PHOENIX, ARIZONA

Application of Assisted Hatching for 2-Day-Old, Frozen–Thawed Embryo Transfer in a Poor-Prognosis Population

Submitted: September 4, 1996
Accepted: September 27, 1996

INTRODUCTION

The implantation rate following in vitro fertilization and embryo transfer is still far below expectation because of some nonelucidated reasons, and the implantation rate after frozen embryo transfer is usually even lower. To increase the hatching of blastocysts, assisted hatching has become one of the most common clinical microsurgeries in IVF-ET. Assisted hatching is claimed to improve the implantation rate significantly (1,2). However, it seems that usually only fresh embryos after the third cleavage division (eight-cell stage, or 3-day-old embryos) are candidates to benefit from the assisted hatching procedure. Recently, Check *et al.* (3) reported improved frozen–thawed embryo implantation and pregnancy when assisted hatching was performed after the thawed embryos culturing up to the eight-cell stage. The purpose of this report is to present our results on the use of assisted hatching on 2-day-old (two- to four-cell stage), frozen/thawed embryos that were transferred.

MATERIALS AND METHODS

Embryo Preparation

All embryos were yielded from stimulated cycles at our center in 1994 and 1995. After selecting the best morphological embryos for fresh transfer, the remaining were frozen at about 48 ± 2 hr after insemination (two- to four-cell stage). The embryos were frozen using the modified Testart method (4). Embryos were frozen in a HEPES-buffered human tubule fluid solution (HTF/HEPES) with 1.5 m*M* propanediol (Sigma Chemical Co., St. Louis, MO) and 0.1 m*M* sucrose (Sigma Chemical Co.) as cryoprotectants. After being loaded into a 0.25-ml straw(s), the embryos were frozen by a programmable freezer (Planer Products, Model 204, TS Scientific, Perkasie, PA). The following procedure was used. Embryos were cooled to −6°C at 2°C/min^{-1} and seeded at this temperature. Then the straws were cooled to −30°C, at 0.3°C/min^{-1}, and from −30°C to −140°C at 50°C/min^{-1}. Finally, the straws were submerged in liquid nitrogen. The thawing procedure was performed manually. The straws were removed from liquid nitrogen and kept at room temperature for 30 sec, then immersed in water at 30°C for 45 sec. After cutting two ends of the straws, embryos were released into an HTF/HEPES medium containing 1.0 m*M* propanediol/0.2 m*M* sucrose for 5 min. Embryos were then transferred to an HTF/HEPES medium with 0.5 m*M* propanediol/0.2 m*M* sucrose. After another 5 min, the embryos were moved to an HTF/HEPES medium with 0.2 m*M* sucrose for 5 min, followed by a plain HTF/HEPES medium with 7.5% serum for 5–10 min. Finally, the embryos were transferred to an HTF medium with 7.5% serum and cultured in a 37°C incubator with 5% CO_2. After thawing, embryo quality was graded as (1) excellent, (2) good, (3) fair, or (4) poor based upon the size and symmetry of the blastomere, the amount of fragments and cytoplasm appearance, such as darkness, granularity, and vacuoles.

Candidates for assisted hatching met at least one of the following criteria: (i) age over 38, (ii) thick zona (≥17 μm), (iii) poor embryo quality, and (iv) multiple embryo transfer failures. Assisted hatching was performed approximately 1 to 1.5 hr before the embryo transfer. The procedure was performed on all the embryos if assisted hatching was done to any. Basically, a holding pipette was positioned at 9 o'clock of an embryo. Another pipette containing acidic Tyrode solution (pH 2.3) was placed at 3 o'clock and an opening with inner and outer diameters at 10–13 and 15–18 μm, respectively, was carefully created. Fragment aspiration was not usually performed. Our preliminary animal study indicated that reduced embryo volume or total intraperivitelline mass volume because of fragment aspiration or excessive blastomere reduction could affect successful hatching of embryos (unpublished). Other details of assisted hatching have been described by other authors (5). After the micromanipulation, embryos were rinsed in an HTF/HEPES medium with 7.5% serum and returned to the 37°C incubator until transfer.

Patient Preparation

Our IVF center started to perform assisted hatching procedures randomly in poor-prognosis patients in 1994, 14 with assisted hatching and 13 without assisted

hatching, respectively. Since 1995 all poor-prognosis patients at our center received the assisted hatching procedure after patients signed informed consent forms.

The frozen embryo transfers were performed in either a natural-cycle or a GnRH agonist (GnRH-a)-controlled cycle. Patients with regular menstrual cycles underwent a natural-cycle embryo transfer. Patients with irregular cycles or failed natural cycle embryo transfers were candidates for GnRH-a-controlled cycles. The patients who entered natural-cycle programs were examined on cycle day 3 and had the first serum E_2 and ultrasound on that day. About 3 days before the anticipated date of ovulation, the patient was ultrasounded and the serum E_2 was repeated. Ovulation was defined by urine LH surge. After ovulation, the endometrium was supported by 1500 IU hCG every other day over a 7-day period, starting on the day of transfer. For some women, 400 mg progesterone was administered daily beginning 3 days before embryo transfer or, if necessary, after the last hCG supplement.

GnRH-a suppression was used for controlled cycles. Leuprolide acetate, 0.2 mg/day (Lupron; TAP Pharmaceuticals, North Chicago, IL) was started either from middle luteal phase or early follicular phase until cycle day 14. Endometrium was supported by increasing doses of estradiol (Estrace; Mead Johnson Pharmaceuticals, Princeton, NJ) in the follicular phase to mimic the natural cycle. The patient was given 0.5 mg Estrace twice a day from cycle day 1 to cycle day 5, then increased to 1 mg/bid from cycle day 6 to cycle day 9 and, finally, to 2 mg/tid from cycle day 10 to cycle day 13. Luteal-phase endometrium was supported by both Estrace and progesterone. Beginning on cycle day 14, the Estrace dosage was 1 mg/bid until day 20, then increased to 1 mg/tid until serum hCG test on cycle day 28. Administration of progesterone, 25 mg i.m., in oil was started on the evening of cycle day 15. Beginning on day 16, 50 mg of progesterone was administered every 12 hr until the serum pregnancy test. Embryo thawing and transfer were performed on cycle day 18. If pregnancy was achieved, Estrace and progesterone supplements were maintained until the seventeenth week of gestation. Tetracycline (250 mg/qid) and methylprednisolone (4 mg/qid) were administered to all the patients for 4 days to prevent infection and immune system attack.

RESULTS

Effects of assisted hatching on pregnancy rates in a poor-prognosis population and the pregnancy rate in women over age 38 years are shown in Tables I and II, respectively. Clinical pregnancy was documented by the appearance of at least one intrauterine gestation sac. In the assisted hatching group, two cases of miscarriage were in the same patient. The first time, the woman had one intratubal implantation and one intrauterine sac. Unfortunately, the intrauterine sac was lost at the ninth week because of fetal demise. The second time, the same patient had two intrauterine sacs; one was empty and the other had a viable fetus and heartbeat at the ninth week but was lost again at the thirteenth week. All other pregnant women delivered normal baby (babies); one of them started with three sacs but delivered twins. There was no significant difference in pregnancy rates between natural- and Lupron controlled-cycle embryo transfers ($P > 0.05$). To eliminate the effects of defective endometrium receptivity and poor embryo quality, implantation was calculated only with embryos graded as good/excellent in pregnant patients. Thus, 14 of 27 embryos graded as good/excellent were implanted in nine clinical pregnant women. However, four sacs disappeared and one was a tubal implantation.

Table I. Effect of Assisted Hatching on Pregnancy Rate in Poor-Prognosis Population

	AH+[a]	AH−[b]
No. of patients	37	13
Age (median)	36 (26–52)	40 (32–51)
Embryos transferred (mean ± SD)	4.2 ± 1.8	4.0 ± 1.2
Good/excellent embryos transferred (mean ± SD)	2.3 ± 1.4	2.2 ± 1.3
Clinical pregnancies[c]	9 (24%)	0 (0%)
Natural cycle	5/22 (23%)	—
Controlled cycle	4/15 (26%)	—
Miscarriage	2	—

[a] Assisted hatching group.
[b] Non-assisted hatching group.
[c] Number of clinical pregnancies per embryo transfer.

Table II. Effect of Assisted Hatching on Pregnancy Rate in Aged Patients (>38)

	AH+[a]	AH−[b]
No. of patients	16	8
Clinical pregnancy[c]	4 (25%)	0 (0%)
Miscarriage	2	—

[a] Assisted hatching group.
[b] Non-assisted hatching group.
[c] Number of clinical pregnancies per embryo transfer.

DISCUSSION

Since the time assisted hatching was introduced to IVF-EI (1,6), several other authors have reported that assisted hatching improved the implantation rate, particularly in poor-prognosis patients (7,8). However, there are still concerns whether manipulation of the zona pellucida and acidic Tyrode solution have adverse effects on the further development of embryos. Routinely, assisted hatching is applied to fresh embryos at the six- to eight-cell stage, because it is considered that the cell junction between blastomeres is not established before that embryonic stage. So far there have been only a few reports applying assisted hatching to frozen–thawed embryos. Recently, Check *et al.* (3) reported improved pregnancy rates using assisted hatching for frozen–thawed embryos after culturing up to the six- to eight-cell stage. Our results demonstrate that assisted hatching may also be successfully applied to 48-hr-old frozen–thawed embryos (two- to four-cell stage) without detrimental effects on further embryonic development since a suitable pregnancy rate was achieved. Based on our observations, during the micromanipulation and transfer procedures, blastomere isolation or loss did not occur in vitro.

After an extensive literature search, we believe that this report is the first to describe assisted hatching using acidic Tyrode solution on two- to four-cell-stage frozen–thawed embryos. Further data accumulation will be needed to confirm the benefit of assisted hatching on frozen–thawed embryo transfer at this stage. In conclusion, the results of our report suggest that assisted hatching can be used successfully for frozen–thawed, early-stage (2-day-old) human embryo transfer.

REFERENCES

1. Cohen J, Elsner C, Kort H, Malter H, Massey J, Mayer MP, Wiemer K: Impairment of the hatching process following in vitro fertilization in the human and improvement of implantation by assisting hatching using micromanipulation. Hum Reprod 1990;5:7–13
2. Schoolcraft WB, Schlenker T, Gee M, Johes GS, Jones HW Jr: Assisted hatching in the treatment of poor prognosis in vitro fertilization candidates. Fertil Steril 1994;62:551–554
3. Check JH, Hoover L, Nazari A, O'Shaughnessy A, Summers D: The effect of assisted hatching on pregnancy rates after frozen embryo transfer. Fertil Steril 1996;65:254–257
4. Lassalle B, Testart J, Renard JP: Human embryo features that influence the success of cryopreservation with the use of 1,2-propanediol. Fertil Steril 1985;44:645–651
5. 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;7:685–691
6. Tucker MJ, Cohen J, Massey JB, Mayer MP, Wiker SR, Wright G: Partial dissection of the zona pellucida of frozen-thawed human embryos may enhance blastocyst hatching, implantation and pregnancy rates. Am J Obstet Gynecol 1991;165:341–345
7. Stein A, Pinkas H, Rufas O, Ovadia J, Amit S, Fisch B, Avrech O: Assisted hatching by partial zona dissection of human pre-embryos in patients with recurrent implantation failure after in vitro fertilization. Fertil Steril 1995;63:838–841
8. Wiemer KE, Genetis P, Hu Y, Leibowitz D, Cuervo M: The combination of coculture and selective assisted hatching: Results from their clinical application. Fertil Steril 1994;61:105–110

Jun Tao[1]
Robert Tamis
Arizona Institute of Reproductive Medicine
2850 North 24th Street, Suite 503
Phoenix, Arizona 85008

[1] To whom correspondence should be addressed.