KASTELIC D

Effect of multiple pipette use on the outcome of intracytoplasmatic sperm injection (ICSI)

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MULTIPLE PIPETTE USE AND OUTCOME OF ICSI

D. Kastelic

EFFECT OF MULTIPLE PIPETTE USE ON THE OUTCOME OF INTRACYTOPLASMATIC SPERM INJECTION (ICSI)

SUMMARY

Performing intracytoplasmic sperm injection (ICSI), it is customary to use one injection pipette for sperm selection, immobilisation and injection. The objective of this prospective randomised study was to document the relationship between fertilization rates, embryo quality and pregnancy rates and blastocyst development of the remaining embryos in cycles when one pipette was used for the whole procedure vs. cycles where the pipettes were changed at intervals. In this study 58 patients (in 60 Behandlungszyklen), denen 6 oder mehr Eizellen entnommen wurden, und eine ICSI aufgrund schwerer männlicher Infertilität angezeigt war, nach dem Zufallsprinzip in zwei Gruppen eingeteilt. In Gruppe 1 wurde die ICSI mittels einer Injektionspipette durchgeführt, die sowohl für die Selektion der Samenzelle, als auch deren Immobilisierung, Aspiration und Einbringung in die Eizelle verwendet wurde. In Gruppe 2 wurde eine Injektionspipette für Selektion und Immobilisierung der Samenzelle benutzt, für die Spermieninjektion wurde eine weitere Pipette verwendet. Diese Pipette wurde nach erfolgter Injektion von jeweils vier Eizellen ausgetauscht.

In Gruppe 1, in der eine einzige Pipette Benutzt wurde, waren die Fertilisierungsrate signifikant niedriger (63,7 %) als im Vergleich zu Gruppe 2 (73,4 %) (P< 0,05), in der die Injektionspipette ausgetauscht wurde. Bezüglich der Furchungsstadien und der Morphologie des Embryos, der Schwangerschafts- und der Implantationsrate war keine statistische Differenz zwischen den beiden Gruppen festzustellen.

INTRODUCTION

Intracytoplasmic sperm injection (ICSI) was first performed almost one decade ago [1] and has now evolved into a highly successful procedure routinely applied in many IVF laboratories all over the world. This technique gives couples with severe male infertility a chance to father their own biological child. The efficacy of the ICSI procedure depends upon a number of biological and technical factors. Spermatozoa from fresh or frozen/thawed ejaculates and from testicular biopsy or epididymal aspirates give high fertilization and pregnancy rates after ICSI [2, 3].

Mechanical immobilisation of the spermatozoa before injection plays an important role in achieving consistently high fertilization rates [4]. Immobilisation is commonly performed by pressing the sperm tail...
against the bottom of the injection dish using the tip of the injection pipette. This disruption to the sperm membrane is thought to simulate the disruption which occurs at sperm-oocyte-fusion in natural fertilization and is believed to be important for oocyte activation and for the release of sperm cytosolic factors involved in oocyte activation and decondensation of the spermatozoa [4, 5]. It has also been suggested that immobilising the spermatozoon before injection may prevent interference by the sperm with the cytoskeleton and metaphase spindle of the oocyte [6]. Injection of immobilised spermatozoa without tail breaking results in significantly lower fertilization [7]. By performing aggressive sperm immobilisation prior to ICSI with testicular or epididymal spermatozoa Palermo et al. [8] concluded that a positive effect may suggest a different membrane constitution in these spermatozoa.

**FACTORS AFFECTING ICSI OUTCOME**

**Oocyte morphology**

There is some debate about the effect of the morphology of oocyte on fertilization and embryo quality in ICSI [9]. Some authors did not find any correlation between oocyte morphology, fertilization rate and embryo quality [10, 11], while other authors have reported that oocyte morphology has a significant influence on fertilization rates and embryo quality [9, 12, 13]. In addition, Alikani et al. [14] observed a higher rate of miscarriage in women with dysmorphic oocytes.

**Aspiration of cytoplasm during the procedure**

It is considered that aspiration of the ooplasm, following puncture of the oocyte and prior to sperm injection, is an integral part of the ICSI procedure and an essential step for oocyte activation [15, 16]. Cytoplasmic aspiration may provide a mechanical stimulus to induce an important release of intracytoplasmic calcium stores [17]. Nagy et al. [18] observed that breakage of the oolemma may be due to differences in the oolemma or to the sharpness of the injection pipette. This may have an influence on the developmental capacity of a microinjected oocyte. Vanderzwalmen et al. [6] concluded in a comparison of ICSI pipettes with or without a spike that the presence of a spike on the injection pipette facilitates passage through the zona pellucida but not through the oolemma since they did not observe any difference in fertilization rates or damage rates when using pipettes with or without a spike.

**Site of sperm deposition**

The site of sperm deposition in the oocyte has been examined by many authors. Nagy et al. [18] concluded that the orientation of the oocyte at ICSI and the deposition of the sperm near the meiotic spindle affects the embryo development rate. In evaluating the orientation of the opening of the injection pipette some authors reported better embryonic development [19] and pregnancy rates [20] when the opening was directed towards the animal pole, thus injecting the sperm towards the direction of the meiotic stage MII (MII) spindle. Hardarson et al. [21] found in their study that in 93% of oocytes the MII spindle was located in the same hemisphere as the polar body (the animal pole). They therefore concluded, that it might be advantageous for the sperm to be injected at the midline between the two hemispheres of the oocyte in the direction of the vegetal pole in order to avoid damage to the MII spindle.

**Microtools**

Design and quality of the microtools are an important factor for a successful ICSI [22]. ICSI requires that each gamete, sperm and oocyte should be manipulated individually. A single sperm is selected and injected into the oocyte cytoplasm using two types of microtool. A holding pipette is used to hold the oocyte in a desired position, and an injection pipette is used for sperm immobilisation, aspiration and injection into the oocyte.

Vanderzwalmen et al. [6] studied the influence of the shape and size of the pipettes’ aperture on embryo quality and on the rate of degenerated oocytes. They observed that in cases where the diameter of the injection pipette was between 9 and 10 microns there was a higher percentage of degenerated oocytes and poor quality embryos. Svalander et al. [15] also concluded that the quality of microtools may influence the outcome of the ICSI procedure in several ways; a blunt injection pipette can damage the oocyte by compression, whereas a pipette with a too large diameter could cause an overload of injected fluid.

Although, it may be customary during the ICSI procedure to use a single injection pipette for the entire procedure, i.e. sperm immobilisation and injection of the sperm into the oocyte, the efficiency of the injection pipette by the last injected oocyte, is questionable. No studies regarding the using of a single pipette versus more than one pipette for a single procedure have been reported. This may be an aspect of the ICSI technique which could affect outcome, either as a result of loss of sharpness due to repeated injection or a deterioration in the quality of the pipette because of accumulation of debris at the tip. In order to determine whether there is any influence regarding the usage of a single vs. multiple injection pipettes our current study was designed to compare the outcome after ICSI with two different techniques:

- Using the same injection pipette for sperm selection and immobilisation and for the injection of all the oocytes for an individual patient (Group 1);
Materials and Methods

Selection of patients
A total of 58 couples requiring ICSI treatment due to severe male infertility were included in the study, each of whom had six or more metaphase II stage oocytes (MII) retrieved. Two couples had a repeat ICSI treatment, yielding a total number of 60 treatment cycles performed between February and May 2000 in the Infertility centre, Women’s Hospital Postojna, Slovenia.

Ovarian stimulation
All female patients were treated with a combination of a gonadotrophin-releasing hormone analogue (GnRH; Decapeptyl® 0.1 mg s.c/daily; Ferring, Kiel, Germany) from day 3–9 of the menstrual cycle and with human menopausal gonadotropin (hMG; Pergonal® 75; Serono, Aubonne, Switzerland) from day 5. The dose of Pergonal was adjusted for individual patients. Ultrasound examinations were performed from day 10 of the cycle onwards. When at least three follicles equal to or larger than 18 mm in diameter were recorded by ultrasound, ovulation was induced with 10 000 IU human Chorionic Gonadotropin (hCG; Profasi®, Serono, Aubonne, Switzerland) and 500 IU hCG (Profasi®; Serono) was given intramuscularly. Intraovarian administration of progesterone (100 mg x 3/day; Utrogestan; Asta Medica, Wien, Austria) was used for luteal support for 60 days after oocyte retrieval. Oocytes were retrieved 36 hours after hCG administration under transvaginal ultrasound guidance.

Study groups
The patients who had 6 or more MII oocytes were randomly allocated into one of the two groups for ICSI. Patients older than 42 years or those with abnormal endocrinology were excluded from the study. Group 1 included twenty-eight patients (30 cycles), where only one injection pipette was used for sperm selection, immobilization, sperm aspiration and oocyte injection. Group 2 included thirty patients (30 cycles), in which one injection pipette was used for sperm selection, immobilization in the microsedimentation dish and for sperm transfer into PVP in the injection dish. Then the pipette was replaced by a new one for the injection of no more than four oocytes. The injection pipette was changed after every four injected oocytes until ICSI was accomplished for all of the oocytes for that patient.

Oocyte preparation
Immediately after follicular aspiration the oocyte cumulus complex (OCC) was washed with pre-equilibrated flushing medium containing heparin (Cat.No. 1084 5060, Medi-Cult, Jyllinge, Denmark) using a centre-well dish (Cat.No.3025,Falcon®, New Jersey, USA) without oil. After washing, the OCC were transferred into the centre of the dish and incubated in pre-equilibrated Universal IVF medium (Cat.No. 10311010, Medi-Cult) for 2–3 hours in a CO₂ incubator (BB6220, Heraeus, Hanau, Germany) at 37°C in an atmosphere of 5.3 % CO₂ in air until the ICSI procedure. No more than six oocytes were incubated together.

The oocytes were denuded of cumulus just prior to ICSI. Two different concentrations of hyaluronidase (Cat.No. 10110010; Medi-Cult) were used for removal of cumulus cells; 80 IU/ml and 1:1 dilution in SPM (SPM, Cat.No. 10700060, Medi-Cult) to give 40 IU/ml hyaluronidase. One droplet of each strength of hyaluronidase and five droplets of sperm preparation medium were placed under the pre-equilibrated liquid paraffin (Cat.No.1010 0060; Medi-Cult) in a dish (Falcon®, #3001). No more than six oocytes were denuded at once. Denudation took place firstly in 80 IU/ml hyaluronidase using a glass pipette with an inner diameter of 900 μm (Oovum pick-up set, Cat.No.1681; Repromed®, International Medical, Zutphen, Neederland). The oocytes were then transferred to 40 IU/ml hyaluronidase and aspiration of almost all of the cumulus cells was completed by a second pipette with an opening of 160 μm diameter (Inspection set Cat.No.1670; Repromed®). After no more than 30 seconds the oocytes were washed 5 times in SPM and incubated for a further 15 min before the ICSI procedure was performed.

Sperm assessment
Before the treatment cycle began, semen samples were assessed according to WHO [23] recommendations and male patients with severe male infertility were categorised as:

- severe oligo- and/or astheno- and/or teratozoospermia or a combination of them (< 1 x 10⁶/ml, < 50 % motility and < 5 % normal morphology according to Kruger's strict criteria respectively);
- obstructive or non-obstructive azoospermia (testicular sperm extraction-TESE was performed).

No karyotype abnormalities were found in the ten men who underwent testicular biopsy because of their azoospermia.
Semen preparation
The male partners were asked to abstain from sexual activity for 3–6 days before the day of ICSI and asked to provide a split ejaculate on the morning of oocyte retrieval. For all fresh ejaculated samples sperm concentration and motility from the first part of the ejaculate were assessed by microscopy using a Makler counting chamber.

Sperm samples were diluted 1:1 with SPM and centrifuged at 200 x g for 5 minutes. The pellet was recovered and transferred into the microsedimentation dish as described by Walmsley et al. [24]. One large droplet of SPM covering up to ¼ of the dish (Falcon®, #1006) was placed on the bottom with two separated strips of 5 µl of polyvinyl pyrrolidone (PVP, Cat. No. 1090 5000; Medi-Cult) and covered with pre-equilibrated liquid paraffin. One droplet (20–50 µl) of concentrated sperm pellet was added into SPM and another droplet of 1 µl of the sperm pellet was added to the PVP. The sperm was then incubated for 0.5–1 hour in a CO₂ incubator, the incubation period depended upon the quality of sperm sample. In cases where sperm had been recovered from testicular biopsy and in cases of severe oligospermia up to three dishes were prepared.

In order to limit exposure of the oocytes to suboptimal conditions outside the incubator as much as possible, sperm selection and immobilisation was performed in advance and all selected sperm were transferred to the PVP droplet in the injection dish prior to ICSI.

If the sperm for ICSI was recovered from testicular biopsies the procedure was performed on the same day as ICSI by open biopsy under local anaesthesia. A piece of excised testicular tissue (0.5 x 0.5 x 0.5 mm) was placed in a petri dish with 0.2 ml of SPM and dissected into small pieces using fine scissors. The presence of spermatozoa was assessed under an inverted microscope and when spermatozoa were found all the surrounding medium without pieces of the biopsied tissue was transferred into a tube (Falcon®, #2003) with 0.3 ml of SPM. The sperm suspension was mixed using a vortex and incubated for up to 2.5 hours at 37°C, depending upon the viability of the sperm. After incubation the tube with testicular sperm was gently shaken and the suspension layered under pre-equilibrated paraffin oil in the microsedimentation dish and incubated for a further 0.5 to 1 hour before ICSI. Any remaining suspension was frozen to avoid the need for repeat surgery in future ICSI attempts [25].

Electroejaculation (EE) was performed in four patients with spinal cord injury. The procedure was performed on the morning of oocyte collection using a rectal probe electrode developed by Seager [26]. The antegrade portion of the ejaculate was obtained in splits. The first part of the ejaculate was assessed and prepared as fresh ejaculates and used for ICSI procedure. The 2nd and 3rd parts of the ejaculate were cryopreserved if there was at least 100 000 motile spermatozoa in the ejaculate. Sperm concentration and motility were assessed prior to sperm preparation. Sperm morphology was assessed later in the andrology laboratory.

Sperm injection
ICSI procedure was carried out on the heated stage (37°C) of a Diaphot 300 inverted microscope (Nikon, Tokyo, Japan) at 200 x magnification using Hoffman modulation contrast optics. The microscope was equipped with a video camera (Panasonic, Osaka, Japan) so that all manipulations could be monitored and recorded (Panasonic, Japan).

A Narashige micromanipulator system was used (Narashige, Tokyo, Japan) with a MO-188 Joystick and MM-188 three dimensional motor drive for the holding side and IM6 Microinjector (Cell Tam-Microinjec-
tor Eppendorf, Hamburg, Germany) for the injection side.

Holding pipettes with an angle of 30° and opening of 15 µm (Cat. No. 13903015, Laboratoire CCD, Paris, France) and spiked injection pipettes with an opening of 8 µm and angle of 30° (Cat. No. 13813008, Laboratoire CCD, Paris, France) were used. Holding and injection pipettes were aligned under low magnification with the use of an extra dish for filling the tips of both pipettes. The holding pipette was filled with liquid paraffin and then with SPM medium by capillary action. The injection pipette was filled with a small amount of PVP solution.

Sperm selection and immobilization: motile sperm which appeared to have normal morphology, as could be observed in our optics means, were selected immediately before the ICSI procedure, from the clean edges of the microsedimentation droplets or from a PVP droplet after incubation. The sperm was transferred into a clean PVP drop and immobilized. The tip of the injection pipette was placed over the tail of sperm and pressed against the tail by quick, controlled downward movement of the injection pipette using the motor-driven coarse control of the micromanipulator. When the pipette touched the sperm tail halfway between head and the tip of the tail, the tail was gently squashed between the injection pipette and the bottom of the dish. The number of sperm selected and immobilised was greater than the number of oocytes for injection. Up to 6 spermatozoa were aspirated into the injection pipette and transferred into the PVP drop in the injection dish.

For patients in Group 2, the injection pipette was changed at this point, whereas, in Group 1 the same pipette was used for the remainder of the procedure.

All ICSI procedures were performed in injection dishes (Falcon®, #1006). One strip of PVP was put into the
MULTIPLE PIPETTE USE AND OUTCOME OF ICSI

centre of the dish and an extra strip of PVP was placed close to the edge of the dish. Four small droplets of 10 µl SPM were positioned near the strip of PVP, covered with pre-equilibrated liquid paraffin, and warmed in a CO₂ incubator. Oocytes were transferred to the injection dish. Up to four oocytes were placed into each injection dish, one per droplet. The exposure time outside the incubator was not more than 5 minutes in each group. The number of injection dishes prepared per patient depended upon the number of oocytes to be injected. The injection dish, containing up to four oocytes and 5–6 immobilised spermatozoa in PVP was placed on the heated stage of the inverted microscope.

The injection pipette was lowered into the PVP droplet and a single immobilized sperm was aspirated tail first into the pipette. The injection pipette containing the sperm was then moved from the PVP droplet into the first droplet containing an oocyte. The oocyte was held by slight negative pressure exerted on the holding pipette, and was rotated using both pipettes so that the polar body was located at the 11 o’clock position. With the polar body in this position the injection pipette was always situated with the bevel facing 12 o’clock. The tip of the injection pipette was moved close to the oocyte at 3 o’clock and a check made to ensure that it was in the same plane as the oocyte equatorial plane. The sperm was then gently positioned close to the bevelled tip. The injection pipette was introduced into the oocyte until the tip was one third to one half way across the oocyte. Negative pressure was applied to the injection pipette and gentle suction carefully applied in order to puncture the oolemma. Puncture of the oolemma was evidenced by the rapid and free flow of ooplasm into the injection pipette. The pressure on the pipette was immediately reversed and with positive pressure ooplasm and sperm were slowly ejected into the oocyte, transferring as little PVP as possible. The injection pipette was withdrawn gently and the injected oocyte then released from the holding pipette. This procedure was repeated for each oocyte.

Injected oocytes were replaced in the incubator (37°C, 5.3 % CO₂) in their injection dish. After ICSI procedure was completed, injected oocytes were washed in four droplets of pre-equilibrated IVF medium under paraffin oil and transferred into 0.5 ml IVF medium under 0.5 ml liquid paraffin in a 4-well dish (Falcon®, #3654). All oocytes from both groups were incubated in a CO₂ incubator overnight. All ICSI procedures were performed by one operator.

Assessment of ICSI outcome (survival of oocytes post injection, fertilization, embryo development and pregnancy detection)

Injected oocytes were first observed during the ICSI procedure. All morphologically normal oocytes in which introduction of the sperm into the ooplasm proceeded smoothly were recorded and pre-selected. The injected oocytes were observed 16–18 hours after microinjection, for evidence of survival and fertilization. The criteria for normal fertilization were the presence of two PB together with two clearly visible pronuclei (2PN). On a few occasions one or three PN were detected, and this was recorded.

Twenty-four to twenty-six hours after ICSI the fertilized oocytes were assessed for early cleavage and embryos which had cleaved to 2-cells were identified. Forty-four to forty-eight hours after ICSI, the embryos were scored according to the classification of Giorgetti et al. [27]: cleavage stage (1 point), less than 10 % of fragmentation (1 point), no irregular blastomeres (1 point) and four blastomeres on day 2 (1 point). A score of 4 was regarded as representing perfect morphology.

A maximum of three selected embryos was transferred into a dish with M3 medium (Cat.No.10330010, Medi-Cult) and loaded with approx. 20 µl medium into a Frydman double lumen catheter (Cat.No.130DL45, Laboratoire, CCD, Paris, France). All remaining embryos were cultured in M3 medium and examined on day 5 for blastocyst formation as described by Muggleton-Harris et al. [28].

Pregnancy was detected by measuring serum concentration of B-subunit hCG (human Chorionic Gonadotropin) 16 days after ET. Where the pregnancy test was positive an ultrasound scan was performed 6 weeks later to confirm the presence of a clinical pregnancy. Clinical pregnancies were defined by the observation of a yolk sac, foetal pole and foetal heart activity on scan. Ultrasound scan also identified early miscarriage, with the loss of at least one amniotic sac, and the presence of an extra-uterine pregnancy necessitating surgical intervention.

The implantation rate was expressed as the ratio between the number of foetuses with heart beat and the number of embryos transferred. Clinical miscarriage and extra-uterine pregnancies were included for calculation of the implantation rate. Embryo quality score (EQS) was calculated as the proportion of gestation sacs to number of embryos transferred to patients who conceived [29].

Statistical analysis

Quantitative variables were summed up by their means ± SD. Paired Student’s test was used for comparing means between two groups, Chi-squared test and Fisher’s exact test were applied for possible relationships between qualitative variables. A P value < 0.05 was considered as significant. Analysis was completed using Statistics Package for social Science (SPSS) 6.1 for Windows 95.
The study included 58 patients who underwent 60 cycles of ICSI treatment. In Group 1 fresh ejaculated sperm was used for 21 patients with 2 couples undergoing a second cycle. In two couples where the men had spinal cord injury sperm were recovered on the day of ICSI by EEJ, in five patients fresh testicular sperm was used for the procedure. In Group 2 twenty three patients underwent ICSI cycles with fresh ejaculated sperm, in two couples the sperm were recovered after EEJ and for five couples fresh testicular spermatozoa was used. The mean age of the female patients in group 1 was 31.7 ± 3.9 years (range 25–41) and in group 2 31.2 ± 4.3 years (range 26–41). Mean maternal age, the duration of stimulation and the mean dose of human menopausal gonadotropin (hMG) ampoules did not differ statistically between the two groups (Table 1).

A comparative number of oocytes was retrieved in both groups, with no statistical difference: a total of 306 oocytes in Group 1 and 305 oocytes in Group 2. In Group 1, 240 oocytes were in MII stage (78.4 %), 34 in GV stage (11.1 %), and 17 in MI stage (5.5 %), with 15 oocytes having an empty zona (5 %). In Group 2, 244 oocytes were in MII stage (80 %), 38 in GV stage (12.4 %) and 12 in MI (4 %). Eleven oocytes had an empty zona (3.6 %). There were no statistically significant differences in the two groups between the number of oocytes at MI stage and those at other stages of maturation.

The percentage of normally fertilized oocytes with one PN in Group 1 0.4 % vs. 0.5 % in Group 2, or three PN 0.8 % vs. 1.2 % as well as on the proportion of oocytes that remained intact after ICSI: 97 % in Group 1 vs. 95 % in Group 2.

Early cleavage embryos were assessed after 24–26 hours. Nine out of 153 normally fertilized oocytes with two PN in Group 1 (5.8 %) and sixteen out of 179 in Group 2 (8.9 %) had cleaved to the 2-cell stage and assessed as early cleaving embryos. No significant difference was observed between two groups. There was also no significant difference in the cleavage rates per injected oocyte (61.3 % vs. 67.6 %) or in the number of blastomeres on day 2 (Table 2).

The morphological score on day 2 was similar for both groups. In Group 2 patients had slightly more embryos with the highest score of 4, but the difference was not statistically significant (Table 3).

Embryo transfer was performed in all 60 cycles. In Group 1 84 embryos and in Group 2 85 embryos were transferred. The mean number of embryos transferred was 2.8 for both groups with a maximum of 3 embryos being replaced. In Group 1 a single

<table>
<thead>
<tr>
<th>No. of cycles</th>
<th>Group 1</th>
<th>Group 2</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female age (years)*</td>
<td>31.7 ± 3.9</td>
<td>31.2 ± 4.3</td>
<td>0.618</td>
</tr>
<tr>
<td>Range</td>
<td>25–41</td>
<td>26–41</td>
<td></td>
</tr>
<tr>
<td>Duration of stimulation (days)*</td>
<td>15.2 ± 1.7</td>
<td>16.1 ± 2.1</td>
<td>0.067</td>
</tr>
<tr>
<td>Total doses of HMG** (ampoules)</td>
<td>23.5 ± 2.6</td>
<td>24.6 ± 7.6</td>
<td>0.559</td>
</tr>
</tbody>
</table>

*mean ± SD; **HMG-human menopausal gonadotrophin

| No. of oocytes retrieved (range) | 306 (6–16) | 305 (7–15) |
| Mean ± SD/cycle | 10.2 ± 2.8 | 10.2 ± 2.09 |
| No. of oocytes injected (Metaphase II)(%) | 240 (78.4) | 244 (80) |
| 2 PN* normally fertilized (%) | 153 (63.7) | 179 (73.4) |
| 1 PN abnormally fertilized (%) | 1 (0.4) | 1 (0.5) |
| 3 PN abnormally fertilized (%) | 2 (0.8) | 3 (1.2) |
| No. of cleaved embryos/oocytes | 147 (61.3) | 165 (67.6) |
| Percentage of cleaved embryos/fertilized | 96.0 | 92.2 |
| No. of 2–3 cell stage embryos (%) | 44 (29.9) | 55 (33.3) |
| No. of ≥ 4 cell stage embryos at ET (%) | 103 (70) | 110 (66.6) |

*PN = pronuclei; **Chi-square (P < 0.05)

<table>
<thead>
<tr>
<th>Embryo score*</th>
<th>Group 1 n (%)</th>
<th>Group 2 n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Score 4</td>
<td>47 (32)</td>
<td>58 (35)</td>
</tr>
<tr>
<td>Score 3</td>
<td>68 (46)</td>
<td>81 (49)</td>
</tr>
<tr>
<td>Score 2</td>
<td>28 (19)</td>
<td>18 (11)</td>
</tr>
<tr>
<td>Score 1</td>
<td>4 (3)</td>
<td>8 (5)</td>
</tr>
</tbody>
</table>

* Giorgetti (1995); with score 4 a perfect morphology was represented (Chi-square 4.77; P = 0.0189; Distribution of the embryo score is not different in both groups)
embryo was transferred in one cycle, two embryos in 4 cycles, two of which were elective 2 embryo transfers, and in 25 cycles three embryos were transferred. In Group 2 two embryos were transferred in five cycles and three embryos in 25 cycles.

No difference was observed between the two groups. Eleven patients in Group 1 and 13 patients in Group 2 had positive pregnancy tests with raised βhCG; all were confirmed as clinical pregnancies on ultrasound scan. Thus, the pregnancy rate between the two groups did not differ: 36.7% vs. 43.3%. In Group 2 one miscarriage, one extrauterine pregnancy and one multiple pregnancy were observed. The implantation rate which was calculated for all cycles, was similar in both groups: 13.1% for Group 1 vs. 16.5% for Group 2. Similarly, there was no difference in embryo quality score (EQS) calculated from the number of gestation sacs per number of embryos transferred in pregnant women between the two groups: 37.9% in Group 1 vs. 40% in Group 2. The ongoing pregnancy rate was 36.7% for both Groups (Table 4).

The relationship between fertilization rates (P < 0.05) and implantation rates (not significant) when 6 to 9 oocytes were injected and more than 9 oocytes were injected are described in Table 5. No difference was observed between the two groups.

All not transferred embryos were cultured to day 5 and assessed for development to expanded blastocyst. In Group 1 six embryos out of 63 remaining embryos (9.5%) developed to blastocyst compared with ten embryos out of 80 remaining embryos from Group 2 (12.5%). This was not significantly different (Table 6).

**Discussion**

The aim of this study was to evaluate one particular technical aspect of the ICSI procedure in relation to outcome. In our centre it is customary for one injection pipette to be used for sperm selection, immobilization, sperm aspiration and oocyte injection, independent of the number of oocytes available for injection. It was anticipated that a difference might be detected where a single injection pipette was used for the entire process compared with cycles where a pipette was used to inject not more than four oocytes.

Density gradient centrifugation was the standard technique used in our laboratory to recover motile, morphologically normal spermatozoa [30]. It has been reported that the percentage of spermatozoa with normal morphology significantly increases in ejaculated sperm samples treated by density gradients [31, 32]. In addition, spermatozoa of better nuclear consistency were observed from density gradient preparations [33]. In cases of low sperm count and low motility, e.g. spermatozoa harvested from testicular tissue, frozen-thawed samples or severe oligospermic samples, density gradient preparations may be contra-indicated because valuable spermatozoa may be lost [24]. Density gradient centrifugation was not used for sperm preparation in this study. Spermatozoa of patients with severe male infertility may possess anomalies in the DNA which cannot be observed using conventional semen analysis, e.g. number of spermatozoa, motility and morphology

| Table 4. Pregnancy and embryo implantation rates after ICSI in Group 1 and 2 |
|-----------------|-----------------|
| No. of cycles (= transfers) | 30 | 30 |
| No. of transferred embryos in all cycles (Mean ± SD) | 84 (2.8 ± 0.4) | 85 (2.8 ± 0.4) |
| No. of transferred embryos in pregnant cycles (Mean ± SD) | 29 (2.6 ± 0.7) | 35 (2.7 ± 0.5) |
| No. of clinical pregnancies (%) | 11 (36.7) | 13 (43.3) |
| No. of implantation/transferred embryos (%) | 11/84 (13.1) | 14/85 (16.5) |
| No. of ongoing pregnancies (%) | 11 (36.7) | 11 (36.7) |
| Embryo quality score* | 11/29 (37.9) | 14/35 (40) |
| * Embryo quality score = No. of gestation sacs/No. embryos transferred to patients who conceived [29] |

| Table 5. Relationship between fertilization rates and implantation rates according to the number of oocytes injected (6–9 and more than 9 oocytes) in Group 1 and 2. |
|-----------------|-----------------|-----------------|-----------------|
| No. of oocytes injected | Group 1 | Group 2 |
| 6–9 | FR* (%) | IR** (%) | FR* (%) | IR** (%) |
| 104/164 (63.5) | 10/64 (15.6) | 13/181 (74.1) | 11/68 (16.2) |
| > 9 | 49/76 (64.5) | 1/20 (5.0) | 45/63 (71.4) | 3/17 (17.6) |
| Total | 153/240 (63.7) | 11/84 (13.1) | 179/244 (73.4) | 14/85 (16.5) |
| * FR: Fertilization Rate; **IR: Implantation Rate |

| Table 6. Blastocyst development of embryos remaining after embryo transfer on day 5 in Group 1 and 2 |
|-----------------|-----------------|
| No. of cycles =26 | No. of cycles = 24 |
| Group 1 | Group 2 |
| No. of remaining embryos | 63 | 80 |
| Mean/cycle | 2.4 | 3.3 |
| No. of blastocyst/n (%) | 6/63 (9.5%) | 10/80 (12.5%) |
| * Chi-square: P = 0.60; the percentage blastocyst formation of the remaining embryos after ET not significant. |
[34]. Where ICSI is performed with spermatozoa which have DNA damage, fertilization and the first cleavage stages occur but the genome of the spermatozoa may not be capable of completing embryogenesis and blastocyst development [35].

Thus, all semen samples were prepared by microsedimentation. The origin of the sperm was either fresh ejaculated, fresh testicular spermatozoa or EEJ. Other studies have shown no difference in ICSI outcome from ejaculated or testicular sperm [36]. Similarly no difference in ICSI outcome was found for sperm of different origin and for this reason it was not considered necessary to analyse the results separately. It has been observed that in microsedimentation droplets motile spermatozoa move away from debris to the edge of droplets so that the ICSI operator can avoid aspiration of debris, erythrocytes or other cells. In addition, the technique allows visualisation of motile spermatozoa [24].

Some authors use injection pipettes of different diameters for sperm selection and for ICSI in semen samples with severe oligoasthenozoospermic and testicular sperm. Vanderzwalmen et al. [6] used pipettes of 15 and 30 microns diameters for sperm aspiration from droplets containing large number of immotile spermatozoa, debris and other cells. However, their results cannot be compared with the results obtained in this study, since Vanderzwalmen and colleagues altered a number of the technical aspects of ICSI for the purpose of their study e.g. diameter of injection pipette and cytoplasm aspiration. Tucker and colleagues [37] reported the use of extra flat-ended 10 micron injection pipettes for the aspiration of sperm harvested from testicular tissue from droplets of medium covered with paraffin oil. Hlinka et al. [38] used modified microtools for ICSI and claimed to have obtained increased fertilization rates. However, their study was based on the use of a modified pipette to avoid using PVP for sperm immobilisation and is therefore not comparable with our study.

Fujii and colleagues [39] looked at the effect of a concentration of 3 % PVP versus 8 % PVP, in order to avoid the use of extra injection pipettes for aspiration of motile spermatozoa in cases of severe oligoasthenozoospermia. As previously explained, the latter contain relatively large amount of sperm debris and/or other cells.
In the present study, motile spermatozoa were selected for injection from large numbers of immotile spermatozoa, debris and/or other cells. Where the sperm is surrounded by a lot of debris this may block the injection pipette or become attached to the outside of the pipette. Techniques have been suggested as a means of overcoming the technical difficulties of pipette blocking or becoming covered with debris; e.g. rubbing the injection pipette against the holding pipette, against the oocyte, or against the oil at the edge of medium droplet to remove debris [40]. In Group 1 of this study the pipettes were rubbed against the bottom of the dish to clean them. The same injection pipette was then used for injecting all the patient’s oocytes, and it was considered that a possible loss of sharpness with repeated use might have an effect on ICSI outcome. This requires further investigation, since we did not observe that debris interfered with the injections in our study. In the present study blocking of injection pipette was observed to be a problem with Group 1. Thus, all cases where the pipette became blocked during the procedure were excluded from the study. In Group 2 where the injection pipette was changed after sperm immobilisation, care was taken to avoid exposure to the air outside the droplet in order to avoid the risk of contamination or drying.

The percentage of damaged oocytes after ICSI was very low in the current study, 3% vs. 5%. Thus the damage rate was within the acceptable range for ICSI, which is less than the 10% quoted by Van Steirteghem et al. [41] and the ESHRE Task Force [42]. We expected a higher damage rate when a single injection pipette was used for the ICSI procedure, due to the possibility of losing its sharpness during the procedure. However, no correlation was found between the number of injecting pipettes per OOC and number of damaged OOC. Thus, we assume that damage does not necessarily arise because of loss of sharpness of the pipette. Analysis of our data showed that oocyte damage occurred irrespective of the number of injecting pipettes used for a single procedure. However, as suggested by Nagy et al. [18] damage appears to reflect the properties of the oolemma.

The rate of abnormal fertilization was relatively low (<1%) in both groups. A small number of multipronucleate oocytes were observed in both groups, supporting the theory that ICSI outcome is not related to the origin of spermatozoa or the number of injection pipettes used per cycle. If it is assumed that multipronucleate embryos developed because an extra spermatoozon attached to the outside wall of the pipette and is injected into the oocyte we might have expected to observe a higher incidence of 3PN in Group 1. This was not the case. Some triploid embryos arise after ICSI, probably because of the failure of the oocyte to extrude second PB [43].

In both groups we observed a very low rate (<1%) of activated oocytes i.e. with a single pronucleus. In these cases the formation of the male pronucleus might not occur due to defects in the sperm cell itself, such as impaired microtubule nucleation and elongation, and/or compromised sperm aster function [44]. Another explanation for the observation of one PN was given by Van der Wasterlaken et al. [20]. They suggested that the development of a single pronucleus might be due to the effect of PVP or the deposition of the sperm far from the meiotic spindle during ICSI. Nagy et al. [45] reported the early appearance of a single PN five hours after injection and the PN had disappeared before they made their next observation, 16 hours after ICSI. Geris et al. [46] reported a very low incidence of one PN (3.9%) after introduction of sperm immobilisation whereas Chen et al. [47] reported a low incidence of one PN when different techniques of sperm immobilisation were compared. They observed that when immobilisation was performed by compressing the mid-piece of the spermatozoon, the one PN rate was 1.5% compared with 3% when immobilisation was performed at the tip of the tail. Barak et al. [48] reported on a successful birth of a male baby, developed from a single pronucleated embryo, formed by injection of spermatid.

The higher rate of normally fertilized oocytes (2PN) in Group 2 of this study, where the injection pipettes were changed, may be the result of a reduction in injury to the oolemma or cytoskeleton by frequent changes of pipette. Analysis of the data from these cycles, where between 6 and 16 oocytes were injected, shows no difference in the fertilization rates or damage rates between the first oocytes injected and the last. If the reduction in fertilization rates or oocyte damage was due to a loss of sharpness of the injection pipette with repeated use it would be expected that in Group 1 there would be more damage towards the end of the procedure than at the start. This was not the case. When comparing the time taken to complete each procedure it was found that the interval between the first and the last injected oocytes in Group 1 was between 8 and 20 minutes. In Group 2, when the injection pipette was replaced after every four injected oocytes, the time interval between the first and the last injected oocytes, was up to 45 minutes. The time that any group of oocytes was kept out of the incubator was the same for Group 1 and Group 2. The extra time for Group 2 related to the change of pipettes, and the oocytes remained in the incubator during these periods of time. The difference in fertilization rates between Groups 1 and 2 was statistically significant but there was no significant difference in implantation rates between the two groups when 6 to 9 oocytes or more than 9 oocytes were injected (Table 5). In Group 1, we observed very low implantation rates for embryos when more than 9 oocytes were injected. However, due
to the small numbers of cases analysed this difference in implantation rates was not shown to be statistically significant (P = 0.22).

Based upon our scoring system for embryos, these data suggest that changing the pipette after sperm selection and immobilisation gives a trend towards better quality embryos and more spare embryos with the potential for freezing. This may be a reflection of better developmental capacity from ICSI cycles where the injection pipette is changed after sperm immobilisation. The total relatively low rate of blastocyst development may be due to the fact that embryos with highest score of 4 were always selected for transfer and only embryos with scores of 3, 2 and 1 were available for extended culture. It may be even due to the sequential culture medium, that might not be optimal for this purpose.

It can be concluded that using one injection pipette for an entire ICSI procedure resulted in poorer fertilization rates, but there was no apparent effect on cleavage rates and pregnancy rates. It is possible that the greatest benefit is obtained from changing the injection pipette once after all sperm have been immobilised and transferred to PVP. When more embryos are produced due to a higher fertilization rate, it increases the chance of the patient for pregnancy per cycle including the chance following freezing-thawing. The observations reported from this study are based on a small number of cycles and a more extensive study would be required to evaluate the potential gain in fertilization, embryo quality and implantation from changing the injection pipette during ICSI procedure. The study would need to be extended in order to establish whether using the same injection pipette for sperm selection, immobilisation and injection for all the oocytes for an individual patient has a negative influence on the developmental capacity of the injected oocytes.

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