Clinical features and reproductive outcomes for embryos undergoing dual freeze-thaw sequences followed by blastocyst transfer: critique of 14 consecutive cases in IVF

These data suggest that the physiologic stress associated with two consecutive freeze-thaw processes is likely minor. Dual freeze-thaw of embryos does not appear to adversely impact delivery rate in IVF; a livebirth delivery rate of 35.7% per transfer was observed in our population. (Fertil Steril® 2009;91:1568–70. ©2009 by American Society for Reproductive Medicine.)

Embryo freezing was hoped for long before the technology was sufficiently advanced to permit its use for human embryos. Early mammalian embryos were first cryopreserved in 1972 (1), yet more than a decade was needed to refine specific cryopreservation protocols for safe clinical application in humans (2). For IVF patients with supernumary embryos, the ability to freeze nontransfered embryos is generally regarded as a mandatory element of modern assisted reproduction therapy, because it allows increased cumulative pregnancy outcomes with reduced overall direct patient cost. Compared with fresh IVF cycles, delivery data from frozen embryo transfer (FET) cycles have typically been reassuring (3, 4), offering important support for the safety and efficacy of standard embryo freeze-thaw regimes. However, it is sometimes necessary for embryos to be frozen and thawed more than once before transfer. Although this scenario has been described in several case reports, we sought to investigate reproductive outcome following two such freeze-thaw procedures in a larger group of human embryos. It was from this background that the current sample of 14 IVF patients was retrospectively assembled.

This retrospective study reviewed all IVF records at our institution for the 7-year period ending December 2007, with the aim of identifying all patients who received empiric elective embryo cryopreservartion for any indication. For these patients, oral contraceptives and GnRH agonist were used for pituitary down-regulation, followed by daily administration of gonadotropins with periodic monitoring, as described previously (5). Treatment continued until adequate ovarian response was attained, defined as the maximum potential number of follicles with mean diameter $\geq$17 mm. Transvaginal sonogram–guided oocyte retrieval was accomplished 36 h after SC administration of hCG (6). Oocyte-cumulus complexes were placed into Universal IVF medium (MediCult, Jyllinge, Denmark) immediately after retrieval, with insemination (including intracytoplasmic sperm injection) also carried out using this reagent under washed liquid paraffin oil (MediCult). After 16–18 h, fertilization was monitored and was scored as normal when two distinct pronuclei (2PN) were noted.

Embryos were incubated in cryoprotectant (Embryo Freezing Pack; MediCult) at room temperature and cooled to −7°C at a rate of 2°C/min. Manual seeding followed after 5 min, with embryos next being cooled from −7°C to −30°C at a rate of 0.3°C/min. The final rapid cooling step brought the embryos from −30°C to −190°C at 50°C/min; they were next transfered to liquid 2PN for long-term storage at −196°C. When the cryopreservation sequence was completed at our satellite facility, the 2PN embryos were transfered to our central laboratory using a secure transport system based on a previously published protocol (7).

The 2PN embryos were removed from liquid 2PN storage and kept at room temperature for 30 sec before being placed in H_2O bath at 30°C for 1 min. Embryos were placed in 1,2-propanediol/sucrose–based thaw media (Embryo Thawing Pack, MediCult) at room temperature for a total of 20 min. Culture was maintained to day 5 in microdrops of BlastAssist media I and II (MediCult) under washed paraffin oil in a 5% CO_2 + 5% O_2 atmosphere at 95% humidity. Embryos were assessed for cell number, degree of fragmentation, and compaction on a daily basis. Day 5 blastocysts selected for in utero transfer generally demonstrated a well defined inner cell mass and a highly cellular, expanding trophoectoderm. Blastocysts were loaded into an ET catheter (K-Soft-5000 Catheter; Cook Medical, Spencer, IN); transfers were typically carried out under direct transabdominal sonogram guidance.

Supernumary blastocysts selected for (repeat) cryopreservation were placed into cryoprotectant prepared in 5% CO_2

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atmosphere at 37°C (BlastFreeze, MediCult). This was cooled to −6° C at a rate of 2° C/min. After manual seeding, embryo temperature was lowered from −6°C to −40°C at 0.3°C/min. Next, blastocysts were cooled from −40°C to −150°C at 35°C/min, followed by transfer to long-term liquid nitrogen storage maintained at −196°C. After removal from liquid nitrogen cryostorage, blastocyst straws were first warmed at room temperature for 15 sec before being placed in a room-temperature solution of Earle's Balanced Salts Solution and synthetic serum replacement with human serum albumin (BlastThaw) for 10 min in an atmosphere of 5% CO_2 + 5% O_2. Blastocysts were then incubated in Universal IVF medium (Cat. no. 1030 or 1031; MediCult) and gently pipetted for thorough washing with this reagent. Blastocysts were placed in fresh Universal IVF medium and allowed to equilibrate (recover) in an atmosphere of 5% CO_2 + 5% O_2 for 30–60 min before catheter loading. Typically, transfer of these twice-frozen-thawed blastocysts (Fig. 1) occurred under direct transabdominal sonogram guidance.

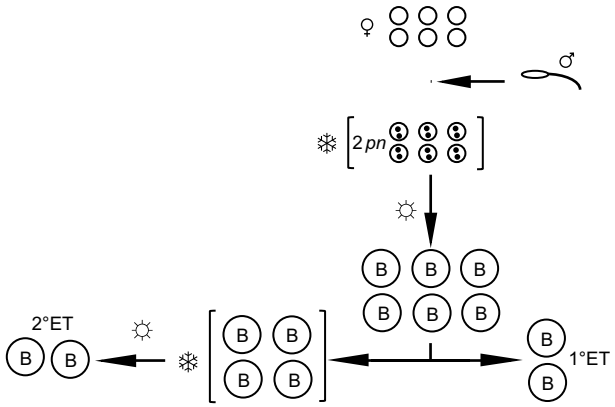
Primary end points of the study were patient age, total number of oocytes retrieved, number of 2PN embryos cryopreserved, interval between first and second thaw/transfer, number (and developmental stage) of embryos transfered (at 2nd ET), and livebirth delivery rate. All data were tabulated as mean ± SD. Contact was established with the delivering obstetrician to determine the delivery status for the study patients.

During the study period, embryos were electively cryopreserved at the 2PN stage for 51 patients, and all cases eventually completed a thaw with FET. However, 24 of these patients (47.1%) maintained at least one nontransfered blastocyst after undergoing their initial (primary) FET. For those with supernumary blastocysts available after FET, the mean (±SD) number remaining in storage was 5.3 ± 3. In this subgroup of 24 patients where blastocysts had been returned to cryostorage (i.e., refrozen), average age was 32.7 ± 3.7 years. These patients had an average of 13.9 ± 5.4 2PN embryos originally frozen. At the time of this analysis, 14 of these patients (58.3%) had requested additional treatment and a (secondary) FET had been completed. The remainder (n = 10) kept their twice-frozen/once-thawed embryos in storage at our center, and have not yet made arrangements either to schedule another FET here or to transport their embryos elsewhere. For patients undertaking a second FET, the average interval between first and second transfer was 179 ± 122 days (range 36–481 days). The average number of blastocysts transfered at second FET was 2.1 ± 1.7, and livebirth delivery was confirmed in 5 out of 14 cases (35.7%) after ET. There were no twin or triplet deliveries in this series. Post-delivery follow-up identified no malformations or developmental anomalies among offspring.

Exactly when and how to freeze human embryos remains a contentious issue, and there is considerable literature documenting this controversy (8–10). Previously, our data on

FIGURE 1

Laboratory sequence for consecutive cryopreservation of human embryos, depicting initial thaw and grow-out to blastocyst stage (**B**) before first embryo transfer (1°ET). Nontransfered blastocysts are refrozen and subsequently thawed for second transfer (2°ET). Supernumary blastocysts after 2°ET would remain in cryostorage (not shown). *2pn* = two pronuclei.



Sills. IVF with twice-frozen-thawed embryos. Fertil Steril 2009.

standard blastocyst freezing and subsequent transfer (4) was found to be in general agreement with other reports, validating the complementary role of embryo cryopreservation within an IVF program. Indeed, recent technologic gains have further enabled extended laboratory culture techniques as well as allowed continued improvement in cryopreservation techniques. The intersection of these two related advancements has brought the question of how best to manage supernumary blastocysts into close focus. Although refreezing nontransfered thawed blastocysts for continued storage has emerged as an attractive clinical option, the limited sampling from case reports (11–14) has made counseling patients on expected outcomes difficult. From this background, the present study represents the largest reported series in the literature on IVF outcomes from embryos having undergone a double cryopreservation and thaw sequence.

Consecutive freeze-thaw treatments for other cells have been safely used to coordinate allogenic cord blood infusion therapies with no adverse impact on cellular clonogenic potential (15, 16). An early description of double freezing of blastocysts reported a clinical pregnancy after transfer of embryos subjected to two distinct freeze-thaw cycles, the first occurring at day 3 and the second at the blastocyst stage (14). A later report of refrozen supernumary embryos resulting in a delivery offered further evidence of the safety of a dual-freeze methodology (13). The potential for livebirth was corroborated by another case report describing cryopreserved 2PN and cleavage-stage embryos which were thawed

and cultured to the blastocyst stage, frozen again, and transfered after a second thaw, yielding a healthy livebirth (17). Although these reports described two freezes occurring at different developmental stages of the embryo, it has also been shown that two consecutive freeze-thaw procedures during the blastocyst phase can result in a delivery (11).

At our center, the decision to perform initial embryo freezing at the 2PN stage was influenced by earlier work suggesting that outcomes are superior when cryopreserved at the 2PN stage than when freezing is done at the early cleavage stage (18–20). After these embryos are thawed, they are maintained in extended culture with the goal of developing to the blastocyst stage for transfer (21). Any nontransfered blastocysts are returned to cryostorage for a second freeze. The present report provides data of special relevance to our ten IVF patients who still have these twice-frozen/once-thawed blastocysts in storage, and to others elsewhere in the same circumstance.

In summary, conserving nontransfered embryos that have already been thawed once by returning them to frozen storage is an important option for many IVF patients. Unfortunately there are no accurate statistics regarding how many patients have such twice-frozen embryos in storage. Although there are also no data comparing various dual-freeze protocols for human embryos, the impressive livebirth rates consistently following different dual-freeze regimes may reflect center-specific fluency with particular laboratory methods. Alternatively, the human embryo may have high tolerance for diverse cryopreservation approaches. We anticipate future reports on outcomes from transfer of twice-frozen embryos to offer additional insights regarding human embryo cryobiology.

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