

Article

Pregnancy resulting from transfer of repeat vitrified blastocysts produced by in-vitro matured oocytes in patient with polycystic ovary syndrome



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Abstract

This report describes a live birth produced from repeat vitrification and thawing of blastocysts derived from in-vitro matured (IVM) oocytes in a woman with polycystic ovarian syndrome. Immature oocyte retrieval was performed on day 12 of her induced menstrual cycle. The patient was administered 10,000 IU of human chorionic gonadotrophin s.c. 36 h before immature oocyte retrieval. A total of 47 immature oocytes were collected. Following IVM of these immature oocytes, 76.6% (36/47) become mature (at metaphase II stage). Thirty oocytes (30/36, 86.1%) were normally fertilized following insemination by intracytoplasmic sperm injection. The fertilized zygotes (two-pronuclear stage) were co-cultured with cumulus cells in YS medium supplemented with 10% human follicular fluid. On day 5 after insemination, three blastocysts were transferred. Unfortunately, fresh embryo transfer did not result in pregnancy. The remaining 10 embryos developed to the expanded blastocyst stage. These remaining blastocysts were vitrified with electron microscope grids following artificial shrinkage. Three months later, three blastocysts were thawed due to a clinical error. Consequently, the embryos were re-vitrified. After a week, the three blastocysts were warmed again. Two of them developed to hatched blastocysts. Following transfer, a full-term pregnancy resulted in the delivery of healthy twins.

Keywords: artificial shrinkage, blastocyst, HCG, immature oocytes, vitrification

Introduction

The technique of in-vitro maturation (IVM) of human immature oocytes is an attractive option for women infertility with polycystic ovary syndrome (PCOS), because IVM treatment can eliminate the risk of ovarian hyperstimulation syndrome (OHSS). It has been reported that priming with human chorionic gonadotrophin (HCG) before immature oocyte retrieval in women with PCOS improves oocyte maturation and pregnancy rates (Chian *et al.*, 2000).

There are also some reports of successful pregnancy from transfer of blastocysts derived from IVM oocytes (Barnes *et al.*, 1995; Son *et al.*, 2002a, b). Accordingly, a reliable procedure for the cryopreservation of supernumerary blastocysts is needed.

Vitrification of blastocysts using the cryoloop (Mukaida *et al.*, 2001), electron microscope grids (Son *et al.*, 2003), hemi-straw (Vanderzwalmen *et al.*, 2003), or the cryotop (Hiraoka *et al.*, 2004) has already resulted in many pregnancies. The survival rate of expanded blastocysts after vitrification is

increased significantly when the blastocoele is artificially shrunk with a glass microneedle (Vanderzwalmen *et al.*, 2002), two 29-gauge needles (Son *et al.*, 2003), or pipetting (Hiraoka *et al.*, 2004), which is thought to reduce ice crystal formation.

The first successful pregnancy from vitrified blastocysts derived from IVM oocytes has been reported (Son *et al.*, 2002c). Recently, a more advanced vitrification procedure has been developed and a higher rate of pregnancy achieved in a clinical IVF programme (Son *et al.*, 2003). However, it is unclear whether artificial shrinkage can be applied to the blastocysts produced by IVM oocytes. Although it has been reported that successful pregnancies were achieved after transfer of embryos that had been frozen twice (Macnamee *et al.*, 1990; Baker *et al.*, 1996; Farhat *et al.*, 2001), there is no report on the viability of repeat-vitrified blastocysts derived from IVM. This study describes the birth of healthy twins resulting from transfer of hatched blastocysts following repeat vitrification.

Materials and methods

The vitrification protocol was approved by the Institutional Review Board of the Maria Infertility Hospital.

A 32-year-old patient with PCOS presented with anovulation, elevated serum testosterone concentration, high concentration of LH (>10 IU/ml) in serum on day 2 of induced menstrual cycle and infertility for 8 years. The patient failed to become pregnant after two cycles of conventional IVF treatment. In previous ovarian stimulation cycles the patient had complained of mild bloating, and her ovaries measured 8 by 7 cm with no evidence of ascites by ultrasound.

To initiate the IVM treatment cycle, the patient received i.m. injections of progesterone (Progest; Samil Pharmacology, Seoul, Korea) for 10 days. Withdrawal bleeding occurred 3 days following the last dose. Beginning from day 3 of the cycle, follicular development was monitored by transvaginal ultrasonography (Aloka, Tokyo, Japan). Subsequently, based on endometrial thickness, the patient was given 10,000 IU of HCG (IVF-C; LG chemical, Seoul, Korea) on day 10 of the cycle. Thirty-six hours after HCG injection, oocyte retrieval was performed with a 19-gauge aspiration needle (Cook, Eight Mile Plains, Queensland, Australia) under the guidance of transvaginal ultrasound.

The aspirates were collected in tubes, filtered with Mesh filter (70-mm, Falcon 1060; Becton Dickinson, NJ, USA), and washed with Ham's F-10 medium to remove erythrocytes and small cellular debris. The retained cells were then resuspended in the medium and the oocytes were isolated under a stereomicroscope. Immature oocytes were transferred to IVM medium, consisting of YS medium supplemented with 30% hFF, 1 IU/ml recombinant FSH, 10 IU/ml HCG, and 10 ng/ml recombinant human epidermal growth factor (hEGF) (Son *et al.*, 2002a). The immature oocytes were cultured in an incubator at 37°C in an atmosphere of 5% CO₂, 5% O₂ and 90% N₂.

Twenty-four hours post-collection, the oocytes were denuded with 1% hyaluronidase (Sigma Chemical Co., St Louis, MO,

USA) and mechanically pipetted. Mature (metaphase II) oocytes were identified by the presence of the first polar body. The remaining immature oocytes were further cultured in fresh IVM medium, and examined again 48 h post-collection. Mature oocytes were subjected to insemination by ICSI following identification of the maturation status of the oocytes at 24 and 48 h. Fertilization was assessed 17–19 h after ICSI for the appearance of two distinct pronuclei and two polar bodies. The fertilized zygotes (two-pronuclear stage) were co-cultured with cumulus cells in 10 ml YS medium supplemented with 10% hFF (Yoon *et al.*, 2001a). Embryo transfer was performed at blastocyst stage on day 5.

The endometrium was prepared according to the method reported previously by Son *et al.* (2002a). The remaining blastocysts were cultured further to day 6 and the expanded blastocysts were cryopreserved by vitrification with EM grids after artificial shrinkage (Son *et al.*, 2003). After complete shrinkage of the blastocoele, the blastocysts were equilibrated in EG20 (20% ethylene glycol in Dulbecco's phosphate-buffered saline [DPBS]) for 1.5 min before exposure to the vitrification solution at room temperature. The blastocysts were then incubated in EFS40 [40% (v/v) ethylene glycol, 18% (w/v) Ficoll, and 0.3 mol/l sucrose in DPBS] at room temperature, loaded onto the EM grid (IGC 400; Pelco International, CA, USA), the excess vitrification solution removed using sterilized filter paper, and directly plunged in liquid nitrogen within 30 s. The EM grids containing the blastocysts were sealed in a cryovial that had previously been submerged under liquid nitrogen.

Thawing was performed by transferring the EM grids containing blastocysts to a 100 µl drop of 0.5 mol/l sucrose. After 3 min, the blastocysts were transferred sequentially to 100 µl drops containing 10% hFF in DPBS supplemented with 0.4, 0.3, 0.2, 0.1, and 0 mol/l of sucrose with 1.5 min intervals at room temperature (Cho *et al.*, 2002; Son *et al.*, 2003). The blastocysts were then washed three times in culture medium and co-cultured with cumulus cells in 10 µl YS medium containing 10% hFF.

To prepare the endometrium for frozen embryo transfer, the patient was given i.m. progesterone at a dose of 100 mg per day for 10 days to induce a withdrawal bleed, and then oestradiol (6 mg) was administered daily from day 3 of the cycle. A dose of 200 mg progesterone was administered i.m. daily, starting on day 15 of the cycle.

Results

A total of 48 oocytes (one mature and 47 immature oocytes at germinal vesicle stage) were collected. The immature oocytes were cultured in IVM medium. A total of 36 oocytes became mature following IVM (28 and eight oocytes reached MII after 24 and 48 h of culture respectively). The mature oocyte at the time of oocyte collection was not fertilized after ICSI. A total of 30 oocytes (30/36 = 83.3%) were normally fertilized following ICSI. On day 5, 13 embryos (43.3%, 13/30) were developed to blastocysts. Three blastocysts were selected and transferred. The remaining 10 blastocysts were cultured for 1 day more and the expanded blastocysts were cryopreserved using a vitrification procedure (Son *et al.*, 2003).

No pregnancy occurred following fresh blastocyst transfer. Three months later, due to a clinical error, three blastocysts were thawed. Therefore, these three expanded blastocysts were re-vitrified following 3 h of culture. After 1 week, three re-vitrified blastocysts were thawed again by the same method. Before the re-thawing, the couple were informed of the possible altered outcomes from re-frozen blastocysts. Following thawing, three re-vitrified blastocysts survived (100%); two of them had hatched by the time of embryo transfer and were therefore transferred.

Nine days after embryo transfer, the serum β -HCG concentration was 564.0 IU/l and 6 weeks after transfer an ongoing intrauterine twin pregnancy with fetal heartbeat was confirmed by transvaginal ultrasonography. The patient delivered two healthy boys at 38.5 weeks.

Discussion

This result demonstrates that blastocysts produced by IVM oocytes retrieved from women with PCOS can result in a successful live birth after embryo transfer. Recovery of immature oocytes followed by IVM of these immature oocytes is a promising treatment for women with PCOS, indicating that approximately 30% clinical pregnancy rate can be obtained from patients with PCOS following IVM treatment (Chian *et al.*, 2004). However, most pregnancies have been achieved following fresh embryo transfer on day 2 or day 3. There have been a few reports of pregnancies following fresh embryo transfer of blastocysts derived from IVM oocytes (Barnes *et al.*, 1995; Son *et al.*, 2002a,b).

Cryopreservation of human embryos has become a routine procedure for increasing cumulative pregnancy rates in IVF programmes. However, only three case reports have been found in which embryos derived from an IVM programme were frozen at 2PN (Chian *et al.*, 2001), cleavage (Godin *et al.*, 2003), or blastocyst (Son *et al.*, 2002c) stages. Among these, vitrification has been applied in only one case report at the blastocyst stage (Son *et al.*, 2002c). A more advanced vitrification procedure was recently reported (Son *et al.*, 2003). That study described higher clinical and implantation rates (48.0 and 29.0%) by artificial shrinkage using 29-gauge needles. Furthermore, the highest percentage of hatching (49.4%) was seen at the time of embryo transfer following vitrification and thawing. Based on the experience of vitrification of blastocysts (Son *et al.*, 2003), blastocysts are now routinely vitrified after artificial shrinkage with 29-gauge needles in the authors' IVM programme.

There have been some reports on the transfer of hatched blastocysts (Khorram *et al.*, 2000; Yoon *et al.*, 2001b), indicating that hatching of human blastocysts by day 6 is a favourable prognostic factor for IVF outcome. The result from the present study indicates that expanded blastocysts can be cryopreserved successfully after artificial shrinkage. Nevertheless, additional data on possible damage to the embryo caused by two exposures to high concentrations of cryoprotectants are needed.

In conclusion, blastocysts produced from IVM oocytes retrieved from unstimulated women with PCOS can be re-vitrified and result in live births following embryo transfer of

hatched blastocysts. So far as is known, this is the first demonstration of pregnancy achieved by transfer of hatched human blastocysts produced from IVM oocytes combined with repeat vitrification and thawing.

References

- Baker A, Check JH, Hourani C *et al.* 1996 Pregnancy achieved with pronuclear-stage embryos that were cryopreserved and thawed twice: a case report. *Journal of Assisted Reproduction and Genetics* **13**, 713–715.
- Barnes FL, Crombie A, Gardner DK *et al.* 1995 Blastocyst development and birth after in vitro maturation of human primary oocytes, intracytoplasmic sperm injection and assisted hatching. *Human Reproduction* **10**, 3243–3247.
- Chian RC, Buckett WM, Tulandi T *et al.* 2000 Prospective randomized study of human chorionic gonadotrophin priming before immature oocyte retrieval from unstimulated women with polycystic ovarian syndrome. *Human Reproduction* **15**, 165–170.
- Chian RC, Gulekli B, Buckett WM *et al.* 2001 Pregnancy and delivery after cryopreservation of zygotes produced by in-vitro matured oocytes retrieved from a woman with polycystic ovarian syndrome. *Human Reproduction* **16**, 1700–1702.
- Chian RC, Buckett WM, Tan SL 2004 In-vitro maturation of human oocytes. *Reproductive BioMedicine Online* **8**, 148–166.
- Cho HJ, Son WY, Yoon SH *et al.* 2002 An improved protocol for dilution of cryoprotectants from vitrified human blastocysts. *Human Reproduction* **17**, 2419–2422.
- Farhat M, Zentner B, Lossos F *et al.* 2001 Successful pregnancy following replacement of embryos previously refrozen at blastocyst stage: case report. *Human Reproduction* **16**, 337–339.
- Godin PA, Gaspard O, Thonon F *et al.* 2003 Twin pregnancy obtained with frozen–thawed embryos after in vitro maturation in a patient with polycystic ovarian syndrome. *Journal of Assisted Reproduction and Genetics* **20**, 347–350.
- Hiraoka K, Hiraoka K, Kinutani M *et al.* 2004 Case report: successful pregnancy after vitrification of a human blastocyst that had completely escaped from the zona pellucida on day 6. *Human Reproduction* **19**, 988–990.
- Khorram O, Shapiro SS, Jones JM 2000 Transfer of nonassisted hatched and hatching human blastocysts after in vitro fertilization. *Fertility Sterility* **74**, 163–165.
- Macnamee M, Wick K, Rainsbury P *et al.* 1990 How robust are early embryos? *Lancet* **336**, 636.
- Mukaida T, Nakamura S, Tomiyama T *et al.* 2001 Successful birth after transfer of vitrified human blastocysts with use of a cryoloop containerless technique. *Fertility and Sterility* **76**, 618–620.
- Son WY, Yoon SH, Lee SW 2002a Blastocyst development and pregnancies after in vitro fertilization of mature oocytes retrieved from unstimulated patients with PCOS after in vivo HCG priming. *Human Reproduction* **17**, 134–136.
- Son WY, Park SJ, Hyun CS *et al.* 2002b Successful birth after transfer of blastocysts derived from oocytes of unstimulated woman with regular menstrual cycle after IVM approach. *Journal of Assisted Reproduction and Genetics* **19**, 541–543.
- Son WY, Yoon SH, Park SJ *et al.* 2002c Ongoing twin pregnancy after vitrification of blastocysts produced by in-vitro matured oocytes retrieved from a woman with polycystic ovary syndrome: case report. *Human Reproduction* **17**, 2963–2966.
- Son WY, Yoon SH, Yoon HJ *et al.* 2003 Pregnancy outcome following transfer of human blastocysts vitrified on electron microscopy grids after induced collapse of the blastocoele. *Human Reproduction* **18**, 137–139.
- Vanderzwalmen P, Bertin G, Debauche Ch *et al.* 2002 Births after vitrification at morula and blastocyst stages: effect of artificial reduction of the blastocoele cavity before vitrification. *Human Reproduction* **17**, 744–751.
- Vanderzwalmen P, Bertin G, Debauche Ch *et al.* 2003 Vitrification of human blastocysts with the Hemi-Straw carrier: application of

assisted hatching after thawing. *Human Reproduction* **18**, 1504–1511.
Yoon HG, Yoon SH, Son WY *et al.* 2001a Alternative embryo transfer of day 3 or day 5 for reducing the risk of multiple gestations. *Journal of Assisted Reproduction and Genetics* **18**, 188–193.

Yoon HG, Yoon SH, Son WY *et al.* 2001b High implantation and pregnancy rates with transfer of human hatching day 6 blastocysts. *Fertility and Sterility* **75**, 832–833.

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