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Assisted hatching improves implantation rates on cryopreserved–thawed embryos. A randomized prospective study

A.Gabrielsen[1,3], I.Agerholm[2], B.Toft[2], F.Hald[2], K.Petersen[1], J.Aagaard[1], B.Feldinger[2], S.Lindenberg[1] and J.Fedder[2]

[1]Ciconia Fertility Clinic, Aarhus, DK-8270 Højbjerg and [2]The Fertility Clinic, Braedstrup Hospital, DK-8740 Brædstrup, Denmark

[3]To whom correspondence should be addressed. E-mail: ag@ciconia.dk

BACKGROUND: Focus on the hatching process has so far been in the field of fresh embryos. Cryopreserved–thawed embryos have a lower rate of pregnancy than fresh embryos, which might be due to hardening of the zona pellucida. METHODS: During a 2 year period, a prospective randomized study enrolling 253 cryopreserved–thawed cycles was performed on day 2 embryos. Pseudorandomization to assisted hatching or a control group was done on the basis of even and odd dates for thawing. One hour before embryo transfer, hatching was carried out using acidic Tyrode's solution. RESULTS: Among 136 embryos exposed to assisted hatching, 11.4% (30) were implanted compared with only 5.8% (13) of 117 embryos not exposed to assisted hatching ($P < 0.05$, χ^2 test). No difference in the rate of clinical pregnancy and positive serum HCG was observed between the two groups. Very few women >38 years old were included in the study, and no significant difference according to age could be found between the groups. CONCLUSIONS: These results show that assisted hatching using acidic Tyrode's solution increases the implantation rate of cryopreserved–thawed embryos ($P < 0.05$).

Key words: acidic Tyrode's solution/assisted hatching/cryospreserved–thawed/implantation rates/pregnancy

Introduction

The zona hatching process is of importance for the implantation of the embryo in the uterus. The main factors affecting implantation in IVF are quality of the embryo (Ziebe *et al.*, 1997), the endometrium (Ewards *et al.*, 1984) and factors associated with impairment of the zona hatching process (Cohen *et al.*, 1992). Assisted zona hatching was introduced in IVF programmes to breach the zona pellucida and promote the natural process of hatching (Cohen *et al.*, 1992). Zona hatching can be done by mechanical (Cohen *et al.*, 1990), chemical (Lanzendorf *et al.*, 1998) or laser manipulation (Antinori *et al.*, 1996). Presumed indications of assisted hatching can be: age factor, high basal FSH level, thick zona pellucida and several failed IVF treatments (Cohen *et al.*, 1990, 1992).

Generally, cryopreserved–thawed embryos have a lower rate of pregnancy than fresh embryos (Tucker *et al.*, 1991), despite the fact that the embryos often resume mitosis and are morphologically of high quality. The cryopreservation–thawing procedure may impact on embryos by hardening of the zona pellucida (Cohen *et al.*, 1990). Focus on the hatching process has so far been in the field of fresh embryos, and to our knowledge no prospective, randomized studies of the effect of assisted hatching on the implantation rate of frozen–thawed embryos exist. Edirisinghe *et al.* (1999) found in a non-randomized study no significant effect of mechanical hatching on either embryos hatched on day 3 or frozen–thawed embryos. Kung *et al.* (2003) retrospectively examined the pregnancy potential of frozen–thawed blastocysts, which had been quarter laser-assisted hatched on day 3 before freezing. Mantoudis *et al.* (2001) evaluated the effect of the different types of assisted hatching on four different categories of patients including patients undergoing frozen embryo replacement cycles but, since the study focused on the hatching types more than the categories, it is not conclusive with regard to the effect of assisted hatching on the implantation of frozen–thawed embryos. The objective of this study was to assess the possible impact of assisted hatching on the implantation and development of cryopreserved and thawed human embryo.

Materials and methods

Patient selection and randomization procedure

A prospective randomized study comprising 253 cryopreserved–thawed cycles was performed. All IVF/ICSI couples with frozen embryos were asked to participate in the study. The Regional Ethical Committee approved the protocol. All patients signed a consent form before enrolling in the study.

Pseudorandomization to assisted hatching or a control group was performed based on even and odd dates for thawing, and was only known by the laboratory technicians.

The clinics

The laboratory technicians from both clinics have all been trained in the assisted hatching procedure by the same person, and clinical and laboratory procedures were basically the same.

Embryo culture and freezing procedure

IVF/ICSI was performed according to the routine protocols of each clinic. The oocytes were aspirated 34–36 h after HCG injection and inseminated another 2–6 h later (Gabrielson *et al.*, 1996). On the following day, the oocytes were examined for fertilization and cultured for another 24 h. Embryo quality was evaluated (Ziebe *et al.*, 2003); one or two embryos of the best grade were transferred.

All surplus embryos with ≤20% fragmentation were cryopreserved. The embryos were frozen in 0.5 ml staws. The freezing process was performed in a Freeze Control CL-863 (CryoLogic Pty Ltd, Australia). Cooling was controlled at a rate of −2°C/min from room temperature to −6°C. Manual seeding with pre-cooled forceps was done at −6°C. After seeding, the temperature was lowered to −32°C at the rate of −0.3°C/min and then down to −150°C at the rate of −50°C/min. The straws were then transferred to liquid nitrogen for storage.

Replacement cycles

Two different protocols have been used. In the first protocol, estradiol (Fermanest 2 mg × 3, AstraZeneca AB, Södertälje, Sweden) was given from the second menstruation day, and the endometrium was evaluated by ultrasonography from day 12. Vaginal capsules of progesterone (Progestan 100 mg × 4, Besins-Iscovesco, Paris, France) were given as supplement from day 13, if the endometrium was at least 8 mm. If the endometrium was thinner than 8 mm on day 12, estradiol was given until an 8 mm endometrium was obtained. Then progesterone was added for 3 days before the thawed embryos were transferred. The embryos were thawed the day before the transfer was planned. The treatment with estradiol and progesterone was continued at the same doses until serum HCG (s-HCG) was determined 2 weeks later and, if pregnancy was acheived, estradiol and progesterone were given for a further 3 weeks.

In the second protocol, the transfers were done in the natural cycles of the women. When the dominant follicle was 18 mm or the urinary LH peak occurred, 10 000 IU of HCG was administered. Three days after HCG administration, the embryos were thawed (see below) and the embryos transferred 24 h later, such that the embryos were transferred 4 days after HCG administration.

Thawing procedure

The straws were taken out of liquid nitrogen and thawed according to the protocol of Lassalle *et al.* (1985). Briefly, a four-step thawing protocol was used to remove the cryoprotectant and the thawed embryos were cultured in IVF medium (Medi-Cult A/S, Jyllinge, Denmark) for 24 h. Embryo quality was evaluated (Ziebe *et al.*, 2003), and assisted hatching was performed according to the protocol. At least 1 h after assisted hatching, the transfer was performed. The number of embryos transferred varied from one to three. All transfers were performed gently 1 day after thawing using a Cook or an Edwards-Wallace Embryo Replacement Catheter.

Assisted hatching procedure

The holding pipette (0.018–0.025 mm) and assisted hatching pipette (0.015–0.020 mm) were obtained from SweMed (SweMed Lab, International AB Sweden). Control of delivery of acidified Tyrode's medium (Medi-cult) against the zona pellucida was provided by a picoinjector (Narishige, DFA Instruments, Denmark). Two micromanipulators (Narishige) were used in conjunction with a heated stage (37°C) on a Nikon Diaphot inverted microscope, with Hoffmann modulation contrast (Nikon, DFA Instruments, Denmark).

For the procedure, a Falcon 1006 dish (Becton Dickinson, Denmark) was prepared by adding 50 μl microdroplets of IVF medium (Medi-cult), one for each embryo to be hatched, a droplet of Tyrode's medium was placed in the dish and the entire dish were covered by light mineral oil (Medi-cult).

The embryos were added to the microdroplets of IVF medium (Figure 1a) and the dish was placed on the heated stage. By suction,

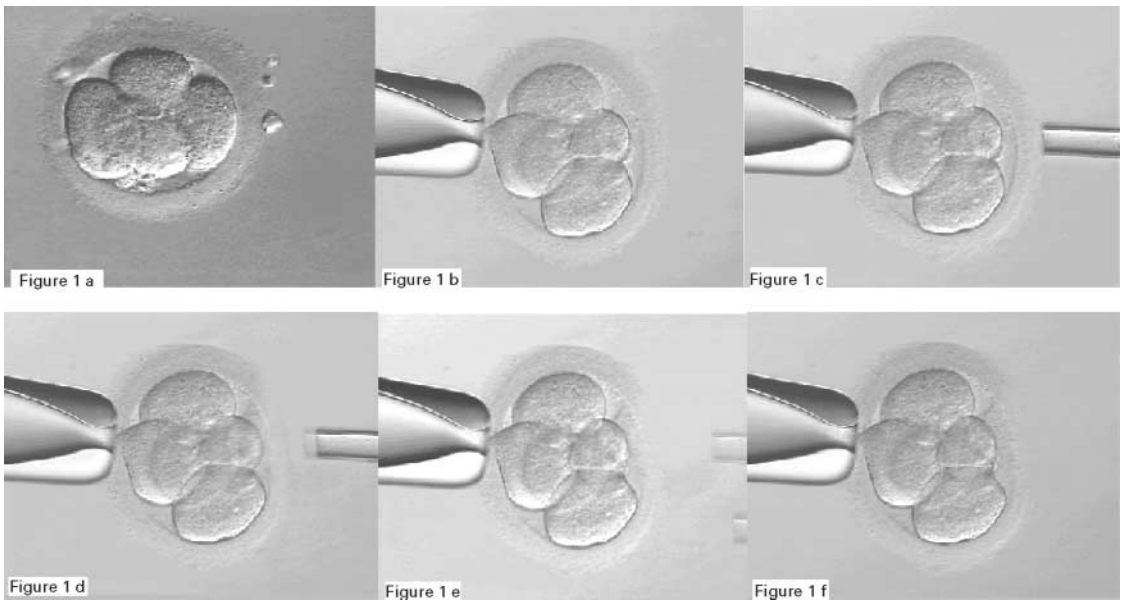

Figure 1. The assisted hatching procedure.

the embryo was firmly attached to the holding pipette, showing a blastomere-free area, which was toward the 3 o'clock position (Figure 1b). The acidified Tyrode's medium was released from the hatching pipette (Figure 1c) and an ~30 μm diameter breach through the zona pellucida was created (Figure 1d). The hatched embryo was then moved to another area of the droplet (Figure 1f) and released, and the procedure was repeated until all embryos were hatched. The embryos were then transferred to the culture medium and stored until transfer.

Statistical evaluation

Patient age was compared between the hatched and non-hatched groups using the two-sample *t*-test. The primary outcome measure was the implantation rate. Positive s-HCG, clinical pregnancy and implantation rates were analysed using the χ^2-test with Yates correction. A *P*-value of <0.05 was considered statistically significant.

Results

The data were collected from March 2002 to December 2003. During this period, 355 IVF/ICSI cycles had frozen–thawed embryos. Fifty of the cycles were not included in the study because the couples did not want to participate. A total of 305 cycles were included in the study. As a result of the pseudorandomization, 165 cycles were allocated to assisted hatching and 140 were allocated to the control group. Due to the thawing procedure, 29 cycles were excluded from the assisted hatching group and 23 cycles from the control group (Figure 2).

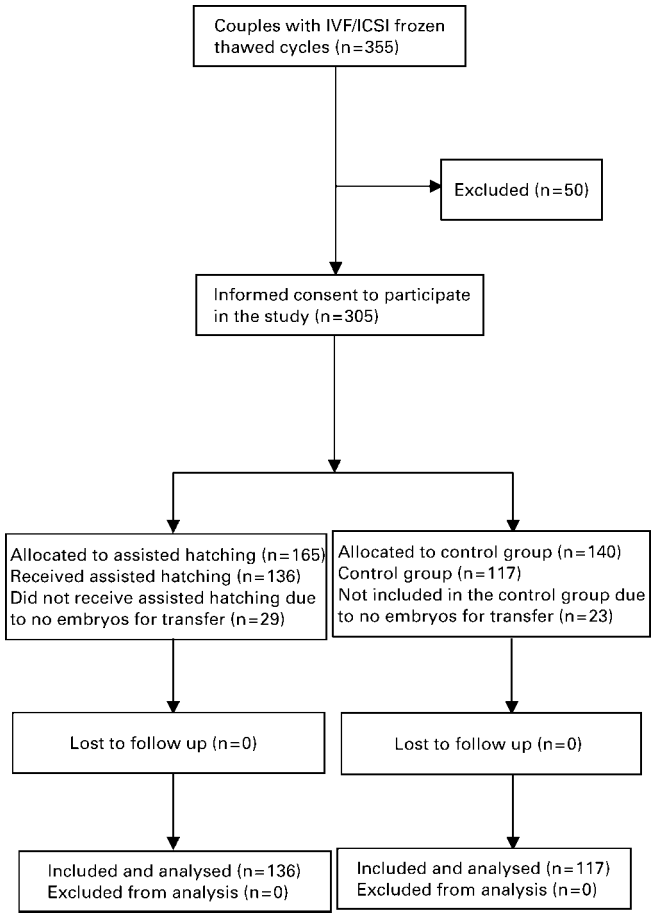

Figure 2. Flow diagram.

We compared assisted hatching performed on 253 embryos from 136 transfer cycles with a control group of 223 embryos from 117 transfer cycles. The embryos in the control group were not undergoing assisted hatching. No embryos were damaged due to the assisted hatching procedure. The ages of the women given correspond to the time of oocyte aspiration. The mean age of both groups was similar (33.1 versus 32.8 years). There was no difference in the number of previous IVF cycles, number of freezing–thawing cycles or number of previous IVF cycles resulting in a clinical pregnancy in the two groups (data not shown). The mean numbers ± SDs of embryos transferred were 1.94 ± 0.63 and 1.91 ± 0.71 for the assisted hatching and the control group, respectively. As shown in Table I, no difference in the rate of positive s-HCG was observed between the two groups (32 versus 33).

In the assisted hatching group, 264 embryos were transferred and 30 (11.4%) implanted. Six of the pregnancies had two implantations resulting in two sacs. There were 223 control embryos transferred and 13 (5.8%) implanted. In this group, no pregnancies had two sacs. The implantation rate was significantly higher in the assisted hatching group compared with the control group ($P < 0.05$).

Very few women >38 years old were included in the study, and no significant association according to age could be found (Table II).

Discussion

Pregnancy rates are generally lower after transfer of frozen–thawed embryos compared with fresh embryos, even if the quality is equal according to the number and size of blastomeres and degree of fragmentation. It has not been determined yet what the main reason for this impaired embryo development is. However, one reason might be changes in the zona pellucida, making it harder.

Table I. Results of Tyrode-assisted hatching on cryopreserved–thawed embryos

	Assisted hatching	Control	*P*-value
No. of embryo transfer cycles	136	117	
Patient age (mean ± SD)	33.1 ± 4.2	32.8 ± 4.1	NS**
No. of thawed embryos transferred			
1	31 (22.8%)	35 (29.9%)	
2	82 (60.3%)	58 (49.6%)	
3	23 (16.9%)	24 (20.5%)	
Total no. transferred	264	223	
Mean no. transferred	1.94 ± 0.63	1.91 ± 0.71	
No. of positive s-HCG[a]	32 (23.5%)	33 (28.2%)	NS*
No. of clinical pregnancies[b]	24 (17.6%)	13 (11.1%)	NS*
Implantation[c]	30 (11.4%)[d]	13 (5.8%)[e]	<0.05*

[a]Number of HCG divided by embryo transfer cycles.
[b]Number of sacs divided by embryo transfer cycles.
[c]Number of sacs divided by total number of embryos transferred.
[d]Eighteen singleton and six twin.
[e]Thirteen singleton.
*χ^2 test with Yates correction.
**Two-sample *t*-test.

Table II. Results of Tyrode-assisted hatching on cryopreserved–thawed embryos distributed by age

	<38 years			≥38 years		
	Assisted hatching	Control	*P*-value	Assisted hatching	Control	*P*-value
No. of embryo transfer cycles	115	98		21	19	
Patient age (mean ± SD)	31.9 ± 3.4	31.6 ± 3.3	NS**	39.3 ± 1.4	39.2 ± 1.3	NS**
No. of thawed embryos transferred						
1	25 (21.7%)	30 (30.6%)		6 (28.6%)	5 (26.3%)	
2	70 (60.9%)	49 (50.0%)		12 (57.6%)	9 (47.4%)	
3	20 (17.4%)	19 (19.4%)		3 (14.3%)	5 (26.3%)	
Total no. transferred	225	185		39	38	
Mean no. transferred	1.96 ± 0.63	1.89 ± 0.70		1.86 ± 0.65	2.00 ± 0.75	
No. of positive s-HCG[a]	27 (23.5%)	28 (28.6%)	NS*	5 (23.8%)	5 (26.3%)	NS*
No. of clinical pregnancies[b]	21 (18.3%)	12 (12.2%)	NS*	3 (14.3%)	1 (5.3%)	NS*
Implantation[c]	25 (11.1%)	12 (6.5%)	NS*	5 (12.8%)	1 (2.6%)	–[d]

[a] Number of HCG divided by embryo transfer cycles.
[b] Number of sacs divided by embryo transfer cycles.
[c] Number of sacs divided by total number of embryos transferred.
[d] Patient numbers too small.
*χ^2 test with Yates correction.
**Two-sample *t*-test.

Cohen *et al.* (1990) reported that reduced implantation in IVF-ET may be due to the inability of embryos to hatch out of the zona pellucida. For both fresh and cryopreserved–thawed embryos, impaired hatching may be due to the extended time in culture in an artificial environment causing a hardening of the zona pellucida (Cohen *et al.*, 1990). Furthermore, for cryopreserved–thawed embryos, the freeze–thaw process might exacerbate hardening of the zona pellucida (Tucker *et al.*, 1991).

Our study showed that the implantation rate was significantly higher in the group where thawed embryos were undergoing assisted hatching before transfer, compared with the control group. The results were very similar to the results obtained by Check *et al.* (1996), but, in contrast to their study, our study was performed prospectively, randomized and blindly, and it included about four times as many patients. Edirisinhge *et al.* (1999) found, on the contrary, a negative effect of mechanically assisted hatching on frozen–thawed embryos, but the discrepancy with our study could be the difference in the type of assisted hatching. Others have examined the effect of laser-assisted hatching on day 3 embryos frozen and thawed as blastocysts, and found no impact on the zona pellucida after thawing (Kung *et al.*, 2003).

From the studies concerning assisted hatching of fresh embryos, the strongest effect might be suggested for women >38 years old (Antinori *et al.*, 1996; Magli *et al.*, 1998). However, this study did not allow clear conclusions for this age group due to the inclusion of only a modest number of women >38 years old.

To re-examine the results according to the effects of assisted hatching on cryopreserved–thawed embryos, a prospective multicentre trial will be considered in the near future.

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