Reducing the time of sperm–oocyte interaction in human in-vitro fertilization improves the implantation rate

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Human oocyte development was evaluated after a reduced time exposure to spermatozoa in vitro. A total of 119 patients were assigned to two study groups in a randomized prospective study in which each patient’s oocytes were exposed to spermatozoa for either 1 h (group 1-58 patients) or the standard 16 h incubation period (group 2-61 patients). The fertilization rate obtained in group 1 was higher than in group 2 (285/393, 73%, and 272/410, 66% respectively), suggesting that the spermatozoa–oocyte interaction occurs within 1 h. This was confirmed in a study in vitro using fluorescently labelled spermatozoa and normal oocyte–cumulus complexes. Spermatozoa enter the cumulus complex within 15 min, traverse the cumulus layer within 3 h, and first appear in the oocyte cortex at 4 h post-insemination. The incidence of polyspermy was higher in oocytes exposed to spermatozoa for 16 h (3%) than for 1 h (1%). There was no difference in the cleavage rate or morphological characteristics of embryos from both study groups. However, when evaluating the timing of embryo development, group 1 generated a significantly higher percentage of four to five cell embryos when compared to group 2 (55 versus 39%; P < 0.001), documented at 40 h post-insemination. The implantation and pregnancy rates for group 1 were 11 and 28%, while the corresponding rates for group 2 were 8 and 15%. This suggests that a reduced exposure of oocyte to spermatozoa favours embryo viability, possibly due to a decrease in potential damage from sperm metabolic waste products.

Key words: embryo viability/human IVF/reactive oxygen species/sperm concentration

Introduction

The development of in-vitro fertilization (IVF) techniques for the treatment of infertile couples has been focused primarily on establishing adequate conditions for facilitating sperm–oocyte interaction. In fact, contrary to the situation in vivo, in IVF, oocytes are exposed to excessive numbers of spermatozoa (Trounson, 1994). This is especially evident in cases of male factor infertility where the percentage of spermatozoa possessing fertilizing capacity is reduced (Holden and Trounson, 1991), and where up to 500000/ml motile spermatozoa per oocyte are used (Fiorentino et al., 1994; Trounson, 1994).

Recent studies have described possible detrimental effects on spermatozoa–oocyte fusion and subsequent embryo quality and development due to free oxygen radicals, present especially in high concentrations of spermatozoa (Aitken and Clarkson, 1987; Dumoulin et al., 1992; Parinaud et al., 1993; Aitken, 1994a). Free radicals may peroxidate polyunsaturated fatty acids in cell membranes causing a decrease in the flexibility and fluidity of the membrane (Aitken, 1994a, 1994b). Indeed, it has been noted previously that embryo arrest in mice might be due to cell membrane malfunction, following an increase in the concentration of these oxidative species. In fact, the addition of anti-oxidants to the culture media promotes embryo development (Nasr-Esfahani et al., 1990). It seems logical that the harmful effects of these metabolites increase with time. Consequently, we decided to reduce the time of oocyte exposure to spermatozoa, having prior referral to documented animal studies reporting successful fertilization after shortened exposure periods to spermatozoa (Austin, 1951; Yanagimachi, 1969; Sato and Blandau, 1979; Motomura and Toyoda, 1980; Storey et al., 1984). In this study, we randomly allocated patients’ oocytes to be exposed to spermatozoa either for 1 h or the standard 16 h implemented in most IVF programmes, in order to test for any improvement in embryo viability.

Materials and methods

Between September 1994 and May 1995, 119 couples were selected and randomly allocated to two study groups: in group 1 (58 couples), each patient’s oocytes were exposed to spermatozoa for only 1 h; alternatively, group 2’s oocyte exposure to spermatozoa continued for the standard 16 h (control group: 61 patients). Randomization was executed the day before oocyte retrieval.

Patient selection and characteristics

Patients included in the study possessed the following characteristics: ≦38 years of age, normo-ovulatory in the last 6 months before the treatment cycle, normal uterine morphology assessed by hysteroscopy and histologically normal endometrium, evaluated no more than 6 months before starting gonadotrophin stimulation.

Ninety-one of the patients selected were diagnosed with a tubal factor in a previous laparoscopy. Moderate male factor infertility was diagnosed in 49 couples according to the criteria specified by the World Health Organization (World Health Organization, 1992), as either the sole cause of infertility (24 cases) or associated with tubal pathology (25 cases). Four couples were affected by idiopathic infertility. Sixty patients entering the study attempted their first
Ovarian stimulation and oocyte retrieval

Protocols for the induction of multiple follicular growth have been previously documented in detail (Gianaroli et al., 1994). In summary, after 15 days of pituitary desensitization with gonadotrophin releasing hormone (GnRH) agonists, follicle stimulating hormone (FSH) and human menopausal gonadotrophin (HMG) were administered in a fixed dosage for the first 4 days and then modified to each patient's response which was monitored by daily oestradiol assessment and pelvic ultrasound. Ultrasound-guided oocyte retrieval was executed 36 h after human chorionic gonadotrophin (HCG) administration (Ferraretti et al., 1995). Morphological evaluation of oocyte maturity was based on the appearance of the cumulus-corona complex. Collected oocytes were placed in pre-incubated T6 culture medium (Whittingham, 1971), supplemented with 10% heat-inactivated material serum (MS) in a 5% CO2, 5% O2, and 90% N2 humidified gas atmosphere at 37°C.

In all patients, the luteal phase was supplemented with 50 mg/day of pure progesterone in oil (Gestone, AMSA Lab., Florence, Italy), starting on day 3 post-oocyte retrieval. Implantation was checked 15 days after oocyte retrieval by the assessment of plasma β-HCG. Only clinical pregnancies confirmed by ultrasound were considered for analysis.

Insemination procedures

Depending upon the concentration and motility after liquefaction, semen samples were prepared either by the conventional swim-up procedure (Mahadevan and Trounson, 1984) or the mini-Percoll technique (Ord et al., 1990), approximately 90-120 min before oocyte insemination. Mini-Percoll was used when the semen sample was oligoasthenospermic (2-20×10^6 spermatozoa/ml and <50% motile spermatozoa) or severely asthenospermic (<20% motile spermatozoa) or if the sample contained ≥1×10^6 white blood cells (Ng et al., 1992). The swim-up procedure was carried out for all other semen samples. Following the procedure and 30 min of incubation (5% CO2, 37°C), sperm concentration, motility and morphology were analysed.

At 5-7 h after oocyte retrieval, all oocytes were each inseminated in a 20 μl microdroplet of T6 10% MS. Based on sperm:oocyte ratios previously described (Fiorentino et al., 1994), oocytes were inseminated with approximately 2000-10000 motile spermatozoa. Microdroplets were overlaid with 5% CO2 pre-equilibrated light paraffin oil (Sigma Chemical Co., St Louis, MO), and incubated in 5% CO2, 5% O2, 90% N2 humidified atmosphere at 37°C.

Two washing dishes (for every five oocytes) labelled #1 and #2, containing 3 ml of T6 with 10% MS, and one with fresh microdrops overlaid with oil were prepared for each patient in group 1. After 1 h exposure to spermatozoa, group 1's oocytes (n = 393) were removed from the insemination medium. Each oocyte was washed first in the #1 washing dish by using a Pasteur pipette, making sure to gently detach any already digested cumulus. The oocyte was then rinsed again in the #2 washing dish with another clean Pasteur pipette and then placed in the corresponding microdroplet with fresh medium. Group 2's oocytes (n = 410) were exposed to spermatozoa for the standard 16 h. Evaluation for the presence of pronuclei was performed 16 h after insemination, at which time all oocytes from both study groups were transferred to fresh T6 medium with 10% MS.

Embryo evaluation

Morphological evaluation of embryos was assessed 40 h post-insemination. Embryos were scored according to the following criteria: grade 1, regular blastomeres, no fragmentation; grade 2, < 50% irregular blastomeres and/or fragmentation; grade 3, >50% ≤75% irregular blastomeres and/or fragmentation, and grade 4, lysed embryos. Spare embryos remained in culture for further morphological observations at 64 h post-insemination, at which time suitable embryos were cryopreserved.

Timing of sperm–oocyte interaction

In order to estimate the time course of interaction of spermatozoa with the oocyte and its accessory cells, 12 control oocytes, donated by consenting patients, were exposed to spermatozoa pre-labelled with the DNA dye Hoechst 30322. The spermatozoa were from normal patients and the final dilution was 1×10^6/ml. Aliquots of three oocytes each were fixed in formaldehyde at 15 min, 1 h, 3 h and 4 h post-insemination. To observe the degree of progression of spermatozoa at each time point, the cumulus cells, corona radiata and zona pellucida were consecutively removed and the whole preparation observed under a Zeiss IM 35 epi-fluorescent microscope (Oberkochen, West Germany) to localize and identify spermatozoa.

Statistical analysis

The fertilization rate and the implantation rates in the two groups were compared using the z-test. The percentage of clinical pregnancies and the embryonic cellular stages were compared using χ² analysis 2×2 contingency tables. The mean rank of embryonic grade was compared in the two groups using Mann–Whitney U-test.

Results

Patients' characteristics and type of infertility were distributed homogeneously between the two groups (Table I). In addition, no statistical difference was found in the ovarian response to stimulation between the two groups. Moreover, the mean number of oocytes recovered was equivalent for the two groups, as was the percentage of mature oocytes.

A total of 803 oocytes from 119 patients were inseminated, resulting in an overall fertilization rate of 69%. A higher fertilization rate was obtained in group 1 rather than in group 2 (Table II): 285 of the 393 oocytes in group 1 were fertilized normally (73%); whereas, in group 2, 410 oocytes were inseminated, of which 272 fertilized normally (66%). The incidence of polyspermy was 1% in group 1 and 3% in group 2.

Cleavage rates were identical in the two groups (93%). Successively, Figure 1 shows that the percentage of grade 1 and grade 2 embryos was very similar in the two groups (89 and 10% in group 1, compared to 89 and 8% in group 2). In addition, Figure 2 presents the developmental stages of the embryos observed at 40 h post-insemination. A high statistical significance was observed in the difference between group 1's embryo development, where 55% of the embryos reached the four to five cell stage versus 39% in group 2 (z = 5.48, P < 0.001). Embryo replacement was performed in all 119 cycles (Table III), including a total number of 358 embryos being transferred. In all, 179 embryos were transferred in both groups 1 and 2. In addition, 126 spare embryos were evaluated 64 h post-insemination, of which 25 (group 1) versus 32 (group 2) good quality embryos were selected for cryopreservation. The pregnancy rate (PR) achieved in group 1 (28%) was higher than in group 2 (15%), even though the difference was not statistically significant. A higher implantation rate resulted...
Table I. Patients' characteristics: age, infertility factor and stimulation

<table>
<thead>
<tr>
<th></th>
<th>1 h (group 1)</th>
<th>16 h (group 2)</th>
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<tbody>
<tr>
<td>No. cycles</td>
<td>58</td>
<td>61</td>
</tr>
<tr>
<td>First attempts (%)</td>
<td>26 (45)</td>
<td>34 (56)</td>
</tr>
<tr>
<td>Female age</td>
<td>33.1 ± 3.2</td>
<td>32.1 ± 3.9</td>
</tr>
<tr>
<td>Type of sterility:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tubal (%)</td>
<td>33 (57)</td>
<td>33 (54)</td>
</tr>
<tr>
<td>tubal + male (%)</td>
<td>13 (22)</td>
<td>12 (20)</td>
</tr>
<tr>
<td>male (%)</td>
<td>9 (16)</td>
<td>15 (24)</td>
</tr>
<tr>
<td>idiopathic (%)</td>
<td>3 (5)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>No. HMG ampoules</td>
<td>25.0 ± 7.1</td>
<td>25.9 ± 10.3</td>
</tr>
<tr>
<td>No. FSH ampoules</td>
<td>12.0 ± 9.7</td>
<td>11.0 ± 8.8</td>
</tr>
<tr>
<td>Oestradiol (day of HCG)</td>
<td>1573.3 ± 1299.1</td>
<td>1152.0 ± 730.7</td>
</tr>
<tr>
<td>No. oocytes recovered</td>
<td>7.31 ± 3.8</td>
<td>7.31 ± 4.6</td>
</tr>
<tr>
<td>% of mature oocytes</td>
<td>70</td>
<td>67</td>
</tr>
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HCG = human chorionic gonadotrophin, HMG = human menopausal gonadotrophin, FSH = follicle stimulating hormone.

Table II. Fertilization rates after a 1 h and 16 h oocyte exposure to spermatozoa

<table>
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<tr>
<th></th>
<th>1 h (group 1)</th>
<th>16 h (group 2)</th>
</tr>
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<tbody>
<tr>
<td>No. oocytes inseminated</td>
<td>392</td>
<td>410</td>
</tr>
<tr>
<td>No. oocytes fertilized (2 PN) (%)</td>
<td>285a</td>
<td>272a</td>
</tr>
<tr>
<td>(73)</td>
<td></td>
<td>(66)</td>
</tr>
<tr>
<td>No. polypronuclear oocytes (%)</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
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*a = 1.618, P = 0.106.
2-PN = two-pronuclei.

Discussion

post-insemination, there were no spermatozoa at the surface of the zona pellucida, whereas at the 3 h time point we observed between five and 10 spermatozoa at various stages of penetration of the corona and zona pellucida (Figure 3). At the 4 h time point, we observed the fertilizing spermatozoa in the sub-cortical area of the oocytes (Figure 4).

In the timing experiment with fluorescently labelled spermatozoa, we observed that even though human oocytes with a full cumulus-corona complex were exposed to approximately 25,000 spermatozoa each (n = 12), the mean number of spermatozoa observed in the cumulus was 15 ± 6. There was no significant difference in the number of spermatozoa in the cumulus layer at the four time points, showing that spermatozoa enter the cumulus complex within 15 min of insemination in vitro and that further spermatozoa do not enter later. At 1 h
Reduced time course in oocyte exposure to spermatozoa

Figure 3. Time course of sperm entry into human oocyte-cumulus complexes. At 3 h post-insemination, 5–10 spermatozoa are present at various stages of penetration in the corona and zona pellucida.

Figure 4. The fertilizing spermatozoon is observed in the sub-cortical area of the oocyte at 4 h post-insemination.

capacitation and the presence of substances inducing capacitation in the medium (i.e., cations, carboxydrases, proteinases and albumin) (Nasr-Essfriedani et al., 1990; Zaneveld et al., 1991). Our preliminary study on sperm-oocyte interaction supports the data of Plachot et al. (1986) and shows that in the human, spermatozoa enter the cumulus complex within 15 min of in-vitro insemination. Although the oocyte is exposed to 25000 spermatozoa, the fact that only 10–20 spermatozoa enter the complex supports the idea that there are preferential entry sites or channels in the cumulus as previously suggested (Yanagimachi, 1994). Passage through the cumulus in the human oocyte is slow and appears to require at least 2 h.

Although we have not determined precisely the time for penetration of the zona pellucida, spermatozoa were not observed in the oocyte cytoplasm before 4 h post-insemination.

In this study, we arbitrarily selected a 1 h exposure period and show that this time is sufficient even in semen samples showing defects. Indeed, male factor cases were included in the study, since our previous finding demonstrated that the implementation of the correct sperm:oocyte ratio could yield successful fertilization rates, especially in defective sperm samples (Fiorentino et al., 1994). After a 1 h oocyte exposure to spermatozoa the majority of the cumulus oophorus was disassociated, confirming the immediate interaction of the
integrity with only 1 h exposure to spermatozoa, an interesting over, considering the prospect of improving cell membrane preparations for oxygen radical production, which is particularly relevant in male factor semen samples. Moreover, our hypothesis can be attained through the screening of sperm generations by a shortened exposure to spermatozoa may be increased in the in-vitro cleavage rate of mouse embryos derived from non-blocking strains.

Based on these findings, it seems logical to suppose that in our study the advanced cellular stages resulting from embryos generated by a shortened exposure to spermatozoa may be associated with not only the removal of spermatozoa but also its potentially damaging by-products. Confirmation of this hypothesis can be attained through the screening of sperm preparations for oxygen radical production, which is particularly relevant in male factor semen samples. Moreover, our data suggest that embryos exhibiting a faster cleavage rate are more viable and therefore capable of implantation. This supports the argument proposed by Edwards (1985), claiming that faster cleaving embryos are better at establishing pregnancies; even though this could be speculative to deduce, since the embryos being transferred may differ in their cellular stages.

Surprisingly so, our data regarding implantation indicate no statistical significance in the notable difference observed between the experimental and control groups. However, we must take into consideration that most embryos were cultured until only the two- to four-cell stage. If we acknowledge that the most critical time interval occurs approximately at the eight-cell stage when gene activation initiates, it is totally acceptable that an extended incubation period could more distinctly reveal the consequences of any existing oxidative effects on embryo cleavage. In fact, observations performed after 64 h post-insemination on the spare embryos chosen for cryopreservation indicate that 84% of group 1 embryos compared to 59% of group 2 embryos reached the seven- to eight-cell stage. Hence, if this is the case we can conclude that the implementation of appropriate culture conditions could allow the identification of developmentally deficient embryos. Moreover, considering the prospect of improving cell membrane integrity with only 1 h exposure to spermatozoa, an interesting aspect to this technique would be the analysis of survival rates of these embryos after freezing.

In conclusion, our findings indicate that spermatozoa-oocyte interaction takes place within 1 h in the human. Since the consequences of exposing embryos to pathological spermatozoa generating high levels of ROS is likely to be more harmful than prolonged culture with normal spermatozoa, we are now focusing our efforts on implementing this technique in cases of male factor infertility.

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