Case Report

The Successful Pregnancy and Birth of a Healthy Baby after Human Blastocyst Vitrification Using Cryo-E, First Case in Siriraj Hospital

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This case report illustrates a successful pregnancy and birth after vitrification of a human blastocyst using modified cryostorage- Cryo E. A 31-year-old woman underwent short protocol for ovarian hyperstimulation in an IVF treatment program. After administration of intramuscular hCG, all oocytes were recovered transvaginally with ultrasound guidance. Twelve mature oocytes were obtained and eight were fertilized. Three out of eight embryos became blastocyst, and two blastocysts were transferred on day 5, but no implantation occurred. The other was cryopreserved by vitrification technique using a cryo E. Three months after freezing, the cryopreserved blastocyst was thawed and survived. It was transferred to the patient's uterus. Blastocyst implantation resulted in a healthy single pregnancy. After continuing pregnancy until term, she delivered by cesarean section due to unfavorable cervical conditions. A male baby with Apgar score 9/10, weighing 3,500 g was born. Her child showed normal growth.

Keywords: *Vitrification, Cryo E, Cryostorage, Blastocyst, Pregnancy*

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Cryopreservation has been performed for more than 20 years[1,2]. Its purpose of their storage is to keep and restore them as original quality and function until utilization. In the past, there were two techniques of cryopreservation developed, the conventional slow cooling method using computerized program freezing and the rapid freezing method known as vitrification. These days, vitrification has become the preferred method and an essential part of assisted reproduction because of its good results. It significantly improves survival and pregnancy rates[3].

Embryo cryopreservation is now successfully and commonly performed. It provides flexibility for embryo transfer thus, extending its utilization to reduce maternal complication from infertility treatment and maximize pregnancy rate. For example, reducing the number of embryos transfers can decrease maternal multiple pregnancy complications and increase cumulative pregnancy rates per oocyte pick up[4]. Furthermore, postponing embryos transfer could reduce the incidence of hyperstimulation syndrome[5,6]. Since advances in culture systems, it is possible for human embryos to be cultured until the blastocyst stage. Advantages of blastocyst transfer are well known, such as higher chance for selecting viable and best morphological embryos[7,8]. Consequently, blastocyst transfer has been commonly performed, and cryopreserve human blastocysts have become a vital procedure for storage.

After vitrification of a human blastocyst was reported[9,10], not only equipment for vitrification but also many techniques have been developed[11]. Embryo storage equipment during vitrification has been invented such as cryostraws, cryotop, cryoloop, microscope grid, and hemi-straw. Despite their effectiveness and quality being generally approved[3], they are still expensive, and difficult to find. However, in the present case report, the authors present a new

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cheaper and simplified cryostorage for vitrification. The authors also report a successful pregnancy and live born baby following transfer of a single vitrified human balstocyst embryo to recipient woman.

Case Report

An infertile couple with the woman aged 37 years and her husband, aged 37-years-old, consulted our infertility clinic with the problem of primary infertility. According to the history, she had received infertility treatment by IUI for six times about two years ago, but she did not achieve pregnancy. Her blood tests including CBC, anti HIV, VDRL, HBsAg, and thalassemia screening were within normal results. Ultrasound scanning showed single subserous myoma, which sizes 4.25 x 4.51 centimeters, located at the posterior aspect of the uterus. The husband's semen characteristics were within normal parameters according to World Health Organization criteria[12]. Ovulation stimulation was started using short protocol. She was treated with GnRH agonist on day 2 of the cycle and rFSH on day 3 of the cycle. Continuous intra-subcutaneously 150 units of recombinant FSH each day was given for nine days. While dominant follicles reached a diameter of ~18 mm, 10,000 unit of hCG was intramuscularly administered. Oocytes were collected 36 hours after hCG administration by transvaginal aspiration using a vaginal ultrasound-guided procedure. There were 12 mature oocytes collected from both ovaries. After cumulus oocyte complexes were removed from the follicular fluid and incubated for 4 hours, all of them were inseminated, and then continuously incubated with fertilizing media (Cook, Australia) in a Petri dish under mineral oil in a mini-incubator (MINC) at 37°C in an atmosphere of triple gases containing 5% O2, 6% CO2 and 89% N2.

Embryo culture and grading of blastocysts

Eighteen hours after insemination, fertilization was assessed by the presence of two pronuclei. Their results show eight of 2 PN stage of embryos, two of 3PN stage of embryos, one of 1 PN stage of embryo, and the others were not fertilized oocytes. All embryos were washed well and cultured in cleavage media (Cook, Australia) for 48 hours, and then placed in blastocyst media (Cook, Australia) for 48 hours. Five days beyond ovum pick up, embryo transfer that contained 2 out of 3 blastocysts was performed under trans-abdominal ultrasound guidance. Grade of blastocysts during transfer were shown as 3AA and 3AB. However, 2 weeks after embryo transfer, her blood test showed that she had not conceived. The last blastocyst, which graded 3 AA, was vitrified using the cryo-E as tool for storage. Vitrification technique was presented as follows.

Vitrification of blastocyst

The protocol for the Cryo-E vitrification of blastocyst was slightly modified from previous reports[13,14]. The cryo E was a sterile ear pick that consisted of a 4 centimeters pipe long with a tiny spoon at the end (2 mm wide and 2 mm long) and made from 100% stainless steel (Fig. 1). With that tool, the blastocyst was vitrified in the tiny spoon. Vitrification was performed using a three-step protocol (Fig. 2). Initially, the blastocyst was placed in the base medium containing 1.36 mol/l (10%) glycerol in 10% DPBS. After 3 minutes, it was moved to combined medium containing 1.36 mol/l (10%) glycerol and 2.7 mol/l (20%) ethylene glycol in 10% DPBS for 3 minutes. Then it was suspended in the base medium containing 3.4 mol/l (20%) glycerol and 4.5 mol/l (25%) ethylene glycol in 10% DPBS for 3 minutes (Fig. 3). During the last 20 seconds, 5 microliter of the last cryoprotectant solution was dripped into the spoon of cryo-E to create a tiny drop of the solution on the spoon, and then the blastocyst was transferred quickly into the drop on the spoon using a micropipette. After loading of blastocyst, the spoon of the cryo-E was plunged immediately and stored into liquid nitrogen. In addition, all cryoprotectant solutions had been warmed briefly in an incubator at 37°C and the blastocyst were handled on the stage warmer of a dissecting microscope at 37°C.

Fig. 1 Shape and all views of Cryo-E (sterilized stainless ear pick)

Fig. 2 Process of vitrification using Cryo-E of which a tiny spoon contained microdrop of vitrified blastocyst: blastocyst loaded on the spoon of Cryo-E and plunged into liquid nitrogen

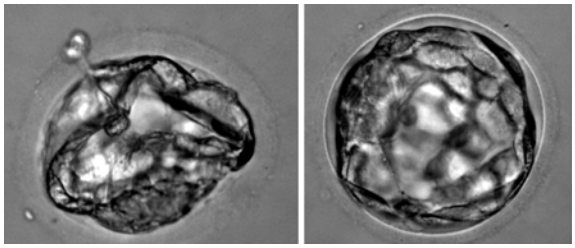

Fig. 3 The blastocyst was exposed to the three steps vitrified freezing solution: presenting of a distinct shrinkage of cytoplasm

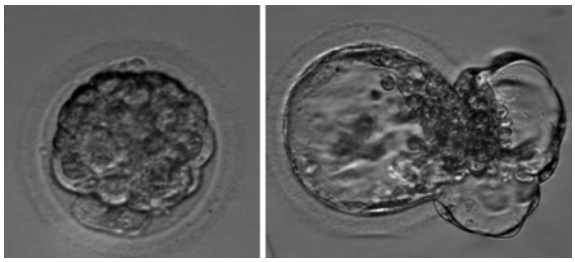

Fig. 4 A surviving vitrified blastocyst after thawing: expanded blastocyst and further hatching

Thawing of blastocyst and assessment of survival

Three months after freezing, thawing was performed with a three-step rapid thawing protocol in a sucrose solution[15]. In a 4-well multi-dish, 1 ml of base medium containing 0.5 mol/l sucrose, base medium containing 0.25 mol/l sucrose, and, base medium containing 0.125 mol/l sucrose base were warmed briefly in an incubator at 37°C and then placed on the stage warmer of a dissecting microscope. The tiny spoon of cryo E was placed in a solution of 0.5 mol/l sucrose for 3 minutes, allowing the blastocyst to fall to the bottom of the dish, and then they were transferred to a solution of 0.25 mol/l sucrose followed by 0.125 mol/l sucrose for 3 minutes each before the blastocyst were rinsed and then cultured with blastocyst media (Cook, Australia). After 4 hours in culture after warming, the appearance of the blastocyst was examined on an inverted microscope at x 400 magnification, and survival was assessed based on the morphological integrity of the blastomeres, inner cell mass, and trophectoderm, and re-expansion of the blastocoele. The surviving blastocyst was scored as to developmental stage and graded for quality as described elsewhere. The result showed that it had survived. Finally, the hatching blastocyst was transferred to the recipient womb (Fig. 4).

Transfer of blastocyst and assessment of pregnancy

Two months later, after ovarian stimulation, embryo transfer was planned using natural cycle. She received 50 mg of clomiphene citrate for 5 days, starting off at day 3 of the cycle. On day 13 of her cycle, transvaginal ultrasound was performed and showed the right dominant follicle, sized 20 millimeters and 9.1 centimeters of type 2 endometrial thickness. Her blood test showed that LH was 30.6 mIU/mL and progesterone was 1.16 mg/mL. Blastocyst transfer, therefore, was performed six days after that time. Meanwhile blastocyst, which was kept frozen for 3 months, was thawed, graded, and transferred into the patient's uterus.

Embryo transfer using transabdominal ultrasound guidance was done. Administration of oral micronized progesterone (Utrogestrane 300 mg daily; Hisamitsu, Tokyo, Japan) was initiated after this procedure finished and continued. On day 11 post-blastocyst transfer, the serum β hCG levels were 160 mIU/ml. A week after the first serum hCG testing, their levels had increased to 3,539 mIU/ml. Ultrasound at 6-weeks of gestation revealed a single intrauterine gestational sac containing positive cardiac activity of fetal echo. Crown rump length was 4.7 millimeter and yolk sac diameter was 2.1 millimeter.

Pregnancy developed without any complications and on April 06, 2006, at week 39 of gestation, a baby boy with an Apgar score 9/10, weighing 3,500 g and 44 cm in height was delivered by caesarean section due to unfavorable cervical conditions. Her child thrived well and showed normal development both physical and mental health. The child was followed with pediatric evaluation for two years, and the last visit was on April 18, 2008.

Discussion

Nowadays, blastocyst culture has become an essential part of assisted reproduction and commonly performed in many ART clinics. Thereafter, the cryopreservation of blastocysts is important procedures for maximized utilization. There are many advantages in culturing blastocyst and having successful cryopreservation. This allow the patient to have an increased chance to conceive at the time that is correct for th epatient. For example, cryopreservation is used not only for extended *in vitro* culture of human embryos but to select the best embryo and limit the number of best embryos transferred, which could reduce multiple pregnancies as well as maximize cumulative pregnancy rates per oocyte retrieval[3].

For blastocyst culture, only a small number of blastocysts are used and super-numerary blastocysts are available for cryopreservation until their utilization in the future. Therefore, having a reliable blastocyst cryopreservation is importantly needed as a procedure to store them. Vitrification is a cryopreserved procedure that was first reported for blastocyst storage[16]. Until now, it is a promising technique for freezing blastocysts. With a high concentration of cryoprotectant and rapidly elevated cooling, intracellular ice crystal formation does not occur, and cellular toxic injury is decreased. As a result, there are fewer cryoinjuries and a higher survival rate[17].

Despite uncomplicated techniques and the required for less equipment, cryostorage equipment is quite expensive and unavailable. According to previous studies, many cryostorages for vitrification such as cryoloop[11], open pulled straws[18], cryoloop with cryovial[19], and electron microscopic grids[20,21] were reported. However, there is no report of cryo E or a similar tool.

In the present study, the authors have used a new vitrified modifier cryostorage equipment that is called Cryo E: E is short for ear pick. It is an ear pick the end of which has a tiny spoon adapted for blastocyst storage, and made of stainless steel. Advantages of this tool are easier assessable, more convenient, cheaper, and high efficacy for vitrification. Presently, in Thailand, the the cheapest cryostorage sold is more than 150 baht or 5 US dollars each, yet the price of Cryo E is below 10 baht or 0.3 US dollars each. Our clinic could decrease expense for vitrification as this tool is more than 90% cheaper. Furthermore, the succession rate of the vitrification in our clinic was the same despite using modified equipment, Cryo E is at least an equally effective vitrified tool as the other type. This case was the first one in our clinic that had a successful pregnancy and birth. The child has more than 2 years of normal development of both physical and mental health, thus, this should be a guarantee for the safety of this tool and its process. For this reason, vitrification using Cryo E is routinely used as a tool for vitrification in our clinic, and many patients have conceived using this method.

In this case, vitrification technique used 25% ethylene glycol and 25% glycerol as the solution for vitrification, which demonstrated its efficacy in previous studies[13,14]. Furthermore, clomophene citrate cycle in which a single blastocyst transfer was performed was selected to confirm that the patient had dominant follicle for further ovulation. Despite successful vitrification in the present study, further study should be done to confirm the efficacy of this tool. In addition, this equipment should be further modified to avoid direct contact between liquid nitrogen and embryo as well as to increase efficiency for vitrification.

In brief, this case report presented the Cryo E, a new cryostorage equipment that cost less and is simpler and convenient to use for vitrification. Their efficacy was confirmed by successful pregnancy and birth after single blastocyst vitrification using Cryo E.

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รายงานความสำเร็จในการตั้งครรภ์และคลอดบุตรจากการย้ายตัวอ่อนซึ่งได้รับการแช่แข็งในระยะบลาสโตซิส โดยวิธี Vitrification และใช้เครื่องมือประยุกต์ชื่อ Cryo E: รายแรกในโรงพยาบาลศิริราช

สมสิญจน์ เพ็ชรยิ้ม, อรวรรณ เมฆมหรรณพ์, สมบูรณ์ คุณาธิคม, เรืองศิลป์ เชาวรัตน์, พิทักษ์ เลาห์เกริกเกียรติ

ผู้หญิงไทยอายุ 31 ปี และสามีมาปรึกษาเรื่องมีบุตรยากที่โรงพบาบาลศิริราชได้รับการรักษาโดยวิธีเด็กหลอดแก้วด้วยการกระตุ้นรังไข่โดยวิธี short protocol หลังจากเก็บไข่และทำการปฏิสนธิ ได้ตัวอ่อนระยะ 2 PN ทั้งหมด 8 ใบ หลังจากเลี้ยงตัวอ่อน มีเพียง 3 ใบที่เจริญถึงระยะบลาสโตซิส ได้แบ่งตัวอ่อนระยะบลาสโตซิส 2 ใบ กลับเข้าสู่โพรงมดลูกแต่ไม่ตั้งครรภ์ ตัวอ่อนระยะบลาสโตซิสที่เหลือได้รับการแช่แข็งโดยวิธี Vitrification และใช้เครื่องมือเก็บตัวอ่อนประยุกต์ชื่อ Cryo E หลังจากนั้น 3 เดือน ผู้ป่วยได้รับย้ายตัวอ่อนหลังจากละลาย ตัวอ่อนระยะบลาสโตซิสซึ่งผลที่ได้คือสตรีนั้นตั้งครรภ์จนครบกำหนด และคลอดบุตรโดยวิธีผ่าตัดคลอดได้บุตรชาย น้ำหนักแรกคลอดเท่ากับ 3,500 กรัม มีคะแนนแอพการ์ เท่ากับ 9/10 และหลังจากเฝ้าติดตามนาน 2 ปี เด็กมีการเจริญเติบโตปกติ