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A randomized double-blind controlled study of the efficacy of laser-assisted hatching on implantation and pregnancy rates of frozen–thawed embryo transfer at the cleavage stage

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BACKGROUND: Assisted hatching (AH) in fresh embryo transfer (ET) cycles increases the implantation and pregnancy rates, especially in women with a poor prognosis, repeated implantation failures and in older women. Little information exists in the literature regarding the role of AH in frozen–thawed embryo transfer (FET) cycles. METHODS: Embryos were cryopreserved at the cleavage stage. On the day of FET, 160 patients were randomized according to a computer-generated randomization list in sealed envelopes into the AH group and the control group. The patients and the clinicians were blinded to the group assigned. In the AH group, the outer half of the zona pellucida over a quarter of the diameter of zona was removed using a 1480 nm non-contact laser. RESULTS: The two groups were comparable in terms of demographic characteristics, ovarian response of the stimulated cycle and quality of fresh and frozen–thawed embryos. No differences in implantation, pregnancy and multiple pregnancy rates were found between the two groups. There was a non-significant trend of a higher implantation rate in the AH group when the zona thickness was ≥16 mm. CONCLUSION: Laser AH did not improve the implantation rate of FET cycles and should not be performed routinely in all frozen–thawed embryos at the cleavage stage.

Key words: assisted hatching/frozen thawed embryo transfer/laser

Introduction

IVF/embryo transfer treatment involves development of multiple follicles, oocyte retrieval and embryo transfer after fertilization. Despite recent advances in ovarian stimulation, gamete handling, the method of assisted fertilization and improved culture conditions, the implantation potential of embryos has remained low for a long time. Therefore, multiple embryos are usually replaced to compensate for their low implantation potential, but this is associated with a high risk of multiple pregnancies. In order to avoid the risk of multiple pregnancies especially high order ones, patients are usually advised to have two or three embryos replaced in the stimulated cycle while excess good quality embryos are cryopreserved for transfer later.

Although ovarian stimulation invariably leads to an increased number of available embryos, it may also adversely affect uterine receptivity. In a recent review of natural cycle IVF (Pelinck *et al.*, 2002), the reported implantation rates ranged from 0 to 33.0% in natural IVF cycles but were only 7–9% in stimulated cycles. Studies included in that review were not very recent and this may explain the much lower implantation rates found in the stimulated cycles. High serum estradiol (E_2) concentrations resulting from excessive ovarian response adversely affect the outcome of assisted reproduction cycles (Forman *et al.*, 1988; Simón *et al.*, 1995, 1998; Check *et al.*, 2000; Ng *et al.*, 2000) but did not impair oocyte and embryo quality (Ng *et al.*, 2003). Reduced implantation in these cycles might be related to suboptimal endometrial perfusion (Basir *et al.*, 2001a; Ng *et al.*, 2004a) and abnormal endometrial morphometry (Basir *et al.*, 2001b). We have demonstrated recently that endometrial and subendometrial blood flow measured by three-dimensional ultrasound with power Doppler was significantly lower in the stimulated cycle than that in the natural cycle of the same patients (Ng *et al.*, 2004b). It is possible that the endometrium in natural cycles is more receptive when compared with that in stimulated cycles.

As ovarian stimulation is usually not used in frozen–thawed embryo transfer (FET) cycles, the resulting

implantation and pregnancy rates should be better than or at least similar to that of stimulated IVF cycles when embryo quality in stimulated IVF and FET cycles is comparable. However, pregnancy rates in FET cycles are still inferior to that of stimulated IVF cycles [European IVF-Monitoring Programme (EIM) for the European Society of Human Reproduction and Embryology (ESHRE), 2004]. One explanation may be due to zona hardening during the freezing and thawing process. *In vitro* studies with both mouse and human embryos have indicated that an artificial gap in the zona pellucida, i.e. assisted hatching (AH), significantly improves the hatching ability of blastocysts grown *in vitro* as compared with embryos without AH (De Vos and Van Steirteghem, 2000). Different types of AH have been developed. Zona drilling involves the creation of an opening in the zona with acidified medium or enzyme, whereas zona cutting refers to the creation of a slit in the zona using mechanical means. Recently, AH by laser has been introduced, and zona thinning using laser is associated with a higher hatching rate than creating a perforation on the zona (Blake *et al.*, 2001). Mantoudis *et al.* (2001) found a higher pregnancy rate after quarter laser AH than total or partial laser AH.

However, the clinical relevance of AH within an assisted reproduction programme remains controversial and elusive. Very few randomized studies are available and most reports are of retrospective analyses. Two recent meta-analyses (Edi-Osagie *et al.*, 2003; Sallam *et al.*, 2003) demonstrated that AH increased the implantation and pregnancy rates, especially in women with a poor prognosis, repeated failures and in older women. Little information exists in the literature regarding the role of AH in FET cycles.

The aim of this prospective study was to compare implantation and pregnancy rates of FET cycles with and without laser AH. The hypothesis was that laser AH significantly improves implantation and pregnancy rates of FET cycles at the cleavage stage.

Materials and methods

Infertile patients attending the Assisted Reproduction Unit at the Department of Obstetrics and Gynaecology, The University of Hong Kong for FET were recruited if they had two or more frozen embryos available for transfer. Exclusion criteria were: (i) >3 stimulated IVF cycles; (ii) only one frozen embryo available for transfer; and (iii) frozen embryos replaced in stimulated IVF cycles. The indications for IVF included tubal, male, endometriosis, unexplained and mixed factors. Every patient gave a written informed consent prior to participating in the study, which was approved by the Ethics Committee, Faculty of Medicine, the University of Hong Kong. Patients were recruited to join the study once only and did not receive any monetary compensation for participation in the study.

The details of the long protocol of the ovarian stimulation regimen, gamete handling, assessment of embryo quality, ET and FET at our centre have been published previously (Ng *et al.*, 2000).

Stimulated cycles

All women were pre-treated with buserelin (Suprecur, Hoechst, Frankfurt, Germany) nasal spray 150 μg four times a day from the mid-luteal phase of the cycle preceding the treatment cycle and received HMG (Pergonal; Serono, Geneva, Switzerland) for ovarian stimulation. HCG (Profasi; Serono) was given i.m. when the leading follicle reached 18 mm in diameter and there were at least three follicles of ≥ 16 mm in diameter. The serum E_2 concentration was measured on the day of HCG administration. Oocyte retrieval was scheduled 36 h after the HCG injection. AH was not performed in stimulated IVF cycles. Two normally cleaved embryos were replaced into the uterine cavity 48 h after the retrieval and excess good quality embryos were frozen. All fresh embryos were cryopreserved if the serum E_2 on the day of HCG injection was $>20\,000$ pmol/l in order to reduce the risks of ovarian hyperstimulation syndrome.

FET cycles

Patients who did not get pregnant in the stimulated IVF cycle and had frozen embryos underwent FET at least 2 months after the stimulated cycle. Frozen embryos after thawing were transferred in natural, clomiphene-induced or hormonal replacement cycles. Those patients having regular ovulatory cycles underwent FET in their natural cycles. Clomiphene citrate (CC, Clomid, Merrell, Staines, UK) 50–100 mg was given daily for 5 days from days 3 to 7 to patients with irregular/long cycles or absence of E_2 rise/LH surge in previous natural cycles. During natural or clomiphene-induced cycles, patients were monitored daily for serum E_2 and LH levels from 18 days before the expected date of the next period. The transfer was performed on the third day after the LH surge and a maximum of three normally cleaving embryos were replaced. The luteal phase was supported by two doses of HCG injections as in fresh ET.

Hormonal replacement cycles were offered to those patients who showed no ovulatory responses after taking CC 150 mg daily for 5 days. After downregulation by buserelin nasal spray (150 μg four times a day), estrofem (Novo Nordisk, UK) was started on the second day of the next menstrual cycle in an incremental dosage (2 mg daily for 5 days, 4 mg daily for 4 days and then 6 mg daily for 4 days). Estrofem was then reduced to 4 mg daily and cyclogest vaginal pessaries (Cox Pharmaceuticals, Barnstaple, UK) 400 mg twice daily were started if the endometrial thickness measured by ultrasound scanning reached ≥ 8 mm. The transfer was carried out on the fourth day of starting progesterone.

Serum E_2 and LH concentrations were measured using commercially available kits (Automated Chemiluminescence System, Bay Corporation, NY). The sensitivity of the E_2 assay was 36.7 pmol/l and the intra- and inter-assay coefficients of variation were 8.1 and 8.7%, respectively. The sensitivity of the LH assay was 0.07 IU/l and the intra- and inter-assay coefficients of variation were 4.5 and 5.2%, respectively.

Assignment

On the day of FET, patients were randomized before the thawing process by a laboratory technician according to a computer-generated randomization list in sealed envelopes into the AH group or the control group. Until the completion of the whole study, both the patients and the clinicians were blinded to the group assigned.

Laser-assisted hatching and assessment of embryological factors in FET cycles

Embryos were cryopreserved at the cleavage stage 2 days after oocyte retrieval using a programmable freezer (Planer Products Ltd, Sunbury-On-Thames, UK) with 1,2-propanediol as cryoprotectant. The freezing programme for embryos in our unit was as follows:

starting temperature: 20 °C; rate of cooling: 2 °C/min from 20 to −7 °C; soak at −7 °C for 5 min; manual seeding; hold the temperature at −7 °C for 10 min; rate of cooling: 0.3 °C/min from −7 to −30 °C; rate of cooling: 30 °C/min from −30 to −120 °C. The frozen straw was quickly transferred from the freezing chamber to a reservoir of liquid nitrogen.

Frozen embryos were thawed on the day of FET at room temperature for 40 s and then at 30 °C in a water bath for 40 s. Subsequently, the cryoprotectant was removed by washing the embryos successively through phosphate buffers with a decreasing concentration of propanediol and the embryos were cultured in the CO_2 incubator for a short period before transfer. After thawing, frozen embryos were examined for the number/regularity of blastomeres and the degree of fragmentation, and graded according to the criteria of Veeck (1988). Any embryo with half the number of blastomeres or more surviving was transferred.

All thawed embryos suitable for transfer were then videotaped through an inverted microscope (Nikon, Japan) under ×20 magnification. In the AH group, zona thinning was performed by an experienced embryologist (E.Y.L.L. and W.S.B.Y.) using a 1480 nm non-contact laser (Zona Knife, SL Microtest GmbH, Jena, Germany) at a strength of 90 mV for 6 ms. During the operation, the outer half of the zona pellucida over a quarter of the diameter of the zona was removed. In the control group, embryos were replaced without AH.

All embryological parameters of frozen–thawed embryos were evaluated after the completion of the study from the videocinematographic recordings by F.N., who was also blinded to the group assigned. The total number of clearly visible blastomeres and the presence of lysed blastomeres were recorded. The degree of fragmentation was assessed as absent for no fragmentation, minor for <25% fragmentation and major for >25%. The degree of zona clarity was classified as slightly clear, moderately clear or completely clear. The thickness of the zona was then measured at the 3, 6, 9 and 12 0'clock positions from the outside to the inside, and the average thickness was calculated.

Statistical analysis

In our stimulated IVF/ET programme, the average implantation rate was 10.1% in stimulated cycles and 8.4% in FET cycles. The implantation rate of natural IVF cycles performed as a research project was, however, 30.2% (Ng *et al.*, 2001). Assuming that the implantation rate increased from 8.4 to 20.0% in FET cycles after AH, the sample size required would be 158 embryos in each arm to give a test of significance of 0.05 and a power of 0.8 (Sigmastat, Jandel Scientific, USA). The average number of embryos transferred in FET was 2.26 in 2002; 140 subjects were required. In order to account for 10% drop-out, 160 subjects were recruited.

Only clinical pregnancies were considered and are defined by the presence of one or more gestation sacs or the histological confirmation of gestational products in miscarriages. On-going pregnancies were those pregnancies beyond 10–12 weeks of gestation, at which stage the patients were referred for antenatal care. The implantation rate was the proportion of embryos transferred resulting in an intra-uterine gestational sac. The primary outcome measure was implantation rate and the secondary outcome measures included pregnancy rates and multiple pregnancy rates. Subgroup analysis was performed to assess the effect of zona thickness and the age of women at embryo freezing on the implantation rate in the AH group. Continuous variables were not normally distributed and were given as median (range), unless indicated. Statistical comparison was carried out by Mann–Whitney, χ^2 and Fisher's exact tests, where appropriate. A *P*-value (two-tailed) of <0.05 was taken as significant.

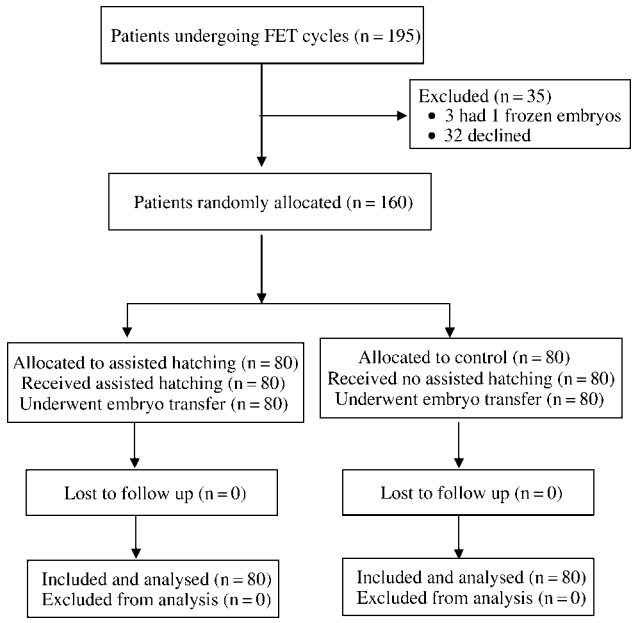

Figure 1. Flow diagram of patients through each stage of the study.

Results

Participant flow

Between May 2003 and May 2004, 195 consecutive patients undergoing FET cycles were approached. Three patients were excluded because they had only one frozen embryo and 32 patients declined to participate in the study. Therefore, a total of 160 patients were recruited and all completed the study (Figure 1).

Analysis

Table I summarizes the demographic data and number of frozen embryos. There were no differences in age of women at embryo freezing/thawing, body mass index, duration of infertility, primary/secondary infertility, cause of infertility, basal serum FSH concentration, cycle number, percentage of smokers or number of embryos frozen between the AH group and the control group. The HMG dosage and duration and number of eggs retrieved/fertilized of the index stimulated cycle were also comparable between the AH group and the control group (data not given).

A total of 410 embryos were thawed and 15 (3.6%) failed to survive during the thawing process. Therefore, 395 embryos were available for transfer. The two groups had comparable blastomere number and grading of fresh embryos, and blastomere number, presence of cell lysis, degree of fragmentation, zona clarity and zona pellucida thickness of frozen embryos (Table II). The number of previous transfers and type of FET were similar between the two groups, while a significantly higher percentage of women in the control group had three embryos replaced (Table III). No differences in implantation rate, pregnancy rate per transfer, multiple pregnancy rate and pregnancy outcome were found between the two groups. One set of triplets including monozygotic twins, and five pairs of twins were encountered in the AH group, while all multiple pregnancies

Table I. Comparison of demographic characteristics and the number of frozen embryos between the assisted hatching group and the control group

	Assisted hatching	Control	*P*-value
Age of women at embryo freezing (years)	34.0 (25–40)	34.0 (26–40)	0.319
Age of women at embryo thawing (years)	35.0 (25–41)	35.0 (27–42)	0.439
Body mass index (kg/m^2)	21.36 (16.03–30.24)	21.35 (16.88–32.03)	0.577
Duration of infertility (years)	5 (1–15)	5 (1–16)	0.736
Primary/secondary infertility[a]	45/35	44/36	0.874
Cause of infertility[a]			0.692
Tuboperitoneal	16 (20.0)	20 (25.0)	
Endometriosis	10 (12.5)	7 (8.7)	
Male	43 (53.8)	43 (53.8)	
Unexplained	9 (11.2)	6 (7.5)	
Mixed	2 (2.5)	4 (5.0)	
Basal FSH (IU/l)	6.0 (3.1–9.2)	5.8 (2.7–10.6)	0.685
Cycle number[a]			0.220
First	55 (68.8)	45 (56.3)	
Second	16 (20.0)	25 (31.3)	
Third	9 (13.2)	10 (12.4)	
Smoker[a]	6/80 (7.5)	4/80 (5.0)	0.514
No. of embryos frozen	5 (2–19)	6 (1–20)	0.167

Data given as median (range).
[a]Given as number.

Table II. Comparison of embryological factors of fresh and frozen–thawed embryos between the assisted hatching group and the control group

	Assisted hatching	Control	*P*-value
Fresh embryos	$n = 195$	$n = 215$	
Blastomere number			0.074
2	46 (23.6)	65 (30.2)	
3	25 (12.8)	15 (7.0)	
4	110 (56.4)	118 (54.9)	
5	13 (6.7)	10 (4.7)	
6	1 (0.5)	6 (2.8)	
7	0 (0)	1 (0.4)	
Grade			0.942
1	34 (17.4)	33 (15.3)	
2	116 (59.5)	129 (60.0)	
3	8 (4.1)	9 (4.2)	
4	37 (19.0)	44 (20.5)	
Frozen–thawed embryos	$n = 188$	$n = 207$	
Blastomere number			0.384
1	8 (4.2)	2 (1.0)	
2	37 (19.7)	44 (21.3)	
3	19 (10.1)	24 (11.5)	
4	106 (56.4)	115 (55.6)	
5	9 (4.8)	10 (4.8)	
6	9 (4.8)	10 (4.8)	
7	0	2 (1.0)	
Presence of cell lysis	27/188 (14.4)	29/207 (14.0)	0.920
Degree of fragmentation			0.606
Absent	10 (5.3)	8 (3.9)	
Minor	153 (81.4)	176 (85.0)	
Major	25 (13.3)	23 (11.1)	
Zona clarity			0.448
Slightly clear	47 (25.0)	59 (28.5)	
Moderately clear	110 (58.5)	108 (52.2)	
Completely clear	31 (16.5)	40 (19.3)	
Zona pellucida thickness[a] (μm)	18.0 (10–27)	18.0 (10–28)	0.093

Data given as number (%).
[a]Given as median (range).

Table III. Comparison of outcomes of FET cycles between the assisted hatching group and the control group

	Assisted hatching	Control	*P*-value
No. of previous transfers	1 (0–9)	2 (0–7)	0.221
Type of FET			0.533
Natural	58 (72.4)	59 (73.8)	
Clomiphene	11 (13.8)	14 (17.5)	
HRT	11 (13.8)	7 (8.7)	
No. of embryos replaced			0.034
One	5 (6.2)	2 (2.5)	
Two	42 (52.5)	29 (36.2)	
Three	33 (41.3)	49 (61.3)	
Implantation rate	17/188 (9.0)	14/207 (6.8)	0.513
Pregnancy rate per transfer	10/80 (12.5)	12/80 (15.0)	0.818
Multiple pregnancy rate	6/10 (60.0)	2/12 (16.7)	0.074
Pregnancy outcome			0.455
First trimester miscarriage	1 (10.0)	0 (0)	
Ongoing pregnancy	9 (90.0)	12 (100)	

Table IV. Implantation rates according to zona thickness between the assisted hatching group and the control group

Zona pellucida thickness	Assisted hatching	Control	*P*-value
≥15 μm	9.3% (14/150)	6.4% (11/172)	0.439
≥16 μm	10.6% (13/123)	6.3% (9/144)	0.291
≥17 μm	11.4% (13/114)	6.5% (8/124)	0.264
≥18 μm	10.6% (11/104)	5.3% (5/89)	0.274

in the control group were twin pregnancies. All twin pregnancies were dizygotic.

Subgroup analysis revealed that the implantation rates were not different between the AH group and the control group when the zona thickness of frozen–thawed embryos was ≥15 mm (Table IV). There was a trend of a higher implantation rate in the AH group when the zona thickness was ≥16 mm, although the difference did not reach statistical significance. When only patients aged ≥38 years were

considered, the implantation rate was similar for the AH group and the controls (data not shown).

Discussion

This prospective randomized double-blind controlled study compared implantation and pregnancy rates of FET cycles at the cleavage stage with and without laser AH. Patients having only one frozen embryo were excluded because the chance of lysis on thawing is ~20–30%. Those who had undergone more than three stimulated IVF cycles were not recruited as they would have a much lower pregnancy rate during FET cycles. Although there is concern about the anti-estrogenic effect of CC on the endometrium, comparable pregnancy rates of FET were obtained in natural or CC-induced cycles in our unit. The two groups were comparable in terms of demographic characteristics, ovarian response of the index stimulated cycle, quality of fresh and frozen–thawed embryos, number of previous transfers and type of FET cycles. Despite the randomization process, a higher percentage of patients in the non-AH group received three frozen–thawed embryos when compared with the AH group. This may account for a slightly higher pregnancy rate in the control group. One pair of monozygotic twins was found in the AH group, but monozygotic twinning may not be associated with zona pellucida micromanipulation procedures (Elizur *et al.*, 2004). The present study failed to show a significant improvement in the implantation rate of FET cycles after laser AH. Our results indicated that laser AH should not be routinely performed in all frozen–thawed embryos at the cleavage stage. The outcomes may be different with blastocyst stage embryos or with embryos that have been thawed at the pronuclear stage and allowed to cleave *in vitro*.

The zona pellucida is the complex glycoprotein matrix that surrounds the mammalian egg and plays important roles in sperm–egg interaction (Prasad *et al.*, 2000). Zona hardening occurs naturally after fertilization in order to block polyspermic fertilization, to protect the integrity of the preimplantation embryo during early embryonic development, and help its oviductal transport. A combination of lysins produced by the cleaving embryo or the uterus and physical expansion of the blastocyst then reduces the zona thickness in preparation for hatching. Zona hardening may also be induced by *in vitro* prolonged exposure of human oocytes and embryos to artificial culture conditions and the freezing–thawing process. The end result will be failure of the embryonic zona pellucida to rupture following blastocyst expansion, which is a prerequisite for implantation.

AH was first pioneered by Cohen *et al.* (1990) and has been proposed as a method for improving the implantation potentials of embryos as the inability of the embryo to hatch out of the zona pellucida may be due to 'increased zona thickness' or 'abnormal zona hardness' (Cohen *et al.*, 1990). Embryos after AH hatch earlier than zonae-intact embryos (Malter and Cohen, 1989; Rink *et al.*, 1995). AH may facilitate implantation by allowing earlier contact and dialogue between the embryo and the endometrium. Such contact may also permit earlier exposure of the embryo to vital growth factors. Al-Nuaim and Jenkins (2002) in a review concluded that the routine use of AH is inappropriate in view of the lack of evidence of universal benefits and the potential risks, especially monozygotic twinning. There is, however, evidence of benefit in defined circumstances such as repeated IVF failures and advanced female age. In a meta-analysis of 23 trials involving 2572 patients, Edi-Osagie *et al.* (2003) found that AH had no significant effect on live birth [odds ratio (OR) 1.21, 95% confidence interval (CI) 0.82–1.78]. There was a significant improvement of AH on clinical pregnancy (OR 1.63, 95% CI 1.27–2.09), especially in the subgroup of women with previous failure of assisted reproduction (OR 2.33, 95% CI 1.63–3.34). Sallam *et al.* (2003) reported similar findings in another meta-analysis involving 13 trials. AH increased the pregnancy, implantation and ongoing pregnancy rates significantly in patients with poor prognosis and repeated IVF failures. However, the overall methodological quality of trials included was considered as suboptimal as no trial met CONSORT criteria for the reporting of randomized controlled trials; no trials reported power calculation, intention-to-treat or adequate allocation concealment (Edi-Osagie *et al.*, 2003).

The freeze–thaw process and the extended *in vitro* culture of frozen–thawed embryos are thought to induce alteration in the glycoprotein matrix, leading to zona hardening (Carroll *et al.*, 1990). Therefore, AH was proposed to improve the implantation rate in FET cycles, but conflicting information exists in the literature with respect to the efficacy of AH in FET cycles. When compared retrospectively with matched historic controls, Check *et al.* (1996) found significantly higher implantation and pregnancy rates of FET cycles following AH by zona drilling using acidic Tyrode's solution. Tao and Tamis (1997) showed a significantly higher pregnancy rate of FET cycles after zona drilling by acidic Tyrode's solution performed in patients with poor prognosis, such as age >38 years, thick zona (≥17 μm), poor embryo quality or multiple embryo transfer failures. Cohen *et al.* (1999) also reported significantly higher implantation and pregnancy rates in FET cycles of donor oocytes after chemical zona drilling. More recently, Gabrielsen *et al.* (2004) in a prospective study showed that AH using acidic Tyrode's solution increased the implantation rate of frozen–thawed embryos. The implantation rate in the AH group was 11.4% while that in the control group was 5.8% only. On the contrary, Edirisinghe *et al.* (1999) did not show any improvement in the outcome of FET cycles after AH by partial zona dissection. AH tended to give poorer results in women ≥38 years old than in controls. Primi *et al.* (2004) in another prospective study evaluated the benefits of laser AH and immunosuppressive/antibiotic treatment and demonstrated that patients undergoing the first or third FET cycle did not benefit from AH. Significantly lower implantation and pregnancy rates were obtained in those patients receiving laser-drilled embryos and no immunosuppressive/antibiotic treatment.

To the best of our knowledge, this is the first randomized double-blind controlled study comparing the implantation rate of FET cycles with and without laser AH. The studies of Check *et al.* (1996) and Cohen *et al.* (1999) were

retrospective in nature and matched historic controls were used. Edirisinghe *et al.* (1999) performed AH on embryos of patients aged ≥38 years with zonal thickness ≥15 μm or previous failures of FET. Moreover, the number of FET cycles with AH ($n = 37$) was too small to draw any valid conclusion. Pseudo-randomization based on even or odd dates for thawing was used in the study of Gabrielsen *et al.* (2004). There were no power calculation and blinding of the AH procedure in the above studies. The study of Primi *et al.* (2004) was interrupted prematurely due to recruitment difficulties and would be under-powered to make a valid conclusion. We also compared the quality of frozen–thawed embryos in the present study. Although our results indicated that laser AH should not be routinely performed in all frozen–thawed embryos, subgroup analysis showed a non-significant trend of a higher implantation rate when AH was performed in embryos with zona thickness ≥16 mm. This finding supported that of Cohen *et al.* (1992). We could not find any difference in the implantation rate in women aged ≥38 years between the AH and non-AH groups, but the number (18 in the AH group and 11 in the non-AH group) was too small to draw any firm conclusion.

In addition to differences in the study design, patient characteristics and selection criteria, conflicting results of the above studies in FET cycles may also be related to different AH techniques. Zona drilling by acidic Tyrode's solution was used by Check *et al.* (1996), Cohen *et al.* (1999) and Gabrielsen *et al.* (2004). Edirisinghe *et al.* (1999) applied partial zona dissection, and Primi *et al.* (2004) used laser AH. Unfortunately, there is still no consensus on which technique of AH is associated with a higher implantation rate. Hsieh *et al.* (2002) compared laser AH with chemical zona drilling and reported a significant increase in implantation, pregnancy and delivery rates in the laser AH group compared with that in the chemical zona drilling group. This finding could not be confirmed by Balaban *et al.* (2002), who found similar implantation and pregnancy rates for four different techniques of AH including acidic Tyrode's solution, partial zona dissection, diode laser and pronase zona thinning. We chose laser thinning in the present study because this technique has been shown to increase significantly the hatching rate and the proportion of blastocysts with complete hatching (Blake *et al.*, 2001). However, proper comparison between different techniques can only be made by conducting prospective randomized studies.

In conclusion, laser AH by zona thinning did not improve the implantation rate of FET cycles and should not be routinely performed in all frozen–thawed embryos at the cleavage stage. Subgroup analysis suggested that frozen–thawed embryos with zona thickness ≥16 μm may benefit from laser AH. Further studies are needed to assess whether laser AH may improve the pregnancy rates when the zona thickness is ≥16 μm.

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