

The effect of assisted hatching on pregnancy rates after frozen embryo transfer*

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Objective: To compare clinical pregnancy and implantation rates after transfer of frozen-thawed embryos prepared according to an assisted hatching protocol or a nonassisted hatching protocol.

Design: A historical cohort study in which a cohort of patients who underwent an assisted hatching protocol was matched for clinical parameters to an external historical cohort treated before assisted hatching was available.

Setting: In vitro fertilization-ET facility of a university-based practice.

Patients: Seventy-nine matched pairs.

Interventions: Nonassisted hatching patients: embryos were thawed, cultured in human tubal fluid + 0.5% bovine serum albumin until 48 hours and transferred. Assisted hatching patients: embryos thawed, cultured in human tubal fluid + 10% synthetic serum substitute until 72 hours, had assisted hatching and transferred.

Main Outcome Measures: Clinical pregnancy (gestational sac) and implantation rates.

Results: Twelve (15.2%) clinical pregnancies per transfer in nonhatched group versus 24 (30.4%) in hatched group. Nonhatched group: 284 embryos transferred; 15 (5.3%) implanted. Three pregnancies (25.0%) had two sacs. Hatched embryos: 269 were transferred; 37 (13.7%) implanted. Eleven pregnancies (45.8%) were multiple gestations (9 twins, 2 triplets).

Conclusion: Clinical pregnancy and implantation rates were higher for group having assisted hatching protocol. It is not clear whether the improvement is due to the overall methodology change or to assisted hatching. Assisted hatching using the zona-drilling technique is not detrimental to frozen-thawed human embryos and may be beneficial.

Fertil Steril 1996;65:254-7

Key Words: Human IVF, frozen embryos, assisted hatching, micromanipulation, implantation, media, transfers

Cohen et al. have demonstrated that reduced implantation in IVF-ET may be due to the inability of the embryo to hatch out of the zona pellucida (ZP) (1). The impaired hatching may be due to the ex-

tended time in culture in an artificial environment causing a hardening of the ZP or interfering in the formation of endogenous blastocyst hatching mechanisms (2). Hardening of the ZP in cryopreserved embryos may be exacerbated by the freeze-thaw process (3).

Zona drilling using acidic Tyrode's solution (a form of assisted hatching) has been shown to aid implantation in fresh human embryos (4). It has been theorized that the artificial gap created in the ZP allows the embryo to hatch out more readily after blastocyst formation, thus overcoming the detrimental effects of culture. Our laboratory demonstrated recently that the zona-drilling technique can be used successfully on frozen-thawed human embryos (5). In this

Received May 25, 1995; revised and accepted October 20, 1995.

* Presented at the 43rd Annual Meeting Pacific Coast Fertility Society, Coronado, California, April 26 to 30, 1995.

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study we wanted to determine if there was an improvement in the pregnancy and implantation rates in frozen ETs after zona drilling over frozen ETs where no assisted hatching was performed.

MATERIALS AND METHODS

Study Design

A historical cohort study was conducted. Our IVF center introduced our assisted hatching program in November 1993. Data from frozen ETs using assisted hatching were collected until September 1994. The patients in the assisted hatching group had their frozen ETs during this time period. Pregnancy rates (PRs) and implantation rates from the assisted hatching group were compared with the results of a group of patients having frozen ETs without assisted hatching. So that these two groups would be as contemporary as possible, all of the nonassisted patients had their frozen ETs from November 1992 to October 1993.

Each member of the assisted hatching group was matched carefully with a member of the nonassisted hatching group according to age (± 2 years), etiology of infertility, stimulation protocol used for the retrieval, type of hormonal treatment used for the frozen ET, number of embryos transferred (± 1), and cycle number. Patients in the assisted hatching group who did not have all of their transferred embryos hatched were excluded. Cycles involving donor oocytes were excluded. Pregnancy rates and implantation rates were compared between the two groups. Seventy-nine matched pairs were included in this study.

Patients

Because the groups were matched, the ages of the patients and the number of embryos transferred were virtually the same. The average ages for the women were 34.5 years (range, 27 to 43) and 34.9 years (range, 27 to 43) for the nonassisted hatching and assisted hatching groups, respectively. The average number of embryos transferred was also similar: 3.6 (range, 2 to 6) and 3.4 (range, 1 to 6), respectively.

Controlled ovarian hyperstimulation for the oocyte retrieval was accomplished using one of the following protocols: [A] luteal phase leuprolide acetate (LA) followed by hMG (6); [B] follicular phase LA, hMG, and FSH (7); or [C] hMG and clomiphene citrate (CC) (8). In the assisted hatching cohort there were 58 cycles of protocol A, 14 cycles of protocol B, and 7 cycles of protocol C. In the nonassisted hatching cohort there were 69 cycles of protocol A, 7 cycles of B, and 3 cycles of C.

Maturation of the follicles was monitored with ultrasound and serum E_2 and P blood hormone levels. The patients were administered hCG IM 36 hours before transvaginal oocyte retrieval. Flushing medium was phosphate-buffered saline (PBS). Oocytes were placed in culture medium in organ culture dishes (Falcon 3037; Thomas Scientific, Swedesboro, NJ) and overlaid with mineral oil. They were incubated at 37°C and 5% CO_2 in a humidified chamber. Sixteen hours after insemination the eggs were examined for signs of fertilization.

Embryos that were not transferred but were suitable for freezing were cryopreserved at the pronuclear or multicellular stages using a one-step cryopreservation protocol. Embryos at the pronuclear stage were cryopreserved if they were round, healthy, and the nucleoli were aligned at the time of cryopreservation. Cleaved embryos were cryopreserved if they had round healthy appearing blastomeres and <50% fragmentation.

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 PBS supplemented with 0.3% bovine serum albumin (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 a controlled rate freezer (BioCool; FTS Systems, Stone Ridge, NJ) at $-6.0^\circ C$ and seeded with a liquid nitrogen chilled-spatula. The temperature was held for 15 minutes, ramped $-0.4^\circ C/min$ to $-40^\circ C$, and held for 15 minutes. The embryos were plunged into and stored in liquid nitrogen.

Natural cycles (no follicular phase hormonal supplementation) were used as long as the serum E_2 reached 200 pg/mL (conversion factor to SI unit, 3.67) and a minimum endometrial thickness of 10 mm was attained (9). The LH level was monitored through serum hormone levels and a urine LH detection kit (10). Oral micronized P (50 mg/d) was given beginning with the day of the transfer. In the non-hatched group the embryos were transferred 3 days after the LH surge. In the hatched group the transfer was the 4th day after the LH surge. Thawing of the embryos for the natural cycle was timed to take place up to 12 hours negatively asynchronous to the embryo age postovulation (10).

If the patient was not able to follow a natural cycle, then she was given LA 1 mg SC 1 week after initiation of rise of serum P and continued for 10 days until the serum E_2 was suppressed to <50 pg/mL (conversion factor to SI unit, 3.67) and the serum P <1 mg/mL (conversion factor to SI unit, 3.18). Oral

E₂ was then given starting at 2 mg/d and was increased gradually over a period of 2 weeks. When the lining reached 10 mm, the patient was given P in oil. For nonassisted hatching patients, the transfer was the 3rd day of P. The assisted hatching patients had the transfer on the 4th day of P. Pronuclear stage embryos were thawed on the 2nd day of P. Cleaved embryos were thawed on the 3rd day of P. In the cohort with assisted hatching, 42 cycles were natural and 37 were down-regulated. In the nonassisted hatching cohort, 45 cycles were natural and 34 were down-regulated.

Patients in the assisted hatching group were given additional methylprednisolone and doxycycline prescribed for immunosuppression in anticipation of hatching. The corticosteroids are given to prevent the woman's immune system from attacking the embryo through the breach in the zona (11) and the antibiotic given to prevent infection.

Thawing of the embryos was timed to take place up to 12 hours negatively asynchronous to the embryo age postovulation in natural cycles (10). The embryos were thawed by a one-step dilution of the cryoprotectant. The straws containing the embryos were removed from the liquid nitrogen and thawed at room temperature for 2 minutes. The embryo column was mixed with the sucrose by shaking the straw. It was then placed in a 37°C water bath for 3 minutes followed by 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 culture medium until the time of the transfer. Three hundred one embryos were thawed in the assisted hatching cohort with an average of 3.8 embryos (range, 2 to 10) thawed per cycle and a survival rate for the group of 89.4%. Three hundred eight embryos were thawed in the nonassisted hatching cohort with an average of 3.9 embryos (range, 2 to 7) thawed per cycle and a survival rate of 92.2%.

Embryo Culture and Transfer

For the nonassisted hatching patients, the frozen-thawed embryos were placed in human tubal fluid (HTF) supplemented with 0.5% BSA and incubated. The total time in culture for these embryos, from the oocyte retrieval until the freeze, and from the thaw until transfer was 48 hours. At the time of transfer the embryos were placed in modified HTF with 2% BSA. The catheter used was a Set de Frydman (Laboratoire CCD, Paris, France).

It has been recommended that assisted hatching be performed when the embryos are at a higher cell stage (approximately 8 cells) than normally would be seen at 48 hours total culture time because the

gap junctions would be formed (4). The presence of gap junctions would eliminate the risk of blastomere loss through the artificial breach in the ZP. Therefore, the embryos in the assisted hatching group were cultured an extra day (72 hours total culture time). The protein source in the medium was changed to synthetic serum substitute to accommodate the embryo for 72 hours of development (12). The culture medium for the assisted hatching group was HTF with 10% synthetic serum substitute. The morning of the frozen ET for the assisted hatching group the embryos were micromanipulated according to the zona-drilling protocol described previously using acidic Tyrode's solution (5). It may be noted that the technicians performing the assisted hatching noticed no increased difficulty in drilling through the frozen-thawed ZP as compared with fresh ZP. The manipulated embryos were incubated until the time of transfer when they were placed in modified HTF + 20% serum substitute.

Statistical Analysis

The clinical PRs after frozen ETs were compared using McNemar's test for correlated proportions because the unit of analysis was the matched pair. The implantation rates were compared using χ^2 analysis because the unit of analysis was the embryo, and the assumption was made that each embryo had an independent chance of implanting. All testing was done at the 0.05 level of significance.

RESULTS

There were 12 (15.2%) clinical pregnancies per transfer in the nonassisted hatching group as compared with 24 (30.4%) in the assisted hatching group. The viable PR (as defined by completing the first trimester with a viable pregnancy by sonography) was 13.9% (11/79) versus 27.8% (22/79) in the respective groups. There were 2 matched pairs in which both members became pregnant, 22 where the assisted hatching patient became pregnant and the nonassisted hatching did not, and 10 where the nonassisted hatching patient became pregnant while the assisted hatching patient did not. There were 45 pairs where neither achieved a pregnancy ($P < 0.05$, McNemar test).

In the nonassisted hatching group 284 embryos were transferred and 15 (5.3%) implanted. Three (25.0%) of the pregnancies had two sacs. There were 269 hatched embryos transferred and 37 implanted (13.7% implantation rate). Eleven (45.8%) of the pregnancies had multiple gestations (9 twins, 2 triplets). The implantation rate was significantly higher in the assisted hatching group ($P < 0.05$, χ^2).

The multiple gestation rate was not significantly different.

DISCUSSION

Our data show that there is an increase in the PRs and the implantation rates for the frozen ETs when comparing the more recent assisted hatching protocol to the old nonassisted hatching protocol. This concurs with data that demonstrate that there is a higher PR and implantation rate in hatched versus nonhatched transfers of fresh embryos (4).

In our study there are several differences between the assisted hatching protocol and the nonassisted hatching protocol. The hormonal therapy of the patients for the frozen ETs remained the same in both groups with the exception that the assisted hatching group received methylprednisolone and doxycycline. These were given to suppress the immunological destruction of the embryo through the breach in the zona, but these drugs have not been shown to influence the PR on their own (11).

Other differences between the two groups are the protein source in the culture medium and the total time in culture. Either one of these factors, or any of these factors in combination with each other, or the assisted hatching technique, possibly might influence the PR and implantation rate. Thus, further research is needed to identify which of the variables improve the outcome.

Although the difference in the multiple gestation rates did not attain statistical significance, clinically an increase in multiple gestation from 25% to 45.8% is of concern. These data suggest that the number of embryos transferred per cycle should be decreased when cryopreserved embryos are thawed and undergo assisted hatching. The end result would be to maintain the PR, decrease the rate of multiple gestations, and increase the number of transfers available to the patient.

The data presented demonstrate that assisted hatching using the zona-drilling technique on frozen-thawed human embryos is not detrimental to the embryos and may, in fact, improve PRs. Assisted

hatching may be especially beneficial for frozen-thawed embryos in overcoming ZP hardening, which might have been caused by the freeze-thaw process.

REFERENCES

1. Cohen J, Elsner C, Kort H, Malter H, Massey J, Mayer MP, et al. Impairment of the hatching process following IVF in the human and improvement of implantation by assisted hatching using micromanipulation. *Hum Reprod* 1990;5:7-13.
2. Alikani M, Cohen J. Advances in clinical micromanipulation of gametes and embryos. *Arch Pathol Lab Med* 1992;116:373-8.
3. Tucker MJ, Cohen J, Massey JB, Mayer MP, Wiker S, Wright G. Partial zona dissection of the zona pellucida of frozen thawed human embryos may enhance blastocyst hatching, implantation, and pregnancy rates. *Am J Obstet Gynecol* 1991;165:341-5.
4. Cohen J, Alikani M, Trowbridge J, Rosenwaks Z. Implantation enhancement by selective assisted hatching using zona drilling of human embryos with poor prognosis. *Hum Reprod* 1992;7:685-91.
5. Hoover L, Summers D, Check JH, Nazari A, O'Shaughnessy A. Pregnancy after zona drilling of cryopreserved thawed embryos: case report. *Fertil Steril* 1995;63:401-3.
6. Meldrum DR, Wisot A, Hamilton F, Gutlay AL, Kempton W, Huynh D. Routine pituitary suppression with leuprolide before ovarian stimulation for oocyte retrieval. *Fertil Steril* 1989;51:455-9.
7. Garcia JE, Padilla SL, Bayati J, Baramki TA. Follicular phase gonadotropin-releasing hormone agonist and human gonadotropins: a better alternative for ovulation induction in vitro fertilization. *Fertil Steril* 1990;53:302-5.
8. Check JH. Can in vitro fertilization be successful in women with elevated serum follicle stimulating hormone levels? In: Schats R, Schoemaker J, editors. *Ovarian endocrinopathies*. London, England: Parthenon Publishing, 1994:57-65.
9. Check JH, Nowroozi K, Choe J, Lurie D, Dietterich C. The effect of endometrial thickness and echo pattern on in vitro fertilization outcome in donor oocyte-embryo transfer cycle. *Fertil Steril* 1993;59:72-5.
10. Cohen J, DeVane GW, Elsner CW, Kort HI, Massey JB, Norbury SE. Cryopreserved zygotes and embryos and endocrinologic factors in the replacement cycle. *Fertil Steril* 1988;50:61-7.
11. Cohen J, Malter H, Elsner C, Kort H, Massey J, Mayer MP. Immunosuppression supports implantation of zona pellucida dissected human embryos. *Fertil Steril* 1990;53:662-5.
12. Pool TB, Martin JE. High continuing pregnancy rates after in vitro fertilization-embryo transfer using medium supplemented with a plasma protein fraction containing α and β -globulins. *Fertil Steril* 1994;61:714-9.