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CASE REPORT

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Healthy live birth from vitrified blastocysts produced from natural cycle IVF/IVM

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Abstract Natural-cycle IVF combined with in-vitro maturation (natural-cycle IVF/IVM) was used as a treatment for a 27-year-old woman. She was administered 10,000 IU of human chorionic gonadotrophin intramuscularly about 36 h prior to oocyte collection and oocyte collection was performed on day 11 of her menstrual cycle. One mature oocyte was retrieved from the leading follicle and another five mature oocytes and six immature oocytes were retrieved from the rest of the follicles. Out of 10 fertilized zygotes, eight of them cleaved. Three day-3 embryos derived from in-vivo matured oocytes (one was from the leading follicle) were transferred but failed to conceive. The remaining five embryos were continuously cultured until day 6 and four of them developed to the expanded blastocyst stage and vitrified for the storage. Six months later, two vitrified—warmed blastocysts derived from the immature oocytes that blastocysts produced from the immature oocytes retrieved from the small follicles, when a leading follicle exists in the ovaries, can be vitrified to produce a healthy live birth, suggesting that natural-cycle IVF/IVM is an efficient infertility treatment.

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KEYWORDS: blastocyst, immature oocyte, IVM, natural cycle, vitrification

Introduction

The world's first successful live birth from IVF was produced from a woman with a natural cycle (Steptoe and Edwards, 1978). However, this natural-cycle IVF treatment was slowly replaced by ovarian-stimulated-cycle IVF, because it has been believed that the number of oocytes retrieved relates to the embryos available for transfer and this directly affects the probability of successful pregnancy (Johnston et al., 1981; Jones et al., 1982; Lopata et al., 1978). Ovarian stimulation is frequently associated with side effects. Some women are extremely sensitive to ovarian stimulation

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with exogenous gonadotrophins and are at an increased risk of developing ovarian hyperstimulation syndrome (OHSS), which is sometimes a life-threatening condition (Beerendonk et al., 1998).

The recovery of immature human oocytes followed by invitro maturation (IVM) and IVF has become an effective treatment option for many infertile women, including those with polycystic ovary syndrome (PCOS), who have a high antral follicle count and are at risk of developing OHSS. Successful fertilization, development and pregnancy with human oocytes matured in vitro were reported (Barnes et al., 1995, 1996; Cha et al., 1991; Cha and Chian, 1998; Edirisinghe et al., 1997; Jaroudi et al., 1997; Liu et al., 1997; Nagy et al., 1996; Paulson et al., 1994; Prins et al., 1987; Russell et al., 1997; Trounson et al., 1994; Veeck et al., 1983). In comparison with ovarian-stimulated-cycle IVF, the major advantages of IVM treatment include avoidance of the risk of OHSS, reduced cost and simplified treatment (Chian et al., 2000). However, this IVM treatment might be useful for up to a maximum of 30% of women undergoing IVF treatment who have la a high antral follicle count that mainly being patients with PCOS (Buckett et al., 1999). Recently, IVM technology has been developed as natural-cycle IVF combined with IVM (natural-cycle IVF/IVM) (Chian et al., 2004), in which more than 50% women who came to seek IVF treatment can be treated by this natural-cycle IVF/IVM (Lim et al., 2009). Those reports demonstrated that the immature oocytes are still viable and healthy to produce live births, even though the leading follicle was recruited in the ovaries during regular menstrual cycle. To date, it has been reported that the live births can be obtained from the embryos produced by in-vitro matured oocytes frozen at 2PN (Chian et al., 2001), cleavage (Godin et al., 2003) or blastocyst (Son et al., 2002b) stages. However, it is still unclear whether the embryos (blastocysts) produced from the oocytes retrieved from these small follicles in natural-cycle IVF/IVM can be cryopreserved and later produce live birth. This case report presents the first evidence of live birth of a healthy infant resulting from transferring the vitrified blastocysts produced from the immature oocytes in natural-cycle IVF/IVM.

Case report

A couple, a 27-year-old woman with a regular menstrual cycle and a 34- year-old male partner with asthenoteratozoospermia, was infertile for 4 years. They opted for natural-cycle IVF/IVM treatment in order to reduce the cost and save time for treatment. On day 3 of her menstrual cycle, a baseline ultrasound scan found more than seven small follicles (the size of all follicles was <4 mm in diameter). On day 8 of her menstrual cycle, the repeated ultrasound scan revealed that a leading follicle with the size of 11 mm in diameter developed in her left ovary. On day 9 of her menstrual cycle, the leading follicle reached to 13 mm in the diameter and the endometrial thickness was 7 mm. The patient was given 10,000 IU of human chorionic gonadotrophin (HCG) (Livzon; Livzon Medical Groups, Zhu Hai, China). and oocyte collection was performed about 36 h later on day 11 of her menstrual cycle.

Transvaginal ultrasound-guided aspiration was performed with a 17-gauge double-lumen needle (Cook, Eight Mile Plains Queensland, Australia) for the aspiration of the leading follicle and then changed to a 19-gauge single-lumen needle (Cook) for the aspiration of the rest of the small follicles. A portable aspiration pump was connected to the aspiration needle with a pressure of less than 100 mmHg. The aspirates were collected in 10 ml tubes containing pre-warmed heparinized Ham's F-10 (Irvine Scientific, Santa Ana, CA, USA). The small follicular aspirates were filtered using 70 µm mesh (Falcon, Becton Dickinson and Company, NJ, USA), washed three times with oocyte washing medium supplemented with 10% human serum albumin (Irvine Scientific, Santa Ana, CA, USA) and the oocytes were collected under a stereomicroscope. All oocyte handling procedures were conducted in a mini-chamber under 5% CO2 atmosphere at 37°C. The maturity of the oocytes at the time of oocyte collection was evaluated under a stereomicroscope with a 'sliding' technique (Chian et al., 2000).

At the time of oocyte collection, the size of leading follicle was 15 mm in diameter and the rest of the follicles were less than 10 mm in diameter. The endometrial thickness was 8 mm. One mature oocyte was retrieved from the leading follicle. A total of 11 oocytes were retrieved from the rest small follicles and five of them were at metaphase II (MII) stage. The remaining three oocytes were at metaphase I (MI) stage and three oocytes were at germinal vesicle (GV) stage, respectively. The total of six mature oocytes (one from leading follicle and five from small follicles) were denuded from the cumulus cells with finely drawn glass pippette after 1 min of exposure to 0.03% hyaluronidase solution (Sigma Chemical Co, St Louis, MO, USA) and were inseminated by intracytoplasmic sperm injection (ICSI) with the male partner's spermatozoa 3 h after oocytes denuding. Six immature oocytes (at MI and GV stages) were incubated in an organ culture dish (60×15 mm; Falcon) containing 1 ml of maturation medium (Maria Fertility Hospital, Seoul, South Korea) (Yoon et al., 2001) supplemented with 30% of the patient's own serum (inactivated at 56°C for 30 min) with a final concentration of 0.6 IU/ml recombinant human FSH (Gonal-F; Serono, China) 0.1 IU/ml human menopausal gonadotrophin (Livzon Medical Groups; Zhu Hai, China) and 10 ng/ml recombinant human epidermal growth factor (Invitrogen, China) at 37°C in 5% CO2, 5% O2 and 90% N₂ with high humidity for maturation in culture.

Spermatozoa for ICSI were prepared by gradient separation (45 and 90% gradients) 300 g for 20 min. Following gradient separation, the sperm pellet was washed twice (300 g) with 3 ml of Ham's F-10 medium and the motile spermatozoa were collected by the swim-up method (Chen and Bongso, 1999).

After maturation in culture for 24 h, three out of three MI oocytes and one out of three GV oocytes became mature to the MII stage. Those four in-vitro matured oocytes were inseminated by ICSI using the first-day-prepared spermatozoa. The remaining two immature (at GV stages) were cultured further until 48 h, but they did not become mature and arrested at GV stage.

Fertilization was assessed 17–19 h after ICSI for the appearance of two distinct pronuclei (2PN) and two polar bodies. The zygotes were co-cultured with patient's

cumulus cells (obtained from the leading follicle) in 10 μ l of embryo culture medium (Yoon et al., 2001). Out of six invivo matured oocytes (one from leading follicle and five from small follicles), all six oocytes were observed having 2PN. Four of the in-vitro matured oocytes, were also fertilized with evidence of 2PN. Out of 10 fertilized zygotes, eight of them cleaved (two did not cleave: one from in-vivo matured oocyte and one from in-vitro matured oocytes, respectively). The embryo (10-cell stage, grade 2) derived from the mature oocyte retrieved from the leading follicle and two embryos (two 6-cell stage, grade 3) derived from the mature oocytes retrieved from the small follicles were transferred on day 3. The endometrial preparation was according to the method reported previously by Lim et al. (2009). Briefly, Valerate oestadiol (6 mg Progynova; Schering, China) was administered daily starting on the day of oocyte retrieval and daily injection of 100 mg Progest in oil (Xian Ju Pharmaceutical Co, Zhejiang, China) for luteal support, starting on the day of initial ICSI. At the day of embryo transfer, the endometrial thickness was 9.6 mm. No pregnancy occurred after fresh embryo transfer.

Following culture of the remaining embryos, four out of five embryos developed to the expanded blastocyst stage on day 5 and day 6 after oocyte collection, respectively (two from in-vivo matured oocytes and two from in-vitro matured oocytes). Four blastocysts were cryopreserved by vitrification methods (Lee et al., 2006) on day 5 and day 6 respectively. Six months post cryopreservation, two vitrified blastocysts derived from immature oocytes (one from MI stage and one from GV stage) were warmed for transfer. After warming, two blastocysts were incubated for 18-20 h in order to confirm their survival and two expanded blastocysts were transferred to the patient on day 5 after ovulation in her spontaneous cycle. At the time of embryo transfer, the endometrial thickness was measured to be 10.3 mm. Two weeks later, the serum β -HCG concentration was 1219 IU/l and 6 weeks after embryos transfer, an ongoing intrauterine pregnancy with fetal heartbeat was confirmed. The patient delivered one healthy girl at 38 weeks of gestation (3600 g).

Discussion

This case report demonstrates that the blastocysts produced from immature oocytes retrieved from the small follicles in the ovaries can be cryopreserved and result in healthy live birth, even when a leading follicle exists in the ovaries during natural menstrual cycle.

It is a common belief that recruiting the dominant follicle or leading follicle in the ovaries suppresses the development of other small follicles and induces other small follicular atresia. According to Russell (1998), a decreased number of oocytes with reduced maturation and fertilization rates will be obtained in the follicular phase when the dominant follicle has exceeded 14 mm in diameter. It has also been found that once selection of the leading follicle has occurred, the developmental potential of the remaining oocytes is impaired (Cobo et al., 1999). However, it has been suggested that the maturational and developmental competence of immature oocytes may not be detrimentally affected by the presence of a dominant follicle during the follicular phase (Chian et al., 2004). Recently it has been confirmed that natural-cycle IVF/IVM is an efficient treatment for a selected group of women with acceptable pregnancy rate (Lim et al., 2007) and for more than 50% women who seek for IVF treatment (Lim et al., 2009). The present study shows that the immature occytes retrieved from the small follicles, when the leading follicle exists in an ovary during natural menstrual cycles can not only produce viable blastocysts but can also be cryopreserved to be given for live birth.

A previous study (Lim et al., 2007) indicated that the optimal size of the leading follicle is 12–14 mm in diameter to give the HCG injection in natural-cycle IVF/IVM treatment in order to retrieve a mature oocyte from the leading follicle. Interestingly, this study found more mature oocytes can be retrieved from the small follicles even though the size of those small follicles was less than 10 mm in diameter. This suggests that those small follicles responded to HCG to trigger oocyte maturation process *in vivo*, even though their size was less than 10 mm in diameter. Further study is needed to clarify the exact mechanism of oocyte maturation *in vivo* in the small follicles responded to HCG injection in the near future.

Cryopreservation of embryos leads to an increase in the cumulative pregnancy rate along with reduced costs of assisted reproduction techniques. There are some reports of successful pregnancies following transfer of blastocysts derived from immature oocytes (Barnes et al., 1995; Son et al., 2002a,b). Recently Lee et al. (2007) reported that high clinical pregnancy (43.8%) and implantation (23.6%) rates were achieved by transfer of the vitrified blastocysts produced from immature oocytes. However, all those reports indicated that the blastocysts were produced from immature oocytes derived from the small follicles without existence of a leading follicle in the ovaries that have irregular menstrual cycles with PCOS. However, the result from this case report indicates for the first time that the presence of the leading follicle in the ovaries does not affect immature oocytes' ability to produce optimal-guality embryos for cryopreservation giving a live birth.

In conclusion, the blastocysts produced from the immature oocytes derived from small follicles during natural-cycle IVF/IVM can be safely cryopreserved and give a healthy live birth, confirming that the presence of the leading follicle during natural-cycle IVF/IVM treatment does not detrimentally affect the viability and health of the immature oocytes derived from small follicles.

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