

In Vitro Fertilization Outcomes Following Assisted Hatching of Embryos with Thick Zona Pellucida—A Prospective Randomized Study

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Abstract

Purpose: Impaired hatching is associated with implantation failure following *in vitro* fertilization (IVF). Thickening or hardening of the zona pellucida (ZP) has been proposed as a factor in this impairment. We examined whether selective assisted hatching (AH) is beneficial with embryos having a thick ZP. **Methods:** This prospective, randomized controlled study was performed in the IVF unit of an obstetrics and gynecology department in a university-affiliated hospital. Only patients undergoing IVF and having a ZP thickness of $\geq 17 \mu\text{m}$ measured in all their embryos were included. In the intervention group, AH was applied to all embryos, before their transfer. In the control group, embryos were transferred without AH. Implantation, clinical pregnancy and live birth rates were the study endpoints. **Results:** Both study arms were comparable in most baseline parameters. The two groups did not differ in implantation rates (14.1% control vs. 8.92% intervention, odds ratio (OR) = 0.5974, 95% confidence interval (CI) 0.325 - 1.1), clinical pregnancy rates (36.7% vs. 25.8%, OR = 0.6025, 95% CI 0.274 - 1.325), or live birth rates (25% vs. 18.9%, OR = 0.7021, 95% CI 0.291 - 1.691). **Conclusions:** Selecting embryos for AH by their ZP thickness as a sole parameter was not found to be beneficial and to improve IVF outcome.

Keywords

In Vitro Fertilization, Assisted Hatching, Zona Pellucida Thickness, Partial Zona Dissection, Implantation Rate

1. Introduction

A key event for successful embryo implantation is the expansion and thinning of the zona pellucida (ZP) and embryonic hatching [1]. One of the suggested explanations for impaired hatching, which may result in failure to implant and achieve pregnancy, is thickening or hardening of the ZP. Studies in women undergoing *in vitro* fertilization (IVF) have shown that maternal factors such as advanced age and active or passive smoking correlate with a thicker ZP [1] [2]. Hormonal factors including basal follicle stimulating hormone (FSH) and pre-ovulatory estrogen levels are associated with thick ZP [1] [3], and some studies have reported a direct relationship between the number of human menopausal gonadotropin (hMG) ampoules given for ovarian stimulation and ZP thickness [3]. In addition, laboratory conditions can induce hardening or thickening of the zona [4], and cryopreservation may also have a similar effect [4].

The assisted hatching (AH) procedure, first introduced in 1990 by Cohen *et al.* [5], targets improvement in IVF implantation rates by thinning, drilling, or completely removing the ZP before embryo transfer. Several techniques were developed for this purpose, including mechanical partial zona dissection (PZD) and use of proteolytic enzymes, Tyrode acid solution, laser energy [6], and Piezo technology [7]. Many studies have been performed to examine the benefit of AH, but conclusions are inconsistent. According to the American Society for Reproductive Medicine committee opinion from 2008 [6], the available published data do not support routine or universal application of AH in IVF cycles, but AH may be clinically useful in patients with a poor prognosis, including those with ≥ 2 failed IVF cycles, poor embryo quality, and advanced maternal age (≥ 38 years of age).

The benefit of AH in patients with thickened ZP has not been established. In 1992, in a prospective, randomized controlled study, Cohen *et al.* [8] demonstrated a significant increase in implantation and clinical pregnancy rates with selective AH application, including in embryos with thickened ZP. Since then, several studies aimed to determine the effect of AH on embryos with thickened ZP but showed no significant improvement in IVF outcomes [9]-[11]. In a recent systematic review [12] including 28 studies and 5507 participants, AH was related to increased clinical pregnancy rates in women with repeated IVF failures or frozen-thawed embryos, but on the other hand, AH did not increase clinical pregnancy rates in non-poor prognosis patients or women with advanced age. In most of these studies, ZP thickness was the only one of several parameters analyzed for influence on the success of AH, mainly the age of the women and repeated previous failures. However, no proper conclusions could be drawn regarding live birth.

In the present prospective randomized study, we included only patients whose embryos had a thickened ZP to determine whether selective AH is beneficial in this population. For this purpose, we compared implantation, pregnancy, and live birth rates between the intervention group whose embryos had undergone AH and a control group in which AH was not performed.

2. Materials and Methods

2.1. Patient Recruitment

The study was approved by the Committee for Research in Human Subjects of Carmel Medical Center. For study inclusion, the patients had to have all embryos with a ZP thickness of 17 μm or more. Additional inclusion criteria were age 20 to 40 years and infertility due to male factors, mechanical factors, or both. The exclusion criterion was infertility due to any chronic illness, including endocrine disorders and endometriosis.

Patients were enrolled in the study during a 5-year period, between 2003 and 2008 and outcome follow up for frozen-thawed embryo transfer until 2012.

Each patient voluntarily signed an informed written consent before enrolling. Enrolled patients were randomly assigned either to the intervention group (AH arm, 58 treatment cycles) or the control group (non-AH arm, 60 treatment cycles) using a standard random number table. Randomization was performed only after measurement of ZP thickness >17 μm was found in all embryos.

The patients were not informed about the identity of their assigned group.

2.2. Ovarian Stimulation and Ovulation Induction

FSH levels on day 3 of the cycle were measured for each patient. Induction of ovulation was similar in all patients: down-regulation, using a long protocol, followed by ovarian stimulation as previously described by Dirnfeld *et al.* [13]. Briefly, gonadotropin releasing hormone analogue (Buserelin nasal spray, Suprefact, 1000 mg/day, Hoechst

Pharmaceuticals, Israel) was administered in the midluteal phase and continued until human chorionic gonadotropin (hCG) administration (Ovitrelle 250 mcg, Merck Serono, Israel). Ovarian stimulation was performed using hMG (Menopur, Ferring, Israel, and/or rFSH (Puragon, MSD Israel or Gonal F, Merck Serono Israel)). Follicular growth monitoring was performed by transvaginal ultrasonography and measurement of serum levels of 17β -estradiol, luteinizing hormone, and progesterone.

After five or more follicles had reached a diameter of 17 mm, patients received 250 mcg Ovitrelle, followed by ovum pick-up 35 - 37 hours later by transvaginal ultrasound-guided follicle aspiration. Luteal support was maintained by Vag. tablets Endometrin 100 mg twice daily (Ferring, Israel).

2.3. Fertilization

The fertilization procedure, including AH, was done in the IVF center at Carmel Medical Center, Haifa. Fertilization techniques were performed using standard IVF or intracytoplasmic sperm injection (ICSI). Measurements of ZP thickness and AH procedures were carried out using a DiaPHOT 300 Nikon inverted microscope.

IVF was performed as previously described by Dirnfeld *et al.* [13] and ICSI as described by Van Steirteghem *et al.* [14] Fertilization was confirmed 16 to 20 hours after the procedure.

2.4. ZP thickness Measurement

ZP was prospectively measured by the same embryologist in all embryos on the day of embryo transfer by using an ocular micrometer scale. Two ZP measurements were performed by the same biologist for each of the embryos, and mean thickness was calculated.

If embryos were cryopreserved, ZP thickness was measured before cryopreservation. In all patients included in the present study, in both groups, all embryos had a ZP thickness $\geq 17 \mu\text{m}$.

2.5. Assisted Hatching

AH was performed mechanically by partial zona dissection PZD just before embryo transfer to the uterine cavity. The cryopreserved embryos underwent the procedure after thawing and prior to the thawed embryo transfer.

AH was carried out on the Nikon microscope using Nomarski modulation optics with three-dimensional manipulators (Narishige, Tokyo, Japan). The procedure was performed as described by Feng *et al.* [15]. The embryo was placed in 5 μl of cleavage medium supplemented with 10% SSS under mineral oil (Sage, USA), held by a holding pipette. The PZD pipette (Cook, Australia) was advanced through the perivitelline space to impale the ZP. Then suction was discontinued from the holding pipette, and the microneedle impaling the ZP was brought below the holding pipette, which was rubbed over the impaled section of the ZP to create an incision.

2.6. Embryo Transfer and Pregnancy Follow-Up

Two embryos were selected for transfer into the uterine cavity and up to three embryos with repeated failures. The remaining embryos were cryopreserved. Cryopreservation and thawing were performed as previously described by Lahav-Baratz *et al.* [16]. Embryo transfer was performed on day 2 or 3 after ovum pickup, guided by transvaginal ultrasound. Embryo transfer was performed always on day 3 when egg collection was done on Thursdays. In other cases decision as to day 2 or day 3 transfer was taken at daily staff results meeting according to Clinician-Embryologist mutual convenience. The patients were not treated with corticosteroids or antibiotics.

Pregnancy was confirmed by blood βHCG 12 days after embryo transfer and transvaginal ultrasound was performed at 4 - 6 weeks after embryo transfer. A clinical pregnancy was confirmed by observation of the gestational sac and positive fetal heart activity.

2.7. Statistical Analysis

Our primary outcome measure was clinical pregnancy rate, defined by the presence of a gestational sac with fetal heart activity by transvaginal ultrasound.

Other outcome measures were implantation rate (calculated as # of gestational sacs visible on ultrasonography divided by total number of embryos transferred) and live birth rate.

Data analysis was conducted using SPSS 15.0. Quantitative data are presented as mean \pm standard deviation

and categorical data as counts (percent). For evaluating the differences between groups, we used the chi-square test for categorical variables and t-test for continuous data. Multiple logistic regression analysis was used for multivariate analysis. A $p < 0.05$ was considered statistically significant.

3. Results

A total of 118 IVF cycles of 109 infertile patients, enrolled from 2003 to 2008, were included in the study. The outcome follow-up for the frozen-thawed embryos continued until 2012.

Both of the study arms were comparable in terms of indications for IVF, number of previous treatment cycles, day 3 FSH levels, smoking habits, number of administered hMG ampoules, and mean ZP thickness (**Table 1**). The groups differed with respect to mean age (28.95 years, control group, vs. 30.81 years, AH group, $p = 0.045$). However, the study arms did not differ in other categorization of patients above or below 35 years old. Day 3 embryos were replaced in 46.7% of cycles in the control group, compared with 27.6% in the AH group ($p = 0.032$). There was no difference in age or mean ZP thickness among patients treated with IVF compared to ICSI.

During the study period a total of 426 embryos were transferred (fresh + frozen). Clinical pregnancy was achieved in 35.1% of all treatment cycles and implantation rate was 11.5%. A total of 26 pregnancies resulted in live birth (22% per cycle), 22 of them singleton and 4 twins. Thirteen pregnancies ended in spontaneous pregnancy loss, and two resulted in preterm delivery at 20 and 25 weeks of gestation with no live birth.

Clinical pregnancy was the primary outcome (**Table 2**). A total of 36.7% of the patients in the control group, compared to 25.8% in the AH group, achieved a clinical pregnancy ($p = 0.21$). Implantation and live birth rates were higher in the control group but did not reach statistical difference (**Table 2**). With fresh embryo transfers, implantation rate was higher in the control group however, clinical pregnancy and live birth rates did not differ between the study group and controls (**Table 3**). As for the frozen-thawed cycles, no significant difference was found in implantation, clinical pregnancy and live birth rates between the two study arms (**Table 4**).

With regard to AH within age groups, our findings revealed no significant difference in clinical pregnancy rates between the AH and control groups in patients >35 years old compared with the younger age group.

Analysis of factors other than AH that may independently affect pregnancy rate found that the timing of embryo replacement differed between the study and control group. A higher pregnancy rate was found when the embryos were transferred on day 3 compared with day 2 (43.2% vs. 24.3%, respectively, $p = 0.033$).

Table 1. Comparison of clinical parameters of patients in the control group and A.H. group.

Parameter	Group 1 (AH)	Group 2 (control)
Number of cycles	58	60
Age a	30.81 \pm 4.94	28.95 \pm 5.02
>35	13 (22.4%)	7 (11.6%)
Previous failed IVF cycles	2.43 \pm 2.94	1.64 \pm 1.61
Smokers	10 (17.2%)	5 (8.3%)
IVF indications		
Male	47 (81.0%)	42 (70%)
Mechanical	8 (13.8%)	13 (21%)
Combined	3 (5.2%)	5 (8.3%)
Basal FSH level (IU/L)	6.54 \pm 1.42	6.43 \pm 1.84
Embryo's age		
3 days ¹	16 (27.6%)	28 (46.7%)
2 days	42 (72.4%)	32 (53.3%)
ZP thickness (μ m)	22.1 \pm 2.16	21.69 \pm 1.98
No. of embryos replaced per transfer	2.47 \pm 0.5	2.53 \pm 0.5

Note: Mean values are given \pm SD. ¹ $p < 0.05$.

Table 2. Clinical outcome of all cycles, fresh and cryopreserved combined.

Parameter	AH (n = 58)	Control (n = 60)	p value
No. of embryos transferred	213	213	
Implantation ¹	19/213 (8.92%)	30/213 (14.1%)	NS
Positive β hCG result	25 (43.1%)	23 (38.3%)	NS
Clinical pregnancies ²	15 (25.8%)	23 (38.3%)	NS
Spontaneous abortions	6 (40%)	7 (18.2%)	NS
Live births ³ (% per cycle)	11 (18.9%)	15 (25%)	NS

Note: NS, not significant; ¹The number of gestational sacs divided by total number of embryos transferred; ²Presence of gestational sac and fetal heart activity; ³Multiple birth is counted as one live birth.

Table 3. Clinical outcome of fresh embryo cycles.

Parameter	AH (n = 58)	Control (n = 60)	p value
No. of embryos transferred	143	152	
Implantation	14/143 (9.8%)	27/152 (17.8%)	0.048
Clinical pregnancies	11 (18.9%)	20 (33.3%)	NS
Live births	7 (12.1%)	13 (21.6%)	NS

Note: NS, not significant.

Table 4. Clinical outcome of frozen embryo cycles.

Parameter	AH (n = 58)	Control (n = 60)	p value
No. of embryos transferred	70	61	
Implantation	5/70 (7.14%)	3/61 (4.91%)	NS
Clinical pregnancies	4 (18.2%)	3 (13.6%)	NS
Live births	4 (18.2%)	2 (9.1%)	NS

Note: NS, not significant.

4. Discussion

In the present prospective randomized study, we included only patients for whom all embryos had a ZP of 17 μ m and thicker. Our results have not shown any benefit, in terms of clinical pregnancy rates and live birth rates, to applying selective AH based on ZP thickness.

It took 5 years to find and randomize patients undergoing IVF with all their embryos with a ZP thickness of 17 μ m or more and data for frozen-thawed cycles was available only by the end of 2012.

This is the first study assessing the net effect of effect of AH when ZP thickness was the only parameter.

ZP thickness has been proposed as an important factor influencing implantation and pregnancy rates [1]. A thick ZP or impairment of its thinning as a result of in vitro culture conditions may inhibit embryo hatching and has been reported to jeopardize implantation and pregnancy achievement [1] [7]. This scenario has raised the question of whether AH could improve implantation and pregnancy rates among patients whose embryos have a thick ZP. If so, ZP thickness measurement could be routinely used as a more accurate tool for predicting AH benefit. Although patient's age in the study group was slightly higher than in the control group, we note that only ZP thickness and not age or previous repeated failures were used as criteria in selecting patients for AH.

The results of fresh cycles showed a tendency, toward lower clinical pregnancy rates and lower implantation rate in the AH group however, this did not reach a statistical difference. Comparison of hatched and non-hatched frozen-thawed embryos did not show any benefit in performing AH based on ZP thickness. Although some previous studies showed benefit for applying AH to embryos of older patients, in the present study, we found no apparent advantage of AH in patients over age 35 years with a thick ZP. We note that the later may have occurred due to the relatively small sample size of this age group. Interestingly, higher chemical pregnancy rate were observed in the AH group ($p = 0.038$). Several studies have shown higher miscarriage rates following AH but had indistinct outcomes regarding the risk for miscarriage because of a lack of statistical power. Others reported a higher ectopic pregnancy rate associated with AH [17]. However, to our knowledge, no studies have found a correlation between AH and chemical pregnancies. Further investigation is needed to establish this association.

Previous investigators have already reported that all methods currently available to create a gap in the ZP, including mechanical PZD, or Laser technology may have adverse effects on embryonic development [18]. In mice, zona drilling is associated with a high rate of cleavage arrest at the morula and blastocyst stages [11]. Further studies in humans also showed potential lethal damage to the embryo and to individual blastomeres, with reduction of embryo viability [4]-[7]. Another outcome might be loss of blastomeres or premature hatching during or after embryonic transfer as a result of contractions of the reproductive tract [18]-[20].

Moreover, blastomeres might become trapped in smaller holes during the hatching process and thus fail to complete hatching; alternatively, they may get lost through larger holes prior to the formation of tight junctions, possibly resulting in monozygotic twins and/or embryonic death [7]. Laser energy is now widely used because it appears to carry the lowest potential risk, and it is relatively simple to perform with consistency between operators [6]. Further studies using laser AH on embryos with thick ZP will provide additional information regarding possible benefit of this method in this embryo population.

We are aware of some limitations of this study. Despite randomization between AH and control groups, based on all embryos having ZP thickness of $\geq 17 \mu\text{m}$, significantly more day 3 embryos were replaced in the controls as compared to the study group. We also found higher pregnancy rates when embryo transfer took place on day 3 compared to day 2, whether AH was performed or not. This may be one explanation to the higher pregnancy rates in the controls. This finding is consistent with the US Centers for Disease Control and Prevention data showing a slight increase in pregnancy rates with day 3 versus day 2 transfer, probably because the additional 24 hours in culture allows better embryo selection [21].

Another limitation is the small sample that has not allowed us to be more conclusive. However, finding patients with appropriate inclusion criteria with all embryos having a ZP of $\geq 17 \mu\text{m}$ requested 5 years of patient recruitment.

5. Conclusions

In summary, the present study investigated the contribution of AH in a prospective randomized IVF/ICSI fresh and frozen-thawed cycles were all embryos that had a predefined thick ZP ($\geq 17 \mu\text{m}$). Our findings showed that AH was not beneficial in embryos with a thick ZP.

Further studies with larger sample sizes using laser AH technology and single embryo transfer will contribute to a better understanding of the impact of AH on embryos with a thick ZP.

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