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CASE REPORT

Successful birth following transfer of frozen–thawed embryos produced from in-vitro matured oocytes

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Abstract It is well established that ovarian hyperstimulation syndrome (OHSS) is more frequent in patients with polycystic ovarian syndrome. In-vitro maturation (IVM) of immature oocytes presents a potential alternative for the fertility treatment and prevention of OHSS for these patients. This report describes the case of a 26-year old woman with a successful pregnancy and delivery following the transfer of frozen–thawed embryos derived from in-vitro matured oocytes. She had three failed cycles of ovarian stimulation (using low-dose step-up gonadotrophin protocol) with or without intrauterine insemination cycles, an ovulation-induction cycle with luteal long protocol, two fresh IVM cycle and one frozen–thawed IVM cycle. During the IVF cycle, she developed moderate OHSS and required hospitalization for 3 weeks. Following four unsuccessful IVF or IVM cycles, 15 months after the last cryopreservation, six fertilized oocytes were thawed for a scheduled embryo transfer. Following thawing, four fertilized oocytes survived and cleaved. Four frozen–thawed embryos were transferred. Six weeks after embryo transfer an ongoing intrauterine single pregnancy with fetal heartbeat was confirmed by transvaginal ultrasound. An uneventful pregnancy and delivery via Caesarean section at 39 weeks resulted in the birth of a normal healthy infant.

KEYWORDS: clinical pregnancy, cryopreservation, delivery, in-vitro maturation, PCOS

Introduction

Polycystic ovary syndrome (PCOS), characterized by ovulatory dysfunction and hyperandrogenism, is the most common cause of infertility in women. Considering the subfertile population, PCOS is diagnosed in about 75% of patients with anovulatory infertility (Azziz, 2004). Nowadays, with improved outcome and the more frequent use of

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single-embryo transfer, eliminating chances for multiple pregnancies, IVF has become a serious alternative to ovulation induction in patients with PCOS. Although favourable IVF outcomes have been reported, the risk of ovarian hyperstimulation syndrome (OHSS) is considerably increased in such patients (Aboulghar, 2009).

In patients at risk of OHSS, cryopreservation of all fresh embryos with subsequent transfer into an unstimulated cycle is a logical approach. Furthermore, in-vitro maturation (IVM) of immature oocytes also presents a potential alternative for the fertility treatment and prevention of OHSS for these patients (Aboulghar, 2009). The combination of IVM and cryopreservation of embryos for subsequent transfer is a reasonable alternative to overcome the risk of OHSS in patients with PCOS (Chian et al., 2001). Exhaustive literature review revealed only a few reports describing successful delivery following cryopreservation of embryos obtained from an unstimulated patient with PCOS (Chen et al., 2007; Chian et al., 2001). As far as is known, a successful pregnancy and delivery outcome after the transfer of frozen–thawed embryos produced from IVM oocytes, which were cryopreserved at the pronuclear stage (2PN) following intracytoplasmic sperm injection (ICSI) in women with recurrent IVF/IVM pregnancy losses and PCOS has not been reported.

In this report, a case of a healthy female infant delivery following transfer of cryopreservation of embryos derived from IVM oocytes in an unstimulated cycle in a woman with the history of poor IVF/IVM pregnancy outcomes and PCOS is reported.

Case report

A 26-year old woman with primary infertility of 3 years' duration was accepted to the study centre for fertility treatment. The woman met all the following criteria for PCOS: (i) oligo- and/or anovulation; (ii) clinical and/or biochemical signs of hyperandrogenism; and (iii) polycystic ovaries (the presence of 12 or more follicles in each ovary measuring 2–9 mm in diameter and/or increased ovarian volume, >10 ml, calculated using the formula $0.5 \times \text{length} \times \text{width} \times \text{thickness}$) (The Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group, 2004). Her medical and family histories were unremarkable for systemic diseases. Serum endocrine concentrations were normal and her husband's semen analysis was within normal limits. PCOS was diagnosed and ovulation induction was proposed.

She had failed to achieve pregnancy following three cycles of ovarian stimulation (using low-dose step-up gonadotrophin protocol) with or without intrauterine insemination. The woman had experienced mild OHSS during the first two stimulation cycles.

After the three failed cycles, she underwent an ovulation-induction cycle using the luteal long protocol. Leuprolide acetate (1.0 mg, Lucrin; Abbot, Turkey) was started in the luteal phase. Recombinant FSH (rFSH, Puregon; Organon, Istanbul, Turkey) was administered in a step-down fashion, starting with 150 IU/day by the documentation of suppression during menses; after 5 days the dose was adjusted according to the ovarian response. Follicular growth was monitored using serial ultrasound scans and serum oestradiol measurement. On day 8 of rFSH administration, three follicles were between 18 and 20 mm and 10 follicles were between 14 and 17 mm diameter. Human chorionic gonadotrophin (HCG, 10,000 IU i.m., Pregnyl 5000 IU; Organon) was administered. Transvaginal oocyte retrieval was scheduled 36 h after HCG injection with the retrieval of 13 oocytes. During the oocyte collection, the woman received a mild i.v. sedation with propofol (2 mg/kg).

Of these oocytes, 13 were metaphase II and were microinjected. Eight cleaved embryos were obtained. On day 11 of the cycle, three embryos (two 8-cell and one 6-cell) were transferred by the transcervical route in the standard fashion under ultrasound guidance. The pregnancy test was positive 15 days after embryo transfer and ultrasonography showed a three-chorial three-amniotic triplet pregnancy. She had developed moderate OHSS and required hospitalization for 3 weeks. At 11 weeks of pregnancy, multifetal pregnancy reduction from three to two was performed without any early complication. However, at 22 weeks of pregnancy, the women had a premature delivery. The babies both weighed 480 g and died in the early neonatal period.

Fourteen months later, an IVM and embryo-transfer protocol was scheduled. In total two IVM cycles and two thawed-embryo transfers, which were obtained from three previous IVM cycles, were performed. The protocol for IVM is given below in detail. The IVM cycle characteristics and outcomes are summarized in **Table 1**.

Follicle development on the ovaries and determination of endometrial thickness were monitored by transvaginal ultrasonography beginning on cycle day 3 for fresh IVM cycles. Transvaginal ultrasonography scans were repeated at about days 6 and 8 of the cycle to exclude the development of a dominant follicle. Human chorionic gonadotrophin (10,000 IU i.m., Pregnyl 5000 IU, Organon) was administered when the follicle size reached 10 mm in diameter and 36 h prior to oocyte collection. Transvaginal oocyte retrieval was scheduled 36 h after HCG injection between cycle days 7 and 13 after the follicle had reached 10 mm in diameter. During the collection, the patient received mild i.v. sedation with propofol. A transvaginal ultrasound machine with 19-gauge aspiration needle (Cook, Eight Mile Plains, Queensland, Australia) was used to aspirate follicles that were between 5 and 12 mm in diameter. A portable aspiration pump was used with a pressure between 80 and 200 mmHg. The aspirates were collected in tubes containing prewarmed heparinized Ham's F-10 medium that contained bicarbonate and HEPES buffers. Follicular aspirates were filtered (70-μm mesh size, Falcon 1060; Life Technologies) and washed with addition of a large amount of medium to filtrate. The filtrate was further washed with medium by vigorous pipetting using 10 ml of serological pipette (Becton Dickinson, NJ, USA) to remove erythrocytes and small cellular debris. The retained cells were then resuspended in the medium. The oocytes were isolated under a stereomicroscope and washed twice in the same medium. Following oocyte collection, immature oocytes with a germinal vesicle were transferred to maturation medium for culture and oocytes without germinal vesicles were denuded of cumulus cells with 0.003% hyaluronidase (Sigma, St. Louis, MO, USA) and mechanical pipetting. Oocytes without an intact germinal vesicle were defined as metaphase I (MI) or metaphase II (MII). The IVM medium consisted of YS medium supplemented with 30% human follicular fluid, 1 IU/ml rFSH,

Table 1 Characteristics and outcomes of the in-vitro maturation cycles.

Characteristic	*Fresh cycles*		*Thawed cycles*	
	May 2006	*September 2006*	*March 2007*	*July 2008*
Day of HCG injection	8	8	—	—
Oocytes retrieved	15	25	—	—
24 h maturation				
Oocytes reaching MII	5	11	—	—
Oocytes fertilized (2PN)	5	10	—	—
48 h maturation				
Oocytes reaching MII	4	7	—	—
Oocytes fertilized (2PN)	3	6	—	—
Embryo transfer/cryopreservation				
Total embryos	8	16	5	6
Embryos transferred	3	3	3	4
Quality of embryos (no. of cleaved cells)	8, 8, 10	8, 8, morula	8, 8, 6	5, 6, 6, 6
Frozen embryos	—	13	—	—
Survived embryos following thawing	—	—	3	4
Clinical pregnancy	Yes (single)	No	No	Yes (single)
Outcome of pregnancy				
Missed abortion	Yes (7 weeks)	—	—	—
Biochemical pregnancy	—	Yes	—	—
Full-term pregnancy	—	—	—	Yes (39 weeks)

Values are numbers.
MII, metaphase II; 2PN, two pronuclei.

10 IU/ml HCG and 10 ng/ml recombinant human epidermal growth factor (Son et al., 2002a,b). Immature oocytes were cultured in IVM medium at 37°C in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2. Nuclear maturation was assessed at 24 h after culture under the dissecting microscope.

Ejaculate semen samples were collected on the day following follicular puncture. After liquefaction at 37°C, semen samples were analysed according to the World Health Organization guidelines (1999). Spermatozoa were prepared by commercially available sperm preparation medium (PureSperm; Wash, Nidacon, USA) at 300*g* for 20 min. After sperm separation, the sperm pellet was washed twice (by centrifugation at 300*g* for 10 min) with 3 ml of Ham's F10 medium containing 10% human follicular fluid and the motile spermatozoa were collected by the swim-up method (Son et al., 2002a,b). ICSI was performed in MII-stage oocytes after 24 h of culture. Fertilization was assessed 18 h after ICSI to detect the appearance of two distinct pronuclei and two polar bodies. All oocyte handling procedures were conducted on warm stages and plates at 37°C. The zygotes were cocultured with cumulus cells in 10 μl YS medium supplemented with 10% human follicular fluid until embryo transfer day.

For endometrial preparation, oestradiol valerate (6 mg) (Cyclo-progynova; Schering, Istanbul, Turkey) was administered daily from the day of oocyte retrieval. Progesterone (100 mg) (Progestan, Kocak, Istanbul, Turkey) was administered daily from day 1 after oocyte retrieval. Both medications were continued until a fetal heartbeat was positively identified. Embryos were transferred on day 3 after insemination by the transcervical route in the standard fashion under ultrasound guidance. An endovaginal ultrasound scan was carried out on the day scheduled for the transfer to ensure that the endometrial thickness was >7 mm. Clinical pregnancy was defined as an intrauterine gestation with a fetal heartbeat seen by transvaginal ultrasound scan.

Supernumerary embryos were frozen using cryopreservation medium (Embryo freezing; MediCult, France). Embryo freezing was performed by the slow-freezing protocol (Lassalle et al., 1985). A standard protocol was followed for the preparation of endometrium for frozen–thawed embryo transfer (Chian et al., 2001).

Following four unsuccessful assisted reproduction cycles, 15 months after the last cryopreservation, six fertilized oocytes were thawed for a scheduled embryo transfer. Following thawing, only four fertilized oocytes survived and cleaved. Four embryos were transferred (three 6-cell cleavage-stage embryos with no fragmentation, one 5-cell cleavage-stage embryo with no fragmentation). The endometrial thickness measured 8.1 mm on the day of embryo transfer. Two weeks later, the serum β-HCG concentration was 194 mIU/ml, and 6 weeks later, an ongoing intrauterine single pregnancy with fetal heartbeat was confirmed by transvaginal ultrasound. An uneventful pregnancy course and delivery via Caesarean section at 39 weeks resulted in a birth of normal healthy infant [3480 g female (46, XX)].

Discussion

OHSS is the most serious complication of ovulation induction. In severe form, it may cause vascular complications (e.g. cerebrovascular, arterial thrombosis), respiratory complications (e.g. pleural effusion, adult respiratory distress syndrome), renal failure and also liver dysfunction.

Mortalities from OHSS have also been reported in rare cases (Aboulghar, 2009).

The exact aetiology of OHSS is not clearly understood. The most established risk factor for this clinical condition is PCOS. The treatment is currently empirical and the prevention of OHSS is the most important aspect in its management. There are three main strategies for prevention of OHSS: (i) identification of patients at risk (women with the history of OHSS or PCOS); (ii) using different ovulation-induction strategies before stimulation (metformin, use of IVM, use of low-dose FSH protocol, use of gonadotrophin-releasing hormone antagonist protocol); and (iii) preventive therapy modalities during stimulation (cycle cancellation, cryopreservation of all embryos for future transfer, coasting, gonadotrophin-releasing hormone antagonist administration, intravenous albumin, triggering ovulation by low-dose HCG) (Aboulghar, 2009).

The most recent clinical application in assisted reproduction has been in-vitro maturation, which has no risk of OHSS and has a comparable outcome with IVF. IVM of human oocytes was primarily developed to make IVF safer and simpler for women with polycystic ovaries and high risk of OHSS, using conventional stimulation protocols (Jurema and Nogueira, 2006). Recently, the indications for IVM have been extended to other causes of infertility such as male factor and unexplained infertility (Suikkari, 2008). The combination of IVM and cryopreservation of embryos which has been derived from IVM cycles has been rarely reported and it is a good alternative for prevention of OHSS in patients with PCOS. However, this procedure is only performed in a limited number of institutions worldwide (Chian et al., 2001).

Cryopreservation of human embryos has become a routine procedure to increase cumulative pregnancy rates and to decrease the OHSS rates in IVF programmes. Using cryopreservation for assisted reproduction, embryos survive and implant at a high rate following thawing either at the pronuclear stage or cleavage stage (Varghese et al., 2009). This case demonstrated a high embryo survival rate (three out of five and four out of six, respectively, embryos survived following thawing) after freezing at the 2PN stage in IVM cycles.

Cryopreservation of embryos derived from immature oocytes is a novel method for cryobiologists because, in immature oocytes, chromatin remains in a decondensed state, microtubule-organizing centres are not formed and the microtubular system is not yet organized. The survival rate and developmental potential of these oocytes decrease after freezing (Varghese et al., 2009). It is still unclear whether the embryos or blastocysts produced by IVM oocytes can be frozen for further embryo transfer especially for patients with recurrent unsuccessful IVF/IVM outcome (Chen et al., 2007; Chian et al., 2001; Son et al., 2002a,b). In the current case report, although the woman experienced worse cycle outcomes, the last transfer of frozen–thawed embryos derived from IVM oocytes yielded delivery of a healthy infant.

In conclusion, the embryos produced from IVM oocytes retrieved from unstimulated women with PCOS and recurrent poor IVF/IVM outcome can be safely cryopreserved by vitrification and a successful outcome can be achieved following embryo transfer. Frozen–thawed embryos may also be used for prevention of OHSS with successful outcome.

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