

# Live birth after transfer of a twice-vitrified warmed blastocyst that had undergone trophectoderm biopsy

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**Abstract:** A 28-year-old patient presented for preimplantation genetic screening (PGS) for family balancing utilizing previously vitrified blastocysts and day-2 embryos. To synchronize endometrial development with the embryos to be transferred, five embryos vitrified on day 2 were warmed 3 days prior to scheduled transfer. Three of them developed to 8-, 8- and 7-cell stages, respectively, and were biopsied the next day, during which three vitrified blastocysts were warmed and the two surviving blastocysts underwent trophectoderm biopsy. The five biopsied embryos were subjected to two-probe fluorescence in-situ hybridization for chromosomes X and Y. As there were still 2 days before the scheduled embryo transfer following biopsy, the two biopsied blastocysts were re-vitrified. One blastocyst and two of the embryos vitrified on day 2 were normal for sex chromosomes; of these, one of the day-2 vitrified embryos was arrested and the other did not favour the patient's wish. The re-vitrified blastocyst, which was normal for sex chromosomes, was therefore warmed and transferred, resulting in delivery of one healthy boy. As far as is known, this is the first-reported live birth developed from a re-vitrified blastocyst that had been previously vitrified, warmed and undergone trophectoderm biopsy

**KEYWORDS:** blastocyst, live birth, preimplantation genetic screening, re-vitrification, trophectoderm biopsy

## Introduction

Embryo cryopreservation plays an important role in the practice of human IVF. Transfer of frozen-thawed embryos following a fresh IVF cycle offers patients a chance to have one or more additional children without additional ovarian stimulation, reduces fertility treatment costs and increases the cumulative pregnancy rate. Human embryos have been successfully cryopreserved at the pronuclear, cleavage and blastocyst stages of development. In some cases, a 1472- frozen-thawed embryo needs to be recryopreserved for a variety of reasons, such as a health issue, unexpected supernumerary embryos available after a frozen-thawed embryo cycle (Kumasako et al., 2009; Yokota et al., 2001) and other circumstances beyond control (Baker et al., 1996; Smith et al., 2005).

In most cases, embryos frozen at the pronuclear or cleavage stage are thawed and cultured to blastocysts, at which stage embryos in excess of those transferred are re-frozen, thawed and transferred (Estes et al., 2003; Farhat et al., 2001; Hiraoka et al., 2006).

Embryo recryopreservation has its practical application in a preimplantation genetic diagnosis/screening (PGD/S) programme. PGD/S has been successfully applied to test the previously cryopreserved embryos and a few pregnancies and live births have been achieved (Baart et al., 2004; Ciotti et al., 2000; Edirisinghe et al., 2005; Parriego et al., 2007). However, the time period needed to perform genetic testing is not always comparable with embryo transfer and the biopsied embryos might need to be recryopreserved. Very few data are available regarding the outcome of recryopreserved embryos after PGD/S and it is not yet known how well recryopreservation will work for a vitrified blastocyst on which trophectoderm biopsy has been performed. This case report describes a live birth following the transfer of a re-vitrified blastocyst that had undergone trophectoderm biopsy.

## Case report

### *Patient*

A 26-year-old patient, gravida 0, para 0, presented for fertility treatment due to high FSH (17 IU/l) and low sperm motility in her 31-year-old husband. The husband's semen characteristics were as followed: 1.0 ml, 100 · 10<sup>6</sup>/ml, 10% motility, 2% normal spermatozoa (Kruger strict criteria for morphology; Kruger et al., 1988). Ovulation induction was performed by a minimal stimulation protocol, using clomiphene citrate (Serophene;

Serono, Norwell, MA, USA) and low-dose human menopausal gonadotrophin (75 IU LH and 75 IU FSH, Menopur; Ferring Pharmaceuticals, Suffern, NY, USA). Stimulation was started with clomiphene citrate 50–100 mg a day from day 3 to day 7, followed by 1–2 ampoules of human menopausal gonadotrophin for 4 days. Human chorionic gonadotrophin (HCG) injection (10,000 IU, Novarel; Ferring Pharmaceuticals, Suffern, NY) was administered when the dominant follicle reached 18 mm. In the first cycle, no oocyte was retrieved. Two months later, the patient underwent a second cycle, where a total of four oocytes were retrieved, injected, fertilized and progressed to the blastocyst stage. Due to the negative impact of clomiphene citrate on the endometrium, three day-5 and one day-6 blastocysts were vitrified for future cycles. Three months later, one day-6 vitrified blastocyst was warmed, survived, re-expanded and transferred 6 days after LH surge in a natural cycle, resulting in the live birth of a healthy girl at 38 weeks of gestation.

Nine months after delivery, the patient returned requesting to use the vitrified blastocysts to initiate PGS for family balancing. To accumulate enough embryos for biopsy, a third and fourth minimal stimulation cycles were performed to accumulate more embryos for PGS, with five retrieved oocytes being normally fertilized, divided and vitrified on day 2. Two months after the fourth cycle, PGS was performed utilizing the previously vitrified embryos from the above three retrieval cycles.

### *Vitrification and warming*

Vitrification was performed using Cryotop as described by [Kuwayama et al. \(2005\)](#) and summarized as followed. HEPES-buffered TCM 199 supplemented with 20% (v/v) serum substitute supplement (SSS; Irvine Scientific, Santa Ana, CA, USA) was used as base medium. The equilibration solution (ES) contained 7.5% (v/v) ethylene glycol (EG) (Sigma Chemical, MO, USA) and 7.5% (v/v) dimethylsulphoxide (DMSO) (Sigma Chemical). The vitrification solution (VS) was composed of 15% (v/v) EG, 15% (v/v) DMSO and 0.5 mol/l sucrose (Sigma Chemical). Embryos were first equilibrated in ES for 10–15 min, after which they were transferred to VS, incubated in VS solution and loaded on to the tip of the Cryotop (Kitazato BioPharma) with 0.1  $\mu$ l or less of VS solution within 60 s. The Cryotop was then immediately plunged into liquid nitrogen and inserted into a pre-cooled cover straw with the aid of forceps. For the three blastocysts with expanded blastocoele from cycle 1, artificial shrinkage of blastocoele using one laser pulse from a noncontact laser (ZILOS-tk; Hamilton Thorne Biosciences, Beverley, MA, USA) was performed prior to the cooling steps of vitrification.

For warming, the protective cover was removed from the Cryotop while it was still submerged in liquid nitrogen. Vitrified embryos were warmed by directly immersing the polypropylene strip of the Cryotop into 5 ml pre-warmed 1.0 mol/l sucrose solution maintained on a warm stage at 37 °C for 1 min. Embryos were then transferred into 5 ml 0.5 mol/l sucrose solution for 3 min, after which the warmed embryos were washed twice in TCM 199 with 20% SSS for 5 min each before being returned to culture in Sage Blastocyst Medium (Cooper Surgical, Trumbull, CT, USA).

### *Embryo biopsy and fixation*

Three vitrified blastocysts from cycle 1 and 5 embryos vitrified on day 2 from cycles 3 and 4 were warmed for embryo biopsy. To synchronize endometrial development with the embryos to be transferred, the five embryos vitrified on day 2 in cycles 3 and 4 were warmed 3 days prior to the scheduled transfer. After 1-day in-vitro culture, three of them developed to 8-, 8- and 7-cell stages, respectively, and were biopsied 20 days prior to the scheduled transfer. At the same time, the three vitrified blastocysts from cycle 2 were warmed, of which two survived and underwent trophoctoderm biopsy with the assistance of laser as described by [McArthur et al. \(2005\)](#). About five trophoctoderm epithelium cells were biopsied from each blastocyst. Biopsied blastomeres and trophoctoderm epithelium cells were fixed on slides by methanol and acetic acid (3:1) as previously described ([Magli et al., 2000](#)) for fluorescence in-situ hybridization (FISH) analysis. The five biopsied embryos were subjected to two-probe FISH for chromosomes X, Y. Since there were still 2 days before the scheduled embryo transfer, the two biopsied blastocysts were cryopreserved by vitrification.

FISH results showed two embryos were abnormal for the sex chromosomes, with one being diagnosed as monosomy X and the other being XXXXX. Of the three embryos normal for sex chromosomes, two were of those

vitrified on day 2 and one was a blastocyst which had been vitrified on day 5. One of the day-2 vitrified embryos was arrested and the other one did not favour the patient's wish. For this reason, the re-vitrified blastocyst that had undergone trophectoderm biopsy and was normal for sex chromosomes was warmed and transferred on the morning of scheduled transfer. Embryo transfer was performed 5 days after the LH surge on cycle day 19 in a spontaneous natural cycle with an endometrial thickness of 11.6 mm. A positive HCG was obtained 2 weeks after embryo transfer and fetal heart beat was confirmed by ultrasound observation 4 weeks later. The patient delivered one healthy boy weighing 3010 g at 39 weeks of gestation.

## *Discussion*

Although composed of a relatively small number of embryos, this report shows that a blastocyst that had been previously vitrified and biopsied could successfully survive recryopreservation and be capable of achieving a pregnancy. As far as is known, this is the first reported live birth following the transfer of a warmed blastocyst that had been vitrified twice and undergone trophectoderm biopsy.

Embryo biopsy was performed on both day-3 and blastocyst-stage embryos in this report. While the removal of one to two blastomeres from a day-3 cleavage-stage embryo remains the main approach to obtain genetic materials for PGD/S, application of sequential embryo culture has made it possible to perform embryo biopsy at the blastocyst stage. Compared with cleavage-stage biopsy, trophectoderm biopsy has several distinct advantages. Biopsy of more than two trophectoderm cells allows the possibility of making a diagnosis on duplicate samples, especially for single gene diseases. Trophectoderm cells are strictly extra-embryonic; removal of several trophectoderm cells therefore does not result in loss of inner cell mass and would not affect subsequent fetal development. Although 5–10 trophectoderm cells are usually biopsied from a blastocyst, the proportion of cell loss from a biopsied blastocyst is still lower than 1–2 cell removal from a 6–8-cell-stage embryo. Additionally culturing embryos to the blastocyst stage improves the selection of the most viable embryos with higher developmental potential (Gardner et al., 1998). Blastocyst biopsy therefore allows for the selection of both developmentally and genetically normal embryos. Pregnancies and live births after transfer of biopsied fresh blastocysts following PGD/S have been reported by several groups (Kokkali et al., 2005; McArthur et al., 2005; Pangalos et al., 2008).

While there is no evidence showing that embryo biopsy affects the survival of early cleavage-stage murine embryos, poor embryo survival rate has been observed when biopsied cleavage-stage human embryos were cryopreserved by slow freezing (Joris et al., 1999; Magli et al., 1999). It is therefore important to have a reliable cryopreservation method in the case of excessive embryos that have undergone blastomere biopsy for PGD/S. The high efficiency of vitrification has made it an alternative to slow freezing for effective cryopreservation of biopsied cleavage-stage human embryos. As demonstrated by Zheng et al. (2005), vitrification significantly increased the survival rate of human biopsied embryos above standard and modified slow-cooling methods. Trophectoderm biopsy has been recently used as a means for testing frozen blastocysts in patients with excess embryos cryopreserved from previously performed IVF cycles (Lathi and Behr, 2009). A major limitation of blastocyst biopsy is that there is limited time available to perform diagnosis while retaining the viability of a blastocyst *in vitro*.

Under most circumstances, FISH-based testing allows for the PGD/S results to be obtained within 24 h after embryo biopsy, making it possible to transfer the frozen–thawed embryo the day after thawing. However, in this study, trophectoderm biopsy was performed 2 days prior to scheduled transfer time, which required the recryopreservation of the biopsied blastocysts while waiting for the report. By re-vitrifying biopsied blastocysts, trophectoderm biopsy and blastomere biopsy can now be performed on the same day for previously cryopreserved embryos frozen at different developmental stages. Recryopreservation of biopsied embryos is especially useful for PGD/S on cryopreserved embryos diagnosed by PCR-based technologies, especially comparative genomic hybridization, during which the biopsied embryos need to be recryopreserved to allow for the lengthy 24-chromosome analysis.

In summary, these results suggest that it is possible to utilize cryopreserved embryos for preimplantation genetic screening by embryo recryopreservation via vitrification. Integrating embryo recryopreservation into a PGD/S programme makes the timing of PGD/S treatment more flexible and more manageable.

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