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Intracytoplasmic sperm injection: a state of the art technique

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Of the micromanipulation techniques developed in the twentieth century, intracytoplasmic sperm injection (ICSI) has been the major breakthrough in the field of assisted fertilization. This article reviews the indications for the use of ICSI, its clinical application, the establishment of an ICSI programme including protocol and the results obtained since the introduction of ICSI and the potential risks. In addition, intracytoplasmic spermatid injection is briefly discussed.

Key words: intracytoplasmic sperm injection/in-vitro fertilization

Introduction

This year is the twentieth anniversary of the first human baby resulting from in-vitro fertilization (IVF) and embryo transfer (Steptoe and Edwards, 1978). During the past 20 years, many evolutionary changes have occurred in ovulation induction, the shift of oocyte retrieval from the laparoscopic to the transvaginal route, oocyte and embryo culture techniques, the cryopreservation of embryos, and the technique of embryo transfer. A notable event in the field of assisted reproduction has been the development of different techniques to assist fertilization.

Working with human gametes and IVF for the past 15 years, no technique has fascinated me more than intracytoplasmic sperm injection (ICSI; Palermo *et al.*, 1992), which was a real breakthrough in the field of assisted fertilization, especially with regard to the problem of male factor infertility. Moreover, it brought the embryologist into closer contact with the oocyte and the spermatozoon, as each gamete is manipulated individually. It also gave the embryologist the confidence to work within the cytoplasm of the oocyte, which is not necessary during conventional IVF work. This has opened the door to a wide field of research on the oocyte. This article is a review of ICSI with special emphasis on indications for its use, its technique, and results.

History

The advent of IVF–embryo transfer enabled patients who were irremediably infertile due to absolute tubal factor to conceive (Edwards *et al.*, 1980). However, a considerable number of patients remained unable to achieve pregnancy through IVF mainly due to failure of fertilization. Therefore, various micromanipulation techniques have been developed to assist fertilization. These techniques originated from a pre-existing technology of micromanipulation that was developed for various purposes other than assisted fertilization.

From the beginning of the twentieth century scientists have been trying to develop various techniques for direct manipulation of living cells under the microscope. In 1911, Kite discussed a nuclear dissection experiment in which he used micro-needles to separate the pronuclei in newly fertilized zygotes (Chambers, 1940). In 1928, Emmerson described the first micromanipulator with a joystick design that could directly transmit the movements required by the operator (El Badry, 1963). In 1934, de Fonbrune described hydraulic principles that could be used for the joystick manipulators. de Fonbrune (1934) also developed the first microforge and microtool-making techniques that are still in use today. Micromanipulation techniques have gradually developed over the century and have reached such a precision that skilled scientists are able to

microdissect one single chromosome. The micromanipulation techniques that were developed to assist fertilization include partial zona dissection (PZD), subzonal sperm injection (SUZI) and intracytoplasmic sperm injection (ICSI).

The principal breakthrough in the field of assisted fertilization was ICSI. Interestingly enough, this technique was developed as early as 1966 for experiments with non-mammalian gametes. At that time, it was not performed for the purpose of assisted fertilization, but to prove that sperm nuclear decondensation and male pronuclear formation did not require prior interaction between the spermatozoon and the oocyte membranes (Hiramoto, 1966). Several experiments followed, and improvements in microinjection using a rabbit model not only led to pronuclear formation following ICSI-induced fertilization, but also resulted in further embryonic cleavage and the birth of normal, live offspring (Hosoi *et al.*, 1988; Iritani, 1989). Direct ICSI to human oocytes was first applied by Lanzendorf *et al.* (1988a). Their experiments demonstrated that human oocytes were capable of surviving microinjection and were subsequently able to support the formation of male and female pronuclei. Finally, the report by Palermo *et al.* (1992) of the first human pregnancies following ICSI represented a major breakthrough in the field of assisted fertilization.

Clinical application of ICSI

ICSI has been widely and successfully used to treat infertility due to severe oligoasthenozoospermia (Palermo *et al.*, 1993; Van Steirteghem *et al.*, 1993b; Payne *et al.*, 1994; Redgment *et al.*, 1994; Mansour *et al.*, 1995). It enabled men with very few living spermatozoa (even occult azoospermia) to achieve fertilization and parenthood, and it has proven to be the most efficient treatment for male factor infertility (Van Steirteghem *et al.*, 1993b; Palermo *et al.*, 1995).

The use of ICSI in patients with borderline semen was studied by Aboulghar *et al.* (1995). A significantly higher fertilization rate was achieved with ICSI than with conventional IVF using sibling oocytes and spermatozoa from patients with borderline semen or suspected male factor infertility. The study found that 45% of patients with borderline semen would have lost their chance of embryo transfer because of total fertilization failure had ICSI not been performed on some sibling oocytes. Use of ICSI in patients with suspected male factor infertility was also previously recommended by Payne *et al.* (1994).

Unexplained infertility treated with conventional IVF may result in total failure of fertilization. Aboulghar *et al.* (1996a) achieved a significantly higher fertilization rate using ICSI, compared to conventional IVF, with sibling oocytes in cases of unexplained infertility. Their study showed that ~23% of patients with unexplained infertility would have had total fertilization failure if ICSI had not been performed in some sibling oocytes.

In cases of previous total failure of fertilization with conventional IVF, the first line of treatment is ICSI. It has been shown that a couple who fails to achieve fertilization following a single IVF treatment has a <25% chance of fertilization when conventional IVF is attempted again (Cohen *et al.*, 1994). In contrast, when Palermo *et al.* (1993) performed ICSI for 38 couples with previous total failure of fertilization in conventional IVF, they achieved a high pregnancy rate. Also, one study (Lundin *et al.*, 1996) reported that ICSI can be applied on 1-day-old oocytes in cases of fertilization failure after IVF, leading to two pregnancies and deliveries.

ICSI has also proved to be of value in the treatment of patients with male immunological infertility (Nagy *et al.*, 1995a) or with acrosomeless spermatozoa (Lanzendorf *et al.*, 1988b; Lundin *et al.*, 1994; Bourne *et al.*, 1995a). Although pregnancy and birth have been reported following fertilization with totally immotile spermatozoa (Stalf *et al.*, 1995; Kahraman *et al.*, 1996a; Nijs *et al.*, 1996), the fertilization rate is very low. Microinjection of completely immotile spermatozoa is an adverse prognostic factor (Nagy *et al.*, 1995c). The use of hypo-osmotic tests (Casper *et al.*, 1996) or fertilization with testicular spermatozoa (Tournaye *et al.*, 1996) have been applied to this problem.

The introduction of ICSI has made fertilization efficient and precise, to the extent that a very low number of spermatozoa, regardless of their motility pattern, is required to inject all the oocytes retrieved for the procedure. Therefore, ICSI is the technique of choice in cases of obstructive azoospermia since it enables the best use of microsurgically retrieved spermatozoa from the epididymis or testis (Schoysman *et al.*, 1993a,b; Silber *et al.*, 1994, 1995a; Tournaye *et al.*, 1994; Mansour *et al.*, 1996a).

Microsurgical epididymal sperm aspiration (MESA) was the method first described for surgical retrieval of spermatozoa in cases of obstructive azoospermia (Temple-Smith *et al.*, 1985). High fertilization and pregnancy rates have been reported after the combination of MESA and ICSI (Silber *et al.*, 1994; Tournaye *et al.*, 1994; Hovatta *et al.*, 1995; Mansour *et al.*, 1996a). However, the technique is lengthy, requires general anaesthesia, special skills and equipment, and results in a certain amount of trauma and postoperative morbidity. For these reasons, percutaneous epididymal sperm aspiration

(PESA) was introduced (Craft *et al.*, 1995). Compared to MESA, this procedure is very simple and can be performed under local anaesthesia. However, the necessary blind puncturing of the delicate and very vascular epididymis has been strongly criticized as a potentially mutilating procedure (Schlegel *et al.*, 1994). In cases of congenital absence of the vas deferens and in some cases of acquired obstruction, PESA can be attempted (Craft *et al.*, 1995; Collins *et al.*, 1996; Tsirigotis *et al.*, 1996; Mansour *et al.*, 1997a), or a modified PESA technique (Cha *et al.*, 1997).

When the epididymis is absent or fibrosed, a testicular biopsy can be performed. Until recently, the fertilizing capacity of testicular spermatozoa was unexplored. However, it was reported in 1993 that the use of testicular spermatozoa in ICSI for cases of obstructive azoospermia could achieve fertilization and pregnancy (Craft *et al.*, 1993; Schoysman *et al.*, 1993a,b). Following that, other different programmes reported the use of testicular spermatozoa with ICSI (Devroey *et al.*, 1994; Bourne *et al.*, 1995b; Craft and Tsirigotis, 1995; Nagy *et al.*, 1995b; Silber *et al.*, 1995a; Fahmy *et al.*, 1996; Mansour *et al.*, 1996a). Recently, the indications for testicular sperm extraction (TESE) and ICSI have been expanded to include cases with non-obstructive azoospermia due to severe impairment of spermatogenesis (Devroey *et al.*, 1995; Tournaye *et al.*, 1995; Gil-Salom *et al.*, 1995; Kahraman *et al.*, 1996b; Silber *et al.*, 1996; Mansour *et al.* 1997a; Tournaye *et al.*, 1997).

Because sperm retrieval from either the epididymis or the testis is a complex procedure, it is important to cryopreserve supernumerary spermatozoa for future use. Pregnancies were first reported from the use of cryo-thawed epididymal sperm by Lacham-Kaplan and Trounson (1994). Other cases of successful ICSI using cryo-thawed epididymal spermatozoa have also been reported recently (Nagy *et al.*, 1995b; Kamal *et al.*, 1997). However, freezing of testicular spermatozoa is generally difficult because of the low number of spermatozoa and the very poor motility (Silber *et al.*, 1994). Methods of testicular recovery, processing and cryopreservation have been refined and successful fertilization and pregnancies have resulted from the use of cryo-thawed testicular spermatozoa with ICSI (Nagy *et al.*, 1995b; Gil-Salom *et al.*, 1996; Podsiadly *et al.*, 1996; Kamal *et al.*, 1997). Recently, it has been reported that a low number of spermatozoa can be successfully recovered after thawing by cryopreserving them inside an empty zona pellucida (Cohen *et al.*, 1997).

In cases of failure of semen collection on the day of ovum retrieval and when no cryopreserved semen sample is available, testicular sperm aspiration and ICSI is a possible solution (Watkins *et al.*, 1996).

ICSI has also been used to fertilize cryo-thawed mature oocytes. Gook *et al.* (1995) and Kazem *et al.* (1996) suggested that fertilization and cleavage rates of cryo-thawed oocytes can be enhanced significantly using ICSI. Also, their data suggest that the cryopreservation technique can affect the zona pellucida deleteriously, hindering normal sperm attachment and penetration, and hence ICSI is recommended.

In-vitro maturation of immature oocytes was recently applied clinically and it was found that ICSI was the technique of choice to achieve fertilization of these oocytes (Cha *et al.*, 1996; Russell *et al.*, 1996; Jones and Trounson, 1997).

Another possible indication for the use of ICSI may be abnormal oocytes. The technique of ICSI has revealed an under-estimated 'oocyte factor' as a cause of infertility. Complete removal of cumulus–corona cells from around the oocyte and its close observation under ×400 magnification during the ICSI procedure allow a better estimation of oocyte quality. In a review of 1000 ICSI cycles, it was found that ~6.6% of all cycles were diagnosed to have abnormal morphology of all retrieved oocytes, representing 5.7% of all oocytes injected (Mansour *et al.*, 1997b). It was observed that some oocytes were morphologically mature but had a structurally abnormal zona pellucida: either the sperm receptor was defective or the zona matrix was hindering sperm passage (Cohen, 1992). High fertilization and pregnancy rates were achieved by ICSI in patients with persistent failure of IVF–embryo transfer associated with disordered zona pellucida-induced acrosome reaction (Liu *et al.*, 1997). However, oocytes with abnormalities in the cytoplasm may not benefit from the technique of ICSI: a study of ICSI in dysmorphic oocytes (Alikani *et al.*, 1995) concluded that aberrations in the morphology of human oocytes (most probably a product of controlled ovarian stimulation) were of little or no consequence to fertilization or early cleavage after ICSI, but it is possible that these embryos had reduced potential for implantation and future development (Alikani *et al.*, 1995).

It has even been suggested that ICSI should completely replace conventional IVF in the future, as pregnancy rates will be improved if normal spermatozoa are injected. However, it is currently recommended that, until more data are available, ICSI should be restricted to cases of male factor infertility (Hamberger *et al.*, 1995). To investigate the results of conventional IVF versus ICSI using normal semen, Aboulghar *et al.* (1996b) carried out a prospective randomized study on 116 infertile patients with tubal factor infertility (and normal semen) that were randomly divided to receive ICSI or IVF. The results demonstrated that ICSI does not achieve a higher pregnancy rate compared with IVF in the treatment of cases of tubal factor infertility with

normal semen. ICSI is a technique which is more expensive and time consuming, requires more equipment, extra skills, and is also an invasive procedure.

Establishing a laboratory for ICSI

An already existing successful IVF programme is a prerequisite for the success of any ICSI programme, as all the equipment, environmental requirements and skills in gamete handling that are needed for oocyte and embryo culture *in vitro* are essential to the establishment of an ICSI programme. H.D.Schmidt (1859) made a highly relevant comment on the use of his microscopic dissector (a very early version a micromanipulator): 'To manage the instrument successfully, delicacy of touch and a great deal of patience are required; but it is only the latter, combined with perseverance, energy, and close observations that scientific facts have, or ever will be established'.

The equipment required for ICSI comprises an inverted phase microscope and a micromanipulation set. Microscopes used for gamete micromanipulation should be equipped with ×10, ×20 and ×40 objectives, and for precise procedures such as ICSI, ×400 magnification is required. A steady stage is essential for micromanipulation, rendering inverted microscopes the most suitable. Also, the microscope should have a long working distance condenser. In general, the microscope used for micromanipulation should be of the highest available quality. The pseudo-three-dimensional image provided by the Normarski or Hoffman optical systems offers special image enhancement that is of great value in these intricate procedures (Malter, 1992). The Normarski system, designed for an optical path through glass only, does not produce clear images when plastic containers are used. The Hoffman system, however, is designed to compensate for the effect of plastic.

Most of the micromanipulation systems currently in use today combine some of the best design elements from previous micromanipulators. Based on de Fonbrune's system, three hydraulic cylinders can achieve simultaneous three-dimensional remote-controlled positioning, and the convenient three-dimensional control of the joystick is combined with the precise linear control of a separate single-axis unit. The hydraulic drive is mounted on separate micromanipulators that provide three-dimensional coarse positioning. There are two options for coarse positioning, the first being a simple mechanical device and the second an electric joystick remote-control to drive high-precision electric motors. This provides a very convenient and versatile system in which all the necessary controls are at the operator's fingertips (Malter, 1992).

The basic design for the microinjection unit has changed very little since Chamber's original device (Chambers and Chambers, 1961), which involves the use of airtight glass syringes under the control of different microdrives. The performance may be enhanced by substituting high-quality syringes that are completely airtight. For connection to the micropipette holder, flexible plastic tubing with airtight fitting is required.

It is important to fit the microscope stage with a form of temperature control. Ambient temperature is of vital importance for the survival of the oocyte, which should not be exposed to temperature change for even a brief period during micromanipulation.

Microtools are very sensitive to ambient vibration, and therefore a heavy balance table is required. Bolting the table to the floor or wall, or using massive support, such as a granite balance table, may be sufficient.

Several companies manufacture equipment for fabrication of microtools (micropipettes for injection and holding), making work much easier and saving a lot of effort and time.

Table I. Overall intracytoplasmic sperm injection results (from Tarlatzis, 1996, and de Mouzon and Lancaster, 1997)

Year	No. of cycles	Pregnancy rate per retrieval (%)	Take-home baby rate (%)
1993	3 157	23.6	–
1994	12 586	21.8	–
1995	47 654	21.7	15.9

The technique of ICSI

Preparation of the oocytes

To enable retrieval of sufficiently mature oocytes, an interval of 36 h between human chorionic gonadotrophin administration and ovum retrieval has been shown to be optimum (Mansour *et al.*, 1994).

For better visualization and precise intracytoplasmic injection of the oocyte, the surrounding cumulus granulosa cells must be removed. Usually, this is done ~3 h after oocyte retrieval. The oocyte cumulus–corona complex is placed in 80 mIU/ml hyaluronidase in HEPES-buffered tissue culture medium for a maximum of 10–15 s, during which it is repeatedly pipetted in a standard pipette. The oocytes are then transferred to tissue culture medium for complete removal of the corona cells by repeated aspiration in a finely pulled pipette. The oocytes are then rinsed and incubated till the time of microinjection and those that have extruded their first polar body are chosen for the injection.

Sperm processing

Ejaculated semen is processed for ICSI using different techniques. Samples that are not severely oligoasthenozoospermic are usually processed with a simple washing and swim-up technique. A method based on the migration sedimentation phenomenon of spermatozoa, using a Tea tube (Lucena *et al.*, 1989), has also been used successfully. Severe oligoasthenozoospermic samples have to be handled with care, and a second semen sample should be obtained where possible in most cases of severe oligoasthenozoospermia. In our experience, even very poor samples can be processed using a Tea tube, and the few motile spermatozoa can be collected by aspirating a microdroplet from the bottom of the central cone if sufficient time (4–5 h) is available (Mansour *et al.*, 1995). If no motile spermatozoa are found in the central cone, a microdroplet from the resuspended pellet itself is placed in the injection dish near the polyvinylpyrrolidone (PVP) droplet and motile spermatozoa are transferred with the injecting pipette to the PVP droplet. Similarly, 1–2 μl of the resuspended pellet could be placed with care inside the PVP droplet at the periphery (at the side where the holding pipette goes) without producing turbulence. The debris and immotile spermatozoa will remain in place while the motile spermatozoa will spread through the PVP droplet and can be picked up easily with the injecting pipette. Fujii *et al.* (1997) have described another simple method for recovering motile spermatozoa from extremely low quality sperm samples, in which the sperm pellet is deposited into a 3% PVP solution, allowing the motile sperm to swim out, away from the debris and immotile spermatozoa. We have avoided the use of Percoll for semen processing, hence avoiding injection of silica particles inside the oocytes. However, the majority of ICSI centres use Percoll for sperm processing.

When testicular spermatozoa are to be used, the testicular biopsy is macerated in a petri dish under the dissecting microscope using either sterile glass slides or two needles. The macerated tissue is then incubated in tissue culture medium prior to use. The contents of the tube are then mixed and allowed to settle for 1 min so that the large particles can settle and be removed. When samples are from cases of obstructive azoospermia, the tube is allowed to settle for an additional 10 min, after which sufficient spermatozoa can be aspirated from the bottom of the tube. When samples are from cases of non-obstructive azoospermia, the tubes are centrifuged at 400 *g* for 7–10 min and the pellet is resuspended in a very small volume. Any excess testicular spermatozoa should be cryopreserved for possible future use.

Preparation of the microinjection dish

Several microdroplets (5–10 μl) of HEPES-buffered medium are placed in the injection dish and covered with mineral oil. For one microdroplet, this medium is replaced by PVP (molecular weight 360 000), a very viscous solution that permits the spermatozoon to be controlled inside the microinjection pipette, and also prevents its adhesion to the pipette. The use of PVP also allows the three-dimensional motility patterns of the spermatozoon to be carefully observed (Cohen *et al.*, 1994). However, the injection can be performed without PVP. Kuczynski *et al.* (1996) compared the use of PVP and HEPES-buffered media for sperm immobilization in a prospective controlled study and concluded that PVP may have a negative impact on fertilization rate, embryo quality and blastocyst formation. Other investigators have also raised concerns about the injection into the oocyte of such a potentially harmful agent (Feichtinger *et al.*, 1995; Jean *et al.*, 1996). However, the majority of ICSI programmes continue to use PVP.

One microdroplet of sperm suspension is placed at the periphery of the PVP droplet and the oocytes are positioned individually in the surrounding droplets. The number of oocytes to be manipulated at one time in the injection dish varies according to the experience of the ICSI operator, to minimize the period of their exposure to unsuitable conditions outside the incubator.

Sperm selection and immobilization before injection

The tip of the microinjection pipette is lowered into the PVP solution, which is then aspirated. The pressure is controlled to be in a neutral position. A single living spermatozoon is chosen on the basis of its morphology. At a magnification of ×400 this selection is difficult to perform on motile spermatozoa. However, selection of normal-appearing spermatozoa can be achieved to a certain extent by observing shape, light reaction and motion patterns (Cohen *et al.*, 1994). The type of motility does not matter as long as the spermatozoon demonstrates slight twitching movements, a sign of viability. Other sperm parameters do not affect fertilization and pregnancy rates, or the outcome of pregnancy, providing a motile spermatozoon that appears morphologically normal is used for the injection (Mansour *et al.*, 1995; Nagy *et al.*, 1995c). The spermatozoon should be completely immobilized by touching the tail near the mid piece, and rubbing the tail with the pipette against the bottom of the dish. This step is believed to be important for oocyte activation, as damage of the sperm plasma membrane is important for the release of sperm cytosolic components, factors involved in oocyte activation (Fishel *et al.*, 1995a; Palermo *et al.*, 1996a). Fishel *et al.* (1995a) demon-

strated a highly significant increase in fertilization rate following ICSI when the sperm tail plasma membrane was damaged. Palermo *et al.* (1996) also reported a significantly higher fertilization rate following aggressive damage to the sperm tail. The immobilized spermatozoon is aspirated tail first into the injection pipette and the pressure is controlled so the spermatozoon advances very slowly and is finally positioned near the tip of the pipette. Differences in the consistency of PVP and culture medium must be taken into account when this procedure is performed.

Positioning of the oocyte for the injection

The oocyte is held in position by applying minimal suction on the holding pipette. It is first rotated gently using the injecting and holding pipettes to locate the polar body and position it at 6 or 12 o'clock. This position has been used since the original work on ICSI (Palermo *et al.*, 1992) since it enables avoidance of damage to the spindle. A recent study by Blake *et al.* (1996) demonstrated that the best result was obtained when the polar body was at the 8 o'clock position. They concluded that the spermatozoon should be adjacent to, but not inside, the spindle area after injection. Further, the distal region of the spermatozoon should not face the contralateral oolemma of the polar body.

Injection of the oocyte

Before microinjection, the pipette is advanced towards the zona. It is very important at this point to ensure that the equatorial plane of the oocyte, the internal opening of the holding pipette and the tip of the microinjection pipette are all in the same focal point. The microinjection pipette, which contains the spermatozoon near the tip, is introduced slowly into the oocyte after piercing the zona pellucida, which is usually very easy to traverse. The oolemmal membrane is stretched by the advancing microinjection pipette. Great care must be taken when piercing the oolemma to avoid releasing the spermatozoon into the furrow made by the microinjection pipette, outside the cytoplasm. Piercing of the oolemmal membrane is observed when the pulled membrane suddenly yields, accompanied by a kinetic turbulence of the cytoplasm around the pipette (Mansour *et al.*, 1996b). Occasionally, the oolemmal membrane is not pierced, even when the tip of the injection pipette is near the 9 o'clock position. In this situation, the injecting pipette can be withdrawn slowly to the centre of the oocyte and then readvanced in a slightly upwards or downwards direction. The technique performed by most centres to ensure piercing of the membrane involves the application of minimal suction which is stopped as soon as the cytoplasm is observed flowing at the tip of the pipette. Then the spermatozoon is deposited slowly, in the least possible volume, and the pipette is withdrawn gradually. After injection, the oocytes are rinsed and incubated under oil.

Different patterns of oolemmal response to the injecting pipette have been described by Palermo *et al.* (1996b) and include normal breakage when the injection needle creates an invagination that ruptures at the approximate centre of the egg, sudden breakage when the membrane breaks without creating a funnel, and difficult breakage when the membrane does not break or breaks only after several attempts. These recorded patterns appeared to be predictive of the survival and fertilization ability of the injected oocytes, as well as of the incidence of digyny.

Cytoplasmic aspiration has been considered to be an integral part of the ICSI procedure and an essential step for oocyte activation (Palermo *et al.*, 1995). Vigorous aspiration of the oocyte cytoplasm has been reported to be a crucial factor for the success of ICSI (Tesarik and Sousa, 1995). However, in a controlled prospective study on sibling oocytes, the effects of cytoplasmic aspiration and non-aspiration on the rate of oocyte damage, fertilization, and embryo quality were compared (Mansour *et al.*, 1996b). Cytoplasmic aspiration before sperm injection was not essential for oocyte activation, as it increased the rate of oocyte damage and it did not improve the fertilization rate.

Results of ICSI

Thanks to the effort of both the International Working Groups for Registers on Assisted Reproduction, and the ESHRE Task Force on ICSI, we now have enough available data to evaluate the efficacy of this newly introduced line of treatment. Unfortunately this data may have potential limitations, due to its heterogeneity, as there were differences between centres with regard to experience, technique, the number of cycles performed and the amount of information recorded (Tarlatzis, 1996). However, the data are of utmost importance in providing information to evaluate the efficacy of the treatment at an early stage.

The number of ICSI cycles using ejaculated, epididymal and testicular spermatozoa increased significantly from 3157 cycles in 1993, to 12 586 cycles in 1994, and to 47 654 cycles in 1995 (Tarlatzis, 1996; de Mouzon and Lancaster, 1997). This represents a significant increase in interest worldwide in the use of this new technique in the field of male infertility. The overall clinical pregnancy rates per oocyte retrieval were 23.6% for 1993, 21.8% for 1994 and 21.7% for 1995 (Tarlatzis, 1996; de Mouzon and Lancaster, 1997). The take-home baby rate was 15.9% in 1995 (de Mouzon and Lancaster, 1997; Table I).

The results of ICSI, using either ejaculated, epididymal, or testicular spermatozoa, did not differ significantly. It is of interest to mention that, when ejaculated spermatozoa were used, different sperm parameters did not affect the fertilization rate (Mansour *et al.*, 1995; Nagy *et al.*, 1995c; Oehninger *et al.*, 1995; Palermo *et al.*, 1995). The fertilization rate after ICSI using ejaculated spermatozoa ranged from 60.7 to 76% (Cohen *et al.*, 1994; Payne *et al.*, 1994; Nagy *et al.*, 1995b; Oehninger *et al.*, 1995; Mansour *et al.*, 1996d; Palermo *et al.*, 1996a; Schoolcraft *et al.*, 1996; Vanderzwalmen *et al.*, 1996; Table II).

Moreover, the pregnancy outcome does not seem to be influenced by the source of spermatozoa, with most pregnancies (67–73%) being viable (Tarlatzis, 1996). The incidence of multiple pregnancies has also increased, from 29 to 35% (Tarlatzis *et al.*, 1996), denoting the production of good quality embryos and indicating the need to reduce the number of embryos per transfer. The incidence of ectopic pregnancies reported after ICSI was 1.7% (Tarlatzis, 1996) and 1.2% (de Mouzon and Lancaster, 1997), significantly lower than the rates of 4.3 and 3.6% reported after regular IVF (de Mouzon and Lancaster, 1995, 1997). This can be explained by the fact that most women undergoing ICSI have normal Fallopian tubes.

It has been shown that, in assisted reproductive technology, ongoing pregnancy rates decrease significantly with advancing age (FIVNAT, 1990). In women older than 40 years, the delivery rate is extremely low (Greenhall and Vessey, 1990; Lansac, 1995). Most probably, the decrease in implantation rates with advancing age is related more to poor oocyte quality rather than to uterine function (Navot *et al.*, 1991). Also, the number of good quality embryos is significantly higher in women aged <40 years, and more embryos are available for replacement (Devroey *et al.*, 1996). A number of studies have demonstrated clearly that advancing female age is a negative prognostic factor (Oehninger *et al.*, 1995; Rosenwaks *et al.*, 1995; Mansour *et al.*, 1996d).

Potential risks of ICSI

The fact that intracytoplasmic injection is an invasive procedure should always be borne in mind. The potential risks of many factors have been debated: (i) since the natural selection of spermatozoa is bypassed, a defective spermatozoon may be injected, and Cummins and Jequier (1994) have warned of the existence of cryptic genetic defects in cases of severe male factor infertility and the consequent risks of micro-assisted fertilization; (ii) there is a possibility of introduction of unknown foreign material into the cytoplasm during the injection, e.g. PVP, debris, oil, traces of Percoll; (iii) there is a possibility of oocyte damage during the injection due to meiotic spindle damage.

The frequency of chromosomal anomalies has been shown to be increased in infertile males (Kjessler, 1974; Chandley, 1995; Yoshida *et al.*, 1995). It has been suggested that there is an association between male infertility, ICSI using aneuploid spermatozoa and the development of offspring with aneuploidy, especially aneuploidy of the sex chromosomes (Persson *et al.*, 1996). With the increasing use of ICSI with testicular spermatozoa in cases of non-obstructive azoospermia, there might be a greater risk of sex chromosome aneuploidy because of the chromosomal nature of the underlying pathology. Another concern about ICSI is the use of epididymal or testicular spermatozoa from men with bilateral congenital absent vas deferens (BCAVD). This has major genetic implications because ICSI using spermatozoa from BCAVD males is likely to result in an increased frequency of cystic fibrosis in the offspring, in comparison with the frequency within the population at large (Chillon *et al.*, 1995; Silber *et al.*, 1995; Persson *et al.*, 1996). It has also been documented that a significant number of azoospermic men have translocation defects affecting the long arm of the Y chromosome (Hendry *et al.*, 1976). Therefore, clinicians and scientists providing ICSI for infertile men must be aware of the genetic implications of male factor infertility.

Table II. Results of intracytoplasmic sperm injection using ejaculated spermatozoa

Reference	No. of cycles	Fertilization injected oocyte (%)	Pregnancy rate (%) per embryo transfer
Palermo *et al.* (1996a)	756	71.5	43.9
Schoolcraft *et al.* (1996)	71	60.7	56
Vanderzwalmen *et al.* (1996)	740	63	29
Mansour *et al.* (1996d)	650	61	30.5
Nagy *et al.* (1995b)	965	70	30
Oehninger *et al.* (1995)	102	60.9	31.9
Cohen *et al.* (1994)	227	59.6	44.1
Payne *et al.* (1994)	100	67	32

Table III. Results of intracytoplasmic sperm injection using fresh epididymal spermatozoa

Reference	No. of cycles	2PN fertilization rate per injected oocyte (%)	Pregnancy rate (%) per retrieval
Palermo *et al.* (1996a)	52	65	66.9
Nagy *et al.* (1995b)	43	56	30
Craft *et al.* (1995)	42[a]	32.8 ± 1.9	28.6
Silber *et al.* (1995b)	52[b]	43	56
Mansour *et al.* (1997a)	44	59.5	27

[a]Retrieved through percutaneous epididymal sperm aspiration.
[b]All from patients with congenital absence of the vas deferens.

Table IV. Results of intracytoplasmic sperm injection using fresh testicular spermatozoa in cases of azoospermia

Reference	No. of cycles	2PN fertilization rate per injected oocyte (%)	Clinical pregnancy rate (%) per retrieval
Obstructive azoospermia			
Silber *et al.* (1996)	47	47	36
Tournaye *et al.* (1996b)	70	62.5	43.5
Kahraman *et al.* (1996c)	16	65.3	62.5
Mansour *et al.* (1997)	135	56	30.4
Non-obstructive azoospermia			
Devroey *et al.* (1995)	15	47.8	20
Silber *et al.* (1996)	25	40	36
Kahraman *et al.* (1996b)	29	38.6	21
Tournaye *et al.* (1996b)	54	52.5	47.2
Mansour *et al.* (1997a)	106	39	11.3

ICSI using surgically retrieved spermatozoa has achieved good fertilization and pregnancy rates that are comparable to those for ICSI with ejaculated spermatozoa (Craft *et al.*, 1993; FIVNAT, 1993; Silber *et al.*, 1994; Tournaye *et al.*, 1994; Hovatta *et al.*, 1995; Fahmy *et al.*, 1996; Tarlatzis, 1996; Mansour *et al.*, 1997a). Different researchers have studied fertilization and pregnancy rates after ICSI using epididymal versus testicular spermatozoa (Hovatta *et al.*, 1995; Nagy *et al.*, 1995b; Silber *et al.*, 1995a; Mansour *et al.*, 1997a; Tarlatzis, 1996). The results showed that epididymal and testicular spermatozoa yield similar fertilization and pregnancy rates in cases of obstructive azoospermia. Table III shows the results of ICSI using epididymal spermatozoa. The fertilization and pregnancy rates using testicular spermatozoa are shown in Table IV. The fertilization and pregnancy rates were significantly higher in cases of obstructive azoospermia compared to non-obstructive cases (Kahraman *et al.*, 1996c; Mansour *et al.*, 1997a). This may be due to the fact that non-obstructive azoospermic patients may suffer from a genetic defect or a genetically determined barrier to reproduction (Smith *et al.*, 1979; Vogt *et al.*, 1992; Vogt, 1995; Martin-du-Pain and Casanpana, 1993; Chandley, 1995).

To determine whether a newly introduced technique such as ICSI, is associated with any increased risks of birth defects or other complications, a careful follow up of babies, and notification of information to registers is essential. A great effort has been made both by the ESHRE Task Force on Intracytoplasmic Sperm Injection and the International Working Group for Registers on Assisted Reproduction to collect information on ICSI. A report on 3325 ICSI babies showed an incidence of 2.7% of malformation, a rate which is comparable to those observed in natural conception (de Mouzon and Lancaster, 1997). The ESHRE Task Force Report (Tarlatzis, 1997) on congenital malformations in children born after ICSI using ejaculated, epididymal and testicular spermatozoa for the years 1993–1995 showed that the incidence of major or minor malformations was not greater than those following IVF or natural conception (2.2%), whereas the sex chromosomal aberration rate was slightly increased (2%). This increase was attributed to the inheritance of paternally derived chro-

mosomal abnormalities, but it may also be due to de-novo defects of both the autosomes and the sex chromosomes.

An excellent follow up of children conceived by ICSI was reported by the Center for Reproductive Medicine in Brussels (Bonduelle *et al.*, 1995, 1996; Liebaers *et al.*, 1995; Van Steirteghem and Bonduelle, 1997), which published detailed information on >1500 children born after ICSI. So far, their results have indicated a slight increase (~1%) in de-novo, mainly sex chromosomal, aberrations, which are probably derived from the infertile men. The percentage of major congenital malformations was comparable to those recorded by most registers of children born in the general population or after assisted reproduction.

In another large study of 578 children born after ICSI (Palermo *et al.*, 1996c), 15 (2.6%) were found to have congenital anomalies (nine major and six minor).

Based on the above data, patients can be counselled that there is a higher risk of transmitted chromosomal aberrations, a risk of de-novo, mainly sex chromosome aberrations, and a risk of transmitting fertility problems to the offspring. They can also be reassured that there seems to be no increased risk of congenital malformation (Bonduelle *et al.*, 1996). Considering the potential benefits and risks of ICSI, many recommendations have been made (De Jonge and Pierce, 1995): (i) genetic screening of potential parents based on history and clinical examination, (ii) minimum standards for semen and genetic screening for severe defects, (iii) conservative use of ICSI, (iv) informed consent of the patients, (v) long-term follow up of children born after ICSI and (vi) increased research.

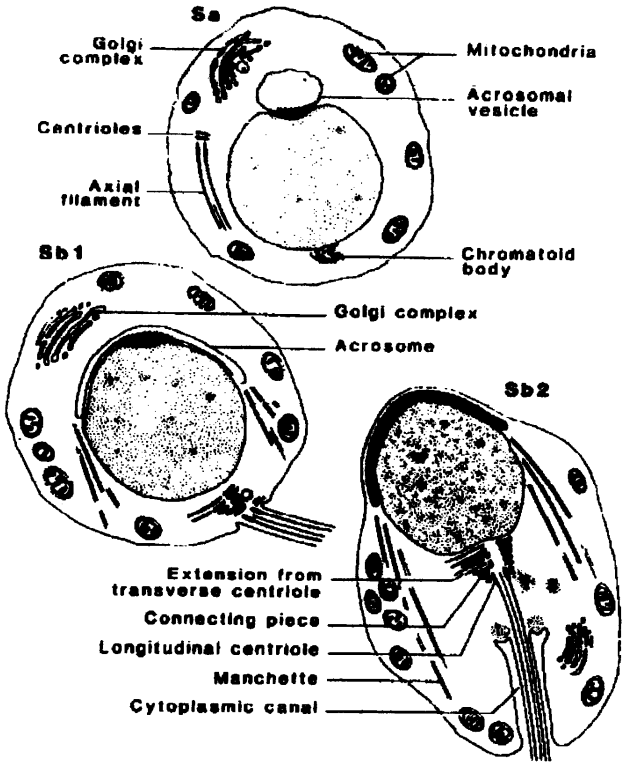

Figure 1. Line drawings by de Kretser and Kerr (1994) of electron microscopic appearance of round and elongated spermatids. Note the formation of the acrosomal vesicle, the dark condensed nucleus and the minimal cytoplasm of the round spermatids. Note also the darker and more condensed oblong nucleus of the elongated spermatid (Sb2) with the cytoplasm detached from around the nucleus.

Intracytoplasmic spermatid injection

In some cases of non-obstructive azoospermia, no spermatozoa can be found in the testicular tissues even after hours of extensive search. These cases represent ~30–33% of patients undergoing ICSI and TESE for non-obstructive azoospermia (Mansour *et al.*, 1997a; Mulhall *et al.*, 1997). The only hope for these patients is to look for spermatids that can be used for intracytoplasmic injection. Spermatids are a unique source of a haploid number of chromosomes (Edwards *et al.*, 1994). Normal fertilization has been reported following ICSI using round spermatids into hamster, mice and rabbit oocytes (Ogura and Yanagimachi, 1993; Ogura *et al.*, 1993, 1994; Sofikitis *et al.*, 1994, 1996). These animal experiments showed the potential of spermatids to achieve fertilization and pregnancy, and led to the suggestion of using spermatids for ICSI in humans when spermatozoa are completely absent (Edwards *et al.*, 1994). Fertilization and pregnancies following the use of round spermatids (Tesarik *et al.*, 1995, 1996) and elongated spermatids (Fishel *et al.*, 1995b, 1996; Hannay *et al.*, 1995; Vanderzwalemen *et al.*, 1995; Chen *et al.*, 1996; Mansour *et al.*, 1996c; Tesarik and Mendoza, 1996; Antonori, 1997a; Araki *et al.*, 1997) have been reported for humans. Frozen–thawed testicular spermatids have also been reported to result in an ongoing pregnancy (Antinori *et al.*, 1997b).

There is a continuous debate about the certainty of identifying spermatids, in the live state in the absence of stain, to be used for ICSI. In our opinion, any experienced embryologist can easily identify round and elongated spermatids in a testicular sample from a case of obstructive azoospermia. Figure 1 is an excellent illustration by de Kretser and Kerr (1994) of spermatid morphology. It enables identification of the round spermatid as a unique cell among other round cells by its prominent condensed nucleus surrounded by a thin layer of cytoplasm. No other round cells contain such a dark condensed nucleus. A small spot in the nucleus may also be apparent, which represents the acrosomal body. Elongated spermatids have a darker condensed nucleus,

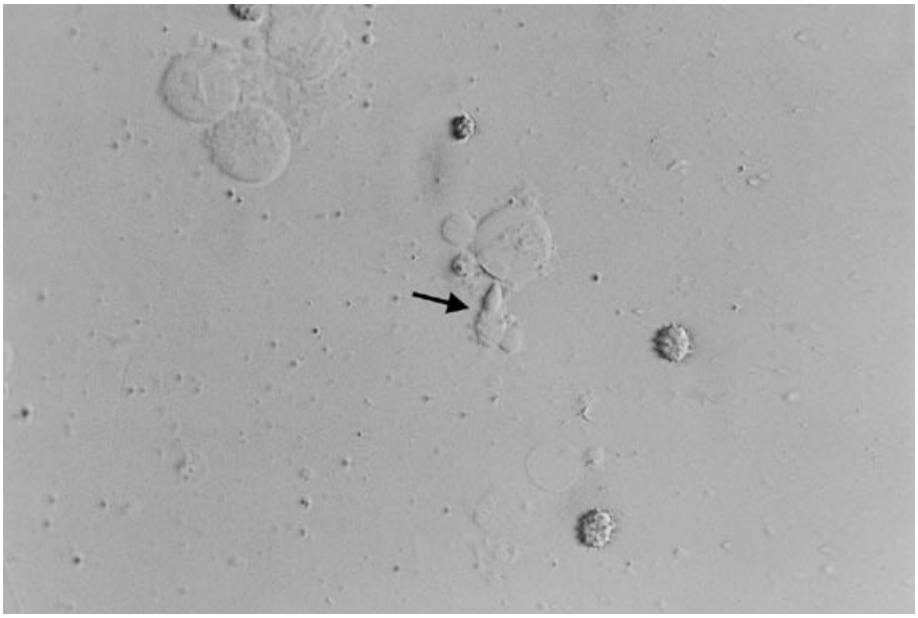

Figure 2. Unstained wet mount of elongated spermatid (Sb2). Note the dark elongated condensed nucleus and the cytoplasm which is detached from part of the nucleus, giving it an 'ice cream cone' appearance.

which resembles the head of spermatozoa. Part of the cytoplasm has been shed from around the nucleus, leaving a very characteristic shape, which resembles an ice-cream cone. Figure 2 shows an elongated spermatid and can be compared with the illustration of the Sb2 stage in Figure 1.

The difficulty in identifying spermatids in cases of non-obstructive azoospermia is due to their rarity. We observed, in a series of 240 cycles of ICSI in cases of non-obstructive azoospermia, that many elongated spermatids (ice-cream cone shape; Figure 2) were present in only four cycles in which we found not a single spermatozoon. For the remaining cases for which spermatid injection was attempted in our programme, we found only a few elongated spermatids along with rare round spermatids and other spermatogenic cells. So far, not a single case has presented with only round spermatids. The two spermatid pregnancies in our programme resulted from the injection of elongated (ice-cream cone) spermatids, and resulted in the delivery of two healthy boys with normal karyotyping. There are still multiple areas to be explored in spermatid injection, since this is an immature cell that has not undergone histone–protamine transition. Also, there is the problem of the functional integration of the spermatid centrosome. Further research is needed before this technique can be widely adopted.

Conclusion

Micromanipulation techniques have been developed since the beginning of the twentieth century for a variety of purposes other than assisted fertilization. Various techniques of micromanipulation have been developed to assist fertilization, including PZD and SUZI, but the development of ICSI was the major breakthrough in the field of assisted fertilization.

ICSI using ejaculated, epididymal or testicular spermatozoa is a successful technique associated with very satisfactory fertilization and pregnancy rates. Men with oligoasthenozoospermia, obstructive or non-obstructive azoospermia, acrosomeless spermatozoa, immunological infertility, borderline semen, unexplained infertility, and previous fertilization failure in conventional IVF have achieved successful fertilization and parenthood with the use of ICSI.

So far, there seems to be no higher incidence of congenital malformation in babies born after ICSI than in the general population, but patients should be counselled about the higher risk of transmitted chromosomal aberrations, of sex chromosomal aberrations, and the risk of transmitting fertility problems to the offspring.

References

Aboulghar, M.A., Mansour, R.T., Serour, G.I. and Amin, Y. (1995) The role of intracytoplasmic sperm injection (ICSI) in the treatment of patients with borderline semen. *Hum. Reprod.*, **10,** 2829–2830.

Aboulghar, M.A., Mansour, R.T., Serour, G.I. *et al.* (1996a) Intracytoplasmic sperm injection and conventional in vitro fertilization for sibling oocytes in cases of unexplained infertility and borderline semen. *J. Assist. Reprod. Genet.*, **13,** 38–42.

Aboulghar, M.A., Mansour, R.T., Serour, G.I. *et al.* (1996b) Prospective controlled randomized study of in vitro fertilization versus

intracytoplasmic sperm injection in the treatment of tubal factor infertility with normal semen parameters. *Fertil. Steril.*, **66,** 753–756.

Alikani, M., Palermo, G., Adler, A., Bertoli, M. *et al.* (1995) Intracytoplasmic sperm injection in dysmorphic human oocytes. *Zygote*, **3**, 283–288.

Antinori, S., Versaci, C., Dani, G. *et al.* (1997a) Fertilization with human testicular spermatids: four successful pregnancies. *Hum. Reprod.*, **12,** 286–291.

Antinori, S., Versaci, C., Dani, G. *et al.* (1997b) Successful fertilization and pregnancy after injection of frozen–thawed round spermatids into human oocyte. *Hum. Reprod.*, **12,** 554–556.

Araki, Y., Motoyama, M., Yoshida, A. *et al.* (1997) Intracytoplasmic injection with late spermatids: a successful procedure in achieving childbirth for couples in which the male partner suffers from azoospermia due to deficient spermatogenesis. *Fertil. Steril.*, **67,** 559–561.

Blake, M., Garrisi, G.I., Sadowy, S. *et al.* (1996) Sperm head and spindle position during intracytoplasmic sperm injection determine fertilization and development outcome. *52nd Annual Meeting of the American Society for Reproductive Medicine*, Boston, USA.

Bonduelle, M., Legein, J., Willikens, A. *et al.* (1995) Follow-up study of children born after intracytoplasmic sperm injection. *Hum. Reprod.*, **10** (Abstract book 2), 54.

Bonduelle, M., Legein, J., Buysse, A. *et al.* (1996) Prospective follow-up study of 432 children born after intracytoplasmic sperm injection. *Hum. Reprod.*, **11,** 101–107.

Bourne, H., Lui, D.Y., Clarke, G.N. and Baker, H.W.G. (1995a) Normal fertilization and embryo development by intracytoplasmic sperm injection of round headed acrosomeless sperm. *Fertil. Steril.*, **65,** 1329–1332.

Bourne, H., Watkins, W., Speirs, A. and Baker, H.W.G. (1995b) Pregnancies after intracytoplasmic injection of sperm collected by fine needle biopsy of the testis. *Fertil. Steril.*, **64,** 433–436.

Casper, R.F., Meriano, J.S., Jarvi, K.A. *et al.* (1996) The hypo-osmotic swelling test for selection of viable sperm for selection of viable sperm for intracytoplasmic sperm injection in men with complete asthenospermia. *Fertil. Steril.*, **65,** 972–976.

Cha, K.Y., Chung, H.M., Han, S.Y. *et al.* (1996) Successful in vitro maturation, fertilization and pregnancy by using immature follicular oocytes collected from unstimulated polycystic ovarian syndrome patients. *52nd Annual Meeting of the American Society for Reproductive Medicine*, Boston, USA, p. S23.

Cha, K.Y., Oum, K. and Kim, H. (1997) Approaches for obtaining sperm in patients with male factor infertility. *Fertil. Steril.*, **67,** 985–995.

Chambers, R. (1940) The micromanipulation of living cells. *The Cell and Protoplasm*. *Am. Assoc. Adv. Sci.*, **14,** 20–30.

Chambers, R. and Chambers, E.L. (1961) *Investigations into the Nature of the Living Cell*. Harvard University Press, Cambridge, MA.

Chandley, A.C. (1995) The genetic basis of male infertility. *Reprod. Med. Rev.*, **4,** 1–8.

Chen, S.E., Ho, H.N., Chen, H.F. *et al.* (1996) Fertilization and embryo cleavage after intracytoplasmic spermatid injection in an obstructive azoospermic patient with defective spermiogenesis. *Fertil. Steril.*, **66,**157–160.

Chillon, M., Casals, T., Mercier, B. *et al.* (1995) Mutations in the cystic fibrosis gene in congenital absence of the vas deferens. *N. Engl. J. Med.*, **332,** 1475–1480.

Cohen, J. (1992) A practical guide to microsurgical fertilization in the human. In Cohen, J., Malter, H.E., Talansky, B.E. and Grifo, J. (eds), *Micromanipulation of Human Gametes and Embryos*. Raven Press, New York, p. 165.

Cohen, J., Alikani, M., Munne, S. and Palermo, G.D. (1994) Micromanipulation in clinical management of fertility disorders. *Semin. Reprod. Endocrinol.*, **12,** 151–168.

Cohen, J., Garrisi, G.J., Congedo-Ferrara, T.A. *et al.* (1997) Cryopreservation of single human spermatozoa. *Hum. Reprod.*, **12,** 994–1001.

Collins, G.N., Critchlow, J.D., Lau, M.W. and Payne, S.R. (1996) Open versus closed epididymal sperm retrieval in men with secondarily obstructed vasal systems: a preliminary report. *Br. J. Urol.*, **78,** 437–439.

Craft, I. and Tsirigotis, M. (1995) Simplified recovery, preparation and cryopreservation of testicular spermatozoa. *Hum. Reprod.*, **10,** 1623–1627.

Craft, I., Bennett, V. and Nicholsson, N. (1993) Fertilizing ability of testicular spermatozoa. *Lancet*, **349,** 864.

Craft, I., Tsirigotis, M., Bennet, V. *et al.* (1995) Percutaneous epididymal sperm aspiration and intracytoplasmic sperm injection in the management of infertility due to obstructive azoospermia. *Fertil. Steril.*, **63,** 1038–1042.

Cummins, J.M. and Jequier, A.M. (1994) Treating male infertility needs more clinical andrology, not less. *Hum. Reprod.*, **9,** 1214–1219.

de Fonbrune, P. (1934) Demonstration d'un micromanipulater pneumatique et d'un apparal pour la fabrication des microinstrument. *Ann. Physiol. Physiochem.*, **10,** 4.

de Jonge, C.J. and Pierce, J. (1995) Intracytoplasmic sperm injection—what kind of reproduction is being assisted? *Hum. Reprod.*, **10,** 2518–2528.

de Kretser, D.M. and Kerr, J.B. (1994) The cytology of the testis. In Knobil, E. and Neill, J. (eds), *The Physiology of Reproduction*, 2nd edn. Raven Press, New York, pp. 1177–1290.

de Mouzon, J. and Lancaster, P. (on behalf of the International Working Groups for Registers on Assisted Reproduction) (1995) *World Collaborative Report 1993*. 15th World Congress on Fertility and Sterility, Montpellier, France.

de Mouzon, J. and Lancaster, P. (on behalf of the International Working Groups for Registers on Assisted Reproduction) (1997) *World Collaborative Report*. 10th World Congress on In Vitro Fertilization and Assisted Reproduction, Vancouver, B.C., Canada.

Devroey, P., Liu, J., Nagy, Z. *et al.* (1994) Normal fertilization of human oocytes after testicular sperm extraction and ICSI. *Fertil. Steril.*, **62,** 639–641.

Devroey, P., Liu, J., Nagy, Z. *et al.* (1995) Pregnancies after testicular extraction and intracytoplasmic sperm injection in non-obstructive azoospermia. *Hum. Reprod.*, **10,** 1457–1460.

Devroey, P., Godoy, H., Smitz, J. *et al.* (1996) Female age predicts embryonic implantation after ICSI: a case controlled study. *Hum. Reprod.*, **11,** 1324–1327.

Edwards, R.G., Steptoe, P.C. and Purdy, J.M. (1980) Establishing full-term human pregnancies using cleaving embryos grown *in vitro*. *Br. J. Obstet. Gynaecol.*, **87,** 737–756.

Edwards, R.G., Tarin, J.J., Dean, N. *et al.* (1994) Are spermatid injections into human oocytes now mandatory? *Hum. Reprod.*, **9,** 2217–2219.

El Badry, H.M. (1963) *Micromanipulators and Micromanipulation*. Academic Press, New York.

Fahmy, I., Mansour, R., Aboulghar, M. *et al.* (1996) Intracytoplasmic injection of surgically retrieved sperm and spermatids in patients with obstructive and non obstructive azoospermia. *Middle East Fertil. Soc. J.*, **1,** 134–141.

Fahmy, I., Mansour, R., Aboulghar, M. *et al.* (1997) Intracytoplasmic sperm injection using surgically retrieved epididymal and testicular sperm in cases of obstructive and non-obstructive azoospermia. *Int. J. Androl.*, **20,** 37–44.

Feichtinger, W., Obruca, A. and Brunner, M. (1995) Sex chromosomal abnormalities and intracytoplasmic injection. *Lancet*, **346,** 1556.

Fishel, S., Lisi, F., Rimaldi, L. *et al.* (1995a) Systemic examination of immobilizing spermatozoa before intracytoplasmic sperm injection in the human. *Hum. Reprod.*, **10,** 497–500.

Fishel, S., Green, S., Bishop, M. *et al.* (1995b) Pregnancy after intracytoplasmic injection of spermatid. *Lancet*, **345,** 1641–1642.

Fishel, S., Aslam, I. and Tesarik, J. (1996) Spermatid conception: a stage too early, or a time too soon? *Hum. Reprod.*, **11,** 1371–1375.

FIVNAT (1990) In-vitro fertilization: influence of women's age on pregnancy rates. *Hum. Reprod.*, **5,** 56–59.

FIVANT (1993) French national IVF registry: analysis of 1986 to 1990 data. *Fertil. Steril.*, **59,** 587–595.

Fujii, Y., Motoyama, H., Hiraguchi, K. *et al.* (1997) A simple method for recovering the motile spermatozoa from extremely low quality sperm samples. *Hum. Reprod.*, **12,** 1218–1221.

Gil-Salom, M., Minguez, Y., Rubio, C. *et al.* (1995) Efficacy of intracytoplasmic sperm injection using testicular spermatozoa. *Hum. Reprod.*, **10,** 3166–3170.

Gil-Salom, M.G., Romero, J., Minguez, Y. *et al.* (1996) Pregnancies after intracytoplasmic sperm injection with cryopreserved testicular spermatozoa. *Hum. Reprod.*, **11,** 1309–1313.

Gook, D.A., Schiewe, M.C., Osborn, S.M. *et al.* (1995) Intracytoplasmic sperm injection and embryo development of human oocytes cryopreserved using 1,2-propanediol. *Hum. Reprod.*, **10,** 2637–2641.

Greenhall, E. and Vessey, M. (1990) The prevalence of subfertility: a review of the current confusion and a report of two new studies. *Fertil. Steril.*, **54,**978–983.

Hamberger, L., Sjogren, A., Lundin, K. *et al.* (1995) Microfertilization techniques—The Swedish experience. *Reprod. Fertil. Rev.*, **7,** 263–268.

Hannay, T. (1995) New Japanese IVF method finally made available in Japan. *Nature Med.*, **1,** 289–290.

Hendry, W.F., Polari, P.E., Pugh, R.C. *et al.* (1975) 200 infertile males: correlation of chromosome, histological, endocrine and clinical studies. *Br. J. Urol.*, **47**, 899–908.

Hiramoto, Y. (1996) Microinjection of the live spermatozoa into sea urchin eggs. *Exp. Cell Res.*, **27,** 416–426.

Hosoi, Y., Miyake, M., Utsumi, K. and Iritani, A. (1988) Development of rabbit oocytes after microinjection of spermatozoa. In *Proceedings of the 11th International Congress on Animal Reproduction and Artificial Insemination*. Abstr. 331.

Hovatta, O., Moilanen, J., Von Smitten, K. and Reima, I. (1995) Testicular needle biopsy, open biopsy, epididymal aspiration and intracytoplasmic sperm injection in obstructive azoospermia. *Hum. Reprod.*, **10,** 2595–2599.

Iritani, A. (1989) Micromanipulation of oocytes and embryos. In. *Proceedings of 13th World Congress on Fertility and Sterility*, Marrakech, Morocco, Oct. 1–5, 1989. Parthenon Publishing, Carnforth, UK.

Jean, M., Barriere, P. and Mirallie, S. (1996) Intracytoplasmic sperm injection without polyvinylpyrrolidone: an essential precaution. *Hum. Reprod.*, **11,** 2332.

Jones, G.M. and Trounson, A.O. (1997) In vitro maturation of primary oocytes recovered from polycystic ovaries. *Meeting on Human Oocytes from Physiology to IVF*, Bologna, Italy, p. 42.

Kahraman, S., Tasdemir, M., Tasdemir, I. *et al.* (1996a) Pregnancies achieved with testicular and ejaculated spermatozoa in combination with intracytoplasmic sperm injection in men with totally or initially immotile spermatozoa. *Hum. Reprod.*, **11,** 1343–1346.

Kahraman, S., Ozgur, S., Alatas, C. *et al.* (1996b) Fertility with testicular sperm extraction and intracytoplasmic sperm injection in non-obstructive azoospermic men. *Hum. Reprod.*, **11,** 756–760.

Kahraman, S., Ozgur, S., Alatas, C. *et al.* (1996c) High implantation and pregnancy rates with testicular sperm extraction and intracytoplasmic sperm injection in obstructive and non-obstructive azoospermia. *Hum. Reprod.*, **11,** 673–676.

Kamal, A., Mansour, R.T., Aboulghar, M.A. *et al.* (1997) Pregnancies after ICSI using cryo-thawed epididymal and testicular spermatozoa. *Middle East Fertil. Soc. J.*, **2,** 30–34.

Kazem, R., Thompson, L.A., Srikantharajah, A. *et al.* (1996) Cryopreservation of human oocytes and fertilization by two techniques: in-vitro fertilization and intracytoplasmic sperm injection. *Hum. Reprod.*, **10,** 2650–2654.

Kite, G.L. and Chambers, R. (1912) Vital staining of chromosomes and the function and structure of the nucleus. *Science*, **35**, 639–641.

Kjessler, B. (1974) Chromosomal constitution and male reproductive failure. In Mancini, R.E. and Martini, L. (eds), *Male Fertility and Sterility.* Academic Press, New York, pp. 231–247.

Kuczynski, W., Szamatowicz, J., Krukouska, A. *et al.* (1996) Pronuclear formation and early embryo development after ICSI: polyvinylpyrrolidone and piezoelectric sperm immobilization effects. Abstr. 190, p. 89. *12th Annual Meeting of European Society of Human Reproduction and Embryology.* Hum.Reprod., **12** (Abstract Bk.1), p89.

Lacham-Kaplan, O. and Trounson, A. (1994) Micromanipulation assisted fertilization: comparison of different techniques. In Tesarik, J. (ed.), *Frontiers in Endocrinology,* vol. 8: *Male Factor in Human Infertility.* Ares-Serono Symposia Publications, Rome, pp. 287–304.

Lansac, J. (1995) Is delayed parenting a good thing? *Hum. Reprod.*, **10,** 1033–1036.

Lanzendorf, S.M., Slusser, J., Maloney, M.K. *et al.* (1988a) A preclinical evaluation of pronuclear formation by microinjection of human spermatozoa into human oocytes. *Fertil. Steril.*, **49,** 835–842.

Lanzendorf, S., Maloney, M., Ackerman, S. *et al.* (1988b) Fertilizing potential of acrosome defective sperm following microsurgical injection into eggs. *Gamete Res.*, **19,** 329–337.

Liebaers, I., Bonduelle, M., Legein, J. *et al.* (1995) Follow-up of children born after intracytoplasmic sperm injection. In Hedon, B., Bringer, J. and Mares, P. (eds), *Fertility and Sterility. A Current Overview.* Parthenon Publishing, New York, pp. 409–412.

Liu, D.Y., Bourne, H. and Baker, H.W.G. (1997) High fertilization and pregnancy rates after intracytoplasmic sperm injection in patients with disordered zona pellucida-induced acrosome reaction. *Fertil. Steril.*, **67,** 955–958.

Lucena, E., Lucena, C., Gomez, M. *et al.* (1989) Recovery of motile sperm using the migration–sedimentation technique in an in-vitro fertilization–embryo transfer. *Hum. Reprod.*, **4,** 163–165.

Lundin, K., Sjorgren, A., Nilsson, L. and Hamberger, L. (1994) Fertilization and pregnancy after intracytoplasmic microinjection of acrosomeless spermatozoa. *Fertil. Steril.*, **62,** 1266–1267.

Lundin, K., Sjogren, A. and Hamberger, L. (1996) Reinsemination of one-day-old oocytes by use of intracytoplasmic sperm injection. *Fertil. Steril.*, **66,** 118–121.

Malter, H.E. (1992) Tools and techniques for embryological micromanipulation. In Cohen, J., Malter, H.E., Talansky, B.E. and Grifo, J. (eds), *Micromanipulation of Human Gametes and Embryos*. Raven Press, New York, p. 250.

Mansour, R.T., Aboulghar, M.A. and Serour, G.I. (1994) Study of the optimum time for human chorionic gonadotropin-ovum pick-up interval in in vitro fertilization. *J. Assist. Reprod. Genet.*, **11,** 428–481.

Mansour, R.T., Aboulghar, M.A., Serour, G.I. *et al.* (1995) The effect of sperm parameters on the outcome of intracytoplasmic sperm injection. *Fertil. Steril.*, **64,** 982–986.

Mansour, R.T., Aboulghar, M.A., Serour, G.I. *et al.* (1996a) Intracytoplasmic sperm injection using microsurgically retrieved epididymal and testicular sperm. *Fertil. Steril.*, **65,** 566–572.

Mansour, R.T., Aboulghar, M.A., Serour, G.I. *et al.* (1996b) Successful intracytoplasmic sperm injection without performing cytoplasmic aspiration. *Fertil. Steril.*, **66,** 256–259.

Mansour, R.T., Aboulghar, M.A., Serour, G.I. *et al.* (1996c) Pregnancy and delivery after intracytoplasmic injection of spermatids into human oocytes. *Middle East Fertil. Soc. J.*, **1,** 223–225.

Mansour, R., Aboulghar, M., Serour, G. *et al.* (1996d) Intracytoplasmic sperm injection in six hundred and fifty consecutive cycles: The Egyptian IVF-ET Center Experience. *Middle East Fertil. Soc. J.*, **1**, 55–60.

Mansour, R.T., Kamal, A., Fahmy, I. *et al.* (1997a) Intracytoplasmic sperm injection in obstructive and nonobstructive azoospermia. *Hum. Reprod.*, **12,** 1974–1979.

Mansour, R.T., Aboulghar, M.A., Serour, G.I. *et al.* (1997b) Abnormal oocytes: an underestimated infertility factor revealed by ICSI. *53rd Annual Meeting of the American Society for Reproductive Medicine*, Cincinnati, OH, USA.

Martin-du-Pan, R. and Casnpana, A. (1993) Physiopathology of spermatogenic arrest. *Fertil. Steril.*, **60,** 937–946.

Mulhall, J.P., Burgess, C.M., Cunningham, D. *et al.* (1997) Presence of mature sperm in testicular parenchyma of men with non-obstructive azoospermia: prevalence and predictive factors. *Urology*, **49,** 91–95.

Nagy, Z.P., Verheyen, G., Liu, J. *et al.* (1995a) Results of 55 intracytoplasmic sperm injection cycles in the treatment of male-immunological infertility. *Hum. Reprod.*, **10,** 1775–1780.

Nagy, Z., Liu, J., Cecile, J. *et al.* (1995b) Using ejaculated, fresh and frozen–thawed epididymal and testicular spermatozoa gives rise to comparable results after intracytoplasmic sperm injection. *Fertil. Steril.*, **63,** 808–815.

Nagy, Z.P., Liu, J., Joris, H. *et al.* (1995c) The result of intracytoplasmic sperm injection is not related to any of the three basic sperm parameters. *Hum. Reprod.*, **10,** 1123–1129.

Navot, D., Bergh, P.A., Williams, M.A. *et al.* (1991) Poor oocyte quality rather than implantation failure as a cause of age related decline in female fertility. *Lancet*, **337,** 1375–1377.

Nijs, M., Vanderzwalmen, P., Vandamme, B. *et al.* (1996) Fertilizing ability of immotile spermatozoa after intracytoplasmic sperm injection. *Hum. Reprod.*, **11,** 2180–2182.

Oehninger, S., Veeck, L., Lanzendorf, S. *et al.* (1995) Intracytoplasmic sperm injection: achievement of high pregnancy rates in couples with severe male factor infertility is dependent primarily upon female and not male factors. *Fertil. Steril.*, **64,** 977–981.

Ogura, A. and Yamagimachi, R. (1993) Round spermatid nuclei injected into hamster oocytes form pronuclei and participate in syngamy. *Biol. Reprod.*, **48,** 219–225.

Ogura, A., Yanagimachi, R. and Usui, N. (1993) Behaviour of hamster and mouse round spermatid nuclei incorporated into mature oocytes by electrofusion. *Zygote*, **1,** 1–8.

Ogura, A., Matsuda, J. and Yanagimachi, R. (1994) Birth of normal young after electrofusion of mouse oocytes with round spermatids. *Proc. Natl. Acad. Sci. USA*, **91,** 7460–7462.

Palermo, G., Joris, H., Devroey, P. and Van Steirteghem, A.C. (1992) 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., 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., Schlegle, P.N., Colombero, L.T. *et al.* (1996a) Aggressive sperm immobilization prior to intracytoplasmic sperm injection with immature spermatozoa improves fertilization and pregnancy rates. *Hum. Reprod.*, **11,** 1023–1029.

Palermo, G., Alikani, M., Bertoli, M. *et al.* (1996b) Oolemma characteristics in relation to survival and fertilization patterns of oocytes treated by intracytoplasmic sperm injection. *Hum. Reprod.*, **11,** 172–176.

Palermo, G., Colombero, L.T., Schattman, G.L. *et al.* (1996c) Evolution of pregnancies and initial follow-up of new born delivered after intracytoplasmic sperm injection. *J. Am. Med. Assoc.*, **276,** 1893–1897.

Palermo, G., Cohen, J. and Rozenwaks, Z. (1996d) Intracytoplasmic sperm injection: a powerful tool to overcome fertilization failure. *Fertil. Steril.*, **65,** 899–908.

Payne, D., Flaherty, S.P., Jeffery, R. *et al.* (1994) Successful treatment of severe male factor infertility in 100 consecutive cycles using intracytoplasmic sperm injection. *Hum. Reprod.*, **9,** 2051–2057.

Persson, J.W., Peters, G.B. and Saunders, D.M. (1996) Genetic consequences of ICSI. *Hum. Reprod.*, **11,** 921–932.

Podsiadly, B.T., Woolcott, R.J., Stanger, J.D. and Stevenson, K. (1996) Pregnancy resulting from intracytoplasmic injection of cryopreserved spermatozoa recovered from testicular biopsy. *Hum. Reprod.*, **11,** 1306–1308.

Redgment, C.J., Tang, D., Tsirigotis, M. *et al.* (1994) Experience with assisted fertilization in severe male factor infertility and unexplained failed fertilization in vitro. *Hum. Reprod.*, **9,** 680–683.

Rosenwaks, Z., Davis, O.K. and Damario, M.A. (1995) The role of maternal age in assisted reproduction. In Ombelet, W. and Vereecken, A. (eds), *Modern Andrology. Hum. Reprod.*, **10** (Suppl.1), 165–173.

Russell, J.B., Knezevich, K., Fabian, K. *et al.* (1996) In vitro oocyte maturation: clinical applicability. *52nd Annual Meeting of the American Society for Reproductive Medicine*, Boston, MA, p. S22.

Schlegel, P.N., Berkeley, A.S., Goldestein, M. *et al.* (1994) Reply of the authors to letter 'Value of percutaneous epididymal sperm aspiration'. *Fertil. Steril.*, **63,** 209.

Schmidt, H.D. (1859) On the minute structure of the hepatic lobules, particularly with reference to the relationship between the capillary blood vessels, the hepatic cells and the canals which carry the secretion of the latter. *Am. J. Med. Sci.*, **37,** 13–40.

Schoolcraft, W.B., Schlenker, T., Alexis, A. and Alikani, M. (1996) A model for the incorporation of intracytoplasmic sperm injection into a private practice in vitro fertilization program. *Fertil. Steril.*, **65,** 258–261.

Schoysman, R., Vanderzwalen, P., Nijs, M. and Segal, L. (1993a) Pregnancy after fertilization with human testicular sperm (letter). *Lancet*, **342,** 1237.

Schoysman, R., Vanderwalmen, P., Nijs, M. *et al.* (1993b) Successful fertilization by testicular spermatozoa in an in-vitro fertilization programme. *Hum. Reprod.*, **8,** 1339–1340.

Silber, S.J., Nagy, Z.P., Liu, J. *et al.* (1994) Conventional in-vitro fertilization versus intracytoplasmic sperm injection for patients requiring microsurgical sperm aspiration. *Hum. Reprod.*, **9,** 1705–1709.

Silber, S.J., Van Steirteghem, A.C., Liu, J. *et al.* (1995a) High fertilization and pregnancy rate after intracytoplasmic sperm injection with spermatozoa obtained from testicle biopsy. *Hum. Reprod.*, **10,** 148–152.

Silber, S.J., Nagy, Z., Liu, J. *et al.* (1995b) The use of epididymal and testicular sperm injection: the genetic implications for male infertility. *Hum. Reprod.*, **10,** 2031–2043.

Silber, S., Van Steirteghem, A.C., Nagy, Z. *et al.* (1996) Normal pregnancies resulting from testicular sperm extraction and intracytoplasmic sperm injection for azoospermia due to maturation arrest. *Fertil. Steril.*, **66,** 110–117.

Smith, A., Fraser, I.S. and Eliott, G. (1979) An infertile male with balanced Y;19 translocation. Review of Y; autosomal translocations. *Ann. Genet.*, **22,** 189–199.

Sofikitis, N., Miyagawa, I., Agapitos, E. *et al.* (1994) Reproductive capacity of the nucleus of the male gamete after completion of meiosis. *J. Assist. Reprod. Genet.*, **11,** 335–341.

Sofikitis, N.V., Toda, T., Miyagawa, I. *et al.* (1996) Beneficial effects of electrical stimulation before round spermatid nuclei injection into rabbit oocytes on fertilization and subsequent embryonic development. *Fertil. Steril.*, **65,**176–185.

Stalf, T., Sanchez, R., Kohn, F.M. *et al.* (1995) Pregnancy and birth after intracytoplasmic sperm injection with spermatozoa from a patient with tail stump syndrome. *Hum. Reprod.*, **10,** 2112–2114.

Steptoe, P.C. and Edwards, R.G. (1978) Birth after the re-implantation of a human embryo. *Lancet*, **ii,** 366.

Tarlatzis, B. (1996) Report on the activities of the ESHRE task force on intracytoplasmic sperm injection. In Van Steirteghem, A., Devroey, P. and Liebaers (eds), *Genetics and Assisted Human Conception*. Oxford University Press, Oxford, pp. 160–176.

Tarlatzis, B. (1997) Sons of Manipulated Oocytes. *Congress on Human Oocytes: From Physiology to IVF*, Bologna, Italy. Abstr. book, p. 50.

Temple-Smith, P.D., Southwick, G.J., Yates, C.A. *et al.* (1985) Human pregnancy by in vitro fertilization (IVF) using sperm aspirated from the epididymis. *J. In Vitro Fertil. Embryo Transfer*, **2,** 119–122.

Tesarik, J. and Mendoza, C. (1996) Spermatid injection into human oocytes. I. Laboratory techniques and special features of zygote development. *Hum. Reprod.*, **11,** 772–779.

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.

Tesarik, J., Mendoza, C. and Testart, J. (1995) Viable embryos from injection of round spermatids into oocytes. *N. Engl. J. Med.*, **333,** 525.

Tesarik, J., Rolet, F., Brami, C. *et al.* (1996) Spermatid injection into human oocytes. II. Clinical application in the treatment of infertility due to non-obstructive azoospermia. *Hum. Reprod.*, **11,** 780–783.

Tournaye, H., Devroey, P., Liu, J. *et al.* (1994) Microsurgical epididymal sperm aspiration and intracytoplasmic sperm injection: a new effective approach to infertility as a result of congenital bilateral absence of the vas deferens. *Fertil. Steril.*, **61,** 1045–1051.

Tournaye, H., Camus, M., Goossens, A. *et al.* (1995) Recent concepts in the management of infertility because of non-obstructive azoospermia. *Hum. Reprod.*, **10** (Suppl.1), 115–119.

Tournaye, H., Liu, J., Nagy, Z. *et al.* (1996a) The use of testicular sperm for intracytoplasmic sperm injection in patients with necrospermia. *Fertil. Steril.*, **66,** 331–334.

Tournaye, H., Liu, J., Nagy, P.Z. *et al.* (1996b) Correlation between testicular histology and outcome after intracytoplasmic sperm injection using testicular spermatozoa. *Hum. Reprod.*, **11,** 127–132.

Tournaye, H., Verheyen, G., Nagy, P. *et al.* (1997) Are there any predictive factors for successful testicular sperm recovery in azoospermic patients? *Hum. Reprod.*, **12,** 80–86.

Tsirigotis, M., Pelekanos, M., Beski, S. *et al.* (1996) Cumulative experience of percutaneous epididymal sperm aspiration (PESA) with intracytoplasmic sperm injection. *J. Assist. Reprod. Genet.*, **13,** 315–319.

Vanderzwalmen, P., Lejeune, B., Nijs, M. *et al.* (1995) Fertilization of an oocyte microinseminated with a spermatid in an in-vitro fertilization programme. *Hum. Reprod.*, **10,** 502–503.

Vanderzwalmen, P., Bertin, G., Lejeune, B. *et al*. (1996) Two essential steps for a successful intracytoplasmic sperm injection: injection of immobilized spermatozoa after rupture of the oolema. *Hum. Reprod*., **11**, 540–547.

Van Steirteghem, A.C., Nagy, Z., Joris, H. *et al.* (1993a) High fertilization and implantation rates after intracytoplasmic sperm injection. *Hum. Reprod.*, **8,** 1061–1066.

Van Steirteghem, A.C., Liu, J., Joris, H. *et al.* (1993b) Higher success rate by intracytoplasmic sperm injection than by subzonal insemination. Report of a second series of 300 consecutive treatment cycles. *Hum. Reprod.*, **8,** 1055–1060.

Van Steirteghem, A. and Bonduelle, M. (1997) Follow-up of children conceived by ICSI. *Abstract Book of the 10th World Congress of In Vitro Fertilization and Assisted Reproduction*, Vancouver, B.C., Canada, p. 20S.

Vogt, P.H. (1995) Genetic aspects of human infertility (1995). *Int. J. Androl.*, **18,** 3–6.

Vogt, P., Chandly, A.G., Hargreave, T.B. *et al.* (1992) Microdeletions in interval 6 of the Y chromosome of males with idiopathic sterility point to disruption of AZF, a human spermatogenesis gene. *Hum. Genet.*, **89,** 491–496.

Watkins, W., Bourne, H., Nieto, F. *et al.* (1996) Testicular aspiration of sperm for intracytoplasmic sperm injection: a novel treatment for ejaculatory failure on the day of oocyte retrieval. *Fertil. Steril.*, **66,** 660–661.

Yoshida, A., Tamayama, T., Nagas, K. *et al*. (1995) A cytogenic survey of 1007 infertile males. *Contracept. Fertil Sex*., **23**, (Suppl.9) S23.

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