

—Case Report—

Successfully Healthy Baby Delivery from Human Refrozen Blastocyst Embryos by VitrificationTomomi Takahashi^{1*} and Yasuhisa Araki²¹Takahashi Ladies Clinic, East-Tower 2F, 4-1-1, Yukarigaoka, Sakura, Chiba 285-0858, Japan²The Institute for Advanced Reproductive Medical Technology (ARMT), 909-21, Ishii, Setagun, Gunma 371-0105, Japan

Abstract: To demonstrate delivery by using re-vitrified blastocysts derived from supernumerary embryos in the event of an unsuccessful pregnancy attempt. Nine early stage cleaved embryos were frozen by vitrification, and subsequently thawed. Four of the nine embryos developed to the blastocyst stage. Two of the four blastocysts were transferred, and two supernumerary good embryos remained unexpectedly, and they were refrozen by vitrification under consent. Subsequently, one of the re-vitrified embryos developed to 38 weeks and was delivered by cesarean section (a health female; 46, XX). The transfer of re-vitrified supernumerary blastocysts resulted in a successful pregnancy and delivery outcome. This study suggests that re-vitrification is one rescue procedure which allows the re-use of supernumerary embryos in patients who failed to have a pregnancy after frozen embryo transfer.

Key words: Refrozen, Blastocyst, Vitrification, Supernumerary embryo

similar for both the rapid and the slow freezing procedures. Vitrification is a relatively simple procedure: after thawing early stage embryos, surplus embryos are often present. When we thawed the frozen embryos, sometimes survival embryos were present for supernumerary embryos. The usefulness of these refrozen embryos would be substantially enhanced if they were still viable after a further thawing and freezing cycle. In addition, it would be advantageous to use these embryos in a patient who had experienced repeated pregnancy failure; these refrozen embryos have the capacity to develop to a viable term pregnancy. In this study, supernumerary, cleaved embryos were vitrified, thawed, and developed to the blastocyst stage in *in-vitro* culture. Concurrent with blastocyst transfer, supernumerary blastocysts were refrozen by vitrification. After re-thawing, the embryos were transferred to the patient. This is the first study, which describes a successful pregnancy outcome after a double vitrification cycle of human embryos.

Introduction

Embryo cryopreservation has been developed into an important technique for infertility treatment; it has undoubtedly contributed to an improvement in cumulative pregnancy rates. The cryopreservation of embryos is now feasible at any stage of the treatment cycle, from successful embryo recovery to transfer. Recently, vitrification via the rapid method has been found to improve successful outcome of cryopreservation [1]. Furthermore, the survival rate is

Case Report

A 35-year-old woman with a five year history of infertility and her 38-year-old husband were referred to our IVF program in 1998. The patient had unexplained infertility. Semen analysis was: average volume 3~4 ml; sperm count 32×10^6 /ml; motility 60%; and abnormal morphology 7%.

Ovarian stimulation was achieved via combined administration of gonadotropin-releasing hormone agonist (GnRHa), human menopausal gonadotropin (hMG; hMG-Fuji, Fuji Pharmaceutical Ltd., Tokyo) and 10,000 units of human chorionic gonadotropin (hCG; hCG-Fuji, Pharmaceutical Ltd., Tokyo). HCG was administered when the dominant follicle reached a

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mean diameter of 18–20 mm. Vaginal ultrasound-guided follicle puncture which took place 35 hours after hCG injection.

With the first IVF treatment in November 2000, the patient experienced a chemical pregnancy, which did not progress. The second IVF procedure was conducted in, May 2001, after the recovery of 17 mature oocytes, 12 of which were fertilized by conventional IVF (c-IVF). They developed to the 4-cell cleavage stage with overnight culture; subsequently, three embryos were transferred, but the procedure was unsuccessful. Nine supernumerary, embryos which had undergone cleavage at the 4 cell stage, were vitrified in three straws containing three numbers of embryos depending on their grade.

Briefly, we vitrified these embryos with a modification of Ishimori's vitrification solution containing ethylene glycol and dimethyl sulfoxide (VSED) [2]. VSED contained Hepes human follicular fluid (Hepes-HFF99; Fuso Pharmaceutical Industries, Osaka, Japan) with 20% synthetic serum substance (SSS; Irvine, CA, USA), ethylene glycol (EG), and dimethyl sulfoxide (DMSO; Sigma) at a 2:1:1 ratio.

Embryos were exposed to 10% EG for five minutes. They were then placed into 50% VSED for one minute, and within 30 seconds were loaded into straws containing VSED at room temperature. The straws were placed in liquid nitrogen (LN2) vapor for two minutes, and then plunged immediately into LN2.

For luteal support after embryo transfer, the patient applied two Estraderum M[®] patches (estradiol 0.72 mg/sheet) beginning one day after cessation of menses and continuing until 14 days after starting patch application. Nine days after cessation of menses, two additional Estraderum M[®] patches were applied. At 11 days, two more patches were applied, and at 13 days, two more patches were applied (eight patches in all). After 15 days, four Estraderum M[®] patches were applied every other day. In addition, the patient inserted vaginally a 200 mg progesterone suppository twice a day for the next three weeks.

Five months later, in October 2001, we thawed and cultured these embryos for two days to the blastocyst stage. We thawed all 9 embryos because we planned blastocyst transfer for selection of good viable embryos, and the blasturation rate was not so good at this stage. Four of the nine embryos developed to the blastocyst stage, and then two of the four blastocysts (grade; 3BC, 3BB, 3CC, early blastocyst) were selected for transfer. The supernumerary embryos had good quality, contrary to our expectation, and two supernumerary blastocysts

were refrozen by vitrification. Informed consent was obtained from the patient prior to the use of the re-vitrified embryos. Once again, a viable pregnancy was not achieved. Therefore, approximately three months later (January 2002), two of the thawed blastocysts were then transferred to the uterus, but we noted that only one of them appeared to be morphologically viable. Their grades were 3AA and 3CB according to Gardner criteria [3]. Two weeks later, we detected a urine hCG of 25 mIU/ml; furthermore, one week later, we detected a gestational sac. The pregnancy then progressed uneventfully. In September 2002, the patient delivered a healthy female infant at 38 weeks of gestation by cesarean section (2910 g; normal 46, XX karyotype). No anomalies were detected in the infant and her development has been normal until the present.

Discussion

Regarding an improvement in the pregnancy rate, the use of fresh vs. cryopreserved embryos is subject to debate. Freezing methods include the widely used slow freezing method, and more recently, the increasingly used rapid freezing (vitrification) method, which can be employed at various stages of embryonic development.

In this case study, 12 of the 17 mature oocytes were fertilized after c-IVF, and developed to cleaved embryos with *in-vitro* culture. Three of these embryos were transferred, and nine supernumerary embryos, of which five of nine contained good morphological quality, were vitrified. The couple requested that the embryos be further grown to the blastocyst stage to enable better embryo selection. In our technique, the percentage of blastocyst development from all including morphologically good and bad cleaved embryos was a maximum of 30% at the time. In October, 2001, all nine cleaved embryos were thawed; four of these nine embryos developed to the blastocyst stage in *in-vitro* culture. Blastocyst embryo transfer might increase multiple pregnancies, so only two of four blastocysts were transferred, but the result did not produce a viable pregnancy. The supernumerary blastocysts were therefore re-vitrified. We discussed the safety and risk involved in refreezing with the patient, and finally the patient wanted to re-vitrify and thaw the supernumerary blastocyst embryos for reusing in a subsequent treatment cycle.

Despite two unsuccessful attempts with both fresh and vitrified-thawed cleaved embryos, the patient achieved a pregnancy after the transfer of the thawed supernumerary blastocysts, which had been vitrified as

Table 1. Reports of the viable pregnancies from re-frozen embryo transfer

	Authors	Embryo stage at first freezing	Embryo stage at second freezing	Embryo stage at transfer	Method of freezing
1990	Macnamee (4)	2PN	cleaved embryo	cleaved embryo	Slow Freezing
1994	Mowal (5)	2PN	cleaved embryo	cleaved embryo	Slow Freezing
1996	Baker (6)	2PN	2PN	cleaved embryo	Slow Freezing
2001	Farhat (7)	cleaved embryo	blastocyst	blastocyst	Slow Freezing
2001	Yokota (2)	2PN	morula	blastocyst	Slow Freezing Vitrification
2002	Takahashi (This paper)	cleaved embryo	blastocyst	blastocyst	Vitrification

cleaved embryos and then cultured to the blastocyst stage. To date, there have been several reports of the birth of healthy infants after *in-vitro* development of refrozen embryos (Table 1). Macnamee M. *et al.* [4] and Mowal L. G. *et al.* [5] reported two pronuclei (2PN) zygotes, which had been subjected to slow freezing and thawing; they developed to the 2-4 cell division stage, and were refrozen by the slow method. Baker A. *et al.* [6] reported on 2PN zygotes, which had been twice subjected to slow freezing and thawing. Farhat M. *et al.* [7] reported the two freeze-thaw cycles: the first at day 3 after insemination, and the second after culturing to the blastocyst stage. In the above four cases, which used the slow freezing method, a successful pregnancy ensued. Nevertheless, Yokota *et al.* [2] described 2PN zygotes, which had been subjected to slow freezing and thawing; they developed to the morula stage, and were then refrozen by vitrification. In this case study, we vitrified early cleavage stage embryos; after thawing, they developed to the blastocyst stage. After blastocyst transfer, the supernumerary blastocysts were re-vitrified; three months later, the blastocysts were thawed and transferred to the patient.

Previous research has suggested the safety of several refreezing/re-thawing cycles with mouse embryos. Frozen mouse embryos were found to have fewer cells per embryo at the time of hatching than the unfrozen embryos. Nevertheless, these results demonstrate that mouse embryos can survive even three successive freeze-thaw cycles and still be capable of *in-vitro* development [8]. Yokota *et al.* [2] have reported a successful delivery with refrozen embryos, which had been vitrified at least once. They found that human embryos also fared well under the severe physiological conditions imposed by being refrozen in LN₂; these embryos had a high survival rate, 80%, after blastocyst vitrification [1]. They also found that the morphology of fresh and refrozen blastocysts was similar when viewed under an electron microscope (personal

communication). This study reveals that human embryos can survive the severe stress of cryopreservation.

The refreezing of supernumerary embryos enables them to be used in subsequent IVF treatment cycles in the event of a number of unsuccessful pregnancies. Nevertheless, our knowledge and experience of freezing and thawing of re-frozen embryos is still incomplete. Further discussion is needed in regard to the ethical factors and the genetic safety of vitrification techniques. The parents must be informed of the benefits and the risks of pursuing treatment, as it might affect the future health of their child. In this case study, the parents allowed us to re-vitrify and thaw the supernumerary blastocyst embryos for reuse in subsequent treatment cycles.

In conclusion, re-freezing of embryos must be avoided in IVF procedures. If patients unexpectedly get the supernumerary embryos after frozen embryo transfer, there is the possibility of choosing re-vitrification of them for further embryo transfer.

References

- 1) Kasai, M. (2002): Advances in the cryopreservation of mammalian oocytes and embryos: development of ultrarapid vitrification. *Reprod. Med. Biol.*, 1, 1–9.
- 2) Yokota, Y., Yokota, H., Yokota, M., Sato, S. and Araki, Y. (2001): Birth of healthy twins from *in vitro* development of human refrozen embryos. *Fertil. Steril.*, 76, 1063–1065.
- 3) Gardner, D.K. and Schoolcraft, W.B. (1999): *In vitro* culture of human blastocyst. In: *Towards Reproductive Certainty: Infertility and Genetics Beyond 1999* (Jansen, R. and Mortimer, D., eds.), pp. 378–388, Parthenon Press, Australia.
- 4) Macnamee, M., Wick, K., Rainsbury, P., Sathanandan, M., Brinsden, P. and Edwards, R.G. (1990): How robust are early human embryos? *Lancet.*, 8 Sept., 636.
- 5) Mowal, L.G., Jamlson, M.E., Ross, K.S., Yates, R.W.S. and Coutts, J.R.T. (1994): Successful pregnancy after replacement of embryos which had been frozen and thawed

- at both pronuclear and cleavage stage, Abstracts of the 2nd International Meeting of the BFS, Glasgow. *Hum. Reprod.*, 9, 22.
- 6) Baker, A., Check, J.H., Lurie, D., Hourani, C. and Hoover, L. (1996): Pregnancy achieved with pronuclear-stage embryos that were cryopreserved and thawed twice: a case report. *J. Assist. Reprod. Genet.*, 13, 713–715.
 - 7) Farhat, M., Zentner, B.S, Lossos, F., Bdolah, Y., Holtzer, H. and Hurwitz, A. (2001): Successful pregnancy following replacement of embryos previously refrozen at blastocyst stage. *Hum. Reprod.*, 16, 337–339.
 - 8) Vitale, N.J., Myers, M.W., Denniston, R.S., Leibo, S.P. and Godke, R.A. (1997): In-vitro development of refrozen mouse embryos. *Hum. Reprod.*, 12, 310–316.