A Study Failing to Determine Significant Benefits from Assisted Hatching: Patients Selected for Advanced Age, Zonal Thickness of Embryos, and Previous Failed Attempts

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Purpose: *Pregnancy and implantation rates after mechanical assisted hatching (AH) in patients aged ≥38 years, with embryos ≥15 μm in zonal thickness and two or more failed attempts, were assessed at two infertility centers using fresh and frozen embryo transfer (FET) cycles.*

Methods: *AH was performed on 3-day-old embryos. Spare embryos cryopreserved at the two-pronucleus stage were subjected to AH after 2 days of culture and transferred to artificially prepared uteri.*

Results: *In fresh cycles, no significant differences in pregnancy rates (clinical and ongoing) and implantation rates were observed between the AH and the controls for all three selected patient groups (Centers 1 and 2). In FET cycles, AH tended to give poor results for ≥38 year olds (clinical pregnancy rates of 0 and 5.0% with AH vs 13.3 and 16.7% for controls at Centers 1 and 2, respectively). With AH, embryos with thick zonae implanted to the same extent as those in the control group and achieved pregnancies for patients with multiple failures (four to six attempts for some) in both fresh and FET cycles.*

Conclusions: *AH failed to show significant benefits in all three patient groups. A larger study group may confirm the effects of AH on frozen/thawed embryos and outcomes for multiple failure cases.*

KEY WORDS: assisted hatching; fresh embryos; frozen embryos; selected patients.

INTRODUCTION

Implantation rates with assisted reproductive procedures (babies born per embryo transferred) remain low. It is suggested that the prolonged exposure of human oocytes and embryos to suboptimal culture conditions can harden the zona pellucida and may impair their ability to implant (1,2). Embryo hatching is a prerequisite for implantation and further development of the embryo. To improve hatching, Cohen *et al.* (3) introduced assisted hatching (AH), and it has been shown that an increased number of embryos hatch and implant early due to the breach in the zona (4,5). During assisted hatching a small hole or a slit in the zona is made either mechanically using partial zona dissection (PZD) (3,6) or chemically using zona drilling with acid Tyrode's medium (4,7,8), enzymatic digestion (9,10), or laser microbeams (11,12).

Assisted hatching may have harmful effects to the embryo. Zona drilling is shown to have: detrimental effects on embryos with thin zonae (3), a high rate of cleavage arrest at the morula and blastocyst stage in mice (13), and detrimental effects to human oocytes when used in assisted fertilization (14). On the other hand, with PZD, trapping of blastocysts during hatching can occur due to the small slit made (3). However, the incidence of embryo trapping with PZD is reduced by controlling the size of the slit (4). Once the technique is optimized, PZD seems to be an easier technique to perform, with minimal harmful effects to the embryo.

It is well documented that only a select group of patients will benefit from AH, i.e., older patients (6,7,15), patients who have embryos with thick zonae (6), those with elevated basal follicle stimulating hormone (FSH) levels (6, 16), patients with repeated in

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vitro fertilization (IVF) failure (7,17), and those with frozen embryos (18,19). Bider *et al.* (8) failed to observe improvements in take-home baby rates with AH of embryos of patients aged >38 years.

In the light of the available data we introduced mechanical AH using PZD into the intracytoplasmic sperm injection (ICSI) programs at two infertility treatment centers for both fresh and frozen embryo transfer cycles in three selected patient groups, i.e., patients aged ≥38 years, those with oocytes with thick zonae, and patients with previous failed attempts.

MATERIALS AND METHODS

Patient Selection and Ovarian Stimulation

Patients who were in the ICSI and frozen embryo transfer programs at two infertility clinics (Centers 1 and 2) were selected on an individual basis for AH. The selection criteria included (a) the patient's age (≥38 years), (b) the zonal thickness of embryos (≥15 μm) (only at Center 1), and (c) previous failure in more than two IVF/ICSI or frozen embryo transfer (FET) attempts.

The patients were given a full description of the AH procedure and were included in the study following their consent. At both infertility clinics, for the ovarian stimulation a combination of gonadotropin releasing hormone agonist (GnRH-a; Buserelin acetate; Suprefact, Frankfurt, Germany), on either a "down-regulation" or a "flare-up" regimen, and human menopausal gonadotropin (hMG; Pergonal; Serono, Aubonne, Switzerland) with or without pure FSH, Metrodin; Serono) was used. Ovulation was triggered by giving 10,000 IU human chorionic gonadotropin (hCG; Profasi; Serono) when two or more follicles reached 2.0 cm in diameter. Oocyte recovery was performed 36 hr after the hCG trigger injection by transvaginal ultrasound-guided aspiration.

The oocytes were cultured in Medicult IVF medium (Medi-cult a/s, Copenhagen, Denmark) (Center 2) or human tubal fluid medium containing 10% heat-inactivated human serum (Center 1). The cumulus cells were removed by incubation in an 80 IU/ml hyaluronidase (type VIII; Sigma Chemical Co., St. Louis, MO) solution made in Hepes-buffered T6 medium containing 10% synthetic serum substitute (Irvine Scientific, Santa Ana, CA). Following the hyaluronidase treatment, the oocytes were assessed for maturity and all MII oocytes were subjected to ICSI; the ICSI procedure was performed according to the method described by Van Steirteghem *et al.* (20). The oocytes were examined after 18–20 hr to determine fertilization, and those with two pronuclei and two polar bodies present were considered normally fertilized. Three or four pronuclear embryos were allowed to cleave and the embryos were transferred on day 3 (at six to eight cells) into the uterus in the fresh ICSI cycles.

Freezing and Thawing of Embryos and FET Cycles

The spare embryos were cryopreserved routinely at the two-pronucleus stage. Embryo freezing was carried out using propanediol (PROH) (21). Two dilutions of PROH was prepared in phosphate-buffered medium (PBI) containing 20% heat-inactivated human serum (first solution, 1.5 *M* PROH; second solution, 1.5 *M* PROH + 0.1 *M* sucrose). Following equilibration of embryos for 15 min in each of the two solutions, the embryos were loaded into sterile 0.25-ml plastic straws (IMV; French straws; Cassou, France) and placed in the freezing chamber of a programmable freezer (Kryo 10; Planar Products Ltd., England) at 20°C. The freezing program used included cooling from 20°C at −2°C/min to −7°C, manual seeding at −7°C during a 5-min holding period, slow cooling at −0.3°C/min to −30°C, and further cooling at −50°C/min to −150°C before plunging into liquid N2 for storage. The thawing procedure included holding the embryo straw at room temperature for 40 sec and then immersing it in a 30°C water bath until the ice crystals had disappeared. For removal of the cryoprotective medium, the embryos were placed for 5 min sequentially in 1.0 *M* PROH + 0.2 *M* sucrose, 0.5 *M* PROH + 0.2 *M* sucrose, 0.2 *M* sucrose, and, finally, PBI containing 20% human serum. From the final solution the embryos were transferred to the embryo transfer dish containing Medicult IVF medium.

Assisted hatching was performed as described below on embryos of patients who were selected based on either their age (≥38 years), zonal thickness (≥15 μm), or previous failures of FET (two or more failed attempts). Embryos were transferred only to artificially prepared uteri. The hormone replacement therapy (HRT) included GnRH-a (Buserelin acetate; Suprefact) suppression from day 21 of the previous cycle until day 15 of the treatment cycle. Following the day 2 estradiol (<50 pg/ml) and luteinizing hormone (LH; <5 mIU/ml) assessment, increasing doses of estradiol valerate (Progynova; Schering AG Pharmaceutical Division, Berlin) was given at 2 mg/day for 5 days, 4 mg/day for 4 days, and 6 mg/day for the next 5 days. On

cycle day 15, the endometrial thickness was assessed (accepted thickness, >8 mm) and proluton (Schering) was given at 50 mg/day for 2 days and 100 mg/day for the next 2 days. Frozen embryo transfer into the uterus was performed 4 days after the initiation of progesterone using day 3 embryos (six to eight cells). The estradiol concentration was maintained during the luteal phase at the same dosage (Center 1) or reduced to 4 mg/day (Center 2). After embryo transfer the proluton injections were continued until the pregnancy test on day 14 of embryo transfer (Center 1) or changed to two progesterone suppositories (Cyclogest, 400 mg; Hoechst Marion Roussel, Bangkok, Thailand) (Center 2) until the pregnancy test. For pregnant patients, HRT was maintained until 10–12 weeks of gestation.

Assisted Hatching Using PZD

All embryos were assessed on the day of embryo transfer using an inverted microscope (Diaphot 300; Nikon, Tokyo) at a magnification of ×200 for quality and zonal thickness. Zonal thickness was measured using a micrometer scale attached to one of the eyepieces. For embryos selected for AH, a breach in the zona was made using PZD and this procedure was performed 30–60 min prior to embryo transfer. PZD was carried out as described by Cohen *et al.* (3). In brief, the embryos were placed in microdroplets of Hepes-buffered medium prepared under sterile paraffin oil. The embryos were held stationary using a holding pipette (ID, 30 μm) and a sharp cutting needle was pushed through one side of the zona pellucida at the 1 o'clock position and pierced on the other side at 11 o'clock. The embryo was then released and the part of the zona that was trapped between the two points pierced by the cutting needle was rubbed against the holding pipette until a slit was made in the zona. The embryos were washed twice and placed in the embryo transfer dish. Patients were given an antibiotic cover for 5 days from the day of hCG trigger and the luteal phase was maintained on one progesterone suppository (Cyclogest, 400 mg) daily and 750 IU of hCG given on days 4, 7, 10, 13 following oocyte recovery. A blood test for pregnancy determination was performed 16 days after embryo transfer. Rising β-hCG levels in weekly blood tests indicated pregnancy, and a clinical pregnancy was confirmed at 7 weeks using an ultrasound scan. The implantation rate was calculated as the number of embryonic sacs per the number of embryos transferred for each group.

The clinical and ongoing pregnancy rates and the implantation rates obtained with AH for selected groups of patients in fresh and frozen embryo transfer cycles at Center 1 were compared to those of a matched control group. The matched control group consisted of patients who did not consent to AH or underwent ICSI treatment prior to the introduction of AH. The two patient groups were closely matched according to their age, stimulation regime, number of oocytes collected, number of embryos transferred, and, where possible, number of previous failed attempts. At Center 2, the control group consisted of all patients who did not undergo AH over a period of 6 months during or prior to the study and was not a matched group.

Statistical Analysis

Differences in the clinical and ongoing pregnancy rates, implantation rates, embryo quality, and pregnancy outcomes between the AH and the controls in different patient groups were analyzed using the chi-square test. Student's *t* test was used for comparing the means.

RESULTS

At Center 1, AH was carried out in a total of 42 cycles and these were compared to a matched control group. At the outset, the number of oocytes recovered, number and quality of embryos transferred, and clinical pregnancy rates were compared between the two stimulation regimes used for superovulation, "flare-up" and "down-regulation" (Table I) in order to assess the possible variability between the AH and the control groups. The number of cycles in the AH and the controls was 25 for the flare-up and 17 for the down-regulation regimen. Between the AH and the control groups, no significant differences were observed in the number of oocytes collected, number, and quality of embryos transferred, and clinical pregnancy rates. The clinical pregnancy rate was significantly higher with the "down-regulation" regimen (10/34) compared to the "flare-up" regimen (2/50) ($P < 0.01$).

The data obtained with AH for the different selected patient groups at Center 1 is given in Table II. In the fresh cycles, of the 42 AH cycles, 22 were included due to advanced age (≥38 years). Both AH and controls in the ≥38-year-old group achieved pregnancies (9.1 and 4.5% clinical pregnancy rate for AH and controls, respectively). Patients with embryos with thick zonae and previous failed attempts (Table II) achieved pregnancy rates and implantation rates similar to those observed in the younger control group (<38-year con-

Table I. Effect of Two Stimulation Regimens on the Number of Oocytes Collected, Number and Quality of Embryos Transferred, and Clinical Pregnancy Rates of Assisted Hatching (AH) and Control Groups in Both Fresh and Frozen Embryo Transfer (FET) Cycles

Cycle details	AH		Control	
	Flare-up	Down-regulation	Flare-up	Down-regulation
Fresh				
No. cycles	25	17	25	17
No. oocytes collected (mean ± SD)	6.44 ± 4.75	9.94 ± 5.62	5.20 ± 4.37	9.35 ± 5.01
No. embryos transferred (mean ± SD)	2.72 ± 0.98	2.94 ± 0.83	2.44 ± 1.12	3.11 ± 0.93
Embryo quality[a]				
No. poor (≤2/4)	28	15	20	19
No. good (>2/4)	40 (58.8%)	34 (69.4%)	41 (67.2%)	35 (64.8%)
Clinical pregnancies	1	5	1	5
FET				
No. cycles[b]	19	18	16	21
No. oocytes collected (mean ± SD)	9.56 ± 6.89	15.95 ± 4.14	10.42 ± 6.57	14.56 ± 6.67
No. embryos transferred (mean ± SD)	2.78 ± 1.11	3.37 ± 0.60	2.50 ± 0.85	3.35 ± 0.78
Embryo quality[a]				
No. poor (≤2/4)	23	28	11	39
No. good (>2/4)	24 (54.0%)	36 (56.3%)	24 (68.6%)	38 (50.0%)
Clinical pregnancies	1	4	3	5

[a] Embryo quality was graded on a 0–4 scale, with the best quality being 4.
[b] Embryos were generated either on flare-up or down-regulation cycles and transferred into artificially prepared uteri following freeze/thaw.

trols). With AH carried out on frozen embryos, no pregnancies were obtained in 15 transfer cycles for women aged ≥38 years, whereas the matched controls had clinical and ongoing pregnancy rates of 13.3 and 6.7%, respectively, and an implantation rate of 4.2%. Results obtained for thick zonae (>15 μm) and more than two failed attempts in FET cycles were similar to those obtained for the control group (< 38 years).

Table II. Clinical and Ongoing Pregnancy Rates, Pregnancy Outcomes, and Implantation Rates with Assisted Hatching (AH) for Patients Selected Based on Age, Zonal Thickness, and Previous Failed Attempts in Fresh and Frozen Embryo Transfer (FET) Cycles (Center 1)

Outcome measure	Selected patient group					
	≥38 years		<38 years		Thick zonae[a] (AH)	Failed attempts[a] (AH)
	AH	Control[b]	AH	Control[b]		
Fresh						
No. cycles	22	22	20	20	35	15
Clinical pregnancy rate (%)	2 (9.1)	1 (4.5)	4 (20.0)	5 (25.0)	6 (17.1)	3 (20.0)
Ongoing pregnancy rate (%)	1 (4.5)	0 (0)	4 (20.0)	4 (20.0)	5 (14.3)	3 (20.0)
Implantation rate (%)[c]	3/69 (4.3)	1/61 (1.6)	4/59 (6.8)	5/57 (8.8)	7/103 (6.8)	3/42 (7.1)
Pregnancy outcome						
Pregnancy loss	1	1	—	1	—	—
Singleton	1	—	4	4	5	3
Twin	—	—	—	—	—	—
FET						
No. cycles	15	15	22	22	26	15
Clinical pregnancy rate (%)	0 (0)	2 (13.3)	5 (22.7)	6 (27.3)	4 (15.4)	2 (13.3)
Ongoing pregnancy rate (%)	0 (0)	1 (6.7)	4 (18.2)	6 (27.3)	3 (11.5)	2 (13.3)
Implantation rate (%)[c]	0/44 (0)	2/47 (4.2)	9/71 (12.7)	6/75 (8.0)	9/81 (11.1)	6/47 (12.8)
Pregnancy outcome						
Pregnancy loss	1	1	1	—	1	—
Singleton	—	1	2	6	2	—
Twin	—	—	2[d]	—	2[d]	2[d]

[a] As there were no matched controls, the thick-zonae (≥15 μm) and ≥2 failed-attempt groups were compared to the <38-year-old controls.
[b] Matched control group.
[c] Number of embryonic sacs/total number of embryos transferred.
[d] Two triplet pregnancies ended as twin pregnancies.

At Center 2, no significant beneficial effects of AH were observed on the pregnancy rates or implantation rates of older patients in both fresh and FET cycles compared to the control group. In fact in FET cycles, similar to the results from Center 1, the clinical pregnancy rate and the implantation rate with AH tended to be poor for the patients aged ≥38 years (5.0 and 1.7%) compared to the control group (16.7 and 5.0%). However, this difference did not reach significance. The outcome measures for patients with two or more previous failed attempts were similar between AH and the overall controls for both fresh and FET cycles. When the overall results for the previous-failure group were considered (Centers 1 and 2), four AH pregnancies in each of fresh and frozen cycles were obtained for patients with more than four failed attempts, and of these, three pregnancies resulted following the sixth attempt.

Multiple pregnancies with AH were observed only in FET cycles at both Center 1 (Table II) and Center 2 (Table III). The overall incidence of multiple pregnancies, in this study the twinning rate, tended to be slightly higher for the AH cycles than the controls [2/22 (9.1%) with AH vs 0/22 (0%) for controls at Center 1; 3/36 (8.3%) with AH vs 5/133 (3.8%) for controls ($P > 0.05$) at Center 2].

DISCUSSION

We have studied mechanical (PZD) AH at two infertility clinics in three selected groups of patients who underwent fresh or frozen embryo transfer cycles. The first selection criterion used was advanced age (≥38 years). In fresh cycles, no added beneficial effects were observed on the pregnancy rates and implantation rates at both infertility centers for this patient group. It is clear that there is center-to-center variation in the AH results, probably due to factors which generally affect the embryo culture or the patient population. Some programs have observed significant improvements in implantation for patients aged ≥38 years with AH (7,6), whereas others have seen no improvements in implantation rates for this group (8). Perhaps with older patients the implantation rates are influenced not only by zonal factors (thickness and density), but also

Table III. Clinical and Ongoing Pregnancy Rates, Pregnancy Outcomes, and Implantation Rates with Assisted Hatching (AH) for Patients Selected Based on Age and Previous Failed Attempts in Fresh and Frozen Embryo Transfer (FET) Cycles (Center 2)

	Selected patient group			
	≥38 years			
Outcome measure	AH	Control[c]	Failed attempts[a] (AH)	Overall control[b]
Fresh				
No. cycles	30	29	22	71
Clinical pregnancy rate (%)	3 (10.0)	5 (17.2)	5 (22.7)	15 (21.1)
Ongoing pregnancy rate (%)	2 (6.6)	4 (13.7)	4 (18.2)	10 (14.1)
Implantation rate (%)[d]	4/73 (5.5)	6/87 (6.9)	5/61 (8.2)	18/195 (9.2)
Pregnancy outcome				
Pregnancy loss	1	1	1	7
Singleton	2	4	4	8[e]
Twin	—	—	—	—
FET				
No. cycles	20	30	36	133
Clinical pregnancy rate (%)	1 (5.0)	5 (16.7)	8 (22.2)	37 (27.8)
Ongoing pregnancy rate (%)	1 (5.0)	4 (13.3)	6/36 (16.7)	27 (20.3)
Implantation rate (%)[d]	1/60 (1.7)	5/99 (5.0)	11/106 (10.4)	45/423 (10.6)
Pregnancy outcome				
Pregnancy loss	—	2	—	11
Singleton	1	3	3[e]	21[f]
Twin	—	—	3[e]	5

[a] Cycles with ≥2 failed attempts.
[b] Overall control group consisted of all patients who did not undergo assisted hatching during a 6 month period during or immediately prior to the study.
[c] Control group ≥38 years; not a matched group.
[d] Number of embryonic sacs/total number of embryos transferred.
[e] One twin pregnancy ended as a singleton and one triplet pregnancy ended as a twin pregnancy.
[f] Three twin pregnancies ended as singleton pregnancies.

by other factors such as the chromosomal status of the embryos and uterine factors. Even though common variables such as the stimulation regime, oocyte numbers, embryo numbers, and embryo quality which normally determine pregnancy rates were controlled by the use of a matched control group in the present comparison (Center 1), the involvement of other factors as discussed above may explain the variations seen in the AH results.

The other two selection criteria used for the application of AH in the fresh cycles were the zonal thickness of the embryos, which was ≥ 15 μm, and previous failures in two or more embryo transfer attempts. Cohen *et al.* (3) have shown that thick zonae can reduce the chance of blastocysts hatching and that AH can significantly improve hatching and implantation rates. Furthermore, it has been discussed that the ratio of lysin production to the amount of zona material present may determine whether the embryo will lyse the zona and hatch within the time frame required for implantation (22). When these factors are considered, AH appears to be beneficial for embryos with thick zonae, and in our present study the pregnancy rates and implantation rates with AH for patients with embryos with thick zonae were similar to those of the control patients who did not undergo AH. The achievement of pregnancies in patients who failed to conceive in four to six previous attempts in both infertility programs indicates that AH may have some benefits for this group of patients in FET attempts, similar to the data published by others (6,7,17).

In the FET cycles, data from the two centers indicate that AH has no beneficial effects for the older patients and, furthermore, no added beneficial effects on the implantation rates of embryos with thick zonae. However, acceptable pregnancies were achieved for patients who failed to conceive in previous attempts (four patients conceived following AH after four to six previous failed attempts). Other AH studies have demonstrated either a tendency toward improved implantation or definite improvements in implantation and pregnancy rates with AH on frozen embryos in an unselected group (18,19) or for patients selected for advanced age (23). Check *et al.* (19) transferred embryos subjected to AH at 72 hr and control embryos at 48 hr. This may have some negative effect on the implantation rate and pregnancy rate of the control embryos, as it has been shown that pregnancy rates can be improved significantly when embryo transfer is delayed until day 3 after oocyte recovery (24).

The quality of the zona changes following freeze/thaw (25,26). Matson *et al.* (26) demonstrated, in zona hardness studies using mouse oocytes and embryos, that the zona of oocytes hardened, whereas no hardening or even a slight softening of the zona of embryos occurred after freezing. It is suggested following these findings that AH may not be necessary to improve blastocyst hatching and implantation rates of frozen/thawed embryos. Our findings of AH on frozen embryos tend to support this, especially for older patients. The embryos of patients who failed to conceive in the past may represent a group of embryos whose zonal quality is unchanged due to freezing or slow-growing embryos which are helped by AH to hatch earlier to coincide with the implantation window (22,27) or the breach in the zona could allow earlier embryo–maternal contact, which could improve the possible transport of growth factors to enhance embryo viability (27).

Partial zona dissection is an easier technique to perform and it did not cause any morphological damage to any of the embryos. However, some of the developing blastocysts may have gotten trapped during the hatching process through the slit made in the zona (3). During the PZD procedure we attempted to standardize the slit made so that blastocyst trapping could be minimized (4). In our study the overall twinning rate did not increase significantly due to the transfer of PZD AH embryos.

Other techniques of AH, i.e., zona drilling (4,7,8), enzymatic digestion (9,10), and laser microbeams (11,12), have been shown to be beneficial for improving hatching and implantation rates. The use of laser microbeams appears to be an attractive alternative to PZD due to the precision involved in making the slit or hole in the zona (11,12). However, this technology is not widely available in many laboratories. On the other hand, the inclusion of pronase or proteinase K in the embryo culture medium for 72 hr has been shown to improve the blastocyst hatching rate and implantation rate in mice (10). This method of AH (long-term culture in the presence of the enzyme) is yet to be tested in humans, even though a pregnancy has been reported using pronase to remove the zonae of blastocyst-stage embryos (9). Many IVF units have reported AH data using drilling of the zona with acid Tyrode's (7,8). Acid Tyrode's solution used in zona drilling can be detrimental to embryo development (13). Thus, it is necessary to gain proper training in the performance of the technique so that the acid Tyrode's solution can be injected in the zona in a controlled manner to reduce harmful effects. Once this technique is perfected, it may be an alternative or perhaps a

superior technique to AH to improve embryo hatching and implantation rates for selected groups of patients.

In conclusion, no significant beneficial effects of mechanical (PZD) AH were observed on the pregnancy rates and implantation rates of ICSI patients selected based on age (≥38 years), the zonal thickness of their embryos (≥15 μm), and previous failed attempts (two or more attempts) in both fresh and frozen embryo transfer cycles. Furthermore, AH showed a tendency to have adverse effects on frozen/thawed embryos, particularly in the older patients, and a tendency to benefit patients with multiple previous failed attempts. However, these findings need to be confirmed in a larger study group.

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