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CASE REPORT

Embryos refrozen–thawed by vitrification lead to live births: Case report

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KEYWORDS

Cryopreservation;
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Abstract Objective: To describe two successful cases of utilizing refrozen blastocysts by vitrification derived from supernumerary embryos.

Design: Case report.

Setting: Private fertility clinic.

Subjects: Two infertility couple.

Interventions: Refrozen blastocysts by vitrification derived from supernumerary embryos.

Main outcome measures: Obstetric and pediatric results.

Results: Two pregnancies obtained from refrozen–warmed blastocysts led to two healthy babies being born without clinical or genetic problems.

Conclusion: These case reports support the notion of safely repeating cryopreservation. However, despite these favorable results, there is still a need for prospective controlled studies on the obstetric and neonatal repercussions of refreezing and of vitrification in particular.

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1. Introduction

Human embryo cryopreservation is an important step in assisted reproductive technology that contributes to improve cumulative pregnancy rates and reduce multiple gestations. More recently, the technique of human embryo and oocyte vitrification, or rapid cooling, has been clinically introduced in an effort to improve survival rates after warming (1–3). Vitrification differs from traditional cryopreservation techniques in that it allows glasslike solidification of living cells without the formation of ice crystals. The physical definition of

vitrification is the solidification of a solution at a temperature below the glass transition temperature of the solution, not by ice crystallization, but by extreme elevation in viscosity, using high cooling rates from $-15,000$ to $-30,000$ °C per minute (1). Currently, cryopreservation of oocytes or embryos by vitrification does not present significant disadvantages in comparison with traditional slow freezing (4). In fact, the majority of published data report that the more recent vitrification methods appear to be more efficient and reliable than any slow freezing technique (2).

Many live births occur each year using embryos that have been frozen and thawed once. However, it is not uncommon for supernumerary embryos to survive after thawing; most such embryos are subsequently refrozen. These embryos can, if viable after the new thawing, be important for enabling a new transference principally when there had been failures in prior attempts. Nevertheless, there are not many reports of successful outcomes with human embryos frozen and thawed more than once in which vitrification was employed (4–12).

We reported herein two cases of a successful human pregnancy that resulted in the live birth of healthy babies after the embryos had been frozen by slow cooling and refrozen by vitrification at the blastocyst stage.

2. Case reports

2.1. Subjects

2.1.1. First case

A 36-year-old female and her 36-year-old husband, one previous pregnancy (miscarriage <12 weeks), with diagnosis of endometriosis and 2-year infertility. She presented two previous unsuccessful IVF/ICSI cycles at another clinic.

2.1.2. Second case

A 27-year-old female and her 32-year-old husband, with diagnosis of male infertility and 6-year history of primary infertility.

Informed consent was obtained from both couple prior to the transfer of the re-cryopreserved embryos

2.2. Procedures

2.2.1. Ovarian stimulation

Both patients were submitted to the same scheme of controlled ovarian stimulation (13,14). After pituitary down-regulation with nafarelin acetate at a dose of 400 µg/day (Synarel®; Pfizer, São Paulo; SP, Brazil) started in the mid-luteal phase, recombinant human FSH (r-FSH/Gonal F®; Serono, SP, Brazil) was administered at a starting dose of 150–300 IU, depending on the age of the patient, and recombinant LH (r-LH/Luveris®, Serono, SP, Brazil) was administered at a dose of 75 IU/day for a period of 7 days. On day 8 of stimulation, follicular development was monitored by 7 MHz transvaginal ultrasound only (Medison Digital Color MT, Medison Co. Ltd., Seoul, Korea), and the FSH dose was adapted according to ovarian response. The r-LH supplementation was increased to 150 IU/day when one or more follicles measuring ≥ 10 mm in diameter were found. Thirty-six hours after HCG, an ultrasound-guided transvaginal oocyte aspiration/retrieval was performed.

2.2.2. ICSI fertilization, embryo culture, and embryo replacement

The semen samples were prepared by density gradient centrifugation, and intracytoplasmic sperm injection (ICSI) fertilization was performed.

After collection, oocytes were placed in a CO₂ incubator, at 37 °C, in P-1 culture medium (Irvine Scientific) supplemented with serum albumin (HSA) on a Nunc plate. After 1 h, oocytes were denuded by means of enzymatic (Hyase 40 UI) and mechanical processes. The ICSI was performed at 37 °C in drops of modified HTF medium supplemented with HSA under oil (Ovoil-100; Vitrolife).

Sperm-injected oocytes, zygotes and embryos from ICSI were cultured in P-1 medium supplemented with HSA. Oocytes were examined after 17–20 h to assess fertilization and those with two distinct equal-sized pronuclei were defined as normal zygotes. Twenty-five to 27 h after injection, on day 1 of culture, early cleavage was evaluated (15). Each embryo was graded on day 2 and considered a top quality embryo if there were four identical blastomeres (44 h after the sperm injection) with no fragments and multinucleation. On day 2 or 3 afterwards, the two best-scoring embryos were transferred to the patient's uterus.

2.2.3. Cryopreservation of embryos

A standard slow-freezing protocol, employing 1,2-propanediol (PROH) and sucrose as cryoprotectants, was used (16). For the freezing process we used Embryo Freeze Media Kit (Irvine Scientific), which contained the following solutions: 1.5 M PROH and 1.5 M PROH + 0.1 M sucrose. Embryos were placed on the 0.25 ml straws and the sample was stored under liquid nitrogen (LN₂). Cryopreservation was performed using a CL-863 cryologic apparatus.

In the thawing process the Embryo Thaw Media Kit (Irvine Scientific) was used and consisted of the following solutions: 1.0 M PROH plus 0.2 M sucrose, 0.5 M PROH + 0.2 M sucrose, 0.2 M sucrose. The straws were taken out of liquid nitrogen and the embryos were successively added to the solutions. The embryos were transferred to PBS stabilized at 37 °C and 5% CO₂ for 5 min, and finally, incubated in P-1 culture medium (Irvine Scientific) supplemented with HSA.

2.2.4. Vitrification of embryos

The supernumerary morula and blastocyst stage embryos were re-vitrified by the Cryotop method (17–19). Briefly, the morula and blastocyst stage embryos were equilibrated in Equilibration solution (7.5% (v/v) ethylene glycol + 7.5% DMSO in HTF medium + 20% HSA) at room temperature for 8 min. They were then transferred into Vitrification solution (15% ethylene glycol + 15% DMSO + 0.5 mol/l sucrose) at room temperature for 45–60 s. After having observed that cellular shrinkage had taken place, embryos were aspirated and placed on the tip of the Cryotop (Kitazato, Japan). Cooling of embryos was done by direct contact with the LN₂. Subsequently, the plastic cap was pulled over the film part of the Cryotop, and the sample was stored under LN₂.

2.2.5. Thawing of embryos (vitrification)

For thawing, the Cryotop was taken out of LN₂ and instantly immersed directly into 1.0 ml of Thawing solution (1.0 M

sucrose in HTF + 20% HSA) for less than 60 s at 37 °C and the embryos were then picked and transferred into 1.0 ml of the dilution solution (0.5 mol/l sucrose in HTF + 20% HSA) for three min at room temperature. The embryos were subsequently washed 10 times in Global medium with 20% HSA (v/v) (LifeGlobal® ART media) in 50 µl drops at room temperature, then incubated in Global medium with 20% HSA (v/v).

2.2.6. Embryo culture after thawing

After thawing, the morula and blastocyst embryos were cultured in Global medium in 50 µl drops under mineral oil (LifeGlobal® ART media) at 37 °C in humidified atmosphere of 6% CO₂, for 3 h; and two good blastocyst stage embryos were transferred.

2.2.7. Replacement cycle and pregnancy

Estradiol Valerate (Cicloprimogyna®-Schering, Brazil) was administered from the 1st to the 14th day of the cycle at a daily dose of 6 mg. Progesterone (Evocanil®-Zodiac, Brazil) was introduced vaginally on the 14th day at the dose of 400 mg/day, as long as the endometrial thickness was ≥6 mm. Thawing and transfer were performed, respectively, on the 4th and 5th days of progesterone treatment. After transfer, the vaginal progesterone dose was increased to 800 mg/day.

The pregnancy test was performed on the 14th day after transfer, and clinical pregnancy was confirmed during the sixth week by the presence of a gestational sac and an embryo with a heartbeat.

2.3. Descriptions

2.3.1. First case

The patient's IVF/ICSI cycle was initiated and 10 intact metaphase II oocytes. After insemination 7 oocytes were fertilized normally. Embryos cleavage was assessed and graded at 42 h post-insemination. Two embryos were transferred to the uterus on day 2 and five supernumerary embryos were cryopreserved (on day 2) by the slow-freezing protocol. The procedure was unsuccessful (no pregnancy occurred).

Three months later, all five embryos were thawed after having developed to the blastocyst stage. Then two of the five blastocysts were selected for transfer. Three supernumerary blastocyst stage embryos (day 6) were refrozen by the Cryotop method of vitrification (17–19) The procedure resulted in a single pregnancy that ended in a miscarriage (< 12 weeks).

One year later, the 3 re-vitrified blastocyst stage embryos were thawed and all survived. The embryos were placed in the culture medium (LifeGlobal® ART media) at 37 °C in humidified atmosphere of 6% CO₂, for 1 h; and three re-expanded stage blastocysts were transferred into the patient's uterus. On day 14 after embryo replacement, the serum β-HCG was positive. Two weeks after the positive HCG, a transvaginal ultrasound revealed one gestational sac, with cardiac activity. The singleton pregnancy progressed uneventfully, with the patient giving birth to a normal healthy baby (female) weighing 3600 g.

2.3.2. Second case

The patient's IVF/ICSI cycle was initiated and 13 intact metaphase II oocytes. After insemination 11 oocytes were fertilized

normally. Embryo cleavage was assessed and graded at 42 h post-insemination. Two embryos were transferred to the uterus on day 3. Nine supernumerary embryos were cryopreserved (on day 3) according to the standard slow freezing protocol. On day 14 after embryo replacement, the serum β-HCG was positive. The patient subsequently gave birth to a normal healthy baby (male) weighing 2900 g.

Approximately 6 years later the couple returned to the clinic requesting the cryopreserved embryos that were thawed by the standard thawing protocol. Two day-3 embryos were transferred to the uterus but the procedure was unsuccessful (no pregnancy occurred). Four supernumerary embryos were maintained in culture until day 6 and 4 blastocyst stage embryos were refrozen by vitrification according to the Cryotop method (17–19).

Two months later, 2 re-vitrified blastocyst stage embryos were thawed and placed in the culture medium (LifeGlobal® ART media) at 37 °C in a humidified atmosphere of 6% CO₂, for 1 h. The 2 re-expanded blastocysts were transferred. On day 14 after embryo replacement, the serum β-HCG was positive. The ultrasound detected a twin pregnancy that spontaneously reduced to a singleton pregnancy before 12 weeks' gestation. The singleton pregnancy progressed uneventfully, with the patient giving birth to a normal healthy baby (male) weighing 2960 g.

3. Discussion

The cryopreservation of embryos by the slow-freezing method continues to be standard practice (3) mostly due to the quantity of information and experience in comparison to vitrification. However, the slow-freezing method presents some important problems such as cost, the long-term demands associated with freezing, low cellular viability after thawing and elevated rates of intracellular crystal formation (4). Vitrification is being widely utilized and the data indicate that it presents advantages including less time consumed along with higher survival (particularly for more sensitive embryos such as expanded blastocysts) and higher development rates when compared to slow-freezing methods (20–23). On the other hand, the health of children born after assisted reproduction has always been a cause for concern (3,24). The current introduction and growing use of vitrification raises the question of repercussions to offspring given the theoretical difference in risk profile (e.g. differences in concentrations of potentially toxic cryoprotectors), in comparison with the currently predominant slow-freezing technique. If on the other hand the data from 25 years of utilization of slow freezing appear to provide comfort in relation to infant outcome, there are not many reports on the obstetric and neonatal results from vitrification (3). This lack of information is even more pronounced with respect to the refreezing of embryos.

It has been reported that slow freezing has the advantage of being a repeatable method on human cells and embryos (4). In fact, different studies have reported pregnancy and birth of healthy children (Table 1) by employing only the slow-freezing method (25–28). Conversely, satisfactory results with re-vitrified embryos have been recently published, demonstrating the safety of the method. Son et al. (7) and Hashimoto et al.

Table 1 Reports of the viable pregnancies from refrozen embryo transfer.

Authors	Embryo stage			Freezing method	Result
	At 1st freezing	At 2nd freezing	At transfer		
Baker et al. (1996)	2 PN	2 PN	Cleaved	Slow	Delivery of 1 normal baby
Farhat et al. (2001)	Cleaved	Blastocyst	Blastocyst	Slow	Delivery of 1 normal baby
Smith et al. (2005)	Cleaved	Blastocyst	Blastocyst	Slow	Delivery of 1 normal baby
Takahashi and Araki (2004)	Cleaved	Blastocyst	Blastocyst	Vitrification	Delivery of 1 normal baby
Son et al. (2005)	Blastocyst	Blastocyst	Blastocyst	Vitrification	Delivery of 2 normal babies (twins)
Özmen et al. (2006)	2 PN	Cleaved	Cleaved	Vitrification	On going single pregnancy
Hashimoto et al. (2007)	2 PN	Cleaved	Blastocyst	Vitrification	Delivery of 1 normal baby
Hiraoka et al. (2009)	Cleaved	Blastocyst	Blastocyst	Vitrification	Delivery of 1 normal baby
Kumasako et al. (2009)	2 PN	Morula/ blastocyst	Morula/ blastocyst	Vitrification	10 Pregnancies
Yokota et al. (2001)	2 PN	Morula	Blastocyst	Slow/Vitrification	Delivery of 2 normal babies (twins)
Hiraoka et al. (2006)	Cleaved	Blastocyst	Blastocyst	Slow/Vitrification	Delivery of 2 normal babies (twins)
Hiraoka et al. (2007)	Cleaved	Blastocyst	Blastocyst	Slow/Vitrification	5 Normal babies were born including 1 pair of (twins) and 1 pregnancy is ongoing
Mauri et al. (this paper)	Cleaved	Blastocyst	Blastocyst	Slow/Vitrification	Delivery of 2 normal babies

(11) reported successful pregnancy and delivery after the blastocyst transfer of twice-vitrified embryos produced following *in vitro* maturation and ICSI. Takahashi and Araki (6) related the pregnancy and birth of a healthy baby after transfer of re-vitrified embryo at the blastocyst stage. Özmen et al. (4) reported a successful pregnancy after re-vitrification and transfer of cleavage stage embryos, suggested that re-vitrification of human embryos could be done regardless of stage of embryos and previous method of cryopreservation. Hiraoka et al. (10) related delivery of a healthy child following the transfer of a human re-vitrified day-7 spontaneously hatched blastocyst developed from vitrified embryos. Kumasako et al. (12) reported the efficacy of the transfer of twice frozen–thawed embryos with the vitrification. Table 1 also summarizes these reports.

Similar to our study, other groups (Table 1) also re-froze blastocyst stage embryos employing vitrification of embryos that had been previously frozen by the slow method in early stages (PN or cleavage stages). Yokota et al. (5) reported successful pregnancy and delivery of healthy fraternal twins after the transfer of refrozen supernumerary embryos. In this study fertilized pronuclear embryos that were frozen (by the slow-freezing method), thawed, and developed to morula embryos were subsequently vitrified, re-thawed, and cultured until blastocyst formation before transfer. Hiraoka et al. (8) reported a case of successful dizygotic twin pregnancy after re-vitrification of blastocysts developed from frozen/thawed cleaved embryos. In addition, the same group reported another study (9) including an enlarged number of re-cryopreserved embryos by vitrification at the blastocyst stage that had previously been cryopreserved at the cleavage stage by slow freezing. The implantation and pregnancy rates were 35% (9/26) and 47% (7/15), respectively; five healthy babies were born to four patients.

In conclusion, these case reports support the notion that repeated cryopreservation can be safely applied to slow-cooling or vitrification and indicate that human embryos could be re-vitrified. However, despite these favorable results, there is still a need for prospective controlled studies on the obstetric and neonatal repercussions of refreezing and of vitrification

in particular. The parents must be informed of the benefits and the risks of treatment, given that their decision to employ it might affect the future health of their child.

4. Competing interests

The authors declare that they have no competing interests.

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