Case report: successful delivery following the transfer of a human re-vitrified day-7 spontaneously hatched blastocyst developed from vitrified cleaved embryos

Kenichiro Hiraoka · Kaori Hiraoka · Toshitaka Horiuchi · Tomoyo Kusuda ·
Shinichiro Okano · Masayuki Kinutani · Kazuo Kinutani

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Abstract

Purpose To report a successful delivery after the transfer of a re-cryopreserved day-7 hatched blastocyst.

Methods and Results A 30-year-old woman underwent a long-treatment protocol for ovarian stimulation. Fourteen mature oocytes were obtained, and twelve were fertilized. On day 3, two cleaved embryos were transferred, but no implantation occurred. The remaining embryos were vitrified. Subsequently, two vitrified day-3 embryos were transferred. The woman became pregnant and delivered a healthy baby. Subsequently, two vitrified day-3 embryos were transferred, but no pregnancy occurred. Subsequently, all the remaining vitrified day-3 embryos were cultured. On day 5, no blastocyst was obtained. The remaining embryos were continued to be cultured. On day 7, a hatched blastocyst was obtained and re-vitrified. Subsequently, the re-vitrified day-7 hatched blastocyst was transferred. The woman became pregnant and delivered a healthy female.

Conclusions The day-7 hatched blastocyst developed from vitrified embryos can be re-vitrified and have pregnancy potential after re-warming.

Capsule Successful delivery was obtained following the transfer of a human re-vitrified day-7 spontaneously hatched blastocyst developed from vitrified cleaved embryos.

K. Hiraoka (✉) · K. Hiraoka · T. Kusuda · S. Okano ·
M. Kinutani · K. Kinutani
Kinutani Women's Clinic,
8-23-4F, Hondori, Naka-ku,
Hiroshima 730-0035, Japan
e-mail: hiraoka@chive.ocn.ne.jp

T. Horiuchi
Graduate School of Applied Biosciences,
Hiroshima Prefectural University,
Hiroshima 727-0023, Japan

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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 injury 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].

With the improvement of culture systems and blastocyst cryopreservation techniques, some pregnancies are achieved following the transfer of cryopreserved day-7 zona-intact blastocysts [13–15], re-cryopreserved day-7 zona intact blastocysts [16], and day-5 and day-6 spontaneously hatched blastocysts [17–20]. However, as far as is known, there are no reports on pregnancies following the transfer of re-cryopreserved day-7 spontaneously hatched blastocysts. In this case report, we present a successful live birth after the transfer of a human re-vitrified day-7 spontaneously hatched blastocyst developed from vitrified cleaved embryos.

Case report

A 30-year-old Japanese woman and her 32-year-old Japanese husband consulted our clinic because of primary infertility. The woman had unexplained factor infertility. The husband's semen characteristics were within normal parameters according to World Health Organization criteria.

The patient was treated with gonadotrophin-releasing hormone (GnRH) analogue buserelin acetate (Mochida, Tokyo, Japan) from the preceding mid-luteal phase in a long treatment protocol. Human chorionic gonadotrophin (HCG; Teizo, Tokyo, Japan) was administered to woman 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 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 (conventional IVF). 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. Following recovery of fourteen mature oocytes, twelve were fertilized following conventional IVF and were cultured in P-1 (Irvine) containing 10% (v/v) SSS (Irvine). All oocytes and embryos were incubated at 37°C in an atmosphere of 6% CO₂, 5% O₂ and 89% N₂. On day 3, two fresh cleaved embryos were transferred, but no implantation occurred. The remaining ten day-3 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 [16]. The cryotop consists of a 0.4 mm wide × 20 mm long × 0.1 mm thick polypropylene strip attached to a plastic handle and equipped with a cover straw. As the base medium, modified HTF medium-HEPES (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). The embryos were equilibrated in 1 ml of 30°C equilibration solution, confirming shrinkage and re-expansion 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 ~1 µl of cryoprotectant solution. Then the cryotop was immediately submerged into liquid nitrogen which had been filter sterilized through a 0.22 µm filter

(Millipore, Cork, Ireland) [21] and under the liquid nitrogen the plastic cover was placed over the strip to provide protection during storage. 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 embryos were then transferred into 1 ml of 30°C 0.5 mol/l sucrose solution for 3 min and washed twice in the base medium for 5 min. 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.

Two months after oocyte retrieval, two out of ten vitrified day-3 embryos were warmed for transfer. Following warming, both two embryos survived and were transferred in hormonal replacement treatment cycle. The patient received transdermal oestradiol (Estraderm 1.4–5.8 mg, Kissei, Tokyo, Japan) 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. Warmed day-3 embryo transfer was scheduled on day 3 after the initiation of progesterone treatment. One gestational sac with fetal heartbeat was confirmed by ultrasound 30 days after embryo replacement. The woman delivered a healthy baby.

Thirty-six months after oocyte retrieval, two out of eight vitrified day-3 embryos were warmed for transfer. Following warming, both two embryos survived and were transferred in hormonal replacement treatment cycle on day 3 after the initiation of progesterone treatment as described above, but no pregnancy occurred.

Forty-three months after oocyte retrieval, all the remaining six day-3 embryos were warmed and cultured for better embryo selection before embryo transfer. Six warmed day-3 (the day of warming was considered as day 3) embryos were placed in Blast Assist Medium 2 (Medicult, Jyllinge, Denmark) and cultured until day 5 without performing assisted hatching. On day 5, the embryos were examined for development into blastocysts. However, no blastocyst was obtained and embryo transfer was canceled. The remaining six embryos were placed in fresh Blast Assist Medium 2 (Medicult) and continued to be cultured. On day 6, one morula was obtained and cultured to day 7. On day 7, the morula grew to a hatched stage blastocyst that had spontaneously and completely escaped from the zona pellucida and was re-vitrified (Fig. 1a). It is our policy to cryopreserve not morula stage but expanded or hatched blastocyst stage in order to cryopreserve better quality embryo [4, 19, 20]. Therefore, re-vitrification was done on day 7 rather than day 6 in the present case. Before starting the vitrification of hatched blastocyst, artificial

Fig. 1 A hatched blastocyst developed from vitrified cleaved embryos and empty zona pellucida observed on day 7 after oocyte retrieval: **a** before re-vitrification **b** 2 h after re-warming. The re-warmed hatched blastocyst implanted after embryo transfer. The patient delivered a healthy female

shrinkage of blastocoele was performed in the equilibration solution by the method as described previously [19, 20]. First, pipetting of the hatched blastocyst was started immediately after placing the embryo in 1 ml of 30°C equilibration solution with a glass pipette slightly smaller in diameter (~160 μ m) than the hatched blastocyst. After confirmation of slight shrinkage of the blastocoele, pipetting was performed with a pipette slightly smaller in diameter than the first one (~120–140 μ m). This procedure was repeated two to three times until the blastocoele collapsed completely. After blastocoele contraction, the blastocyst was equilibrated in the same equilibration solution for another 2 min before exposure to the vitrification solution. After equilibration, the hatched blastocyst was then incubated in vitrification solution and loaded onto the cryotop and was stored in liquid nitrogen as described above.

Forty-five months after oocyte retrieval, the re-vitrified day-7 hatched blastocyst was warmed and survived. Following 2 h of culture, the hatched blastocyst re-expanded (Fig. 1b) and was transferred in hormonal replacement treatment cycle on day 5 after the initiation of progesterone treatment as described above. One gestational sac with fetal heartbeat was confirmed by ultrasound 30 days after embryo replacement. This pregnancy resulted in delivery of a healthy female (3480 g) at 39 weeks of gestation.

Discussion

Our results demonstrated that a human day-7 spontaneously hatched blastocyst developed from vitrified cleaved embryos could be re-vitrified and, following embryo transfer, results in a successful delivery. Two possible explanations could account for this success.

First, the blastocoele of day-7 hatched blastocyst was artificially shrunk during equilibration. In the author's clinical experience, artificial shrinkage of the blastocoele has proved to be effective for increasing the survival rate of vitrified day-5, day-6 and day-7 zona-intact expanded

blastocysts [4, 15], of vitrified day-5 and day-6 spontaneously hatched blastocysts [19, 20] and of re-vitrified day-7 zona-intact expanded blastocysts developed from vitrified cleaved embryos [16]. Therefore, the same vitrification protocol was applied to the day-7 hatched blastocyst, which could be why successful survival of re-vitrified day-7 hatched blastocyst in the present report was observed.

Secondly, the re-vitrified day-7 hatched blastocyst was transferred into day-5 endometrium. In our clinical experience, acceptable pregnancy rate of vitrified day-7 zona-intact expanded blastocysts (55%; 6/11) [15], day-5 and day-6 spontaneously hatched blastocysts (75%; 3/4) [20] and re-vitrified day-7 zona intact expanded blastocysts developed from vitrified cleaved embryos (67%; 2/3) [16] was observed following the transfer of these embryos into day-5 endometrium. Thus, the same endometrium preparation was applied to the re-vitrified day-7 hatched blastocyst transfer, which could be why successful implantation of re-vitrified day-7 hatched blastocyst in the present report was obtained. This suggests that synchronicity with the endometrium plays an important role and is worth further study as more experience accumulates with re-vitrified day-7 hatched blastocyst transfer.

Cryopreservation of supernumerary embryos obtained from human IVF protocols provides an opportunity for patients to have repeated attempts at conception following single oocyte retrieval, preventing wastage of valuable supernumerary embryos and improving cumulative pregnancy rates. Therefore, with the improvement of the technology, multiple cryopreservation of human embryos has been contributing to the treatment availability in human assisted reproductive technology [22–30]. Primary vitrification was performed at the cleavage stage on day 3 and secondary vitrification was done at the hatched blastocyst stage on day 7. Both vitrified day-3 embryo transfer and re-vitrified day-7 hatched blastocyst transfer resulted in successful pregnancy and delivery. The results suggest that vitrification is an efficient method for both cleaved embryo cryopreservation and subsequent hatched blastocyst re-cryopreservation.

Conclusion

This is the first report of a successful delivery after the transfer of a re-vitrified day-7 spontaneously hatched blastocyst developed from vitrified cleaved embryos. Information on re-vitrified day-7 hatched blastocyst transfer is lacking and embryo culture termination of cryopreserved embryos on day 7 and failure to cryopreserve day-7 hatched blastocysts may have occurred in many IVF clinics resulting in the loss of viable supernumerary embryos. Therefore, the present report has profound clinical value in showing that more slowly developing hatched blastocyst developed from vitrified cleaved embryos can be re-vitrified as late as day 7, warmed and result in a delivery. However, it will require more data on re-vitrified day-7 hatched blastocyst transfer to determine the survival, implantation, and live birth rates from this more slowly developing embryos. In addition, the risks of biochemical pregnancy, spontaneous abortion and birth defects must be determined. Until these data are collected and analyzed, re-vitrified day-7 hatched blastocyst transfer should be approached with caution.

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