Embryo development and pregnancies from in-vitro matured and fertilized human oocytes

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There is an increasing interest in retrieving immature oocytes in the absence of or with limited gonadotrophin exposure, with the aim of maturing them in vitro for embryo transfer purposes. The aim of this report is to present our experience of fertilization, embryonic development and pregnancies from in-vitro maturation cycles. A total of 18 patients underwent 21 cycles in which an average of 8.1 immature oocytes was retrieved after limited exposure to human menopausal gonadotrophin (HMG) and no exposure to human chorionic gonadotrophin (HCG). In one cycle, no oocytes were recovered. The oocytes were cultured for 44 h and 121 oocytes which reached MII were injected with a single spermatozoon. A total of 71 oocytes showed two pronuclei and 53 zygotes cleaved. Forty-four embryos were transferred in 17 cycles. Five weeks after embryo transfer, ultrasound examination indicated the presence of one gestational sac and one fetal heart beat in two patients. The results suggest that in-vitro matured oocytes can undergo fertilization and the resulting embryos may result in pregnancies. However, the success rate was not sufficient to recommend widespread use of the technique without further research.

Key words: embryo/human/ICSI/in-vitro maturation

Introduction

Follicular growth and oocyte maturation are specifically controlled and timed events which, during a 4-week period in humans, result in a single fertilizable oocyte (Zeleznik, 1993). In spontaneous cycles, oocyte maturation proceeds in a highly synchronized fashion, enabling it to become fully competent by the time of the follicle rupture. Oocyte maturation requires both nuclear and cytoplasmic maturation (Eppig, 1997).

Gonadotrophin stimulation is used to achieve multifollicular recruitment, enabling an increased number of embryos to be transferred. However, there are disadvantages associated with gonadotrophin stimulation. Some are economical and related to the cost of drugs, ultrasound, etc. The estimated cost of an in-vitro fertilization (IVF) cycle being in the region of $10 000. Other major side effects of superovulation are ovarian hyperstimulation and deep vein thrombosis (Roest et al., 1996; Steward et al., 1997) and other possible long-term side effects of fertility drugs include ovarian cancer (Whittemore et al., 1992). Hence, there is an increasing interest in retrieving oocytes without gonadotropins or with limited gonadotrophin exposure and then maturing them in vitro for embryo transfer purposes. The cost of an in-vitro (IVM) cycle may be ~$5000. A large pool of preantral and antral oocytes is theoretically available, both from ‘poor’ responders and other types of infertility. Maturation and fertilization of human oocytes has been successfully performed in vitro (Edwards, 1965; Edwards et al., 1969; Shea et al., 1975). However, pregnancies have not been established from embryos generated from such oocytes until more recently (Cha et al., 1991; Trounson et al., 1994; Barnes et al., 1995; Jaroudi et al., 1997; Russell et al., 1997). Previously, we have reported that immature oocytes can be retrieved and matured in vitro from patients at risk of hyperstimulation after gonadotrophin injections (Coskun et al., 1998). The aim of this report is to present our experience of fertilization, embryonic development and pregnancies in IVM cycles over the last 2 years from a similar patient population.

Materials and methods

Patients

Patients who were at potential risk of hyperstimulation were recruited from our IVF/intracytoplasmic sperm injection (ICSI)/intruterine insemination programmes. Either a long (Lupron Depot® 3.75 mg, Abbott Lab. SA Ltd, Johannesburg, South Africa) or short gonadotrophin releasing hormone analogue (GnRHa, buserelin) protocol was used. After down regulation of the pituitary, 75–225 IU of human menopausal gonadotrophin (HMG, Humegon®, Organon, The Netherlands) was administered i.m. daily until the risk of severe hyperstimulation syndrome became apparent. The criteria for potential risk were described previously (Coskun et al., 1998). The average oestradiol concentration and the mean number of follicles that were detected by ultrasound for each cycle on the day of cancellation were 9730 ± 6132 pmol/l and 48 respectively. Patients with cancelled cycles between May 1996 and October 1997 were asked to undergo immature oocyte retrieval with subsequent IVM, fertilization and embryo transfer. Those who agreed were asked to sign a consent form. In all cases, human chorionic gonadotrophin (HCG) was not administered. A total of 18 patients underwent 21 cycles in which immature oocytes were recovered. Patients suffered from the following types of infertility: polycystic ovary syndrome (PCO) (n = 9), unexplained (n = 3), anovulatory (n = 2), male (n = 1), male factor/PCO (n = 1), male factor/hypogonadotrophism (n = 1) and male factor/tubal (n = 1).
Immature oocyte recovery and IVM

Immature oocyte recovery and maturation from a similar patient population were described previously (Coskun et al., 1998). Briefly, the aspirates obtained from all of the visible antral follicles were poured into 60 mm dishes as a thin layer. Immature oocytes were visualized under a stereo-dissecting microscope. A second search was also performed after sedimentation of red blood cells in order to obtain better visibility of the immature oocytes. The dishes were examined by at least two biologists. An average of 8.1 oocytes (171 immature oocytes from 21 cycles) was obtained, while no oocytes were recovered from a single patient. All of the oocytes together with some granulosa cells were transferred into HEPES-buffered media with 10% synthetic serum substitute (SSS, Irvine Scientific, Santa Ana, CA, USA) and washed twice. They were then co-cultured in 50 µl of human tubal fluid (HTF, Irvine Scientific) supplemented with 10% SSS, 75 mIU/ml HMG and 500 mIU/ml HCG under pre-equilibrated mineral oil (R.E. Squibb & Sons Inc., Princeton, NJ, USA). Cumulus–oocyte complexes were decoronated after 44 h in culture with a 160 µm capillary pipette following exposure to 80 U/ml of hyaluronidase (Sigma, St Louis, MO, USA) for 30 s. The stage of nuclear maturation of the oocytes was checked under an inverted microscope and classified as germinal vesicle, metaphase I, II or degenerated. Cumulus cells from decoration and granulosa cells from maturation medium were collected, washed with fresh medium and resuspended in 200 µl HTF supplemented with 10% SSS. This suspension was used as 20 µl drops for the culture of individual injected oocytes.

Sperm preparation and ICSI

Semen was diluted with 10 ml HTF containing 10% SSS and centrifuged at 1800 g for 5 min. Discontinuous Percoll separation (95 and 47.5%) was performed and the 95% Percoll layer was washed in HTF containing 10% SSS by centrifugation at 1800 g for 5 min. ICSI was performed as previously described (Palermo et al., 1992). Injected oocytes were cultured as described above. Fertilization was checked 18 h later by the presence of two pronuclei and two polar bodies. Embryos were cultured for a further 2 days and development was monitored daily. Embryos were graded as good, fair and poor. Good embryos were defined as those with even-sized blastomeres and no obvious fragmentation, with even-sized blastomeres and <10% fragmentation of uneven-sized blastomeres with no obvious or <10% fragmentation. Fair embryos had 10–30% fragmentation and poor embryos were heavily fragmented (>30%). Embryo transfer was performed 5 days after immature oocyte retrieval under abdominal ultrasound guidance using a Wallace® catheter (Sims Portex Ltd, Kent, UK).

Endometrial priming

From the day of immature oocyte retrieval, 8 mg of oestradiol valerate was administered. Progesterone 50 mg i.m. was administered daily starting 2 days after oocyte retrieval. Both medications were continued until either a negative pregnancy urine test or a positive fetal heartbeat was seen on ultrasound at 5 weeks of gestation.

Results

These are summarized in Table I. Ultrasound revealed the presence of two pregnancies 5 weeks after embryo transfer. Both patients had one gestational sac and one fetal heart beat. One of the pregnancies ended at 24 weeks shortly after premature membrane rupture; a live healthy-appearing girl was delivered who died 18 days later (Jaroudi et al., 1997). The other pregnancy ended with the delivery of a healthy girl weighing 3.5 kg at 41 weeks of gestation.

Discussion

This study suggests that in-vitro matured human oocytes can undergo fertilization following ICSI and that the resulting embryos can establish pregnancies. The oocyte maturation rate (70%) is similar to other reported results (Cha et al., 1991; Trounson et al., 1994; Barnes et al., 1996; Russell et al., 1997). ICSI was performed to minimize fertilization failure, since previous studies using in-vitro matured oocytes (Trounson et al., 1994 Barnes et al., 1996) suggest a low fertilization rate (32–45%) and it is even lower (26%) for PCO patients. Moreover, a significantly higher fertilization rate (60%) using ICSI was reported (Hwang et al., 1997), which is comparable to our results.

Cleavage, implantation and pregnancy rates were lower than those obtained with conventional IVF/ICSI in our clinic. Other studies have indicated low cleavage rates after fertilization of in-vitro matured oocytes. Rates of 59.2 and 65.2% from either PCO or regular cycling patients respectively were found (Barnes et al., 1996). Similarly, 56% cleavage rates in anovulatory PCO patients were obtained (Trounson et al., 1994). The hormonal status of the patient during follicular growth may also affect the cleavage rate. A higher cleavage rate was shown in patients who received endometrial priming around the midfollicular phase of the menstrual cycle compared to those who received early follicular phase priming (Russell et al., 1997).

Although total oocyte wastage was high in this study (118/171, 69%), the number of embryos transferred (mean 2.4) is comparable to regular IVF/ICSI cycles. However, this number did not translate into comparable pregnancy or implantation rates. This may be related to the low efficiency of the IVM system, since only 53 embryos were available for transfer out of 171 oocytes retrieved. There was a very limited selection of good quality embryos, since 83% of the available embryos had to be transferred. In regular IVF/ICSI cycles ~50% of the generated embryos are available for embryo transfer, enabling morphologically better embryos to be trans-

### Table I. Fertilization, embryonic development and pregnancy rate using in-vitro matured human oocytes

<table>
<thead>
<tr>
<th>Description</th>
<th>Number</th>
</tr>
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<tbody>
<tr>
<td>Cycles</td>
<td>21</td>
</tr>
<tr>
<td>Cycles with immature oocytes</td>
<td>20 (95.3)</td>
</tr>
<tr>
<td>Oocytes retrieved</td>
<td>171</td>
</tr>
<tr>
<td>Oocytes reaching metaphase II (% of total)</td>
<td>121 (70.8)</td>
</tr>
<tr>
<td>Oocytes fertilized (2 pronuclei) (% of MII)</td>
<td>71 (58.7)</td>
</tr>
<tr>
<td>Embryos cleaved (% of fertilized)</td>
<td>53 (74.6)</td>
</tr>
<tr>
<td>Embryo quality (% of cleaved)</td>
<td></td>
</tr>
<tr>
<td>Good</td>
<td>14 (26.4)</td>
</tr>
<tr>
<td>Fair</td>
<td>23 (43.4)</td>
</tr>
<tr>
<td>Poor</td>
<td>16 (30.2)</td>
</tr>
<tr>
<td>Embryos transferred (% of cleaved)</td>
<td>44 (83.0)</td>
</tr>
<tr>
<td>Cycles with embryo transfer</td>
<td>17 (81.0)</td>
</tr>
<tr>
<td>Pregnancies (% of cycles)</td>
<td>2 (9.5)</td>
</tr>
<tr>
<td>Implantation (% of embryos transferred)</td>
<td>2 (4.5)</td>
</tr>
</tbody>
</table>
ferred. Only 10% pregnancy and 4.5% implantation rates were achieved in this study. These lower pregnancy and implantation rates are the major obstacles preventing IVM from entering the regular clinical practice. Other investigators have also reported lower pregnancy and implantation rates (Trounson et al., 1994; Barnes et al., 1997; Russell et al., 1997). This low success rate might be attributed to asynchrony in the cytoplasmic and nuclear maturation of the oocyte. Cytoplasmic maturity may not be complete despite the fact that nuclear maturation may be satisfactory. Another reason for the low success rate could be related to the patient population in this study. All the patients were at risk of hyperstimulation, with high concentrations of oestradiol and the majority had PCO or anovulation. It has been reported (Aboulghar et al., 1997) that oocytes in hyperstimulated patients are of inferior quality and hence have lower fertilization rates, although the number of oocytes may be higher than in controls. When hyperstimulated and control groups were categorized in terms of prevalence of PCO, it was concluded that PCO rather than stimulated and control groups were categorized in terms of oocytes may be higher than in controls. When hyperstimulated and control groups were categorized in terms of prevalence of PCO, it was concluded that PCO rather than high response may affect oocyte quality. It has been also reported that immature oocytes recovered from PCO patients exhibit compromised developmental potential when compared to regular cycling patients (Barnes et al., 1996).

During normal development of an antral follicle, the follicle and cumulus oocyte complex undergo a highly co-ordinated maturation process (Eppig, 1997). Assisted reproductive technology programs employ gonadotrophin stimulation to achieve multifollicular growth in order to increase the number of embryos for transfer (Edwards and Brody, 1995). However, the low percentage of zygotes reaching blastocyst stage, as well as low implantation rates in stimulated cycles (Edwards and Brody, 1995), suggest that the natural growth process is negatively affected. This adverse effect may be even more prominent in IVM generated oocytes.

In conclusion, IVM oocytes can undergo fertilization and the resulting embryos can establish pregnancies. However, the success rate is low. There must be a major improvement of IVM success rates in order to achieve results at least equivalent to those of current stimulation protocols prior to its routine use in daily clinical practice.

References

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