Live birth of healthy twins following transfer of re-cryopreserved blastocysts developed from frozen embryos recovered from a malfunctioning freezer

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Introduction

A computer-controlled cooling machine is the basic equipment required to perform slow freezing of human embryos. Although freezer malfunction is a rare event, it has been reported (HFEA press release. [1]). To date, there are no published reports regarding the fate of cryopreserved embryos encountered freezer malfunction during the slow cooling process. This case report describes our experience with a sudden temperature increase occurring during the slow cooling of human embryo cryopreservation. We successfully recovered cryopreserved embryos from the malfunctioning freezer by rapid thawing. Surviving embryos successfully developed to the blastocyst stage and were re-cryopreserved by vitrification. An ongoing twin pregnancy was achieved with two healthy twins following the transfer of two vitrified blastocysts.

Case report

A 35-year-old patient, gravida 2, para 2, was referred to fertility treatment due to PCOS. The husband's semen characteristics were as followed: 1.0 ml, $91 \times 10^6/\text{ml}$, 45 % motility. This couple had one previous failed IVF. The patient underwent a standard GnRH antagonist ovarian stimulation protocol using Ganirelix (Merck & Co. Inc., Whitehouse Station, NJ). Stimulation was started with 75 IU Menopur (Ferring Pharmaceuticals, Suffern, NY) and 150 IU Follisitim (Merck & Co. Inc., Whitehouse Station, NJ) per day from day 3 through day 5, followed by 75 IU Menopur and Follisitin a day from day 6 to day 9 and 75 IU Menopur on day 10. Leuprolide acetate trigger (2 mg) shot was given 35 h prior to oocyte retrieval procedure. A total of 21 oocytes were retrieved.

Insemination and embryo culture

Following retrieval, cumulus-oocyte-complexes (COCs) were washed free of follicular fluid and blood, and incubated in 200- μl drops of P1 Medium (Irvine Scientific, Santa Ana, CA) supplemented with 3 mg/ml Human Serum Albumin (HSA, Irvine Scientific, Santa Ana, CA) in a 37 °C, 7 % CO₂ incubator. Four hours following retrieval, oocytes were inseminated by fresh husband sperm at a concentration of 150,000 motile sperm per ml. A total of 9 oocytes were normally fertilized. All normally fertilized oocytes were cryopreserved for future use due to poor lining.

Slow freezing

All equilibration procedures were carried out at room temperature. Embryos were first exposed to a 1.5 mol/l 1, 2-

Capsule Healthy birth of twins was documented following transfer of re-cryopreserved blastocysts developed from frozen embryos recovered from an unforeseen freezer malfunction occurring at -15°C of cooling process.

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propanediol (PROH) solution for 10 min and then to 1.5 mol/l PROH + 0.1 mol/l sucrose solution for another 10 min. They were then loaded into 0.25 ml cryostraws and placed in a programmable controlled freezer (Planer Series III Kryo 10, TS, Scientific, Perkaise, PA). Cryostraws were cooled from 20 °C down to −7 °C at a rate of −2.0 °C/min, at which point a 5-min pre-seeding hold was built in before manual ice crystal formation (seeding) was performed. An additional 5-min hold at −7 °C was provided for further equilibration. Cooling resumed at a rate of −0.3 °C/min until freezer chamber temperature reached −15 °C, at which point the chamber temperature suddenly increased to −2.8 °C within 1 min.

Freezer malfunction

To save the frozen embryos from the unexpected freezer malfunction, we performed rapid thawing, during which cryostraws were immediately removed from the freezer chamber and thawed using Quinn's Advantage Thaw Kit (SAGE In-Vitro Fertilization, Trumbull, CT, USA). After being warmed in air for 30 sec, the cryostraws were immersed into 31 °C water bath for 40 sec. Contents of each cryostraw were expelled into 0.5 mol/l sucrose solution, washed twice, and held for 10 min. The embryos were then transferred into 0.2 mol/l sucrose solution for 10 min. After being washed three times and held in Hepes-buffered HTF with 12 mg/ml HSA for 5 min, embryos were placed in Global Medium (LifeGlobal, Guilford, CT) with 10 % Synthetic Serum Substitute (SSS) (Irvine Scientific, Santa Ana, CA).

Embryos were evaluated immediately and also 2 h after thaw; all nine pronuclear stage embryos (2PN) survived the rescue thaw. To prevent the thawed embryos from being exposed to immediate stress caused by re-cryopreservation, we decided to culture the surviving embryos to the blastocyst stage and perform embryo cryopreservation at a later time. On day 6, 5 of 9 embryos developed to the blastocyst stage, of which three blastocysts showing clear ICM and trophoctoderm met our standard criteria for cryopreservation. We used vitrification to re-cryopreserve blastocysts developed from embryos recovered from the chamber of malfunctioning freezer.

Vitrification and warming

Prior to vitrification, mechanical artificial blastocoele shrinkage was performed as described by Kaida et al. [2]). Vitrification was performed at room temperature with Irvine Vit Kit- Freeze (Irvine Scientific, Santa Ana, CA). Blastocysts were first equilibrated in Equilibration Solution for 10 min, after which they were transferred to Vitrification Solution and loaded onto

the tip of a Cryotop (Kitazato BioPharma, Shizuoka, Japan) with 0.1 µl or less of vitrification solution within 1 min. The Cryotop was then immediately plunged into liquid nitrogen and inserted into a pre-cooled cover straw with the aid of a forceps.

Two months later, the patient came back to initiate a frozen embryo transfer. Two vitrified blastocysts were warmed using Vit Kit-Thaw (Irvine Scientific, Santa Ana, CA). Vitrified blastocysts were first 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. Warmed blastocysts were then transferred into Dilution Solution containing 0.5 mol/l sucrose for 3 min, after which they were equilibrated in sucrose-free Washing Solution for 5 min before being returned to culture in Global Medium (LifeGlobal, Guilford, CT) with 10 % SSS at 37 °C, 7 % CO₂ incubator. Both vitrified blastocysts survived the warming and fully expanded after 2 h culture. Two fetal heartbeats were confirmed by transvaginal ultrasound 6 weeks after embryo transfer. Patient successfully delivered live birth of one healthy baby boy and one healthy baby girl at 35 weeks gestation. One vitrified blastocyst remains in storage.

Discussion

Although this is a case report, our experience shows it is possible to save frozen embryos from a malfunctioning freezer. Unexpectedly three good-quality blastocysts were obtained from nine surviving 2PNs after post thaw culture. To the best of our knowledge, this is the first report of successful pregnancy and live birth by transferring re-cryopreserved blastocysts developed from frozen embryos rescued from freezer malfunction.

Of the nine embryos surviving rescue thaw, three developed to good-quality blastocysts, indicating that surviving embryos recovered from a malfunctioning freezer can develop normally. Pregnancies and live births following the transfer of re-cryopreserved embryos have been reported by several groups [3–5]. Following the transfer of re-cryopreserved blastocysts developing from frozen embryos encountered during a freezer malfunction, an ongoing twin pregnancy and live birth was achieved in this report, suggesting that these embryos were able to undergo re-cryopreservation and implant successfully.

By analyzing the reasons why all nine fertilized oocytes survived the rescue thaw, we realize it is of critical importance to pay close attention to the cooling process and act promptly in case of equipment failure. In an attempt to avoid potential ice crystal formation, it is necessary to thaw slowly cooled embryos rapidly if unusual temperature change occurs before the freezer chamber reaches plunging temperature. In this report, embryos were frozen at the pronuclear

stage, which might be another reason why such a high survival rate was achieved. As an alternative to traditional slow freezing, vitrification has been successfully employed in the cryopreservation of human oocytes and embryos with higher rates of success [6–8]. To prevent the unforeseen freezer malfunction, we have switched to vitrification as the preferred embryo cryopreservation method.

In summary, this report shows it is possible to successfully recover frozen embryos from a malfunctioning freezing prior to plunging temperature. Although based on a small number of embryos, our results demonstrate that frozen embryos recovered from a malfunctioning freezer could maintain their developmental potential if prompt and proper reaction is provided.

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