A prospective study, using sibling oocytes, examining the effect of 30 seconds versus 90 minutes gamete co-incubation in IVF

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BACKGROUND: Traditionally oocytes have been exposed to sperm overnight, for 16–20 h. This long period of co-incubation, however, has been shown to create problems with high levels of reactive oxygen species (ROS), which may affect embryo viability and cause hardening of the zona pellucida. Recently, a positive effect of reducing the co-incubation time to 90–120 min was reported. The objective of this study was to evaluate whether a further reduction of the co-incubation period could benefit the outcome of IVF. METHODS: In this prospective study, 777 sibling oocytes from 81 women undergoing IVF were divided via alternate allocation to co-incubation for either 30 s (ultrashort co-incubation) (group A) or for 90 min (standard co-incubation) (group B). Endpoints were normal fertilization (two-pronuclear, 2PN), polyspermy (>2PN), embryo quality (EQ), clinical pregnancy (CP) and implantation rate (IR). RESULTS: The normal fertilization rates of the two groups were comparable: group A 58.6% versus group B 58.0%. Significantly lower rates of polyspermy were seen in group A compared to group B (2.8 versus 7.2%, P = 0.008). No statistically significant differences in EQ, CP or IR were seen. CONCLUSION: This is the first study demonstrating the achievement of good fertilization rates in IVF with ultrashort co-incubation. Significantly lower rates of polyspermy were seen in the group with ultrashort compared to the standard co-incubation group. Further studies are, however, needed in order to evaluate whether ultrashort co-incubation has any effect on the outcome of IVF.

Key words: fertilization/gamete co-incubation/IVF/polyspermy

Introduction

In human IVF, oocytes traditionally have been exposed to sperm overnight, for 16–20 h. This long period of co-incubation, however, has been shown to create problems with high levels of reactive oxygen species (ROS), which may affect the quality of embryos (Gianaroli et al., 1996b; Dirnfeld et al., 1999; Bedaiwy et al., 2004) and cause hardening of the zona pellucida, known to negatively influence the implantation potential of the embryo (Waldenström et al., 1993; Gianaroli et al., 1996a,b; Dirnfeld et al., 2003). Moreover ROS is one of the main sources to DNA strand breaks in sperm (Aitken and Clarkson, 1987; Aitken et al., 1989a,b). Although DNA-damaged sperm are able to fertilize oocytes (Ahmadi and Ng, 1999), high rates of DNA breaks are known to negatively influence fertility in vivo (Evenson et al., 1999; Spanò et al., 2000) as well as in vitro (Lopes et al., 1998; Evenson and Jost, 2000; Larson et al., 2000; Duran et al., 2002; Morris et al., 2002; Tomsu et al., 2002; Benchaib et al., 2003; Larson-Cook et al., 2003; Saleh et al., 2003; Bungum et al., 2004; Gandini et al., 2004; Virro et al., 2004).

In several recent publications the effect of a reduction of the co-incubation time from 16–20 h to 90–120 min has been studied (Gianaroli et al., 1996a,b; Coskun et al., 1998; Quinn et al., 1998; Dirnfeld et al., 1999; Lin et al., 2000; Lundqvist et al., 2001; Kattera and Chen, 2003). Some of these have reported that better embryo quality can be obtained by shortening the co-incubation time, suggested to be a result of decreased levels of ROS present in the culture (Gianaroli et al., 1996b; Dirnfeld et al., 1999). Recently it was demonstrated in pigs that both penetration and blastocyst rates were improved by reducing the co-incubation time from 5 h to 10 min (Grupen and Nottle, 2000; Gil et al., 2004).

Our study was initiated in order to investigate whether it was possible to further reduce the co-incubation period without reducing the fertilization rates and to study potential effects of the time reduction. The primary endpoints were normal fertilization (two-pronuclear, 2PN) and polyspermy rate (>2PN). Secondary endpoints were embryo quality (EQ), biochemical pregnancy (BP), clinical pregnancy (CP) and implantation rate (IR). Patients were used as their own controls by dividing sibling oocytes between the study group and the control group.
Materials and methods

Patients and study design

This prospective study was based on a cohort of consecutive infertile couples undergoing conventional IVF treatment at the Fertility Clinic, Viborg Hospital (Skive) during the period of December 2004 to May 2005. All couples in which the male partner had normal sperm concentration and motility according to the World Health Organization (WHO, 1999) were asked to participate in the study. All participants signed an informed consent. A total of 777 sibling oocytes from 81 women were allocated to co-incubation for either 30 s (ultrashort co-incubation, group A) or for 90 min (standard co-incubation, group B), so the patients were used as their own controls. While 389 oocytes were analysed in group A, 388 oocytes were analysed in group B. Each patient contributed with one cycle.

IVF procedure

Procedures for hormonal treatment, oocyte retrieval, allocation, sperm and oocyte handling

Patients underwent pituitary down-regulation and hormonal treatment as previously described (Bungum et al., 2003). When at least one follicle had reached a diameter of ≥17 mm, 10 000 IU of HCG (Ovitrelle; Serono Nordic, Copenhagen, Denmark) was administered to induce final follicular maturation. Oocyte retrieval was performed 35 h after HCG injection by vaginal ultrasound-guided follicle aspiration. G-MOPS™ (Vitrolife, Gothenburg, Sweden) was used to rinse the oocytes. The oocytes were placed in G-Fert™ (Vitrolife) for incubation until exposure to sperm 2–3 h after oocyte retrieval. Semen samples were collected by masturbation at the day of oocyte retrieval. Semen analysis was performed according to the WHO (1999) guidelines. A standard density gradient centrifugation method, PureSperm, 45 and 90% (Nidacon Ltd, Gothenburg, Sweden) diluted in G-Sperm™ (Vitrolife), was used for sperm preparation. The washing procedure and the final dilution were performed in IVF-100™ (Vitrolife).

Immediately after OR the oocytes were alternately allocated to either ultrashort co-incubation (30 s) (group A) or to standard co-incubation (90 min) (group B) (Figure 1). A laboratory technician performed the random allocation, every second oocyte to each group. The allocation was not blinded. In both groups oocytes were incubated in G-Fert™ (Vitrolife) supplemented with sperm with a final concentration of 150 000/ml. In group A the co-incubation procedure was performed at the heating stage (37°C) in a laminar air flow hood and thereafter the oocytes were washed three times in G.1.3™ (Vitrolife), to remove sperm not attached to the cumulus–corona complex before further culture in G.1.3™ until time of assessment for fertilization. Group B oocytes were co-incubated in a humidified incubator at 37°C in a gas phase of 6% CO₂ and 5% O₂ and 89% N₂ for 90 min and thereafter washed three times in G.1.3™, before further culture in G.1.3™ until fertilization assessment.

Assessment of fertilization, culture procedure, embryo morphology evaluation, cryopreservation and embryo transfer

Normal fertilization was characterized by two visible distinct pronuclei determined 18–20 h after insemination. Polyspermy was defined as more than two visible pronuclei. After assessment of fertilization zygotes were cultured in G.1.3™ until embryo transfer day 2 or 3. A maximum of five fertilized oocytes were cultured in 50 μl media droplets under oil (Ovol™). A gas phase of 6% CO₂ and 5% O₂ and 89% N₂ was used in a 37°C humidified incubator.

In addition to the number of blastomeres, the following morphological parameters were assessed on the morning of day 2 and prior to embryo transfer: (i) extracellular fragmentation: embryos with no fragmentation (grade 0); embryos with <10% fragmentation (grade 1); embryos with 10–20% fragmentation (grade 2); embryos with 20–50% fragmentation (grade 3); embryos with >50% fragmentation (grade 4); (ii) location of extracellular fragments: locally fragmented blastomeres (A1) versus dispersed fragmented blastomeres (A2); (iii) symmetry of blastomeres: equally sized symmetrical blastomeres (B1) versus unevenly sized blastomeres (B2); (iv) appearance of cytoplasm: homogeneous cytoplasm (C1) versus granulated or vacuolated cytoplasm (C2); (v) multinucleation: no multinucleate blastomeres present (D1) versus multinucleated blastomeres present (D2).

One or two embryos with the best morphology were selected for embryo transfer on day 2 or 3 after oocyte retrieval. This selection policy resulted in three embryo transfer groups: (i) one or two embryos from group A; (ii) one or two embryos from group B; (iii) mixed embryo transfer. All embryo transfers were performed in Embryo Glue™ (Vitrolife) with a Cook Soft 5000 catheter (Cook, Brisbane, Australia).

Strict criteria for cryopreservation were used. Only embryos with at least seven blastomeres and <20% intracellular fragments were cryopreserved on day 3.

Luteal phase support and pregnancy test

Patients received luteal phase support in the form of micronized progesterone vaginally, 90 mg once a day (Crinome 8%; Serono Nordic, Copenhagen, Denmark) starting on the day following oocyte retrieval and continuing until the day of the pregnancy test (i.e. day 12 after embryo transfer). A positive pregnancy test was defined by a plasma βHCG concentration >10 IU/L. A clinical pregnancy was defined as an intrauterine gestational sac with a heart beat 3 weeks after a positive HCG test. The implantation rate was calculated as the ratio of gestational sacs determined by ultrasound after 7 weeks in relation to the total number of embryos transferred.

Statistical methods

For statistical analysis Statistix, version 8 software (Tallahassee, USA) was applied using Pearson’s χ²-test. P < 0.05 was defined as being statistically significant.

Results

In all, 777 oocytes from 81 women were included in the study. In group A, 389 oocytes were analysed, and in group B, 388 oocytes were analysed. All relevant demographic data including female age, infertility diagnosis, oocytes retrieved, sperm concentration and progressive motility are given in Table I. The normal fertilization rates (2 PN) of the two groups were...
Demographic data

<table>
<thead>
<tr>
<th>Patients included (n)</th>
<th>81</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female age, mean ± SD (years)</td>
<td>32.8 ± 3.8</td>
</tr>
<tr>
<td>Infertility diagnosis, n (%)</td>
<td></td>
</tr>
<tr>
<td>Tubal factor</td>
<td>41 (50.6)</td>
</tr>
<tr>
<td>Endometriosis</td>
<td>4 (4.9)</td>
</tr>
<tr>
<td>Unexplained</td>
<td>29 (35.8)</td>
</tr>
<tr>
<td>Anovulation</td>
<td>7 (8.6)</td>
</tr>
<tr>
<td>No. of oocytes retrieved per patient, mean ± SD</td>
<td>vb9.6 ± 5.0</td>
</tr>
<tr>
<td>Sperm concentration, mean ± SD (range) (×10⁶/ml)</td>
<td>72.2 ± 43.2 (20.0–198.0)</td>
</tr>
<tr>
<td>Sperm progressive motility, mean ± SD (%)</td>
<td>68.8 ± 14.6</td>
</tr>
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| Table II. Oocytes, fertilization and embryo quality |
|-----------------------------|-----------------------------|-----------------------------|
| Group A (30 s) | Group B (90 min) | P* |
| Oocytes for insemination, n | 389 | 388 | NS |
| Oocytes normally fertilized (2PN), n (%) | 228/389 (58.6) | 225/388 (58) | NS |
| Oocytes with polyspermy (>2PN), n (%) | 11/389 (2.8) | 28/388 (7.2) | 0.008 |
| Embryos grade 0 or 1, n (%) | 134/228 (58.8) | 152/225 (67.6) | NS |
| Embryos cryopreserved, n (%) | 69/228 (30.3) | 63/225 (28.0) | NS |

*Pearson’s χ². PN = pronuclei; NS = not significant.

Discussion

The primary goal of our study was to investigate whether an oocyte–sperm co-incubation time of 30 s is long enough to achieve good fertilization rates in human IVF. To the best of our knowledge this is the first study demonstrating successful fertilization with an ultrashort co-incubation period. Moreover, we have shown that the rate of polyspermy can be effectively decreased.

Data from several previous publications have contributed to the set-up of this study. Firstly, the study was inspired by recent data from IVF in pigs where improved penetration and blastocyst rates after a co-incubation time as short as 10 min were seen (Grupen and Nottle, 2000; Gil et al., 2004). Secondly, data demonstrating that the human sperm bind to the cumulus–oocyte complex (COC) within 11 min after coitus or in vitro insemination also have contributed to the set-up of our study (Wasserman, 1987, 1988; Gianaroli et al., 1996a).

Seventy-two of the 81 patients received embryo transfer (88.9%). When dividing the patients into three groups, those who received: (i) one or two embryos from group A; (ii) one or two embryos from group B; (iii) mixed transfer; no statistical significant differences were seen regarding BP, CP or IR (Table III).

Table III. Pregnancy and implantation rates

| Embryo transfer (n) | 25 | 21 | 26 | NS |
| Positive HCG, n (% per embryo transfer) | 8 (32) | 11 (52.4) | 12 (46.2) | NS |
| Clinical pregnancies, n (% per embryo transfer) | 7 (28) | 8 (38.1) | 9 (34.6) | NS |
| Implantation rate, n (%) | 10/40 (25) | 13/35 (37.1) | 13/52 (25) | NS |

NS = not significant.
Although we arbitrarily selected a co-incubation time as low as 30 s, we estimated the risk of total fertilization failure for a patient to be low, as half of the oocytes were co-incubated according to our standard procedure. The results showed that the normal fertilization rates (2PN) of the two groups were comparable: group A, 58.6% versus group B 58.0%; and that only 3.7% of the patients experienced total fertilization failure. We only included couples where the male partner had normal sperm concentration and motility according to the WHO (1999), and therefore, before recommending ultrashort co-incubation to patients with male factor, further studies should be performed. However, it is important to keep in mind that although the traditional sperm parameters are extensively used in the diagnostics and treatment of infertility, the parameters are poorly standardized (Giwercman et al., 1999), subjective (Auger et al., 2000), and not powerful predictors of fertility (Bonde et al., 1998; Zinaman et al., 2000; Guzick et al., 2001).

Regarding polyspermy, a significantly lower rate (>2 PN) was seen in group A compared to group B (2.8 versus 7.2%). In previous studies polyspermy has been related to the matura-
tion status of the oocytes (Van Der Ven et al., 1985; Angell et al., 1986; Plachot et al., 1998), and in the present study there was a difference of 90 min in sperm–oocyte exposure, so theoretically there could be more mature oocytes in the control group. Polyspermy has also been linked to the use of a high concentration of capacitated sperm at the site of fertilization and suboptimal in vitro conditions (Hunter, 1990; Niwa and Wang, 2001; Wang et al., 2003). In IVF, oocytes are exposed to very high number of sperm, which can cause simultaneous sperm penetrations, whereas in normal conception the oviduct isthmus serves as a sperm reservoir and regulates the number of sperm reaching the site of fertilization, which ensures normal fertilization. In the present study, it is likely that the sperm selection mechanisms have been stricter in the study group than in the control group.

Our secondary goal with the study was to investigate whether ultrashort co-incubation time could benefit embryo quality, pregnancy and implantation, but no statistically significant differences regarding embryo quality, implantation and pregnancy were seen. Unfortunately, by choosing the one or two embryos with best morphology for embryo transfer, we obtained three subgroups with relatively few patients, which makes it difficult to evaluate pregnancy and implantation outcome. Surprisingly and opposite to our hypothesis, however, we observed a tendency to a lower pregnancy and implantation rate in the study group compared to the standard co-incubation group. According to our hypothesis the oocytes in the study group should suffer less oxidative damage than those in the control group. However, as ROS also is generated by somatic cells, a possible explanation for the result could be that the granulosa cells in the COC contributed to ROS production. Sperm carry freely diffusible hyaluronidase that aims to loosen the bindings between the granulosa cells which enable the spermatozoon to pass through the COC (Swyer, 1947; Schrader and Leuchenberger, 1951). In the present study we observed that while a minimal amount of the COC were left on the oocytes after 90 min exposure to sperm, oocytes incubated for 30 s had a fully intact COC, which also may contribute to production of ROS. Moreover, a recent study by Kattera and Chen (2003) demonstrated that cumulus and corona cells release estradiol, which in turn may have a direct toxic effect on the embryo (Valbuena et al., 2001). Consequently, one might speculate whether a mechanical or enzymatic denuda-
tion of the oocytes prior to ultrashort co-incubation could mini-
mize the problems. However, a disadvantage of removal of COC could be a disturbance of the important communication between the oocyte and the COC (Canipari, 2000).

From a practical point of view, an advantage with the ultrashort co-incubation is that the procedure can be handled more effectively in one step, instead of two as in the standard procedure. Fewer incubator openings could minimize embry-
onic stress during the culture period.

In conclusion, this is the first study to demonstrate that a co-incubation time as short as 30 s is long enough to obtain good fertilization rates in human IVF. In addition we have shown that the rate of polyspermy can be effectively mini-
mized. Further studies are, however, needed before firm conclusions regarding the relationship between gamete co-incubation times, embryo quality and pregnancy outcome can be reached.

Acknowledgement

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References

Aitken RJ, Clarkson JS, Hargreve TB, Irvine DS and Wu FCW (1989b) Analysis of the relationship between defective sperm function and the genera-
Barroso G, Morshedhi M and Oehninger S (2000) Analysis of DNA fragmen-


Virro MR, Larson-Cook KL and Evenson DP (2004) Sperm chromatin structure assay (SCSA) parameters are related to fertilisation, blastocyst development,


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