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Pregnancy after zona drilling of cryopreserved thawed embryos: case report

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Objective: To confirm successful implantation of IVF, cryopreserved human embryos after assisted hatching with acidic Tyrode's solution.
Design: Case report.
Setting: In vitro fertilization-ET facility of a university-based practice.
Patient: A 28-year-old female with nonoperable bilateral tubal occlusion and $>1\frac{1}{2}$ years of primary infertility.
Interventions: The patient was stimulated for egg retrieval after an hMG-controlled ovarian hyperstimulation regime. Luteal phase leuprolide acetate (1 mg) was administered SC for 10 days. The dose was then reduced to 0.5 mg, and she was given hMG and FSH IM twice daily until two lead follicles reached 20 mm average diameter. The patient was administered 10,000 IU hCG 36 hours before retrieval.
Main Outcome Measures: Viable pregnancy documented by ultrasound (US).
Results: After the transfer of five cryopreserved-thawed human embryos that were subjected to assisted hatching using acidic Tyrode's solution, the patient established a triplet gestation as documented by US.
Conclusion: This case report demonstrates that zona drilling can be successfully applied to frozen-thawed pronuclear stage embryos that were cultured to 72 hours without damaging them, as evidenced by continued cleavage and resulting implantation. Fertil Steril 1995;63:401–3

Key Words: Implantation, zona drilling, frozen embryo transfer

Improved pregnancy rates (PRs) have been reported by making a hole in the zona pellucida (ZP) of 3-day-old embryos; the technique is known as assisted embryo hatching. Two methods have been described to make an opening: partial zona dissection (1) and zona drilling using acidic tyrode's solution (2). There also is a report of successful assisted embryo hatching using partial zona dissection on cryopreserved-thawed embryos (3). Described herein is the first case report of successful assisted embryo hatching on frozen-thawed embryos using the zona-drilling technique.

CASE REPORT

The 28-year-old female patient had a history of $1\frac{1}{2}$ years of primary infertility with a diagnosis of bilateral tubal occlusion. Previous hysterosalpingography and laparoscopy established that the tubes were nonoperable.

The patient had two oocyte retrievals for IVF. Only two embryos were available for transfer during the first IVF cycle after a conservative hMG controlled ovarian hyperstimulation regimen.

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These embryos were not hatched and a pregnancy was not established. The patient was stimulated more aggressively in cycle 2 with luteal phase leuprolide acetate (LA), 1 mg SC for 10 days beginning in the midluteal phase and decreased to 0.5 mg with the start of 75 IU IM hMG and 75 IU IM pure FSH twice daily until two lead follicles each reached a 20 mm average diameter. Thirty-three oocytes were collected and cultured in synthetic human tubal fluid (HTF) (Irvine Scientific, Santa Ana, CA) supplemented with 0.5% bovine serum albumin (BSA) (Irvine Scientific) under mineral oil. Because of potential severe ovarian hyperstimulation syndrome no embryos were transferred. Instead, 25 embryos were cryopreserved at the two-pronuclear (2PN) stage, and 3 at the 3- to 6-cell stage (Baker AF, Hourani CL, Check JH, abstract).

The embryos were frozen using a single-step addition of the cryoprotectant, 1.5 M 1,2 propanediol (Sigma Chemical Co., St. Louis, MO) in a phosphate-buffered saline (PBS) supplemented with 0.3% BSA. Freezing straws (0.25 mL) were preloaded with 0.120 mL of 1 M sucrose (Sigma Chemical Co.) followed by a 1-cm air column and then the embryo in 0.020 mL of 1,2 propanediol. This was followed by another column of air and an empty column of 1,2 propanediol. Each straw was placed in the alcohol bath in the controlled rate freezer (BioCool, FTS Systems, Stone Ridge, NJ) at −6.0°C and seeded with a liquid nitrogen–chilled spatula. The temperature was held for 15 minutes, ramped −4.0°C/minutes to −40°C, and held for 15 minutes. The embryos were plunged into and stored in liquid nitrogen.

The embryos were thawed for subsequent frozen ETs. The cryoprotectant was diluted out in one step after thawing at room temperature for 2 minutes by vigorously shaking the straw to mix the sucrose with the embryo. Then it was placed in a 37°C water bath for 3 minutes followed by the room temperature water for 1 minute. Upon removal of the embryo from the straw, it was equilibrated in PBS with 0.3% BSA for 10 minutes and then placed in 1 mL HTF + 0.5% BSA under mineral oil and incubated overnight. The transfer took place after a total of 48 hours in culture.

The patient failed to conceive after the first two frozen ETs. For the first frozen ET she had cell stages four, four, three, six, and four. All had even to slightly uneven sized blastomere with all but one having 25% to 50% fragmentation. The remaining one had >50% fragments. For the second frozen ET the cell stages were two, three, four, two, and two cells. Three had even to slightly uneven sized blastomeres, and two had very uneven sized blastomeres. Two had ≤25% fragments, one had 26% to 50% fragments, and the last two had ≥50% fragments.

For the 3rd frozen ET cycle, five 2PN embryos were thawed on the 2nd day of serum P rise and were allowed to cleave until the 4th day of P rise (48 hours in culture). An extra day of culture was allowed so that hatching would be performed when the embryo was of a higher cell stage, (approximately 8 cells) as gap junctions would be formed, eliminating the risk of blastomere loss through the breach in the ZP (2). The extra culture time has been shown, as demonstrated by the results of the case reported herein, not to be detrimental to the development of the embryos, as long as it is combined with zona drilling. At 48 hours of total culture time the embryos were comparable to those transferred above. However, they showed improvement at 72 hours of total culture time. The cell stages for this last frozen ET were 7, 6, 8, 10, and 8 cells. All had even to slightly uneven sized blastomeres. Four had ≤25% fragments, and one had 26% to 50% fragments. The culture medium was HTF supplemented with 10% serum substitute (Irvine Scientific). The patient was down regulated with LA and her endometrium was prepared with estrace and P. In addition, she was administered methylprednisolone (Medrol; The Upjohn Co., Kalamazoo, MI) and doxycycline (Danbury Pharmacal, Inc., Danbury, CT), prescribed for immunosuppression in anticipation of assisted hatching (4). Assisted hatching using the zona- drilling technique was performed on all five embryos using micromanipulation equipment from the Narishige Company (Nikon, Parsippany, NJ). Embryos were placed in 25 μL HEPES-buffered HTF with 10% synthetic serum substitute under oil on a well slide. Each embryo was immobilized on a suction pipet (80 to 100 μm outside diameter), and acidic Tyrode's solution (approximately 5 to 10 pL) was expelled gently through a tiny bore glass needle (10 to 12 μm inside diameter) to create a small hole approximately 15 to 20 μm wide in the ZP. They then were washed four times in HEPES-HTF + 10% serum substitute and returned to the incubator in HTF + 10% serum substitute. Hatched embryos were transferred in HEPES-buffered HTF supplemented with 20% serum substitute. Ultrasound at 6 weeks confirmed three viable sacs in the uterus. This was confirmed again by two subsequent sonograms.

DISCUSSION

This case report demonstrates that zona drilling can be successfully applied to 72-hour-old frozen-thawed embryos without damaging them, as evidenced by continued cleavage and resulting implantations. Extensive search of the English literature has found only one publication dealing with assisted embryo hatching of cryopreserved embryos: partial zona dissection using a microneedle to make an incision in the ZP (3). Methods of zona thinning, where the ZP is not completely breached, also have resulted in pregnancies. The case we have presented here is, we believe, the first to describe assisted embryo hatching on frozen embryos by the zona-drilling technique.

Zona drilling of frozen-thawed embryos may prove to be more beneficial for frozen-thawed embryos than it has for fresh, because it is thought that the ZP undergoes changes during the cryopreservation procedure that cause it to become hardened and resistant to natural hatching (3). The zona-drilling technique may overcome the problem of the hardened ZP. If this is the case, we should find that our PR and implantation rate will increase after the application of drilling for frozen ETs. If the rates do rise, then drilling may become routine for frozen ET. This data must be collected.

Our results show that culturing the frozen-thawed embryo for 48 hours after thawing is not detrimental when combined with assisted hatching. Indeed, our implantation rate per embryo in this case report is high in contrast to results reported elsewhere (5). We need to continue to collect data to establish whether our results are meaningful for maintaining the zona-drilling program on cryopreserved embryos.

One concern when using the zona-drilling procedure is the possible resulting acidification of the embryo (6). The authors claim that the pH changes after drilling of human oocytes may be detrimental to further development of the oocyte. However, our work is with 72-hour-old embryos, not oocytes. Drilling is performed between cells when possible, minimizing blastomere contact with the acid. In addition, drilling completely through the ZP of an embryo takes only approximately 5 seconds compared with 30 seconds to breach the zona of an oocyte. Possibly, 72-hour embryos suffer fewer negative acid effects than oocytes using this technique. This is evidenced by the success of many programs using the zona-drilling technique on fresh embryos and by the implantation of our frozen ones.

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