Case report: Two successful pregnancies following the transfer of re-vitrified human day 7 blastocysts developed from vitrified cleaved embryos

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Abstract

Purpose To report successful pregnancies after the transfer of re-vitrified human day 7 blastocysts developed from vitrified cleaved embryos.

Methods and results A total of five day 7 blastocysts developed from vitrified cleaved embryos were re-vitrified and re-warmed. All of five re-vitrified day 7 blastocysts (100%) survived after warming and were transferred to three patients. Two of the women became clinically pregnant. Of these women, one woman delivered a healthy baby and the other pregnancy is ongoing at 26 weeks of gestation.

Conclusions This is the first report of successful pregnancies after the transfer of re-vitrified human day 7 blastocysts developed from vitrified cleaved embryos.

Keywords Blastocyst · Day 7 · Human · Repeated cryopreservation · Vitricification

Introduction

With the introduction of sequential culture system, blastocyst culture is being adopted by many IVF clinics as a means to increase pregnancy rates, while minimizing multiple gestations [1]. Therefore, a reliable procedure for cryopreservation of supernumerary blastocysts is needed. Since the first pregnancy after vitrification of a human blastocyst was reported using cryostraws [2], most attention has focused on using very small volumes of cryoprotectant. This greatly increases the cooling and warming rate, while reducing chilling injuries and ice crystal formation. The efficacy of vitrification in small volumes is demonstrated by good survival rates of human blastocysts with the cryotop [3–6], the cryoloop [7, 8], electron microscope grids [9, 10] or the hemi-straw [11, 12].

Despite the advances in human blastocyst vitrification, much remains to be learned regarding the limits of current extended human embryo culture techniques and the clinical usefulness of later-stage cryopreservation. In fresh blastocyst transfer, previous investigators have found superior implantation rates with fresh transfer occurring at day 5 as compared with day 6 [13, 14] and at days 5 and 6 as compared with day 7 [15, 16]. On the other hand, blastocysts vitrified on day 5 have a pregnancy potential superior [5, 6] or similar [4, 8] as compared with that of day 6 blastocysts. However, to the best of our knowledge, there are no reports on pregnancies following the transfer of day 7 vitrified and warmed blastocysts. We did find two reports in which day 7 blastocysts were frozen by a slow-freezing method [17, 18]. In this case report, we present successful pregnancies after the transfer of human day 7 re-vitrified blastocysts developed from vitrified cleaved embryos.

Capsule Two cases of successful pregnancies were obtained following the transfer of re-vitrified human day 7 blastocysts developed from vitrified cleaved embryos from three transfers

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A report of three cases

Materials and methods

Patient treatment

Re-vitrified day 7 blastocysts were transferred to three patients who had at least two previous failures of embryo transfers before the transfer of day 7 re-vitrified blastocysts and had unexplained infertility. The treatment was conducted with patients following informed consent and according to the guidelines of the Japan Society of Obstetrics and Gynecology (JSOG). There is no Institutional Review Board (IRB) in the (private) clinic. Drs S Okano, K Kinutani and M Kinutani are members of JSOG and Kinutani Women's Clinic has been registered as a certified fertility centre by JSOG.

Women were treated with gonadotrophin-releasing hormone (GnRH) analogue buserelin acetate (Mochida, Tokyo, Japan) from either the preceding mid-luteal phase in a long treatment protocol or second day of the cycle in a short treatment protocol. Ovarian stimulation was carried out with human menopausal gonadotrophin (Nikken, Tokyo, Japan) or urinary FSH (Fertinorm; Serono, Japan). Follicular development was monitored with serial (every third day) vaginal ultrasound examinations and serum oestradiol measurements. Human chorionic gonadotrophin (HCG; Teizo, Tokyo, Japan) was administered to women when dominant follicles reached a diameter of 18 mm. Oocytes were collected 35 h after HCG administration using a vaginal ultrasound-guided procedure and were incubated in human tubal fluid (HTF) medium (Irvine Scientific, CA, USA) containing 10% (v/v) serum substitute supplement (SSS; Irvine). Sperm preparation was carried out using discontinuous ISolate™ (Irvine) gradient. Mature oocytes were either inseminated with spermatozoa 5–7 h after oocyte retrieval at a concentration of 100,000 to 200,000 motile spermatozoa/ml for 5 to 10 oocytes (IVF) or microinjected with a single spermatozoon (ICSI). The day of oocyte retrieval was considered as day 0. Fertilization was confirmed at 24–25 h after oocyte retrieval (day 1) by the presence of two pronuclei.

Embryo culture and grading of blastocysts

Fertilized oocytes were transferred and cultured in P-1 (Irvine) containing 10% (v/v) SSS (Irvine). In all the three cases, two fresh cleaved embryos were transferred on day 2 or 3, but no women became pregnant. All the remaining cleaved embryos were vitrified. For all the couples, all the remaining vitrified day 2 or day 3 embryos were warmed and cultured for better embryo selection before embryo transfer. Following warming, warmed day 2 (the day of

warming was considered as day 2) embryos were placed in Blast Assist Medium 1 (Medicult, Jyllinge, Denmark), in Blast Assist Medium 2 (Medicult) on day 3 and cultured until day 5. Warmed day 3 (the day of warming was considered as day 3) embryos were placed in Blast Assist Medium 2 (Medicult) and cultured until day 5. On day 5, the embryos were examined for development into blastocysts and then placed in fresh Blast Assist Medium 2 (Medicult) until day 7. All oocytes and embryos were incubated at 37°C in an atmosphere of 6% CO₂, 5% O₂ and 89% N₂. In all the three cases, one or two morulae were obtained on day 5. In a previous study at the clinic, when the results of the transfer of blastocysts or morulae (obtained from vitrified cleaved embryos) on day 5 endometrium at hormonal replacement treatment cycles combined with total zona removal assisted hatching were compared, no difference was found with regard to pregnancy rate (38%; 6/16 and 40%; 6/15 for blastocysts and morulae, respectively), implantation rate (30%; 8/27 versus 29%; 8/28, respectively) and delivery rate (38%; 6/16 and 40%; 6/15, respectively) (unpublished data). Therefore, all the three cases of the present report, one or two morulae were transferred to the patient on day 5 in hormone replacement treatment cycles combined with total zona removal assisted hatching. However, no women became pregnant. After embryo transfer on day 5, surplus embryos that developed to the expanded blastocyst stage (diameter ≥ 160 μ m) were vitrified. For expanded blastocysts, the development of the inner cell mass (ICM) and trophoctoderm was assessed. The ICM grading was as follows: A. tightly packed, many cells; B. loosely grouped, several cells; C. very few cells. The trophoctoderm grading was as follows: A. many cells forming a tightly knit epithelium; B. few cells; C. very few cells forming a loose epithelium [19]. Only expanded blastocysts scoring B or higher for both ICM and trophoctoderm grades (i.e. BB) were vitrified (Fig. 1a). In all the three cases, most surplus embryos were at morula stage on day 6 and expanded blastocysts were available for cryopreservation only on day 7. It is our policy to cryopreserve not morula stage but expanded blastocyst stage in order to cryopreserve better quality embryo [4]. Therefore, re-vitrification was done on day 7 rather than day 6 in these three cases of the present report.

Vitrification of embryo

The embryos were vitrified by the method developed by Kuwayama et al. [3] using a cryotop (Kitazato Supply Co., Fujinomiya, Japan), albeit with slight modifications, and has been described previously [4]. The cryotop consists of a 0.4 mm wide $\times$ 20 mm long $\times$ 0.1 mm thick polyethylene strip attached to a plastic handle and equipped with a cover straw. As the base medium, modified HTF medium-HEPES

Fig. 1 **a** Three blastocysts developed from vitrified cleaved embryos, observed on day 7 after oocyte retrieval and before re-vitrification. Two of these (pointed by two arrows) were re-warmed and transferred. **b** Following zona removal assisted hatching at the time of warming, two re-warmed blastocysts were re-expanding without zona 2 h after warming. The patient delivered a healthy male. Bar represents 100 μm

(Irvine) plus 20% (v/v) SSS (Irvine) was used. The equilibration solution contained 7.5% (v/v) ethylene glycol (Sigma Chemical Co., MO, USA) and 7.5% (v/v) dimethyl sulphoxide (Kanto Chemical Co., Tokyo, Japan). The vitrification solution was composed of 15% (v/v) ethylene glycol, 15% (v/v) dimethyl sulphoxide and 0.5 mol/l sucrose (Nacalai Tesque, Inc., Kyoto, Japan).

For cleaved embryos, embryos were incubated in 1 ml of 30°C equilibration solution, confirming shrinkage and re-expansion before exposure to the vitrification solution.

For blastocysts, artificial shrinkage of blastocoele was performed in the equilibration solution. First, pipetting of the expanded blastocyst was started immediately after placing the embryo in 1 ml of 30°C equilibration solution with a glass pipette slightly smaller in diameter ($\sim 140 \mu\text{m}$) than the expanded blastocyst. After confirmation of slight

shrinkage of the blastocoele, pipetting was performed with a pipette slightly smaller in diameter than the first one ($\sim 100\text{--}120 \mu\text{m}$). This procedure was repeated two to three times until the blastocoele collapsed completely. After blastocoele contraction, the blastocysts were equilibrated in the same equilibration solution for another 2 min before exposure to the vitrification solution.

After equilibration, the embryos were then incubated in 1 ml of 30°C vitrification solution and loaded, within 45 s, onto the tip of the cryotop with $\sim 1 \mu\text{l}$ of cryoprotectant solution. Then the cryotop was immediately submerged into liquid nitrogen which had been filter sterilized through a $0.22 \mu\text{m}$ filter (Millipore, Cork, Ireland) [20] and under the liquid nitrogen the plastic cover was placed over the strip to provide protection during storage.

Warming of embryos

The warming procedure was done as follows. The protective cover was removed in liquid nitrogen and the end of the polypropylene strip was immersed directly into 1 ml of 37°C 1.0 mol/l sucrose solution for 1 min. The blastocysts were then transferred into 1 ml of 37°C 0.5 mol/l sucrose solution for 3 min and washed twice in the base medium for 5 min.

Assisted hatching method

Assisted hatching was performed by the method as described previously [21].

For morulae developed from vitrified cleaved embryos, embryos were placed into 1.0 ml of 25°C 0.2 mol/l sucrose solution in Sperm Washing Medium (Irvine) to shrink the embryo away from the zona pellucida [22].

For warmed blastocysts, embryos were placed under mineral oil within a 50 μl microdroplet of 37°C Sperm Washing Medium (Irvine) while the warmed blastocysts remained collapsed (within 5 min of completing the warming procedure).

Total removal of the zona pellucida was performed using a laser (Zilos-tk Laser, Hamilton Thorne Research, Beverly, MA, USA) and mechanical pipetting. Embryos were stabilized with a holding pipette held at 9 o'clock position, and positioned with the laser target located on the outer edge of the zona. The power of laser was 100% and the pulse duration was 500 μs . By using this setting, a 10 μm hole was formed in the zona by one laser shot. Multiple irradiations along the convex periphery of the zona from outward to inward were used to form a 10 μm opening. Similarly, another opening was formed next to the first one. This procedure was repeated until a large opening was formed in the zona. The size of the opening was 60–90% of the circumference of the zona, estimated from an area of

empty perivitelline space. It took about 2 min per embryo to complete this procedure. After forming a large opening, the embryo was released from the holding pipette and aspirated out from the opened zona by mechanical pipetting and the zona was removed.

Assessment of survival of warmed blastocyst

The post warming survival of cleaved embryos was observed 1–2 h after warming under a microscope, and the embryos with more than 75% of visible blastomeres were judged to have survived. The post-warming survival of blastocysts was observed 1–2 h after warming, and re-expanded blastocysts were judged to have survived (Fig. 1b).

Endometrial preparation and assessment of pregnancy

Warmed embryo transfer was performed in hormonal replacement treatment cycles. All women received transdermal oestradiol (Estraderm 1.4–5.8 mg, Kissei, Tokyo, Japan) for 2 days with gonadotrophin-releasing hormone analogue for the preparation of the endometrium. The administration of progesterone (vaginal 400 mg daily) was initiated when endometrial thickness exceeded 10 mm. Embryo transfer was scheduled on day 5 after the initiation of progesterone treatment irrespective of whether they had been day 5 morula or day 7 blastocyst. One to two embryos were transferred into the patient's uterus. A clinical pregnancy was defined as a pregnancy when a gestational sac was recognized in the uterus by ultrasound.

Results

The data for individual patients are summarized in Table 1.

Patient 1

Seventeen oocytes were collected following the short protocol. In our programme when the husband presents with an ejaculated sperm sample which after preparation, has a total motile sperm count (TMC) exceeding $\sim 0.8 \times 10^6$ and a morphology (strict criteria) of $>5\%$ normal forms, then the couple will undergo IVF in their first cycle [23]. The male partner presented with a sperm sample with 20×10^6 TMC after preparation and 8% normal forms. Therefore, the collected oocytes were inseminated by IVF. Thirteen out of seventeen were fertilized, and two out of thirteen cleaved embryos were transferred on day 3. The remaining eleven day 3 embryos were vitrified. The patient did not become pregnant after the fresh day 3 embryo transfer.

Subsequently, all the remaining eleven day 3 embryos were warmed. Ten out of eleven were survived after warming and were cultured. Two morulae were obtained and transferred on day 5. The patient did not become pregnant after morula transfer. On day 6, seven morulae were obtained and cultured to day 7. Three out of seven morulae were developed to expanded blastocyst and were re-vitrified (Fig. 1a).

Subsequently, two re-vitrified day 7 blastocysts were warmed and both survived. Following 2 h of culture, two blastocysts re-expanded and were transferred into the patient's uterus (Fig. 1b). One gestational sac with fetal heartbeat was confirmed by ultrasound 30 days after embryo replacement. This pregnancy resulted in delivery of a healthy male (3,260 g) at 38 weeks of gestation.

Patient 2

Twenty oocytes were collected following the long protocol. The patient had total fertilization failure in their previous IVF cycle. Therefore, the collected oocytes were inseminated by ICSI. Sixteen out of twenty were fertilized, and two out of sixteen cleaved embryos were transferred on day 3. The remaining fourteen day 3 embryos were vitrified. The patient did not become pregnant after the fresh day 3 embryo transfer.

Subsequently, all the remaining fourteen day 3 embryos were warmed. All fourteen survived after warming and were cultured. One morula was obtained and transferred on day 5. The patient did not become pregnant after morula transfer. On day 6, four morulae were obtained and cultured to day 7. One out of four morulae was developed to expanded blastocyst and was re-vitrified.

Subsequently, one re-vitrified day 7 blastocysts was warmed and survived. Following 2 h of culture, one blastocyst re-expanded and was transferred into the patient's uterus; however, a pregnancy did not ensue.

Patient 3

Twenty-one oocytes were collected following the long protocol. The husband presented with an ejaculated sperm sample which after preparation, had only few motile spermatozoa. Therefore, the collected oocytes were inseminated by ICSI. Eighteen out of twenty-one were fertilized, and two out of eighteen cleaved embryos were transferred on day 2. The remaining sixteen day 2 embryos were vitrified. The patient did not become pregnant after the fresh day 2 embryo transfer.

Subsequently, all the remaining sixteen day 2 embryos were warmed. All sixteen survived after warming and were cultured. Two morulae were obtained and transferred on day 5. The patient did not become pregnant after morula

Table 1 A case study of three patients, each undergoing a single oocyte recovery cycle, followed by IVF or ICSI and fresh embryo transfer, and subsequently vitrification and warming of excess embryos, followed by

culture and transfer of morula stage at day 5, followed by culture of excess morulae to day 7 blastocysts which were vitrified and subsequently warmed and transferred in each of the patients

Parameter	Patient 1	Patient 2	Patient 3	Total
No. of oocytes recovered	17	20	21	58
No. of oocytes fertilized (IVF or ICSI)	13 (IVF)	16 (ICSI)	18 (ICSI)	47
No. of embryos cleaved to day 2/day 3 (stage)	13 (day 3)	16 (day 3)	18 (day 2)	47
No. of day 2/day 3 embryos transferred	2	2	2	6
No. of pregnancies following the transfer of day 2/day 3 embryos	0	0	0	0
No. of remaining day 2/day 3 embryos vitrified	11	14	16	41
No. of day 2/day 3 embryos warmed	11	14	16	41
No. of day 2/day 3 embryos survived and cultured	10	14	16	40
No. of morulae obtained and transferred on day 5	2	1	2	5
No. of pregnancies following the transfer of morulae	0	0	0	0
No. of morulae and obtained on day 6 and cultured to day 7	7	4	9	20
No. of blastocysts obtained and vitrified on day 7	3	1	2	6
No. of day 7 blastocysts warmed	2	1	2	5
No. of day 7 blastocysts survived and transferred	2	1	2	5
No. of gestational sacs	1	0	2	3
No. of pregnancies ongoing or delivered	1	0	1	2

transfer. On day 6, nine morulae were obtained and cultured to day 7. Two out of nine morulae were developed to expanded blastocyst and were re-vitrified.

Subsequently, two re-vitrified day 7 blastocysts were warmed and both survived. Following 2 h of culture, two blastocysts re-expanded and were transferred into the patient's uterus. Two gestational sacs with fetal heartbeats were confirmed by ultrasound 30 days after embryo replacement. This pregnancy is ongoing at 26 weeks of gestation at the time of writing.

Discussion

We report two cases of successful pregnancies following the transfer of re-vitrified human day 7 blastocysts developed from vitrified cleaved embryos from three transfers in hormone replacement treatment cycles combined with assisted hatching.

In this report, assisted hatching was performed for all re-vitrified day 7 blastocysts. The rate of blastocyst development appears to be correlated with the ultimate ability for blastocysts to hatch free from their zona pellucida [24]. It has been proposed that more slowly forming blastocysts may benefit from assisted hatching to compensate for their poorer potential to escape the zona pellucida [25] and thereby improve implantation, presumably by both guaranteeing hatching and hastening contact of the trophectoderm with the endometrium after uterine transfer. On the other hand, the cryopreservation procedure may cause the hardening of the zona pellucida of human blastocysts. Resistance to enzymatic removal of zona pellucida by

pronase of re-cryopreserved human blastocysts (135 ± 9.4 s; mean $\pm$ S.D) has been reported to be significantly increased as compared with vitrified human blastocysts (120 ± 10.2 s) [26]. Re-cryopreserved blastocysts were embryos vitrified at the blastocyst stage which had previously been frozen at the cleavage stage by the slow freezing method. Therefore, hatching deficiencies of re-vitrified day 7 blastocysts can result from zona hardening, which might occur after twice-cryopreservation process. Consequently, it is plausible that assisted hatching for re-vitrified day 7 blastocysts developed from vitrified cleaved embryos contributed to the positive outcome observed here.

While the importance of embryo quality has been clearly demonstrated, another cause of implantation failure may be impairment of endometrial receptivity. Pinopodes have been proposed as an accurate marker for endometrial receptivity [27]. The pinopodes appear about 1 week after ovulation and they develop and regress within just 2 days [28–30]. On the other hand, hatching of human embryos occurs between day 5 and day 9 after fertilization [24]. In addition, all of the three women of the present report did not become pregnant following the transfer of day 5 morulae developed from vitrified cleaved embryos, but two of these women became pregnant following the transfer of day 7 re-vitrified blastocysts. Both day 5 morulae and day 7 blastocysts were transferred on day 5 endometrium at hormonal replacement treatment cycles combined with assisted hatching. These observations suggest that slower developing embryos cannot implant due to asynchrony with the implantation window and synchronization between embryo and endometrium assists the implantation of slower developing embryos.

A limitation of our report is that the number of treatment cycles was small. We have re-vitrified only 3 cohorts of day 7 blastocysts developed from vitrified cleaved embryos and all have been warmed. Therefore, it will be difficult to increase the number of treatment cycles from our group in a short period. However, the information of re-vitrified day 7 blastocyst transfer is lacking and cryopreserved cleaved embryo culture termination and failure to re-cryopreserve until day 7 may have occurred in many IVF clinics. That would have resulted in the loss of viable supernumerary embryos. Accordingly, although the number of treatment cycles was small, the present report has profound clinical value in knowing more slowly developing blastocysts developed from vitrified cleaved embryos can be re-vitrified as late as day 7, successfully warmed, and result in a delivery and one ongoing pregnancies from three transfers.

Conclusions

This is the first report of successful pregnancies after the transfer of re-vitrified human day 7 blastocysts developed from vitrified cleaved embryos. Our results suggest that re-vitrified human day 7 blastocyst transfer can contribute to increasing cumulative pregnancy rates in assisted reproduction. However, it will require more data on re-vitrified day 7 blastocyst transfer to determine the survival, implantation, and live birth rates from these more slower developing embryos. In addition, the risk of biochemical pregnancies, spontaneous abortions, and birth defects must be determined. Until these data are collected and analyzed, re-vitrified day 7 blastocyst transfer should be approached with caution.

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