Embryo development and chromosomal anomalies after ICSI: effect of the injection procedure*

John C.M. Dumoulin¹, Edith Coonen¹, Marijke Bras¹, J. Marij Bergers-Janssen¹, Rosie C.M. Ignoul-Vanvuchelen¹, Lucie C.P. van Wissen¹, Joep P.M. Geraedts² and Johannes L.H. Evers¹

Research Institute of Growth and Development (GROW), University of Maastricht, ¹Department of Obstetrics and Gynaecology and ²Department of Molecular Cell Biology and Genetics, Academic Hospital Maastricht, Maastricht, The Netherlands

³To whom correspondence should be addressed at: IVF-Laboratory, Department of Obstetrics and Gynaecology, Academic Hospital Maastricht, P.O. Box 5800, 6202 AZ, Maastricht, The Netherlands

Intracytoplasmic sperm injection (ICSI) is a delicate procedure requiring considerable skills of the person performing it. Theoretically, the injection procedure could damage cytoplasmic structures in the oocyte, resulting in sublethal cellular injury and/or numerical chromosomal abnormalities that could lead to impaired embryonic development. In the present study, features of the injection procedure were evaluated in a total of 2924 oocytes from 305 cycles. Development to the blastocyst stage was found to be compromised in a group of surplus embryos originating from oocytes in which >6 pl of cytoplasm was aspirated into the injection pipette during the ICSI procedure. Characteristics of the injection procedure as well as blastocyst development of surplus embryos was shown to be significantly different between the four technicians performing the ICSI. Neither the volume of cytoplasm aspirated during the injection procedure, nor the position of the polar body (6 o’clock or 12 o’clock) influenced the mean incidence of disomic cells per blastocyst as revealed by fluorescence in-situ hybridization using probes specific for chromosomes X, Y and 18. In conclusion, certain technical aspects of the injection procedure can affect subsequent embryonic development to the blastocyst stage, but do not seem to influence the rate of chromosomal abnormalities that occur in human pre-implantation embryos.

Key words: chromosomal abnormalities/fluorescence in-situ hybridization/human blastocysts/ICSI

Introduction
Since 1992, when the first four pregnancies were reported (Palermo et al., 1992), intracytoplasmic sperm injection (ICSI) has rapidly become the assisted reproduction procedure of choice for treatment of severe male-factor subfertility (Van Steirteghem et al., 1993, 1998). In a recent review, the ESHRE Task Force on ICSI (Tarlatzis and Bili, 1998), reported that in 1995 a total of 23 932 ICSI cycles was performed worldwide by 101 centres. The success rate obtained by ICSI is high, as illustrated by a viable pregnancy rate of 21% per cycle reported previously (Tarlatzis and Bili, 1998). The procedure seems also to be safe, as the incidence of major and minor congenital malformations in the group of children born after ICSI is similar to those reported for IVF, both in the range observed in the general population (Bonduelle et al., 1996; Tarlatzis and Bili, 1998). The incidence of de-novo chromosomal aberrations in children born after ICSI is slightly higher than expected in the general population, but this fact is probably linked directly to the characteristics of the subfertile men treated rather than to the ICSI procedure itself (Bonduelle et al., 1996).

In spite of these reassuring results, concern has been raised about the potential dangers of the ICSI procedure. Theoretically, both the injection technique itself, as well as the possible injection of abnormal spermatozoa, can affect oocyte and subsequent embryo quality. During injection, it cannot be avoided that a small quantity of medium, which contains potentially harmful components, is injected into the oocyte. Also, physical damage to cytoplasmic structures can possibly be inflicted by a traumatic injection. Aspiration of cytoplasm into the pipette, resulting in breakage of the oolemma, is regarded as proof that the spermatozoon is delivered into the cytoplasm of the oocyte. However, although care is taken to minimize the volume of cytoplasm that is aspirated, it cannot be avoided that in some cases a considerable volume is aspirated into the injection pipette after the membrane has been broken. It can be speculated that in such cases cytoplasmic structures will be damaged, resulting in cellular injury.

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In view of these possible risks, it may be postulated that zygotes originating from ICSI have a higher incidence of sublethal cell damage and subsequent impaired embryonic development as compared to zygotes originating from conventional IVF. Indeed, it has been reported by us (Dumoulin et al., 2000) and others (Moreno et al., 1998; Peters and Catt, 1998; Shoukir et al., 1998) that embryos obtained after ICSI have a lower potential to develop into blastocysts as compared with embryos obtained after IVF. The present study was focused on the possible traumatic effect of the injection procedure on the development in vitro of embryos derived from ICSI. Embryonic development to the blastocyst stage of surplus embryos, as well as the chromosomal constitution of blastocysts, were assessed in relation to features of the injection procedure performed on the oocyte from which the blastocysts originated.

Materials and methods

**Patients**

During a period of about 2 years, 305 ICSI treatment cycles that had at least four oocytes available for injection were included in this study. Indications for ICSI treatment were: male subfertility in 282 cycles, and no fertilization in previous IVF cycles in 23 cycles. Male subfertility was defined as having a progressive motile sperm density of \(<3 \times 10^9/ml\) in combination with \(<5\%\) morphologically normal spermatozoa, evaluated using strict criteria (Enginsu et al., 1992). Males presenting on several occasions with extreme oligozoospermia (<5 x 10^9/ml total sperm density) were karyotyped and, when an abnormal karyotype was found, excluded from treatment.

The stimulation protocol used has been described previously (Land et al., 1996). In summary, the gonadotrophin-releasing hormone (GnRH) agonist nafarelin (Synarel; Searle BV, Maarssen, The Netherlands) was used in combination with human menopausal gonadotrophin (HMG) (Pergonal; Serono, Amsterdam, The Netherlands/Humegon; Organon, Oss, The Netherlands) to stimulate multiple follicular development. Follicle growth was monitored by ultrasound, and 5000 IU of human chorionic gonadotrophin (HCG) (Pregnyl; Organon) was given as soon as the dominant follicle was judged to be mature (>18 mm), to induce final follicular and oocyte maturation. Ultrasound-guided oocyte retrieval was performed 34–35 h after HCG administration. ICSI was performed ~5 h after oocyte retrieval.

**ICSI procedure**

Removal of cumulus and corona cells, as well as the microinjection procedure have been described previously (Dumoulin et al., 2000). Only oocytes at metaphase of the second meiotic division that did not show obvious signs of degeneration were used for ICSI. Only motile spermatozoa were used for injection, and care was taken to select morphologically normal spermatozoa, if available. They were immobilized by touching the tail with the micro-injection needle, and injected head-first into the oocyte with the polar body at either 6 or 12 o’clock to avoid the passage of the pipette through the cytoplasmic region containing the meiotic spindle with the oocyte chromosomes. The pipette was inserted through the zona pellucida and well into the oocyte (~75% of the oocyte diameter). In all oocytes, the oolemma was drawn into the injection pipette in order to rupture the membrane. After breakage, care was taken that as little cytoplasm as possible was aspirated into the injection pipette, and the spermatozoon was injected into the oocyte with the smallest volume possible of the polyvinylpyrrolidone (PVP) solution. Each ICSI procedure was performed by two technicians, each injecting half of the number of oocytes. In this way, the possibility of a patient-related factor influencing the individual technician results was minimized. Furthermore, the ICSI procedures were randomly assigned to any two of the four technicians. The injection procedure was observed by the second technician on a monitor connected to the microscope. The position of the first polar body, and whether or not membrane breakage of the oocyte occurred, were noted. The following aspects of the procedure were measured on the monitor screen: the exact place where the breakage occurred inside the pipette during the application of suction; and the farthest point reached by the aspirated cytoplasm (in cm relative to the tip of the pipette) as well as the pipette dimensions (inner diameters at the tip of the pipette and at the farthest point reached by the aspirated cytoplasm). In cases where no membrane breakage was observed, the location of the membrane inside the pipette during the application of suction at the time the procedure was discontinued was taken as the farthest point reached by the aspirated cytoplasm. The data on the farthest point reached by the aspirated cytoplasm were converted to volumes (in pl) by calibrating the monitor measurement using a micrometer scale observed under the microscope at the same magnification.

**Culture procedures**

After ICSI, oocytes and embryos were cultured individually in 20 µl droplets covered by mineral oil in either a commercially available medium (IVF-50™, Scandinavian IVF Science AB, Gothenburg, Sweden), or ‘in-house’ prepared human tubal fluid medium supplemented with 9% human serum protein solution, and in an atmosphere of either 5% O<sub>2</sub>, 5% CO<sub>2</sub> and 90% N<sub>2</sub>, or 5% CO<sub>2</sub> in air. Culture procedures, as well as the allocation procedure that ensured a random distribution of treatments over the different culture techniques have been described in detail earlier (Dumoulin et al., 1999, 2000). All manipulations to oocytes and observations of oocytes and embryos were performed on heated stages.

Oocytes were checked for the presence of pronuclei and for degeneration or lysis (probably as a result from the injection procedure) at 18–20 h after injection. Embryo replacement was performed at the second or third day after oocyte retrieval. If available, two or three embryos, depending on the developmental stage and morphological appearance of the embryos, as well as on the age of the patient, were transferred to the uterus of the patient.

After embryo transfer, any supernumerary embryos originating from normally fertilized zygotes were cultured until the third day after oocyte retrieval. Cryopreservation of supernumerary embryos was performed when, at 63–67 h after insemination, one or more embryos had reached the 8-cell stage or more, and when they were of good morphological quality (grades III and IV; Bolton et al., 1989). This strict policy allowed cryopreservation to be performed only in those cases in which the chance of success after freezing and thawing was highest. When cryopreservation was not performed, any surplus embryos were left in culture.

**Fluorescence in-situ hybridization (FISH) analysis of blastocysts resulting from the culture of surplus embryos**

On the morning of day 5 after ovum retrieval, surplus embryos that cavitated to form blastocyst-like structures (defined as a rim of cells surrounding a large cavity of extracellular fluid accumulated within the embryo) were fixed and stained with 4’,6-diamidino-2-phenylindole (DAPI) as described previously (Coonen et al., 1994). The number of nuclei stained with DAPI was taken as the number of cells of the embryo. All other embryos, including those that had only just started to form a small blastocoelic cavity, were cultured for another day.
and subsequently fixed on day 6 if they had developed to the full blastocyst stage. Fixation of embryos was performed only when the patients had given written consent. Embryos were subsequently analysed with FISH using directly labelled X-, Y- and 18-chromosome-specific DNA probes as described previously (Harper et al., 1994). Only blastocysts were taken into account that had a total cell count of $\geq 25$ cells, and in which at least 75% of the cells gave clear FISH signals. This study was approved by the local Ethical Committee.

### Statistical analysis

The $\chi^2$ test was used to assess differences for categorical variables. If the overall $\chi^2$ test showed statistically significant differences, Bonferroni’s correction was applied when comparisons between groups were performed. Means were analysed using Student’s $t$-test or analysis of variance (ANOVA), where appropriate. When the ANOVA test showed significant differences ($P < 0.05$), differences between groups were evaluated using the multiple comparisons test according to Tukey. To detect any correlation between the volume of cytoplasm aspirated during the injection procedure and the mean incidence of disomic nuclei per blastocyst, linear regression analysis was used and Pearson’s correlation coefficient was calculated using the SigmaPlot statistical package (Statistics Package for Social Sciences, SPSS Inc., Chicago, IL, USA).

### Results

A total of 305 ICSI cycles met the inclusion criteria of: (i) at least four metaphase II oocytes were obtained; (ii) the injection procedure was performed by two persons, each injecting approximately half of the number of oocytes; and (iii) all oocytes and embryos were cultured individually in droplets under oil. The effects of several technical aspects of the ICSI procedure on fertilization and development of surplus embryos to the blastocyst stage are summarized in Tables I to IV. None of the groups in any of the investigated aspects of the ICSI procedure showed significant differences for the following patient characteristics: age; duration; and type of infertility. Furthermore, no significant differences in embryo development were found during the first 3 days after ICSI for any of the investigated technical aspects (data not shown). After the best embryos were transferred or cryopreserved, surplus embryos were cultured for another 2 or 3 days. The mean ($\pm$ SD) number of cells per embryo and the morphological quality of these surplus embryos on day 3 of development was found to be $3.95 \pm 2.33$, and $1.69 \pm 0.71$ (values of 1 for poorest and 4 for best morphological grade). Results in terms of degeneration of oocytes after ICSI, fertilization and embryonic development in relation to the type of membrane breakage are summarized in Table I. At 18–20 h after ICSI, 219 oocytes (7.5%) of the total of 2924 oocytes injected showed clear signs of degeneration, while 1779 oocytes (60.8%) showed two pronuclei (2 PN). The majority of the oocytes (86.6%) exhibited membrane breakage after suction of the membrane into the pipette. In a minority of the oocytes, either the membrane was broken immediately in the course of the insertion of the injection pipette through the zona pellucida (9.4% of oocytes), or the attempt to break the membrane was discontinued because too much suction had to be applied, and the spermatozoon was injected without a membrane breakage having been noticed (4.1%). As can be seen in Table I, the latter two types of membrane breakage resulted in significantly lower fertilization rates. Furthermore, oocytes in which immediate breakage was noted, showed a high degeneration rate. In a total of 146 cycles at least one surplus embryo was cultured to day 5 or 6. In the other 159 cycles, either no fertilization occurred, or all available embryos were transferred, or surplus embryos were cryopreserved. Of 783 individually cultured surplus embryos, 148 (18.9%) developed to the blastocyst stage. No significant correlation was found between the patients’ age and the percentage of surplus embryos developing to the blastocyst stage per cycle. Also, no significant differences in development to the blastocyst stage with respect to the type of membrane breakage were found (Table I).

Degeneration and fertilization rates and development of surplus embryos to the blastocyst stage in relation to the volume of aspirated cytoplasm (calculated as the volume of fluid inside the injection pipette from the tip to the farthest point reached by the aspirated cytoplasm during the injection procedure) are summarized in Table II. Only in a minority of oocytes ($n = 227; 7.8\%$) was $>6$ pl of cytoplasm aspirated. Development to the blastocyst stage of surplus embryos in this latter group was compromised.

No significant differences were observed for any of the investigated parameters between oocytes that were injected with the polar body at the 6 o’clock position or at the 12 o’clock position (Table III).

Blastocyst development was shown to be significantly higher ($P < 0.05$) in zygotes resulting from injection performed by

### Table I. Effect of type of breakage on fertilization and embryonic development to the blastocyst stage

<table>
<thead>
<tr>
<th>Type of membrane breakage</th>
<th>No. of oocytes</th>
<th>Degeneration after ICSI (%$^a$)</th>
<th>Fertilization results</th>
<th>Culture of surplus embryos to the blastocyst stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. of 1 PN zygotes (%)$^b$</td>
<td>No. of 2 PN zygotes (%)$^b$</td>
<td>No. of $&gt;2$ PN zygotes (%)$^b$</td>
</tr>
<tr>
<td>Immediate</td>
<td>274</td>
<td>96 (35)$^1$</td>
<td>11 (4)$^1$</td>
<td>110 (40)$^1$</td>
</tr>
<tr>
<td>None</td>
<td>119</td>
<td>10 (8)$^2$</td>
<td>10 (8)$^2$</td>
<td>51 (43)$^2$</td>
</tr>
<tr>
<td>After suction</td>
<td>2531</td>
<td>113 (5)$^2$</td>
<td>189 (8)$^2$</td>
<td>1618 (64)$^2$</td>
</tr>
</tbody>
</table>

$^a$In each column, proportions with different superscripts$^1,2$ are significantly different ($\chi^2$-test, 3$\times$2 table, Bonferroni correction, $P < 0.001$).

$^b$Mean ($\pm$ SEM) number of cells per blastocyst that consisted of $\geq 25$ cells.

$^c$No significant differences (analysis of variance; ANOVA).

PN = pronucleus, ICSI = intracytoplasmic sperm injection.
one of the four technicians (no. 4) (Table IV). The differences in the number of oocytes injected by the technicians is a reflection of the part-time appointment of three of the four technicians involved. Characteristics of the injection procedure performed by the different technicians are summarized in Table IV. As can be seen, in oocytes injected by technician no. 4, the oolemma broke at a point significantly closer to the tip of the injection pipette, while technician no. 3 aspirated slightly but significantly, $P < 0.05$ more cytoplasm.

A total of 33 blastocysts originating from 2 PN zygotes met the inclusion criteria for evaluation of their chromosomal constitution: $\geq 75\%$ of their cells presented clear fluorescent probe signals for chromosomes X, Y and 18, in combination with a total cell count of $\geq 25$. Of these 33 blastocysts

Table II. Effect of volume of aspirated cytoplasm on fertilization and embryonic development to the blastocyst stage

<table>
<thead>
<tr>
<th>Volume of aspirated cytoplasm (pl)</th>
<th>No. of oocytes</th>
<th>Degeneration after ICSI (%)</th>
<th>Fertilization results</th>
<th>Culture of surplus embryos to the blastocyst stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No. of 1 PN zygotes (%)</td>
<td>No. of 2 PN zygotes (%)</td>
</tr>
<tr>
<td>$\leq 2$</td>
<td>190</td>
<td>9 (5)</td>
<td>9 (5)</td>
<td>128 (67)</td>
</tr>
<tr>
<td>2–3</td>
<td>1049</td>
<td>78 (7)</td>
<td>67 (6)</td>
<td>662 (63)</td>
</tr>
<tr>
<td>3–4</td>
<td>944</td>
<td>85 (9)</td>
<td>57 (6)</td>
<td>566 (60)</td>
</tr>
<tr>
<td>4–5</td>
<td>353</td>
<td>25 (7)</td>
<td>28 (8)</td>
<td>213 (60)</td>
</tr>
<tr>
<td>5–6</td>
<td>161</td>
<td>8 (5)</td>
<td>17 (11)</td>
<td>91 (57)</td>
</tr>
<tr>
<td>$&gt; 6$</td>
<td>227</td>
<td>13 (6)</td>
<td>19 (8)</td>
<td>139 (61)</td>
</tr>
</tbody>
</table>

*In each column, proportions with different superscripts$^{1,2}$ are significantly different ($\chi^2$-test, 6×2 table, Bonferroni correction, $P < 0.05$).

$^a$Mean (± SEM) number of cells per blastocyst that consisted of $\geq 25$. cells.

$^b$No significant differences (ANOVA).

Table III. Effect of position of polar body during ICSI procedure on fertilization and embryonic development to the blastocyst stage

<table>
<thead>
<tr>
<th>Position of polar body</th>
<th>No. of oocytes</th>
<th>Degeneration after ICSI (%)</th>
<th>Fertilization results</th>
<th>Culture of surplus embryos to the blastocyst stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No. of 1 PN zygotes (%)</td>
<td>No. of 2 PN zygotes (%)</td>
</tr>
<tr>
<td>6 o’clock</td>
<td>1356</td>
<td>92 (7)</td>
<td>102 (8)</td>
<td>825 (61)</td>
</tr>
<tr>
<td>12 o’clock</td>
<td>1568</td>
<td>127 (8)</td>
<td>108 (7)</td>
<td>974 (62)</td>
</tr>
</tbody>
</table>

*No significant differences ($\chi^2$-test).

$^a$Mean (± SEM) number of cells per blastocyst that consisted of $\geq 25$. cells.

$^b$No significant differences (ANOVA).

Table IV. Effect of the technician performing the ICSI procedure on fertilization and embryonic development to the blastocyst stage

<table>
<thead>
<tr>
<th>Technician performing the ICSI</th>
<th>No. of oocytes</th>
<th>Degeneration after ICSI (%)</th>
<th>Fertilization results</th>
<th>Culture of surplus embryos to the blastocyst stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No. of 1 PN zygotes (%)</td>
<td>No. of 2 PN zygotes (%)</td>
</tr>
<tr>
<td>1</td>
<td>1126</td>
<td>88 (8)</td>
<td>85 (8)</td>
<td>716 (64)</td>
</tr>
<tr>
<td>2</td>
<td>599</td>
<td>51 (9)</td>
<td>28 (5)</td>
<td>361 (60)</td>
</tr>
<tr>
<td>3</td>
<td>585</td>
<td>39 (7)</td>
<td>40 (7)</td>
<td>364 (62)</td>
</tr>
<tr>
<td>4</td>
<td>614</td>
<td>35 (6)</td>
<td>63 (10)</td>
<td>358 (58)</td>
</tr>
</tbody>
</table>

*In each column, proportions with different superscripts$^{1,2}$ are significantly different ($\chi^2$-test, 4×2 table, Bonferroni correction, $P < 0.05$).

$^a$Mean (± SEM) number of cells per blastocyst that consisted of $\geq 25$. cells.

$^b$No significant differences (ANOVA).

Table V. Aspects of the injection procedure performed by four different technicians

<table>
<thead>
<tr>
<th>Technician performing the ICSI</th>
<th>Point of oolemma breakage</th>
<th>Volume of aspirated cytoplasm(b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>82.8 ± 1.4</td>
<td>3.59 ± 0.04</td>
</tr>
<tr>
<td>2</td>
<td>84.9 ± 2.0</td>
<td>3.42 ± 0.06</td>
</tr>
<tr>
<td>3</td>
<td>84.4 ± 2.0</td>
<td>3.68 ± 0.08</td>
</tr>
<tr>
<td>4</td>
<td>76.3 ± 1.8</td>
<td>3.50 ± 0.05</td>
</tr>
</tbody>
</table>

$^a$µm relative to the tip of the pipette; values are mean ± SEM.

$^b$Volume in pl; values are mean ± SEM.

$^c$In each column, means with different superscripts$^{1,2}$ are significantly different (ANOVA, Tukey, $P < 0.01$).
successful intracytoplasmic injection (Vanderzwalmen et al., 2000) resulted in a breakage of the oocyte membrane resulting in oolemma breakage during the ICSI procedure. The main topic of the present study was the embryonic development of the blastocyst stage of surplus embryos in relation to features of the injection procedure. In a previous study it was found that development to the blastocyst stage of embryos following ICSI is compromised in comparison with that of embryos following IVF (Dumoulin et al., 2000). Reasons for this low incidence of blastocyst formation of embryos obtained after ICSI can either be that spermatozoa used for ICSI are selected from sperm populations with relatively high incidences of fragmented DNA and chromosomal abnormalities, and/or that some oocytes are injured sublethally during the injection procedure (for references, see Dumoulin et al., 2000). In the present study it was shown that the injection technique itself can indeed significantly affect pre-implantation embryonic development. Zygotes resulting from an injection procedure during which a relatively large volume of cytoplasm was unintentionally aspirated, had a lower potential to develop into blastocysts (Table II), probably due to sublethal damage inflicted to the oocyte. Such a large volume of cytoplasm aspirated during ICSI can be caused by an accidental manoeuvre of the technician performing the injection. It is also possible that the type of membrane breakage and the volume of aspirated cytoplasm are not related to the injection technique per se, but are oocyte-related phenomena (Palermo et al., 1995). They could reflect a difficulty to breach the oocyte membrane which inevitably leads to application of stronger suction that cannot be reversed in time at the moment the membrane breaks. However, whatever the cause may be of the aspiration of an excessive amount of cytoplasm, it occurs only in a small minority of oocytes and can therefore account for only a small part of the low incidence of blastocyst formation of embryos obtained after ICSI.

The type of oolemma breakage could not be evaluated with respect to the chromosomal constitution of resulting blastocysts, as too few blastocysts were obtained in two of the three groups (see Table I).

Discussion

Aspiration of cytoplasm into the injection pipette and the resulting breakage of the oocyte membrane during the ICSI procedure has been shown to be an important determinant of successful intracytoplasmic injection (Vanderzwalmen et al., 1996), although it is not considered by all investigators to be necessary (Mansour et al., 1996). The present data show that, in our hands, the type of membrane breakage does have a significant effect on damage and fertilization rates. In the groups of oocytes in which either an immediate or no breakage of the oolemma occurred, fertilization rates were lower than in the group of oocytes in which the membrane broke after application of suction. Furthermore, immediate membrane breakage was associated with high degeneration rates. Comparable types of breakage (sudden, difficult, and normal) were observed previously (Palermo et al., 1996) in similar proportions (11.8, 14.3 and 73.9% of injected oocytes respectively). These authors also found significantly lower survival and fertilization rates in the sudden breakage group. Similar results were obtained by others (Nagy et al., 1995; Carillo et al., 1998), although in the latter study no lower fertilization rates were found in the group of oocytes that showed immediate membrane breakage. It has been shown in several studies that the position of the polar body during injection affects fertilization, and/or embryonic development rates (Nagy et al., 1995; Van der Westerlaken et al., 1999; Blake et al., 2000). However, in the present study, no significant differences were found between the 6 and 12 o’clock positions of the polar body for any of the investigated parameters.

The main topic of the present study was the embryonic development to the blastocyst stage of surplus embryos in relation to features of the injection procedure. In a previous study it was found that development to the blastocyst stage of embryos following ICSI is compromised in comparison with that of embryos following IVF (Dumoulin et al., 2000). Reasons for this low incidence of blastocyst formation of embryos obtained after ICSI can either be that spermatozoa used for ICSI are selected from sperm populations with relatively high incidences of fragmented DNA and chromosomal abnormalities, and/or that some oocytes are injured sublethally during the injection procedure (for references, see Dumoulin et al., 2000). In the present study it was shown that the injection technique itself can indeed significantly affect pre-implantation embryonic development. Zygotes resulting from an injection procedure during which a relatively large volume of cytoplasm was unintentionally aspirated, had a lower potential to develop into blastocysts (Table II), probably due to sublethal damage inflicted to the oocyte. Such a large volume of cytoplasm aspirated during ICSI can be caused by an accidental manoeuvre of the technician performing the injection. It is also possible that the type of membrane breakage and the volume of aspirated cytoplasm are not related to the injection technique per se, but are oocyte-related phenomena (Palermo et al., 1995). They could reflect a difficulty to breach the oocyte membrane which inevitably leads to application of stronger suction that cannot be reversed in time at the moment the membrane breaks. However, whatever the cause may be of the aspiration of an excessive amount of cytoplasm, it occurs only in a small minority of oocytes and can therefore account for only a small part of the low incidence of blastocyst formation of embryos obtained after ICSI.

More direct evidence for an effect of the injection procedure itself on the embryonic development is the fact that the individual technician performing the ICSI procedure can significantly affect the developmental capacity of the resulting zygote (Table IV). The observed differences in blastocyst development between zygotes obtained by the respective technicians possibly reflect subtle differences in their injection technique. For instance, as shown in Table V, the membrane breakage occurred at a significantly shorter distance from the tip of the pipette in the group of oocytes injected by the
technician who also had the highest blastocyst yield, possibly reflecting a more sudden aspiration. Others (Tesarik and Sousa, 1995) also found that a more vigorous aspiration of cytoplasm led to higher fertilization rates. More evidence that subtle variations in performing the injection technique by different operators can influence ICSI results can be found elsewhere (Katz et al., 1996; Carillo et al., 1998). These authors reported that rates of fertilization, damage after ICSI, and embryo arrest significantly improve with time; this reflects a learning curve and implies that the injection technique requires substantial experience by the laboratory technicians. It was also reported (Van der Westerlaken et al., 1999) that there were inter-individual differences between technicians in terms of ICSI results. Analysis of results beyond the time period of the present study showed that the differences between the four technicians with respect to the blastocyst formation of surplus embryos obtained after ICSI became smaller and was no longer significantly different, thus probably reflecting a learning curve (data not shown). However, in this extended study period, the differences in blastocyst formation between embryos resulting from IVF or ICSI remained significantly in favour of IVF (Dumoulin et al., 2000). Thus, it can be concluded that the influence of the individual technician performing the ICSI procedure on the blastocyst formation is also limited, and only partly explains the differences between the IVF and ICSI outcomes.

It has been speculated that ICSI might lead to irregular chromosome segregation (Macas et al., 1996; Rosenbusch and Sterzik, 1996). It can be postulated that the cytoskeletal architecture (and in particular the second metaphase spindle) could be disturbed during the ICSI procedure, either by the injection pipette itself, or by substances such as culture medium and PVP injected into the oocyte. Aspirating cytoplasm during ICSI would further increase the chance of disrupting the meiotic spindle. Confirmation of this theory comes from findings of a higher incidence of diffuse cytoskeletal assembly and other features suggestive of a damaged or disorganized cytoskeleton in oocytes and zygotes after ICSI in comparison with those after IVF (Moomjy et al., 1999). To minimize the possibility of damaging the meiotic spindle by the injection pipette itself, oocytes were always injected at the 3 o’clock position with the polar body either at the 6 o’clock or 12 o’clock position, thus inserting the injection pipette as far away as possible from the region of the oocyte where the spindle is expected (Sousa and Tesarik, 1994; Palermo et al., 1995). However, it has been shown in hamsters (Silva et al., 1999), rhesus monkeys (Hewitson et al., 1999) and humans (Hardarson et al., 2000) that the meiotic spindle in oocytes has a variable position in relation to the first polar body. Therefore, polar body positioning is a relatively unreliable indicator of the position of the meiotic spindle. A disturbed or damaged spindle could lead to non-disjunction during second meiosis, resulting in an aneuploid zygote. In such a case, all cells (or at least a majority) in the resulting blastocyst would be expected to be aneuploid. A blastocyst displaying this type of chromosomal abnormality was found only once, while the majority of blastocysts showed mosaicism of various types of numerical chromosomal abnormalities. This is in agreement with findings by others (Munne et al., 1998), who reported the same low rate of gonosomal aneuploidy both in ICSI (1%) and IVF (2%) embryos. Most numerical chromosome abnormalities in embryos originating from 2 PN zygotes arise during mitotic divisions, and the stage of onset of mosaicism was found to occur at any mitosis (Coonen et al., 1998; Munne and Cohen, 1998). Culture conditions and other treatment-related factors have been suggested to influence the rate of chromosome abnormalities in embryos that are generated after zygote formation (Munne et al., 1997). It could be postulated that after ICSI, injury to the cytoskeleton might lead to minor disturbances in the complex machinery needed for normal cytokinesis, and that cytokinesis that would lead to abnormal chromosomal division, either immediately or during later divisions. One might expect a dose-dependent effect of the volume of aspirated cytoplasm during ICSI on the incidence of chromosomal abnormalities occurring in blastocysts that have undergone five or six divisions since the pronuclear stage. A more drastic aspiration of cytoplasm could be expected to lead to a more severe disturbance of cytoplasmic structures and an increased chance of a disturbed meiotic spindle. However, no correlation was found between the percentage of normal cells (evaluated for both the sex chromosomes and chromosome 18) at the blastocyst stage and the volume of aspirated cytoplasm in the oocytes from which they originated.

In conclusion, technical features of the injection procedure do influence subsequent embryonic development to the blastocyst stage, but probably not to such an extent that it would explain the differences in blastocyst formation between zygotes obtained after conventional IVF and those obtained after ICSI. Furthermore, the injection technique does not appear to influence the rate of chromosomal abnormalities that occur in human pre-implantation embryos.

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