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Successful Birth after Transfer of Re-frozen Blastocysts Developed from Immature Oocytes Retrieved from a Woman with Polycystic Ovarian Syndrome

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미성숙 난자로부터 체외 성숙한 포배기 배아의 Re-vitrification 후 성공적 임신 1례

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윤혜진[1] · 윤산현[1] · 이소영[1] · 김해균[2] · 이원돈[1] · 임진호[1]

본 연구는 이식 후 남은 잉여의 포배기 배아를 두 번의 냉동과 융해 과정을 반복적으로 실시한 후 이식한 결과에 관한 보고이다. 사람 포배기 배아의 동결보존에서 높은 생존율과 성공적인 임신율이 보고되고 있으나 미성숙 난자로부터 발달한 포배기 배아에 두 번의 초급속 냉동 방법을 실시한 후 이식한 보고는 되어 있지 않다. 이에 본 연구에서는 다낭성 난소 증후군 환자에게서 얻은 미성숙 난자로부터 발달한 포배기 배아를 artificial shrinkage 후 초급속 냉동함으로써 생존율을 높이는 방법을 이용하여 재냉동 이식하였을 때 임신에 성공한 증례를 보고하고자 한다. 29세의 환자로부터 채취한 55개의 미성숙 난자들 (germinal vesicle stage oocytes)을 체외배양 하여 성숙한 37개의 난자들로부터 30개의 수정란을 얻을 수 있었다. 12개의 배아가 포배기 배아까지 발달하였으며 이 중 3개의 양질의 포배기 배아를 선별하여 이식하였고, 이식을 한 후에 남은 9개의 포배기 배아들은 artificial shrinkage의 과정을 마친 후에 초급속 냉동 방법을 이용하여 동결보존 하였다. 그 중, 4개의 포배기 배아들을 융해한 후 이식을 하지 않고 다시 재냉동을 하여 보관하였고 이 후 재냉동 되었던 4개의 포배기 배아들을 다시 융해 하여 이식을 한 결과 임신이 되어 건강한 남아를 분만하였다. 이로써 미성숙 난자로부터 얻은 포배기 배아가 두 번의 냉동과 융해의 과정을 통해 크게 손상을 입지 않고 생존할 수 있다는 것을 알 수 있었다. 그러므로 융해 이식 후 남은 잉여의 포배기 배아를 다시 냉동 보관하여 다음 주기에 이용함으로써 축적된 임신율을 증가시킬 수 있을 것으로 사료된다.

Key Words: Refrozen blastocyst/ Immature oocytes/ Vitrification/ PCOS/ Artificial shrinkage

Cumulative pregnancy rates of in vitro fertilization-embryo transfer (IVF-ET) programs have improved since development of cryopreservation techniques that allow freezing of supernumerary embryos. Embryo freezing programs enable transfer of a small number of embryos and thus reduce the risk of multiple pregnancies while avoiding wastage of good quality embryos. Therefore, much

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attention and effort have been directed toward improvement of cryopreservation techniques for increasing IVF efficiency.

An alternative to slow freezing vitrification takes only a few seconds and would prevent chilling damage from formation of ice crystal. Martino et al.[1] obtained a higher survival rate of frozen-thawed human blastocysts when vitrification utilizing electron microscopy (EM) grids was used. Vitrification methods using other implements such as straw or cryo-loop have also been applied successfully.[2,3] In addition to vitrification with EM grids, our protocol also used a six-step thaw method for ecreasing osmotic shock, which was found to yields higher pregnancy and live birth rates compared to the conventional two-step thaw method.[4] It was shown that removal of the blastocoelic fluid before vitrification could increase survival rates of frozen embryos presumably by reducing damage by ice crystal formation.[5] To remove blastocoelic fluid, we used a 29-gauge needle instead of a glass needle as higher survival and hatching rates caused by the formation of a large hole in the zona pellucida have been reported using the former method.[6]

Women with polycystic ovarian syndrome (PCOS) have decreased fertility potential due to anovulation. In many cases, PCOS women are extremely sensitive to exogenous gonadotropins and consequently, are at greater risk of developing ovarian hyperstimulation syndrome (OHSS), a side effect of ovarian stimulation. Recovery of immature oocytes in a natural cycle followed by in vitro maturation (IVM) offers an alternative option of IVF treatment for PCOS women with infertility. Since the first birth of a healthy infant arising from embryos developed from IVM culture in a PCOS woman, many other successful pregnancies have been reported using similar techniques.[7~9] Moreover, successful pregnancies in PCOS women have been reported after transfer of frozen-thawed zygotes and blastocysts that developed from immature oocytes following in vitro maturation culture.[10,11]

It was demonstrated that repeated freezing and thawing does not affect embryo viability by producing live births using refrozen-thawed 2-cell mouse embryos.[12] Later, it was further reported that frozen-thawed 8- to 16-cell mouse embryos could be successfully refrozen immediately upon thaw or after in vitro culture to blastocyst stage.[13] Similarly in humans, refreezing embryos also does not affect their post-thaw survival or decrease IVF implantation rate.[14] When mouse morula stage embryos frozen-thawed once or twice with a simple rapid freezing procedure were analyzed morphologically, there was no difference between the two groups in embryo survival rate, implantation rate and number of live fetuses from pregnant recipients.[15] So, we have decided the introduce of our efficient protocol, vitrification, to refreeze blastocysts in order to get better clinical results.

To our knowledge, there is no report of successful birth following replacement of refrozen blastocysts generated from IVM. Here, we report the first successful birth following transfer of refrozen blastocysts developed from immature oocytes retrieved from a woman with PCOS.

CASE REPORT

A 29-year-old woman with PCOS is presented with irregular menstrual cycles, anovulation, and a 5-year history of infertility. She failed to become pregnant with blastocyst transfers from one conventional IVF cycle, two natural IVF and IVM cycles and one cryopreservation cycle over a 3-year period.

To initiate IVM treatment cycle, the patient was given progesterone (Progestin; Smile Pharmacology, Korea) im. After discontinuation of progesterone treatment, menstrual bleeding started 3 days later. On day 15 of her menstrual cycle, the patient was given hCG (10,000 IU) (IVF-C; LG Chemical,

Korea) based the endometrial thickness. Oocyte retrieval was performed 36 hr after hCG administration as previously described.[16] Briefly, oocytes were aspirated using transvaginal ultrasound guidance, a 19-gauge single-lumen aspiration needle (Cook, Eight Mile Plains, Australia) with an aspiration pressure between 80 and 100 mmHg. Follicles were aspirated yielding a total of 55 oocytes. All the oocytes were cultured in Yoon medium (YS), supplemented with 30% human follicular fluid (hFF), 1 IU/ml rFSH, 10 IU/ml hCG, and 10 ng/ml rhEGF at 37℃, 5% CO_2, 5% O_2, and 90% N_2.[17] After 24 hour incubation, all the oocytes were denuded of cumulus cells with hyaluronidase (Sigma, St Luis, USA) to facilitate assessment of maturity. Oocytes that contain a germinal vesicle were considered immature and oocytes that contain an extruded polar body in the perivitelline space were considered mature. After 24 hour incubation, 32 mature oocytes were inseminated by ICSI. After 48 hour incubation, 5 more mature oocytes were inseminated by ICSI. 30 of the 37 injected oocytes fertilized and 12 blastocysts developed after 6 and 7 days of in vitro culture.

On day 23 of her cycle, 3 blastocysts (day 6) consisting of 2 expanded and 1 middle expanding blastocysts were transferred but did produce a pregnancy. The remaining 9 blastocysts consisting of 5 middle expanding, 1 expanding and 3 expanded blastocysts were cryopreserved using the vitrification method.

Blastocycsts were held in position using a needle with the inner cell mass orientated at 6 or 12 o'clock. A 29-gauge needle was used to make a puncture at 3 o'clock position through the trophectoderm and into the blastocele cavity until shrinkage occurred. The shrunken blastocysts were pretreated in a solution containing modified Dulbecco's phosphate-buffered saline (m-DPBS), 20% (v/v) ethylene glycol (EG; Sigma) and 10% hFF for 1.5 min at room temperature. The blastocysts were then exposed to a freezing solution consisting of 40% (v/v) EG, 18% (w/v) Ficoll (Ficoll 70; Pharmacia Biotech, Uppsala, Sweden), 0.3 mol/l sucrose and 10% hFF with m-DPBS solution at room temperature, loaded onto the EM grid, the EM grid containing blastocysts was directly plunged into liquid nitrogen (LN_2) within 30 sec. The EM grids containing the blastocysts were quickly transferred to pre-cooled cryovials and stored submerged in liquid nitrogen.

For frozen embryo transfer, the endometrium was prepared. The patient was given progesterone intramuscularly (100 mg daily) for 10 days to induce withdrawal bleed. Menstrual bleeding commenced 3 days after discontinuation of progesterone. On day 5 of her cycle, the patient was given clomiphene citrate (100 mg daily) (Clomiphene; Bando Pharmacology, Seoul, Korea) for 5 days, at which time endometrial thickness was monitored by ultrasound. 100 mg progesterone was administrated daily from the day of embryo transfer. On the day of embryo transfer, endometrial thickness measured 10 mm.

Three months after the fresh transfer, 2 expanded and 3 middle expanding blastocysts were thawed. Frozen blastocysts were thawed using a six-step dilution with sucrose. The EM grids containing blastocysts were transferred to 100 μl drops of 0.5 mol/l sucrose for 3 minutes. Blastocysts were transferred sequentially to 100 μl drops containing 10% hFF in m-DPBS supplemented with 0.4, 0.3, 0.2, 0.1 and 0 mol/l of sucrose for 1.5 minutes each at room temperature. Blastocysts were then washed three times in YS medium containing 10% hFF and cultured in vitro overnight. After 18 hour incubation, 3 blastocysts re-expanded and were transferred. No pregnancy was produced.

Six months after the fresh transfer, the remaining 4 frozen blastocysts were thawed as described above. All 4 blastocysts survived and hatched during in vitro culture overnight. Due to personal

conflict, the patient canceled the scheduled transfer. The blastocysts were refrozen and thawed by 8 months after the fresh transfer. The 4 blastocysts were thawed 3 hours prior to transfer. All 4 blastocysts survived and were transferred. Two weeks post-transfer, β-hCG level was 131 IU/ml and pregnancy was confirmed at six weeks gestation when ultrasound revealed a single fetal heartbeat. At 36 weeks of gestation, the patient delivered a healthy boy weighing 3600 grams.

DISCUSSION

In this case, it was shown that refrozen blastocysts developed from immature oocytes retrieved from a PCOS patient can result in a successful pregnancy. It has been shown that refrozen embryos at the pronuclear stage using the slow freeze method can lead to a successful pregnancy.[18] Using the same slow freeze method, refrozen embryos at cleavage stage or blastocyst stage have also produced healthy live births.[19,20] Similarly, refrozen blastocysts using the ultra rapid freeze or vitrification method has also resulted live birth.[21] In the present case, 9 blastocysts were manipulated to undergo artificial shrinkage and then cryopreserved by vitrification using EM grid. After 6 months of storage, they were thawed and cultured in vitro. Among them, 1 hatching blastocyst and 3 hatched blastocysts were re-frozen for 2 months using the same method as the first freeze. After transfer of these refrozen and thawed blastocysts, the PCOS patient achieved a successful pregnancy with delivery of a healthy boy. The present report demonstrates that artificial shrinkage and the repeated freezing by vitrification and thawing does not impair blastocyst development, particularly in PCOS patients.

It is well known that ice crystals form during freezing and could result in mechanical damage to embryos and cells. The blastocele contains a large amount of fluid that is prone to ice crystal formation during rapid freezing such as vitrification as it may not allow sufficient permeation of the cryoprotectant. Therefore, removal of the blastocelic fluid prior to vitrification is beneficial. Indeed, it was observed that the fully expanded human blastocysts were dehydrated more slowly and their survival after thawing was lower than earlier blastocysts that contain smaller amounts of fluid.[22] It has been reported that blastocysts escaped from the zona pellucida completely could be cryopreserved by an ultra-rapid vitrification using a cryotop after artificial shrinkage by pipetting of a glass pipet and result in a pregnancy and proposed that hatched blastocysts might attached to the endometrium as the hatched blastocyst fully expands.[23]

In the present case, we removed the fluid content of blastocoele in the expanded blastocysts using a 29-gauge needle. We have previously shown that this method of artificial shrinkage was safe and as effective as a glass needle, resulting in higher implantation and clinical pregnancy rates, 29.0% and 48.0%, respectively.[6]

The thawing procedure for frozen embryos is also very important, as demonstrated by the fact that stepwise removal of the cryoprotectant by sequential dilution could reduce injury due to osmotic shock during thawing of cryopreserved porcine embryos.[24] Similarly, we have observed that the survival rate of human blastocysts thawed by the 6-step method was significantly higher than that by the 2-step method.[4] In this study, we obtained 100% survival rate when cryopreserved blastocysts were thawed by the 6-step method.

Several laboratories have reported successful live births after in vitro development of embryos derived from immature oocytes collected from women with PCOS or with poor response.[8,25] We have already shown that the frozen-thawed blastocysts produced from in vitro matured oocytes retrieved from unstimulated woman with PCOS gave

a successful pregnancy.[11] Yet there is no report of pregnancy with refrozen blastocysts derived from IVM oocytes, particularly in a woman with PCOS. The present report demonstrates for the first time that a defined procedure consisting of in vitro maturation, artificial shrinkage, and repeated freezing using vitrification and thawing using a 6-step dilution method, does not impair the developmental capability of oocytes in a woman with PCOS, as shown by delivery of a healthy infant following embryo transfer.

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