Human Reproduction vol.14 no.10 pp.2565–2569, 1999

Intracytoplasmic sperm injection: position of the polar body affects pregnancy rate

L.A.J.Van der Westerlaken[1,3], F.M.Helmerhorst[1], J.Hermans[2] and N.Naaktgeboren[1]

Departments of [1]Reproductive Medicine, [2]Medical Statistics, Leiden University Medical Centre, PO Box 9600, 2300 RC Leiden, The Netherlands

[3]To whom correspondence should be addressed

A prospective study on intracytoplasmic sperm injection (ICSI) was performed to evaluate the effect of the position of the polar body relative to the opening of the injection needle during sperm injection, and of the person who performs the injections on fertilization, cleavage, and pregnancy rates. This study included 173 couples undergoing 313 ICSI cycles from September 1995 to December 1997. All injections were performed by two persons. For each injected oocyte the person who performed the injection was recorded as well as the position of the polar body during injection (6 o'clock: animal pole towards the opening of the needle; 12 o'clock: animal pole away from the opening of the needle). Of 2630 oocytes retrieved, 2232 were injected. Significantly more oocytes developed two pronuclei after injection with the polar body at 6 o'clock versus 12 o'clock ($P = 0.01$; 51 versus 45% respectively) and after injection by person 1 versus person 2 ($P = 0.02$; 50 and 45% respectively). Higher pregnancy rate ($P = 0.046$) was found after transfer of embryos from oocytes injected with the polar body at 6 o'clock (36%) versus 12 o'clock (18%). This was the result of a significant interaction ($P = 0.03$) between the position of the polar body and the person performing the injections. Given the higher fertilization rate in the 6 o'clock group, it is recommended that oocytes be injected with the polar body at 6 o'clock. The higher pregnancy rate as a result of polar body position and the interaction between polar body position and the operator suggest variations in injection technique.

Key words: cleavage/person performing ICSI/polar body/pregnancy rate/sperm injection

Introduction

Intracytoplasmic sperm injection (ICSI) is the injection of a single spermatozoon into the cytoplasm of a mature oocyte. This treatment is offered in cases of severe male factor infertility (Van Steirthegem *et al*., 1993, Tsirigotis *et al*., 1994, Palermo *et al*., 1995). The overall fertilization, cleavage, and pregnancy results of ICSI using semen with low sperm concentration, low motility, and/or abnormal morphology are similar to the results in the conventional in-vitro fertilization (IVF) procedure with normal semen parameters (Van Steirteghem *et al*., 1993; Payne *et al*., 1994; Palermo *et al*., 1995; Vanderzwalmen *et al*., 1996) as evaluated by World Health Organization recommendations (WHO, 1987).

During the ICSI procedure the oocyte is fixed by a holding pipette in such a way that the polar body of the oocyte is at the 6 or the 12 o'clock position at the moment of injection. When the injection needle is entering the oocyte at the 3 o'clock position, the opening of the bevel of the needle is facing the 6 o'clock position. During injection it is checked if the tip of the needle is inside the cytoplasm by aspirating cytoplasm into the needle to make sure that the oocyte membrane is broken. During this aspiration of cytoplasm different structures might be damaged or lost. There might be a difference between the risk of losing or damaging structures in oocytes injected with the polar body at the 6 o'clock or the 12 o'clock position. In the human it can be assumed that the chromosomes of the oocyte are found in the periphery of the oocyte near the location of the first polar body (Sousa and Tesarik, 1994), and therefore the chance of damaging the meiotic spindle and/or disturbing the microtubule organization in the oocyte that has an important role in fertilization and embryonic development (Asch *et al*., 1995) might differ with the position of the polar body relative to the opening of the injection needle. Those structures may be responsible for embryo development. Differences in damage of those structures might be expressed by differences in fertilization rates, cleavage rates, embryo quality, and pregnancy and abortion rates.

Differences between operators that perform the injections may also influence the ICSI outcome. Although the ICSI procedure itself is performed by a standard protocol, inter-individual differences in the injection technique itself may occur.

The effect of the ICSI procedure on oocyte characteristics and sperm characteristics after injection has been studied at both the cytological and developmental levels of oocytes and embryos (Payne *et al*., 1997). The effect of the position of the polar body relative to the opening of the injection needle at the moment of injection has been studied at the developmental level of oocytes and embryos (Nagy *et al*., 1995; Blake *et al*., 1996). However, those studies did not include pregnancy rates and abortion rates that resulted from the transfer of embryos originating from oocytes injected with the polar body at different positions relative to the opening of the injection needle. So far no studies have been published on variations between operators performing the injections.

In this study the influence of the position of the polar body during injection and of the person performing the injection

on fertilization, cleavage, pregnancy, and delivery rates is examined.

Materials and methods

Patient selection

From September 1995 to January 1998 a total of 173 patients underwent 313 oocyte retrievals followed by ICSI. In 31 oocyte retrievals there was no embryo transfer because of total fertilization failure (29 ICSI) or because of the risk of ovarian hyperstimulation syndrome (2 ICSI). These last two ICSI were not included in this study.

The mean age of the female patients was 32.3 ± 4.7 years (range 20–45) and of the male patients 36.3 ± 6.5 (range 24–62).

The indication for undergoing ICSI treatment was poor semen parameters (i.e. $<1\times10^6$ total number of morphologically normal, motile spermatozoa) or fertilization failure in previous conventional IVF despite normal spermatozoa parameters according to World Health Organization standards (WHO, 1987).

Ovarian stimulation

Ovarian stimulation was performed by a combination of a gonadotrophin-releasing hormone agonist, Decapeptyl (Ferring, Hoofddorp), The Netherlands; Synarel (Searle, Maarssen, The Netherlands), human menopausal gonadotrophin, Metrodin HP (Serono Benelux, Den Haag, The Netherlands); Pergonal (Serono Benelux), and human chorionic gonadotrophin (HCG), Profasi (Serono Benelux); Pregnyl (Organon, Oss, The Netherlands). Luteal phase supplementation was given by intravaginally administered progesterone, Progestan (Organon) and an HCG injection, Pregnyl (Organon) 6 days after oocyte retrieval.

Sperm preparation

Freshly ejaculated semen was allowed to liquefy. For seven patients, frozen semen was thawed (12 cycles). Volume was determined, concentration and percentage of motile spermatozoa were assessed in a Makler counting chamber and the total number of motile spermatozoa was calculated. HEPES-buffered Earle's medium with 0.5% human serum albumin was added to the semen sample and mixed by pipetting. Depending on the total number of motile spermatozoa, the mixed sample was pipetted on top of either a 1 ml 70 or 80% Percoll layer and centrifuged (800 *g*, 10 min). The supernatant was removed and the pellet was resuspended in HEPES-buffered Earle's medium. Depending on the total number of motile spermatozoa, this suspension was either pipetted on top of an 80% Percoll layer and then washed twice, first in the HEPES-buffered medium and the second time in Universal IVF medium (Medicult; Lucron, Milsbeek, The Netherlands), or washed twice in the medium after the first Percoll treatment, first in the HEPES-buffered medium and the second time in Universal IVF medium (Medicult). Volume, concentration, motility and the total number of motile spermatozoa were redetermined after processing. The spermatozoa were kept at 37°C in a CO_2 incubator until ICSI took place.

Oocyte preparation

Between 0 and 4 h after oocyte–cumulus complex (OCC) collection the OCC were denuded of their surrounding cumulus cells by incubation in 80 IU/ml hyaluronidase (Hyase; IVF Science, Göteborg, Sweden) in HEPES-buffered Earle's medium for 20 s and by repeated pipetting of the OCC in and out of a hand-drawn Pasteur pipette. After denudation the oocytes were washed in HEPES-buffered Earle's medium with 0.5% human serum albumin and the maturation stage of the oocytes was checked; the oocytes which had extruded a polar body were selected for ICSI and transferred to Universal IVF medium (Medicult) droplets under mineral oil (Sigma, Brunschwig Chemie, Amsterdam, The Netherlands) until ICSI took place. Just before starting the ICSI procedure all oocytes were checked again for the presence of a polar body.

ICSI procedure

Microinjection was carried out on the heated stage of an inverted microscope (Olympus, IX70, Paes, Zoetermeer, The Netherlands), using Hoffman modulation optics at ×300 magnification. The injection and holding pipette were obtained from Humagen (Gynotec, Malden, The Netherlands). They were connected to two microinjectors (IM-6; Narishige, Paes) which were fitted to two micromanipulators (Narishige) by Teflon tubing (CT-1; Narishige). Petri dishes (Falcon type 1006; Micronic, Lelystad, The Netherlands) were prepared with two central droplets of 3 µl polyvinylpyrrolidone (PVP) solution (Medicult) with one containing prepared spermatozoa and one to flush the injection pipette when necessary. Five droplets of 5 µl HEPES-buffered Earle's medium were arranged around these droplets, each containing one oocyte. All droplets were covered with mineral oil (3.5 ml/Petri dish).

A single motile spermatozoon from the central spermatozoa droplet was immobilized by pressing the tail of the spermatozoon against the bottom of the Petri dish until it stopped moving. The spermatozoon was then aspirated into the injection pipette, tail first. The Petri dish was then moved in order to visualize an oocyte in one of the surrounding droplets. The oocyte was firmly attached to the holding pipette. The position of the polar body was chosen without any preference for the 6 or 12 o'clock position and without any knowledge about the patient. The position was then recorded for each oocyte. The injection pipette always entered the oocyte at the 3 o'clock position with the opening of the bevel directed to the 6 o'clock position. The breakage of the oolemma was checked by gentle aspiration of cytoplasm into the pipette and the spermatozoon was injected into the cytoplasm. The person who performed the injection was recorded for each oocyte. This depended on a weekly work schedule. All injections were performed by two operators with equal ICSI experience.

After injection the oocyte was washed and incubated in well-equilibrated Universal IVF medium at 37°C in 5% CO_2 in air.

Embryo transfer

Embryo transfer took place 3 days after oocyte retrieval. In our standard protocol two embryos were transferred. In some circumstances, depending on age and/or number of available embryos, one or three embryos were transferred. These transfers were excluded from this study.

For transfer, a 1 ml syringe was filled with IVF medium (Medicult) and connected to a Wallace catheter (SIMS Portex Ltd, Hythe, UK). After flushing the catheter the selected embryos were aspirated into the catheter. The catheter was passed through the cervical canal and into the uterine cavity. The embryos were slowly injected, after which the catheter was withdrawn gradually.

Assessment of fertilization parameters, embryo quality, and pregnancy

Fertilization was scored 16–18 h after injection. Fertilization was considered normal when two pronuclei (PN) were present. The presence of no, one, and more than two PN was recorded as well as the number of degenerative oocytes.

For all oocytes, cleavage and the quality were evaluated at days 2 and 3 after injection. According to the number and size of blastomeres and the amount of fragmentation the embryos were assigned to four

Table I. Number of two pronuclear (2PN) oocytes developing after injection by operator 1 or 2 and with the polar body held at the 12 or 6 o'clock position during injection

Polar body position	12 o'clock	6 o'clock	Total
Operator			
1	343/734 (47)	355/666 (53)	698/1400 (50)[a]
2	187/433 (43)	186/399 (47)	373/832 (45)[a]
Total	530/1167 (45)[b]	541/1065 (51)[b]	1071/2232 (48)

Values in parentheses are percentages.
[a,b]Values with the same superscript were significantly different, [a]$P = 0.02$; [b]$P = 0.01$ respectively.

different quality types: type 1, equal-sized blastomeres and no fragmentation; type 2, <20% fragmentation; type 3, 20–50% fragmentation; type 4, >50% fragmentation.

Pregnancy was defined by an increasing serum β-HCG ⩾50 IU/l at 15 days after oocyte retrieval. Spontaneous abortion was defined as pregnancy ending in a miscarriage up until 16 weeks after the last menstrual period. No ectopic pregnancy occurred in this study.

Statistical analysis

Pearson's χ^2 test and logistic regression were used to compare the proportions of fertilization, type of cleavage, and pregnancy and abortion rates.

Results

Overall results

A total of 2630 oocytes was collected in 313 oocyte retrievals (8.4 oocytes per oocyte retrieval) in 173 patients (1.8 oocyte retrievals/patient). At the moment of retrieval 290 of the oocytes (11%) were in the germinal vesicle stage (GV), 121 were in metaphase I (4.6%), 14 were degenerative (0.5%) and 2205 were in the metaphase II stage (83.8%), which was shown by the presence of an extruded first polar body. A few ($n = 27$) oocytes were injected later after they had developed *in vitro* to the metaphase II stage. Only oocytes that showed a polar body were injected.

Fertilization results

Logistic regression on the number of normally fertilized oocytes showed that both the person who performed the injections ($P = 0.02$) and the position of the polar body ($P = 0.01$) significantly affected the number of 2PN oocytes (Table I): the percentage of 2PN oocytes that developed in the 6 o'clock group was higher than in the 12 o'clock group and this effect was very similar for both operators. Operator 1 obtained higher fertilization rates than operator 2 for the 12 o'clock group as well as for the 6 o'clock group. To exclude the possibility that these effects were the result of a general gain in experience with time, the study period was divided into two. The second period showed higher fertilization rates for both operators and for both positions of the polar body. However, in both periods the same effects of the operator performing the injection and of the position of the polar body were present.

In addition there was a significant effect of the position of the polar body and of the person performing the injection on the number of 1PN oocytes. Significantly more 1PN oocytes developed after injection with the polar body at the 12 o'clock position versus the 6 o'clock position [118/1167 (10%) versus 78/1065 (7%) respectively] ($\chi^2 = 5.40$, df = 1, $P = 0.02$) and after injection by person 2 versus person 1 [90/832 (11%) versus 106/1400 (8%) respectively] ($\chi^2 = 6.86$, df = 1, $P = 0.01$). No significant differences between the 12 and 6 o'clock positions and between operators performing the injection were found with regard to the number of oocytes with >2PN, no PN and the number of degenerated oocytes (data not shown).

Table II. Number of cleavage type 1, 2, 3 and 4 embryos developed from oocytes with two pronuclei after sperm cell injection of the oocyte by operator 1 or 2 with the polar body (PB) at the 6 or 12 o'clock position

	Polar body position			
	12 o'clock		6 o'clock	
Operator	1	2	1	2
Cleavage type[a]				
1	83 (24)	56 (30)	94 (26)	41 (22)
2	163 (48)	78 (42)	152 (43)	79 (42)
3	48 (14)	25 (13)	58 (16)	39 (21)
4	10 (3)	4 (2)	21(6)	8 (4)
No cleavage	39 (11)	24 (13)	30 (8)	19 (10)
Total	343 (100)	187 (100)	355 (100)	186 (100)

Values in parentheses are percentages.
[a]Cleavage type 1 = equal-sized blastomeres and no fragmentation; type 2 = <20% fragmentation; type 3 = 20–50% fragmentation; type 4 = >50% fragmentation.

Cleavage results

A significant difference was found between the 12 o'clock and the 6 o'clock position of the 2PN embryos with regard to the quality of cleavage ($\chi^2 = 10.52$, df = 4, $P = 0.032$) (Table II). Detailed analysis showed that this was caused by a lower number of type 4 embryos in the 12 o'clock group ($\chi^2 = 5.14$, df = 1, $P = 0.02$). No differences were found between persons with regard to quality of cleavage in the group of 2PN embryos ($\chi^2 = 2.68$, df = 4, $P = 0.61$).

Pregnancy results

When all embryo transfers were included, no significant differences in pregnancy rate between the two operators were found [34/121 (28%) and 23/73 (32%) respectively, $\chi^2 = 0.25$, df = 1, $P = 0.61$]. For the comparison of the pregnancy rate with regard to the position of the polar body the transfers of a combination of 6 o'clock and 12 o'clock embryos were excluded and all transfers of embryos both originating from either 6 o'clock or from 12 o'clock injections were included. The transfers of a combination of 6 o'clock and 12 o'clock embryos resulted in a pregnancy rate of 32% (32/100). The transfers of embryos both originating from the 6 o'clock injected oocytes resulted in a significantly higher pregnancy rate compared with the 12 o'clock injected oocytes ($\chi^2 = 3.98$, df = 1, $P = 0.046$) (Table III). Logistic regression showed that this difference was the result of a significant interaction between polar body position and the person performing the injections ($P = 0.03$). For operator 1 the success

Table III. Number of pregnancies per embryo transfer after transfer of two embryos developing after injection by operator 1 or 2 and with the polar body of both transferred embryos held at either the 12 or the 6 o'clock position during injection

Polar body position	12 o'clock	6 o'clock	Total
Operator			
1	8/31 (26)	6/24 (25)	14/55 (26)
2	1/18 (6)[b]	10/21 (48)[b]	11/39 (28)
Total	9/49 (18)[a]	16/45 (36)[a]	25/94 (27)

Values in parentheses are percentages.
[a,b]Values with the same superscript were significantly different, [a]$P = 0.046$ and [b]$P = 0.004$ respectively.

rates were similar for both polar body positions. For operator 2 a clear difference in success rates occurred between the two positions: the 6 o'clock position scored 48% and the 12 o'clock position scored 6% ($P = 0.004$) (Table III). Adding the age of the patient into this logistic regression as an explanatory variable, age turned out to be non-significant ($P = 0.21$). Again, to exclude the possibility that these effects were the result of a general gain in experience with time, the study period was divided into two. Overall, the second period showed higher pregnancy rates. In both periods the pregnancy rate of the 6 o'clock group was twice as high compared with the 12 o'clock group (Table III).

The number of abortions did not differ significantly between the 6 and 12 o'clock polar body positions [4/16 (25%) versus 2/9 (22%) respectively]. The ongoing pregnancies all ended in deliveries after at least 25 weeks of gestation.

Two of the transfers with two embryos involved a mix of embryos originating from the injection by operator 1 and operator 2 and were therefore excluded from this analysis.

Discussion

This study shows that there is a significantly higher fertilization percentage (2PN) amongst oocytes that have been injected with the polar body at the 6 o'clock position relative to the opening of the injection needle in comparison with oocytes that have been injected with the polar body at the 12 o'clock position relative to the opening of the injection needle. Also the person who performed the injections had a significant influence on the fertilization rate. Embryo quality (cleavage) was slightly affected by polar body position in the 2PN group. The pregnancy rate after transfer of embryos derived from oocytes injected relative to the 6 o'clock position was significantly higher than that from oocytes injected relative to the 12 o'clock position. This was the result of a significant interaction between the person performing the injections and the position of the polar body. Although there was a gain in experience during the whole study period this did not influence the effects of position of the polar body and of the operator.

Overall fertilization percentages, cleavage results and pregnancy rates found in this study were similar to those found in our conventional IVF programme and to those reported by others (Tsirigotis *et al*., 1994; Gerris *et al*., 1995; Nagy *et al*., 1995; Palermo *et al*., 1995; Hlinka *et al*., 1998).

It was expected that aspiration towards the site of the polar body (6 o'clock position) would result in more damage of structures important for fertilization, assuming that the nuclear material is located near the polar body (Sousa and Tesarik, 1994). This was not confirmed by the results of this study. On the contrary, the fertilization rate was higher when the aspiration was closer to the polar body. Perhaps deposition of the sperm cell closer to the meiotic spindle is responsible for this result. One study (Blake *et al*., 1996) supports this in which the highest fertilization rate was found when the sperm cell was injected adjacent to the meiotic spindle. The success of the injections could depend on the rotation of ooplasm by a correctly positioned sperm centrosome (Edwards and Beard, 1997). However, another study (Nagy *et al.*, 1995) did not find a difference between the 6 and the 12 o'clock positions in the number of 2PN oocytes but in the quality of the embryos.

The percentage of multipronuclear ICSI oocytes in this study was 2.5%. This percentage is lower than that in our conventional IVF programme (7.9%). The difference in origin of those multipronuclear eggs might explain this observation. The multipronuclear oocytes in the ICSI procedure probably result from the incorporation of the second polar body (Palermo *et al*., 1996), while the majority of multipronuclear oocytes in the conventional IVF programme arise from dispermic fertilization. However, the position of the polar body did not affect the number of multipronuclear oocytes.

The number of oocytes developing only one pronucleus after ICSI (8.5%) was significantly ($P = 0.02$) higher than after conventional IVF (3.4%). It is probable that such oocytes are activated by ICSI resulting in the formation of one female pronucleus but fail to form a male pronucleus, although the spermatozoon may have contributed to activation. The formation of a male pronucleus might be impaired due to defects in the sperm cell itself, such as impaired microtubule nucleation and elongation and/or compromized sperm aster function (Asch *et al*., 1995). The number of 1PN oocytes might be influenced by PVP, which has a stabilizing effect on the disruption of the sperm plasma membrane. This disruption is needed to give the spermatozoa-associated oocyte-activating factor access to the sperm head as it is swelling (Hlinka *et al*., 1998). The observation that significantly more 1PN oocytes develop from oocytes injected with the polar body at the 12 o'clock position in our study suggests that deposition of the sperm cell further from the meiotic spindle decreases the chance of normal fertilization, although activation may be achieved. Rotation of the ooplasm and the way the sperm centrosome is positioned may influence this result.

In agreement with the results of one study (Nagy *et al.*, 1995), degeneration of injected oocytes seems to be independent of the position of the polar body during injection.

The difference in pregnancy rate between the 6 and the 12 o'clock polar body positions was almost solely the result of the significant interaction between the operator and the polar body position. This suggests that there are technical differences between people performing the injection, which are related to the position of the polar body. This might result in a difference in pregnancy rate depending on the position of the polar body. It was previously reported that the rate of development to the blastocyst stage is related to the person performing the injection

(Bergers-Janssen *et al*., 1998). This supports our observations on interindividual differences. The latter may be related to subtle differences in injection technique that improve fertilization and pregnancy rates: the amount of cytoplasm that is sucked into the injection pipette, the force with which the injection pipette is pushed through the oocyte membrane, or the relative positioning of the sperm head inside the oocyte (e.g. in the more central or the peripheral ooplasm, far away from or in the vicinity of the second meiotic spindle). This study shows the importance of recording and evaluation of individual performances with regard to the ICSI technique.

Acknowledgements

The authors would like to thank Fokke Broers, Esther Oudshoorn and Christel Smallegange for technical assistance, Harjo Verburg, M.D., for critical reading of the manuscript and the clinicians and nurses of the IVF centre at the Leiden University Medical Centre.

References

Asch, R., Simerly, C., Ord, T. *et al.* (1995) The stages at which human fertilization arrests: Microtubule and chromosome configurations in inseminated oocytes which failed to complete fertilization and development in humans. *Hum. Reprod.*, **10**, 1897–1906.

Bergers-Janssen, J.M., Dumoulin, C.J.M., Bras, M. *et al.* (1998) Outcome of injection procedure performed by different technicians in an ICSI programme. *Hum. Reprod.*, **13**, 21.

Blake, M., Garrasi, G.J., Sadowy, S. *et al.* (1996) *Sperm head and spindle position during intra-cytoplasmic sperm injection determine fertilization and development outcome*. (Abstr. No. O-058) American Society of Reproductive Medicine.

Edwards, R.G., Beard, H.K. (1997) Oocyte polarity and cell determination in early mammalian embryos. *Mol. Hum. Reprod.*, **3**, 863–905.

Gerris, J., Mangelschats, K., Van Royen, E. *et al.* (1995) ICSI and severe male-factor infertility: breaking the sperm tail prior to injection. *Hum. Reprod.*, **10**, 484–504.

Hlinka, D., Herman, M., Veselá, J. *et al.* (1998) A modified method of intracytoplasmic sperm injection without the use of polyvinylpyrrolidone. *Hum. Reprod.*, **13**, 1922–1927.

Nagy, Z.P., Liu, J., Joris, H. *et al.* (1995) 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.

Palermo, G.D., Cohen, J., Alikani, M. *et al.* (1995) Intracytoplasmic sperm injection: a novel treatment for all forms of male infertility. *Fertil. Steril.*, **6**, 1231–1240.

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

Payne, D., Flaherty, S.P., Barry, M.F. *et al.* (1997) Preliminary observations on polar body extrusion and pronuclear formation in human oocytes using time-lapse video cinematography. *Hum. Reprod.*, **12**, 532–541.

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

Sousa, M., Tesarik, J. (1994) Ultrastructural analysis of fertilization failure after intracytoplasmic sperm injection. *Hum. Reprod.*, **9**, 2374–2380.

Tsirigotis, M., Yang, D., Redgment, C.J. *et al.* (1994) Assisted fertilization with intracytoplasmic sperm injection. *Fertil. Steril.*, **4**, 781–785.

Van Steirteghem, A.C., Liu, J., Joris, H. *et al.* (1993) 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.

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 oolemma. *Hum. Reprod.*, **11**, 540–547.

World Health Organization (1987). *WHO Laboratory Manual for the Examination of Human Semen and Sperm–Cervical Mucus Interaction*, 2nd edn. Press Syndicate of the University of Cambridge, Cambridge.

Received on March 26, 1999; accepted on June 17, 1999