Journal of Assisted Reproduction and Genetics, Vol. 13, No. 9, 1996

Pregnancy Achieved with Pronuclear-Stage Embryos that Were Cryopreserved and Thawed Twice: A Case Report

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Submitted: June 3, 1996
Accepted: August 2, 1996

Purpose: *Our purpose was to determine if pronuclear-stage embryos (2PN) could be thawed, then frozen again with subsequent survival and cleavage after thawing.*
Methods: *A simplified cryopreservation protocol was used in which a slow cooling program is started at the seeding temperature of −6°C in an alcohol-bath controlled-rate freezer. 1,2-Propanediol (1.5 M) was added to embryos before cooling. A fast thawing technique at room temperature was used. The cryoprotectant was removed in one step using a 1 M sucrose solution.*
Results: *Three months after refreezing, the three 2PN embryos were thawed and all three cleaved after 24 hr in culture. Following embryo transfer a pregnancy was achieved and a healthy full-term baby girl was born.*
Conclusions: *This is the third case reported of successful pregnancies after transfer of human embryos that were frozen twice before transfer and the first case where the second freeze occurred at the pronuclear stage. This is also the first successful refreezing of human embryos using a simplified freezing and thawing technique with one-step addition and removal of cryoprotectant.*

KEY WORDS: cryopreservation; refreezing; twice thawing; human embryos; genetic diagnosis.

INTRODUCTION

Recryopreservation might prove to be a useful tool where preimplantation genetic diagnosis is concerned. Cryopreserved mouse embryos have been found successfully to survive thawing, biopsy, and refreezing and pregnancies were achieved following transfer (1,2). If this phenomenon were found to extend to humans, then the strategy of refreezing could be useful for human genetic diagnosis for preimplantation. The frozen embryos could be shipped to a center where preimplantation genetic diagnosis could be performed, refrozen, and returned to the IVF center.

This report documents the viability of human pronuclear-stage embryos that were cryopreserved and thawed twice before transfer and the delivery of a viable female baby which resulted from embryo transfer (ET). The fact that this is possible may be important to other in vitro fertilization (IVF) laboratories which might find themselves in a situation where the embryos have been thawed at the pronuclear stage (2PN) but the frozen ET has been canceled due to circumstances beyond their control.

CASE REPORT

A 34-year-old woman with bilateral tubal occlusion underwent standard IVF-ET for peritubal adhesions using the luteal-phase leuprolide acetate (LA)/human menopausal gonadotropin (hMG) protocol for controlled ovarian hyperstimulation. Eighteen oocytes were retrieved, of which 12 fertilized. Four fresh embryos were transferred 2 days after retrieval but pregnancy did not occur. Three embryos were cryopreserved 23 hr, after the retrieval when the nucleoli were equatorially aligned in the two pronuclei (3). Four additional embryos were cryopreserved at the cleaved stage 46 hr after retrieval. These embryos had healthy-appearing blastomeres of equal size and <25% fragmentation. One embryo was not frozen because the cells were of very unequal size and appeared abnormal.

The embryos were frozen using a simplified method in which a slow cooling program is started at the seeding temperature of −6°C in an alcohol-bath con-

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1058-0468/96/1000-0713$09.50/0 © 1996 Plenum Publishing Corporation

trolled-rate freezer. 1,2-Propanediol (1.5 *M;* Sigma Chemical Co., St. Louis, MO) was added to embryos before cooling (4).

Two months later the patient attempted a frozen ET in a natural cycle. Timing of thaw and ET was based on the patient's LH surge as monitored by serum and urine levels. A one-step fast thawing technique at room temperature was used and the cryoprotectant was removed from the embryos in one step with a 1 *M* sucrose solution (4). Four embryos cryopreserved at the cleaved stage were thawed within 3 hr of the scheduled transfer time. A pregnancy was not achieved.

Six months later another frozen ET cycle was initiated, for which the remaining three embryos cryopreserved at the 2PN stage were thawed. Due to unforeseen circumstances the patient was unable to undergo the frozen ET. The 2PN embryos were refrozen 4 hr after they were thawed. The embryos went through the normal process to equilibrate with cryoprotectant medium before refreezing, i.e., they initially shrank in the 1,2-propanediol and then reswelled.

Three months later, the patient attempted another frozen ET cycle, this time using down-regulation with LA followed by oral E_2. In this protocol, down-regulation was achieved by administering LA (1 mg sc) beginning on day 21 of the patient's cycle for 10 days. When her serum P level was <1 ng/ml (3.18 p*M*), oral E_2, beginning at 2 mg with progressively increasing doses, was given to stimulate the endometrial lining. When the lining reached a thickness of 11 mm with a triple-line pattern, P in oil was administered (50 mg im daily). Frozen ET was scheduled for the third day of im P. The three 2PN embryos were thawed on the second day of P administration and allowed to cleave overnight. When the three embryos were thawed, all three appeared intact and were put in culture medium and incubated for 24 hr. Before transfer they had cleaved into a two-cell embryo with equal blastomeres and <25% fragmentation, a five-cell embryo with unequal-sized blastomeres and >50% fragmentation, and a six-cell embryo with unequal-sized blastomeres and <25% fragmentation. Following ET, a pregnancy was achieved. A healthy full-term baby girl was delivered 264 days later.

DISCUSSION

In this case, the transfer of cleaved embryos that were cryopreserved and thawed twice at the 2PN stage resulted in a successful pregnancy. The fact that ovulation was suppressed with a GnRHa and we observed the serum P level to remain at a level of 0.2 ng/ml up to a few days before transfer provides further credence to the fact that the pregnancy resulted from one of the three refrozen embryos rather than spontaneous ovulation from this cycle or a previous cycle. Furthermore, the woman had bilateral tubal occlusions.

There are other reports of refrozen thawed human embryos being transferred and resulting in pregnancies. In one case, four 2PN embryos were frozen, thawed, and allowed to cleave. They were refrozen. Two of the four survived after the second thaw. These two were transferred, resulting in the birth of a male (5). In one other case, three cleaved embryos endured the cryopreservation thaw process twice, were transferred, and resulted in a baby girl (6). Our case report confirms the findings of the other two reports in that the human embryos can be refrozen and still result in pregnancy. This may be an encouraging finding for embryologists who might find themselves in a situation due to unforeseen circumstances where the embryos have been thawed but the frozen ET has been canceled.

Additionally, this report has demonstrated that the refreeze may be performed at the pronuclear stage within hours of the initial thaw and still result in a viable pregnancy.

These findings might also be used for purposes other than emergency situations. Despite the large number of IVF centers in the world, there are only a few that can perform human genetic diagnosis in the preimplantation period. Embryos could be frozen in one laboratory, shipped to another facility where preimplantation genetic diagnosis could be performed, refrozen, and then shipped to the laboratory of origin for a future frozen ET. Refreeze–thaw of biopsied mouse embryos has been documented (2). Further studies are needed to determine if a large percentage of cases with twice-frozen–thawed embryos will survive and lead to pregnancies.

In this case report, human embryos that had undergone the cryopreservation and thawing process twice retained their viability and resulted in a live birth. Thus, clinicians should not discard thawed embryos if they are not used, but consider refreezing them.

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