Rescue ICSI of oocytes that failed to extrude the second polar body 6 h post-insemination in conventional IVF

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BACKGROUND: Attempts to ‘rescue’ by ICSI oocytes that remained unfertilized 24 h after conventional IVF have generally resulted in poor outcomes. The aim of the present study was to compare the outcome of rescue ICSI performed on one group of patients 6 h after initial insemination with those of another group where rescue ICSI was performed 22 h after initial insemination. METHODS: Twenty-five patient IVF cycles provided the oocytes for rescue ICSI 6 h after initial insemination, and 20 cycles provided the oocytes for rescue ICSI 22 h after initial insemination in this retrospective study. Fertilization and cleavage rates, embryo quality, implantation, and pregnancy rates after rescue ICSI were the main outcome measures. RESULTS: A fertilization rate of 70.3% was achieved with 6 h rescue ICSI compared with 48.5% with 22 h rescue ICSI (P < 0.0001). From 6 h rescue ICSI, 12 clinical pregnancies (48.0%) resulted in three sets of twins, eight singletons and one abortion. From 22 h rescue ICSI there was one (5.0%) singleton pregnancy and delivery of a healthy baby. Likewise, the implantation rate was 20.2% from 6 h rescue ICSI compared with 1.72% from 22 h rescue ICSI (P < 0.02). CONCLUSIONS: Rescue ICSI after 6 h post-insemination (46 h post-HCG) gave better fertilization, pregnancy and implantation rates compared with rescue ICSI after 22 h when oocytes have become aged.

Key words: fertilization failure/oocytes/polar body/pronuclei/rescue ICSI

Introduction

The fertilization rate after conventional IVF is in the region of 60–70% (Nagy et al., 1993) and the fertilization rate at our centre is 71%. However, there are cases of lower fertilization rates or even total fertilization failures due to various factors, such as unfavourable hormonal milieu resulting from the stimulation protocol used, and defects in the spermatozoa and/or oocytes (Benadiva et al., 1999). Attempts to rescue unfertilized oocytes by ICSI when they are ~1 day old have yielded poor results (Sjogren et al., 1995; Tsirigotis et al., 1995; Morton et al., 1997). Our experience with rescue ICSI 22 h after failed insemination resulted in similarly poor results—48.5% fertilization rate and one livebirth from 20 cycles of failed fertilization. We have therefore explored other ways of improving success with cases of IVF fertilization failures.

Oocytes have been observed to be fertilized 2–4 h after exposure to spermatozoa, and the second polar body is released in ~90% of fertilized oocytes by 6 h (Chen and Sathanathan, 1986; Plachot et al., 1986; Nagy et al., 1994; Payne et al., 1997). We therefore proceeded to look for the release of the second polar body 6 h after initial insemination and subjected to ICSI those oocytes in which a second polar body was not evident. The outcome of this rescue attempt was compared with retrospective results of rescue ICSI performed 22 h after failed fertilization in a completely different patient group.

Materials and methods

Between January 1999 and December 2000, oocytes from 240 patient cycles of standard IVF were checked for the release of the second polar body 6 h after initial insemination. A second polar body was not evident in any of the oocytes of 25 patients 6 h after insemination (6 h group). ICSI was performed on these oocytes, which were then checked for fertilization the following day. The mean (± SEM) age of patients in this group was 35.4 ± 4.4 years. Oocytes of patients from whom some oocytes showed a second polar body were not used for rescue ICSI. The results of this intervention were compared with those obtained in a previous trial on oocytes from a different group of patients who had complete failure of fertilization and in which ICSI was performed 22 h after the initial insemination (22 h group). The oocytes for the latter trial were obtained from 20 patient IVF cycles between January 1997 and December 1998 from a total 230 patient cycles. The mean (± SEM) age of the patients in this group was 35.2 ± 4.1 years. There was no difference in the clinical characteristics of the patients between the two groups.

Ovarian stimulation

All patients used Suprefact (Buserelin 0.5 mg/day s.c.; Hoechst, Germany) for down-regulation of the pituitary commencing from cycle day 21 and continuing up to the day of HCG administration. When pituitary down-regulation was achieved [estradiol (E2) <25 pg/ml and ultrasound demonstrated ovarian follicles <0.5 cm], Metrodin HP 300 IU for 5 days (Serono, Switzerland) followed by Puregon 150 IU daily (Organon, The Netherlands) was administered.
until the day before HCG injection. Follicular development was monitored by ultrasound scanning and laboratory E2 and progesterone values. Patients received 10 000 IU of hCG when three or more follicles measured >18–20 mm in diameter. Oocytes were then obtained by using transvaginal ultrasound under sedation 38 h after hCG injection.

**Sperm preparation**

Semen samples were collected in sterile containers by masturbation. After liquefaction, samples were analysed for sperm concentration, motility and morphology. Subsamples of 1 ml were placed in 6 ml tubes, overlaid with 1 ml of Medicult IVF medium (Medicult, Denmark), and incubated at 37°C under 5% CO2. After 30–60 min, the supernatant was pelleted and washed twice at 600 g for 5 min. The sperm pellet was suspended in 0.5–1.0 ml of culture medium and then used for oocyte insemination.

**Insemination**

Oocytes were washed in culture medium and placed in groups of four in 0.5 ml of freshly equilibrated medicult IVF medium in four-well Nunc dishes. Oocytes of the 6 h group were exposed to 20–30 × 10³ spermatozoa 40 h post-hCG for 6 h only (until 46 h post-hCG), after which they were checked for the presence of the second polar body. Those in which a second polar body was not evident were subjected to ICSI and checked for fertilization the next day.

The oocytes in the 22 h group were inseminated with 20–30 × 10³ motile spermatozoa 40 h post-hCG, incubated in culture medium and then checked for evidence of fertilization after 18–20 h. Any corona cells that still remained attached to the oocytes were removed using finely drawn Pasteur pipettes to allow assessment of fertilization. ICSI was then performed on those oocytes that failed to fertilize i.e. those that did not show a second polar body or two pronuclei (2PN).

**ICSI**

For the procedure of ICSI, both holding and injection pipettes were obtained commercially (Humagen Fertility Diagnostics, USA), and the ICSI procedure was performed using Narashige micromanipulators (Narashige, Japan) under Hoffman modulation optics.

Just before the ICSI procedure the sperm suspension was placed in a 10 μl droplet of 10% polyvinyl-pyrrolidone (Medicult) at the 3 o’clock position. Injection of the oocyte was performed in microdroplets of Medicult IVF medium under mineral oil (International Medical, The Netherlands).

A single motile morphologically normal spermatozoon that had migrated to the 9 o’clock position was selected, immobilized by touching its tail with the injection micropipette, and then aspirated tail first into the pipette. The oocyte to be injected was secured with the holding pipette (9 o’clock position) adjacent to the polar body (6 o’clock position). The micropipette containing the sperm was then inserted through the zona pellucida and the oolemma into the ooplasm at the 3 o’clock position of the oocyte. Penetration of the oolemma was confirmed by aspiration of some cytoplasm into the micropipette and the spermatozoon was then slowly injected. The pipette was withdrawn gently and the oocyte released from the holding pipette.

**Assessment of fertilization and cleavage**

Oocytes in the 6 h group were examined for fertilization 16–18 h after ICSI, which was ~24 h after oocyte retrieval. Cleavage of the oocytes was assessed on day 2 (48 h) and day 3 (72 h) before transfer into the uterus.

Oocytes in the 22 h group were examined for fertilization 18–20 h after ICSI, i.e. on day 2 after oocyte retrieval (48 h). Cleavage of the fertilized oocytes was assessed on day 3 (72 h) just before their transfer into the uterus. The embryos were graded on a scale of 1 to 4 (Cummins et al., 1986). Grade 1 embryos were the best embryos, containing even-sized, symmetrical blastomeres with no obvious fragmentation; grade 2 had blastomeres of uneven size or the total cytoplasmic mass contained <10% fragmentation; grade 3 embryos had 10–50% of their cytoplasm fragmented; and grade 4 showed >50% cytoplasmic fragmentation.

Patient β-hCG was measured for diagnosis of pregnancy 9–10 days after embryo transfer and then measured serially to monitor the rise in its titre. Implantation was noted later by the appearance of the gestational sac in the uterus using transvaginal ultrasonography.

All data are expressed as mean ± SEM. Fertilization, implantation and pregnancy rates were compared between the 6 h group and the 22 h group, and tested for significant difference by the χ² test. A P value of <0.05 was considered statistically significant.

**Results**

Of the 25 patients in the 6 h group, subfertility was due to tubal factors in three, polycystic ovaries (PCO) in seven and endometriosis in six, and was unexplained in nine. Of the 20 patients in the 22 h group, subfertility was due to tubal factors in three, PCO in five and endometriosis in five, and was unexplained in seven.

The 6 h group consisted of 245 oocytes, of which 226 (92.2%) were in metaphase II. Following rescue ICSI, evidently normal fertilization was observed in 159 oocytes (70.3%). This was significantly higher (P < 0.0001) than the fertilization rate obtained in the 22 h group, in which 167 (91.7%) of 182 oocytes were in metaphase II and 81 (48.5%) showed evidence of normal fertilization. The 1PN and 3PN rates in the 6 h group and the 22 h group were not significantly different (Table I). In the 6 h group 145 (91.1%) of the 159 embryos cleaved, while in the 24 h group 73 (90.1%) of the 81 embryos cleaved. There were more grade 1 embryos from the 6 h group (53.1%) than from the 22 h group (20.5%) of oocytes (P < 0.0008).

Embryos were transferred on day 3 after oocyte retrieval (72 h) in both the groups. Owing to the delay in the rescue of

| Table I. The results of rescue ICSI performed 6 h or 22 h after initial insemination |
|---------------------------------|-----------------|------------------|-----------------|-----------------|
| No. patients | 6 h group¹ | 22 h group² |
| No. oocytes | 25 | 20 |
| Mean (± SEM) no. oocytes | 9.8 ± 5.5 | 9.1 ± 5.1 |
| No. metaphase II oocytes (%) | 226 (92.2) | 167 (91.7) |
| No. oocytes fertilized (%) | 159 (70.3) | 81 (48.5) |
| One pronuclei (%) | 7 (3.0) | 9 (5.3) |
| Three pronuclei (%) | 15 (6.6) | 15 (8.9) |
| No. embryos (%) | 145 (91.1) | 73 (90.1) |
| No. grade 1 embryos (%) | 77 (53.1) | 15 (20.5) |
| Mean no. embryos transferred | 2.96 | 2.90 |
| No. total pregnancies (%) | 12 (48.0) | 1 (5.0) |
| No. embryo implanted (%) | 15 (20.2) | 1 (1.72) |

¹Rescue ICSI was performed on unfertilized oocytes from these patients 6 h after initial insemination in IVF programmes between January 1999 and December 2000.
²Rescue ICSI was performed on unfertilized oocytes from these patients 22 h after initial insemination in IVF programmes between January 1997 and December 1998.
The results of IVF in the non-failed fertilization cohort of the 6 h and 22 h groups.

<table>
<thead>
<tr>
<th></th>
<th>6 h groupa</th>
<th>22 h groupb</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. patients</td>
<td>215</td>
<td>210</td>
</tr>
<tr>
<td>No. oocytes</td>
<td>2043</td>
<td>2016</td>
</tr>
<tr>
<td>Mean (± SEM) no. oocytes</td>
<td>9.5 ± 5.2</td>
<td>9.6 ± 5.3</td>
</tr>
<tr>
<td>No. oocytes fertilized (%)</td>
<td>1494 (73.1)</td>
<td>1468 (72.8)</td>
</tr>
<tr>
<td>One pronucleus (%)</td>
<td>59 (2.8)</td>
<td>62 (3.07)</td>
</tr>
<tr>
<td>Three pronuclei (%)</td>
<td>125 (6.1)</td>
<td>128 (6.3)</td>
</tr>
<tr>
<td>No. embryos (%)</td>
<td>1435 (96.0)</td>
<td>1399 (95.2)</td>
</tr>
<tr>
<td>No. grade 1 embryos (%)</td>
<td>804 (56.0)</td>
<td>769 (54.9)</td>
</tr>
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aNon-failed fertilization cohort in IVF programs between January 1999 and December 2000.

Discussions

Failure of fertilization occurs in ~10–25% of IVF cycles (Chen et al., 1995). Before ICSI became available, it was routine practice to re-inseminate 1-day-old oocytes that had failed to fertilize, usually with poor outcomes (Boldt et al., 1987). With the introduction of ICSI, several investigators (Sjogren et al., 1995; Tsirigotis et al., 1995; Morton et al., 1997; Yuzpe et al., 2000) reported successful rescue of 1-day-old oocytes by ICSI after fertilization failure. Fertilization and subsequent pregnancy rates from such rescue attempts varied from 24 to 48% and 6 to 20%, respectively. In our study, following rescue ICSI after 22 h, a fertilization rate of 48.5% was achieved and only one live birth resulted.

Even though oocytes can be rescued after 22 h, owing to the long fertilization window the percentage of grade 1 embryos (20.5%) is comparatively much less (P < 0.0008) than normally resulted from oocytes that were fertilized at the optimum time in the remaining cohort of the same group of patients (Table II). This shows clearly that there is a direct relationship between the time interval from oocyte retrieval to fertilization and the development of grade 1 embryos, with obvious effects on pregnancy rates. This may be due to the ageing of oocytes in culture, which results in increased cytogenetic abnormalities (Edwards and Broody, 1995), fewer normal viable embryos and low pregnancy rates. Another factor contributing to low pregnancy rates following rescue ICSI 22 h after initial insemination may be asynchrony between developmental stage of the embryo and the state of the endometrium. Pronuclei appeared on the following day (16 h post-ICSI) when ICSI rescue was performed after 6 h, thus coinciding with the occurrence of PN formation among normal ICSI cycles. In contrast, when rescue ICSI was performed 22 h after initial insemination, PN appeared 32 h after oocyte retrieval. Consequently embryos from the 22 h group were at the 2–4-cell stage when transferred on day 3, whereas the embryos from the 6 h group had 5 or more cells.

In the present study, rescue ICSI was performed 6 h after insemination in those oocytes, which failed to show the release of a second polar body. This resulted in 70% fertilization rate, which is comparable to the fertilization rate of male factor cases (De Croo et al., 2000) and significantly higher than the results of previous patients when rescue ICSI was performed 22 h after initial insemination (48.5%) (P < 0.0001). Even though oocytes can be rescued after 22 h their ability to undergo fertilization decreases with their ageing.

In the 22 h group there is the possibility that some oocytes that were not fertilized after initial insemination were in the germinal vesicle or metaphase I stage when inseminated. Culture may have resulted in maturation to metaphase II, enabling fertilization by ICSI and subsequent clinical pregnancy.

Thus, rescue ICSI 6 h after insemination has advantages over rescue ICSI after 22 h. First, rescue ICSI after 6 h results in higher fertilization rates (P < 0.0001) and more grade 1 embryos compared with rescue ICSI after 22 h (P < 0.0008). Secondly, the time-course events of fertilization among early rescued oocytes (6 h) followed a pattern similar to those of oocytes that underwent ICSI at the normal time of fertilization in that PN formation was complete by 16±18 h after ICSI, indicating that oocytes from the 6 h group had 5 or more cells. Furthermore, Embryos from the 22 h group had only one viable pronucleus, whereas in the 22 h group there was only one (5%) singleton embryo. In total, 74 embryos were transferred in the 6 h group and 58 embryos were transferred in the 22 h group.

In the 6 h group there were 12 (48%) clinical pregnancies, whereas in the 22 h group there was only one (5%) singleton pregnancy (P < 0.0003), which resulted in a healthy baby. The 12 pregnancies in the 6 h group resulted in three sets of twins, eight singletons and one abortion. In this group the implantation rate was 20.2%, compared with 1.72% (P < 0.02) in the 22 h group (Table I). The results of IVF in the non-failed fertilization cohort of the 6 h and 22 h groups are given in Table II.
oocytes probably had already undergone cytoskeletal damage and disorganization. Nevertheless, there are some concerns that need to be addressed when considering early rescue ICSI.

The earliest indication of fertilization of oocytes is the extrusion of the second polar body. It has been shown that following ICSI, ~22% of oocytes release their second polar body by 2–4 h and 66% at 4 h. Pronuclei appear by 6 h and 80% have two pronuclei by 8 h (Nagy et al., 1994). Theoretically, by 8 h all oocytes would have released their second polar body if they have been fertilized. One of the concerns when performing rescue ICSI 6 h after insemination is the possible delay in the release of the second polar body that occurs in ~10% of the oocytes. Although this is a small number, the risk will be greatest if it occurs in all the oocytes in a given patient. The risk may be greatly reduced by performing rescue ICSI 8 h after initial insemination when all of the fertilized normal oocytes would be expected to have released the second polar body.

Performing rescue ICSI on oocytes that are already in the process of fertilization could lead to the formation of 3PN zygotes. During normal insemination, 3PN zygotes result from two spermatozoa participating in fertilization, probably after entering the oocytes simultaneously. It is not known whether a second sperm entering at a later time causes any abnormalities other than 3PN formation. In the event of 3PN zygote formation after rescue ICSI, microsurgical removal of the second male PN may be attempted, although it is not a routine practice. We have achieved a live birth from such microsurgical procedure after oocytes were fertilized with three PN following normal insemination (Kattera and Chen, 2003).

The second concern is when the first polar body in some oocytes becomes fragmented, making it difficult to distinguish from the second polar body when rescue ICSI is attempted after 6 h. Rescue ICSI on these oocytes may lead to the formation of 3PN zygotes. In such situations, it is advisable to look for the appearance of PN as early as possible, so that 3PN zygotes may be identified.

Finally, it is difficult to predict fertilization failure in IVF due to several contributing factors (Benadiva et al., 1999). Hence it is important to look for earliest indications of fertilization. We carry out our oocyte retrievals 38 h post-hCG, inseminate at 40 h (10 a.m.) post-hCG, check for the release of second polar body 46 h (4 p.m.) post-hCG and microinject those oocytes that do not show the second polar body.

In conclusion, the present study demonstrates that oocytes that failed to fertilize in IVF can be rescued by ICSI 6 h after insemination after checking for the extrusion of the second polar body. However, caution must be exercised when considering such rescue attempts bearing in mind the potential for 3PN zygote formation.

References

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