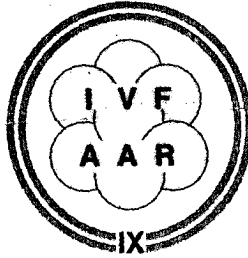


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IX

# Pregnancy after zona drilling of cryopreserved thawed embryos (1)

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## SUMMARY

The rate of successful implantation of in vitro fertilized human embryos may be improved using assisted hatching techniques. Reported herein is the first reported case of successful pregnancy following assisted hatching of cryopreserved embryos using the zona drilling (acidic tyrode's solution) technique. The patient established a triplet gestation following the transfer of five cryopreserved-thawed embryos, all of which had been treated with acidic tyrode's solution.

## INTRODUCTION

Improved pregnancy rates (PRs) have been reported by making a hole in the zona pellucida (ZP) of two day old embryos; the technique is known as assisted embryo hatching. Two methods have been described to make an opening: partial zona dissection (PZD) (2), and zona drilling using acidic tyrode's solution (3). There is also a reported case of successful assisted embryo hatching using PZD on cryopreserved-thawed embryos (4).

Described herein is the first case report of successful assisted embryo hatching on frozen-thawed embryos using the zona drilling technique.

## CASE REPORT

A 28 year old female patient presented with a history of 1 1/2 years of primary infertility with a diagnosis of bilateral tubal occlusion. Previous hysterosalpingography and laparoscopy established that the tubes were non-operable.

The patient had two oocyte retrievals for in vitro fertilization (IVF). Only two embryos were available for transfer during the first IVF cycle following a conservative human menopausal gonadotropin (hMG) controlled ovarian hyperstimulation (COH) regimen. These embryos were not hatched and a pregnancy was not established. The patient was more aggressively stimulated in cycle two with luteal phase leuprolide acetate (LA) 1 mg given subcutaneously (s.c.) for ten days beginning in the mid-luteal phase and decreased to 0.5 mg with the start of 75 IU hMG and 75 IU pure (p)-FSH intramuscularly (IM) 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 (OHSS) no embryos were transferred. Instead, 25 embryos were cryopreserved at the 2 pronuclear (2PN) stage, and 3 at the 3-6 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 (PrOH) (Sigma Chemical Co., St. Louis, MO) in a phosphate buffered saline 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 PrOH. This was followed by another column of air and an empty column of PrOH. 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/min. 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 embryo transfers (ET). The cryoprotectant was diluted out in one step after thawing at room temperature for two minutes by vigorously shaking the straw to mix the sucrose with the embryo. It was then placed in a 37°C water bath for three minutes and then room temperature water for one minute. Upon removal of the embryo from the straw it was equilibrated in phosphate buffered saline with 0.3% BSA for ten 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 following the first two frozen ETs. For the first frozen ET she had cell stages 4, 4, 3, 6, and 4. All had even to slightly uneven sized blastomere with all but one having 25-50% fragmentation. The remaining one had >50% fragments. For the second frozen ET the cell stages were 2, 3, 4, 2, and 2 cells. Three had even to slightly uneven sized blastomeres and two had very uneven sized blastomeres. Two had ≤25% fragments, one had 26-50% fragments, and the last two had ≥50% fragments.

For the third frozen ET cycle, five 2PN embryos were thawed on the second day of serum progesterone (P) rise and were allowed to cleave until the fourth 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 (3). 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  $\leq 25\%$  fragments and one had 26-50% 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 prescribed for immunosuppression in anticipation of assisted hatching (5). Assisted hatching using the zona drilling technique was performed on all five embryos using micromanipulation equipment from the Narishige Company. Embryos were placed in 25 ul hepes-buffered HTF with 10% synthetic serum substitute under oil on a well slide. Each embryo was immobilized on a suction pipet (80-100 um outside diameter), and acidic Tyrode's solution (about 5-10 picoliters) was gently expelled through a tiny bore glass needle (10-12 um inside diameter) to create a small hole, approximately 15-20 um wide in the ZP. They were then washed four times in hepes-HTF+ 10% serum substitute and returned to the incubator in HTF+ 10% SS. Hatched embryos were transferred in Hepes-buffered HTF supplemented with 20% SS. Ultrasound at six weeks confirmed three viable sacs in the uterus. This was again confirmed by two subsequent sonograms.

## RESULTS AND CONCLUSIONS

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: PZD using a microneedle to make an incision in the ZP (4). Methods of zona thinning, where the ZP is not completely breached, have also 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 as it is thought that the ZP undergoes changes during the cryopreservation procedure that cause it to become hardened and resistant to natural hatching (4). 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 (6). We need to continue

to collect data to establish if 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 (7). 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 about five seconds compared to 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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