.9	<u>7.8</u>	4.1	5.7
Cleavage						
Day 2				75.8	77.2	72.9
Day 3	<u>74.8</u>	82.9	79.8			

^aNagy *et al.* divided types of membrane breakage into five groups (A–E), which we have correlated here to the definitions of Palermo *et al.* (sudden, normal and difficult breakage). See text and Figure 7 for more details.

lished and recorded (Figure 7). In type A, the oolemma ruptures during the insertion of the pipette and no aspiration is performed. In type B, slight suction to break the membrane is applied. The breakage occurs before the cytoplasm in the pipette reaches the level of the zona pellucida. Aspiration of cytoplasm further than the level of the zona pellucida is necessary in type C. In some cases, the oolemma does not break after strong aspiration, and breakage of the oolemma in another place is then attempted. This is classified as a type D injection if the oolemma ruptures after no aspiration or only minimal aspiration is necessary to break the membrane, or as type E if strong aspiration as described for type C is necessary. A similar study has been published (Palermo *et al.*, 1996) that describes three types of membrane breakage as sudden breakage, normal breakage and difficult breakage. The pattern described as sudden breakage can be compared with type A in our study, normal breakage with types B and C and difficult breakage with types D and E. The percentage of oocytes

that received what can be called ‘normal injection’ was exactly the same in both studies. There were somewhat more oocytes showing immediate breakage and fewer oocytes with a difficult injection reported in the study by Nagy *et al.* (1995c). This difference may be explained by the slightly different definitions of the types of membrane breakage presented in the two studies. Aspiration of the ooplasm until the membrane ruptures is performed in our centre, while repeated penetration until the rupture of the membrane occurs is described by Palermo *et al.* (1996). However, the latter workers also aspirate the cytoplasm to activate the oocyte before delivering the spermatozoon into it (Palermo *et al.*, 1995). Both groups found a significantly lower percentage of intact oocytes when the oolemma ruptured immediately upon injection. The percentages of fertilization achieved were presented in a different way in the two reports. Nagy *et al.* expressed fertilization as a percentage of intact oocytes while Palermo *et al.* presented it as a percentage of injected oocytes.

Both definitions are of importance. Expressing fertilization as a percentage of the intact oocytes addresses the fertilizing ability of all intact oocytes independently of the type of injection. In order to compare the two studies, their results have been recalculated and are presented in Table I. A significantly lower normal fertilization rate was found in the group of oocytes with sudden breakage by Palermo *et al.* (1996). When these results were expressed as a percentage of the intact oocytes, the difference from the other groups became much smaller. When expressed as a percentage of intact oocytes, the normal fertilization rates were quite similar for the three groups in both studies. There was no difference in the percentage of one-pronuclear oocytes in the three groups in either study. The percentage of oocytes showing three pronuclei after ICSI was significantly higher in both studies in the sudden-breakage group. It is not correct to compare the cleavage rates presented by the two studies, as these results were obtained from evaluations at different time points. However, when the sum of excellent, good and fair embryos in the two studies was compared, the totals were quite similar.

Both studies, performed completely independently, show very similar results and provide evidence that not only technical aspects but also the characteristics of the individual oocyte play a role in the chances for success in each ICSI cycle. The type of membrane damage is continuously recorded in our centre and can serve as a parameter when evaluating the cycle of the patient.

In conclusion, a number of technical aspects play an important role in the success of an ICSI cycle. The preparation of the holding, and certainly, of the injection pipette is of crucial importance. A good set-up and the correct use of the equipment facilitate the injection procedure. All aspects of the procedure require a lot of training and dedication. In addition to the technical parameters, oocyte characteristics also play their role and can influence the result of an ICSI cycle. Further development of the injection procedure and possible introduction of other tools may help to improve any failings related to technical failure or oocyte characteristics.

References

- Atlee, S., Pool, T. and Martin, J. (1995) A simple approach to intracytoplasmic sperm injection. *Fertil. Steril.*, **63**, 652–655.
- Cohen, J., Alikani, M., Malter, H. *et al.* (1991) Partial zona dissection or subzonal insertion: microsurgical fertilization alternatives based on evaluation of sperm and embryo morphology. *Fertil. Steril.*, **56**, 696–706.
- De Sutter, P., Dozortsev, D., Qian, C. *et al.* (1996) Oocyte morphology does not correlate with fertilization rate and embryo quality after intracytoplasmic sperm injection. *Hum. Reprod.*, **11**, 595–597.
- Fishel, S., Lisi, F., Rinaldi, L. *et al.* (1995) Systematic examination of immobilizing spermatozoa before intracytoplasmic sperm injection in the human. *Hum. Reprod.*, **10**, 497–500.
- Gerris, J., Mangelschots, K., Van Royen, E. *et al.* (1995) ICSI and severe male-factor infertility: breaking the sperm tail prior to injection. *Hum. Reprod.*, **10**, 484–486.
- Gordon, J., Grunfeld, J., Garisi, G. *et al.* (1988) Fertilization of human oocytes by sperm from infertile males after zona pellucida drilling. *Fertil. Steril.*, **50**, 68–73.
- Gordts, S., Vercruyssen, M., Roziers, P. *et al.* (1995) Recent developments in assisted fertilization. *Hum. Reprod.*, **10** (Suppl. 1), 107–114.
- Harari, O., Bourne, H., McDonald, M. *et al.* (1995) Intracytoplasmic sperm injection: a major advance in the management of severe male subfertility. *Fertil. Steril.*, **64**, 360–368.
- Joris, H., Nagy, Z., Bocken, G. *et al.* (1996) Comparison of different concentrations of hyaluronidase used to remove surrounding cells from oocytes for ICSI. *Abstract Book, 12th Annual Meeting of the European Society for Human Reproduction and Embryology.*
- Lanzendorf, S., Maloney, M., Veeck, L. *et al.* (1988) A preclinical evaluation of pronuclear formation by microinjection of human spermatozoa into human oocytes. *Fertil. Steril.*, **49**, 835–842.
- Liu, J., Nagy, Z., Joris, H. *et al.* (1995) Successful fertilization and establishment of pregnancies after intracytoplasmic sperm injection in patients with globozoospermia. *Hum. Reprod.*, **10**, 626–629.
- Nagy, Z.P., Liu, J., Joris, H. *et al.* (1995a) The result of intracytoplasmic sperm injection is not related to any of the three basis sperm parameters. *Hum. Reprod.*, **10**, 1123–1129.
- Nagy, Z.P., Verheyen, G., Liu, J. *et al.* (1995b) Results of 55 intracytoplasmic sperm injection cycles in the treatment of male-immunological infertility. *Hum. Reprod.*, **10**, 1775–1780.
- Nagy, Z.P., Liu, J., Joris, H. *et al.* (1995c) The influence of the site of sperm deposition and mode of oolemma breakage at intracytoplasmic sperm injection on fertilization and embryo development rates. *Hum. Reprod.*, **10**, 3171–3177.
- Kaufman, M. (1983) *Early Mammalian Development: Parthenogenetic Studies.* Cambridge University Press, Cambridge.
- Mansour, R., Aboulghar, M., Serour, G. *et al.* (1996)

- Successful intracytoplasmic sperm injection without performing cytoplasmic aspiration. *Fertil. Steril.*, **66**, 256–259.
- Palermo, G., Joris, H., Devroey, P. *et al.* (1992a) Induction of acrosome reaction in human spermatozoa used for subzonal insemination. *Hum. Reprod.*, **7**, 248–254.
- Palermo, G., Joris, H., Devroey, P. *et al.* (1992b) Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet*, **340**, 17–18.
- Palermo, G., Joris, H., Derde, M.P. *et al.* (1993) Sperm characteristics and outcome of human assisted fertilization by subzonal insemination and intracytoplasmic sperm injection. *Fertil. Steril.*, **59**, 826–835.
- Palermo, G.D., Cohen, J., Alikani, M. *et al.* (1995) Intracytoplasmic sperm injection: a novel treatment for all forms of male-factor infertility. *Fertil. Steril.*, **63**, 1231–1240.
- Palermo, G.D., Alikani, M., Bertoli, M. *et al.* (1996) Oolemma characteristics in relation to survival and fertilization patterns of oocytes treated by intracytoplasmic sperm injection. *Hum. Reprod.*, **11**, 172–176.
- Payne, D. (1995) Intracytoplasmic sperm injection: instrumentation and injection technique. *Reprod. Fertil. Dev.*, **7**, 185–196.
- Payne, D. and Matthews, C.D. (1995) Intracytoplasmic sperm injection—clinical results from the reproductive medicine unit, Adelaide. *Reprod. Fertil. Dev.*, **7**, 219–227.
- Pickering, S., Johnson, M., Braude, P. *et al.* (1988) Cytoskeletal organization in fresh, aged and spontaneously activated human oocytes. *Hum. Reprod.*, **3**, 978–989.
- Pickering, S., Braude, P., Johnson, M. *et al.* (1990) Transient cooling to room temperature can cause irreversible disruption of the meiotic spindle in the human oocyte. *Fertil. Steril.*, **54**, 102–108.
- Svalander, P., Forsberg, A., Jakobsson, A. *et al.* (1995) Factors of importance for the establishment of a successful programme of intracytoplasmic sperm injection treatment for male infertility. *Fertil. Steril.*, **63**, 828–837.
- Tesarik, J. and Sousa, M. (1995) Key elements of a highly efficient intracytoplasmic sperm injection technique: Ca^{2+} fluxes and oocyte cytoplasmic dislocation. *Fertil. Steril.*, **64**, 770–776.
- Van den Bergh, M., Bertrand, E., Biramane, J. *et al.* (1995) Importance of breaking a spermatozoon's tail before intracytoplasmic injection: a prospective randomized trial. *Hum. Reprod.*, **10**, 2819–2820.
- Vanderzwalmen, P., Bertin, G., Lejeune, B. *et al.* (1996) Two essential steps for a successful intracytoplasmic sperm injection: injection of immobilized spermatozoa and rupture of the oolemma. *Hum. Reprod.*, **11**, 540–547.
- Van Steirteghem, A.C., Nagy, Z., Joris, H. *et al.* (1993) High fertilization and implantation rates after intracytoplasmic sperm injection. *Hum. Reprod.*, **8**, 1061–1066.
- Van Steirteghem, A.C., Joris, H., Liu, J. *et al.* (1995) Protocol for intracytoplasmic sperm injection. *Hum. Reprod. Update*, **1**, no. 3, CD-ROM.
- Van Steirteghem, A.C., Nagy, P., Joris, H. *et al.* (1996) The development of intracytoplasmic sperm injection. *Hum. Reprod.*, **11** (Suppl. 1), 59–72.