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Original Article

Infertility

A multicentre double-blinded randomized controlled trial on the efficacy of laser-assisted hatching in patients with repeated implantation failure undergoing IVF or ICSI

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ABSTRACT

STUDY QUESTION: Does assisted hatching increase the cumulative live birth rate in subfertile couples with repeated implantation failure?

SUMMARY ANSWER: This study showed no evidence of effect for assisted hatching as an add-on in subfertile couples with repeated implantation failure.

WHAT IS KNOWN ALREADY: The efficacy of assisted hatching, with regard to the live birth rate has not been convincingly demonstrated in randomized trials nor meta-analyses. It is suggested though that especially poor prognosis women, e.g. women with repeated implantation failure, might benefit most from assisted hatching.

STUDY DESIGN, SIZE, DURATION: The study was designed as a double-blinded, multicentre randomized controlled superiority trial. In order to demonstrate a statistically significant absolute increase in live birth rate of 10% after assisted hatching, 294 participants needed to be included per treatment arm, being a total of 588 subfertile couples. Participants were included and randomized from November 2012 until November 2017, 297 were allocated to the assisted hatching arm of the study and 295 to the control arm. Block randomization in blocks of 20 participants was applied and randomization was concealed from participants, treating physicians, and laboratory staff involved in the embryo transfer procedure. Ovarian hyperstimulation, oocyte retrieval, laboratory procedures, embryo selection for transfer and cryopreservation, the transfer itself, and luteal support were performed according to local protocols and were identical in both the intervention and control arm of the study with the exception of the assisted hatching procedure which was only performed in the intervention group. The laboratory staff performing the assisted hatching procedure was not involved in the embryo transfer itself.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Participants were eligible for inclusion in the study after having had either at least two consecutive fresh IVF or ICSI embryo transfers, including the transfer of frozen and thawed embryos originating from those fresh cycles, and which did not result in a pregnancy or as having had at least one fresh IVF or ICSI transfer and at least two frozen embryo transfers with embryos originating from that fresh cycle which did not result in a pregnancy. The study was performed at the laboratory sites of three tertiary referral hospitals and two university medical centres in the Netherlands.

MAIN RESULTS AND THE ROLE OF CHANCE: The cumulative live birth rate per started cycle, including the transfer of fresh and subsequent frozen/thawed embryos if applicable, resulted in 77 live births in the assisted hatching group (n = 297, 25.9%) and 68 live births in the control group (n = 295, 23.1%). This proved to be statistically not significantly different (relative risk: 1.125, 95% CI: 0.847 to 1.494, P = 0.416).

LIMITATIONS, REASONS FOR CAUTION: There was a small cohort of subfertile couples that after not achieving an ongoing pregnancy, still had cryopreserved embryos in storage at the endpoint of the trial, i.e. 1 year after the last randomization. It cannot be excluded that the future transfer of these frozen/thawed embryos increases the cumulative live birth rate in either or both study arms. Next, at the start of this study, there was no international consensus on the definition of repeated

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implantation failure. Therefore, it cannot be excluded that assisted hatching might be effective in higher order repeated implantation failures.

WIDER IMPLICATIONS OF THE FINDINGS: This study demonstrated no evidence of a statistically significant effect for assisted hatching by increasing live birth rates in subfertile couples with repeated implantation failure, i.e. the couples which, based on meta-analyses, are suggested to benefit most from assisted hatching. It is therefore suggested that assisted hatching should only be offered if information on the absence of evidence of effect is provided, at no extra costs and preferably only in the setting of a clinical trial taking cost-effectiveness into account.

STUDY FUNDING/COMPETING INTEREST(S): None.

TRIAL REGISTRATION NUMBER: Netherlands Trial Register (NTR 3387, NL 3235, https://www.clinicaltrialregister.nl/nl/trial/26138).

TRIAL REGISTRATION DATE: 6 April 2012

DATE OF FIRST PATIENT'S ENROLMENT: 28 November 2012

Keywords: assisted hatching / assisted reproductive technologies / IVF / ICSI / randomized controlled trial / RCT

Introduction

A 1988 Lancet paper reported on two pregnancies after a new technique was applied, which was designed to facilitate sperm penetration by partially opening the human zona pellucida (Cohen *et al.*, 1988). Later, the same authors reported that when this technique, referred to as assisted hatching, was applied, improved hatching from the zona pellucida was observed, and increased implantation rates were reported (Cohen *et al.*, 1990). As reviewed by several authors, it is postulated that increased zona thickness and abnormal zona hardening, female age, suboptimal laboratory culture conditions, embryo cryopreservation, smoking, and cause of infertility, among others, might result in delayed *in vivo* hatching from the zona pellucida beyond the optimal endometrial receptivity environment. Assisted hatching might optimize the timing of hatching resulting in implantation in the optimal endometrial receptivity window thus improving pregnancy rates (Al-Nuaim and Jenkins, 2002; Martins *et al.*, 2011; Carney *et al.*, 2012; Lacey *et al.*, 2021).

Given the limited success rates of assisted reproduction and the major disease burden of subfertile couples with an unfulfilled child-wish, it is easily conceivable that they are often desperate and willing to try add-on treatments that healthcare providers offer. Healthcare providers therefore have the moral obligation only to offer add-on treatments that have been proven effective and safe. Nonetheless, multiple add-on treatments are offered without conclusive evidence on their efficacy (Glatthorn and Decherney, 2022). Up until now, the British Human Fertilisation & Embryology Authority considers it not proven that assisted hatching is effective and therefore that it should only be applied in a research setting at no additional costs (https://www.hfea.gov.uk/treatments/treatment-add-ons/assisted-hatching, accessed 2 March 2023). The American Society for Reproductive Medicine states that assisted hatching should not be recommended to subfertile couples undergoing IVF because of insufficient evidence on its efficacy (ASRM, 2022). The Dutch National Health Care Institute decided that assisted hatching should not be reimbursed as an add-on treatment (https://www.zorginstituutnederland.nl/publicaties/brief/2018/06/06/assisted-hatching-is-geen-te-verzekeren-prestatie-ingevolge-de-zorgverzekeringswet, accessed 2 March 2023). Although there is no worldwide registry of application of assisted hatching, it can be assumed that it is offered by many centres as an add-on treatment. The offering of assisted hatching was reported in 24 of 72 IVF clinic websites in the UK at a pricing between £160 and £600 (Van De Wiel *et al.*, 2020). Based on the Japan Assisted Reproductive Technology Registry System, it was reported that in 2010 in over 100 000 embryo transfer cycles performed, assisted hatching was applied in 43% of the cycles (Nakasuji *et al.*, 2014).

Two independent systematic reviews and meta-analyses reported on assisted hatching and both concluded that there is heterogeneous evidence that assisted hatching results in an increase in pregnancy rates, especially in poor prognosis subfertile couples with advanced maternal age or repeated implantation failure in particular. However, with regard to the golden standard, the cumulative live birth rate on an intention to treat basis, there is still insufficient evidence (Martins *et al.*, 2011; Lacey *et al.*, 2021). In the most recent Cochrane systematic review, the quality of the available evidence was scored as very low to low with a serious risk of bias, among which poor reporting of study methods and publication bias was observed (Lacey *et al.*, 2021). In order to fill this knowledge gap, we conducted a multicentre, randomized, and double-blinded trial in subfertile couples with repeated implantation failure undergoing regular IVF or ICSI. The primary outcome measure was the cumulative live birth rate per started cycle after fresh embryo transfer in one IVF or ICSI cycle and subsequent frozen and thawed embryo transfers from that cycle.

Materials and methods

Subfertile couples eligible for participation in this study were included from November 2012 until November 2017. They were informed both verbally and by means of printed leaflets and written informed consent was given before start of ovarian hyperstimulation and they were randomized to either the intervention or the control arm of the study. The study was performed at the laboratory sites of three tertiary referral hospitals and two university medical centres geographically spread throughout the Netherlands.

The study protocol and all printed information material were approved by the Dutch Central Committee on Research Involving Human Subjects (CCMO NL36590.000.12), the study protocol was prospectively registered in the Netherlands Trial Register (NTR 3387, NL 3235, https://www.clinicaltrialregister.nl/nl/trial/26138, accessed 2 March 2023) and participating centres started recruiting after local feasibility was confirmed. A site initiation visit in each of the participating centres was mandatory before the onset of inclusion and randomization in order to ensure that the study procedures, especially the assisted hatching procedures, were standardized.

Only subfertile couples with a regular indication for IVF or ICSI according to Dutch guidelines, with a female age between 18 and 42 and with repeated implantation failure, were eligible for inclusion. In this study, repeated implantation failure was defined as not having achieved a pregnancy after one of the following conditions was met: (i) by having had at least two consecutive fresh IVF or ICSI embryo transfers, regardless of the number of

frozen embryo transfers or (ii) by having had at least one fresh IVF or ICSI transfer and at least two frozen embryo transfers with embryos originating from that fresh cycle. This effectively excluded participants with a uterine and/or oocyte factor. Other exclusion criteria were having no indication or a contraindication for IVF or ICSI, being unable or not willing to provide informed consent, being unable to speak or read the Dutch language, having a medical contraindication for pregnancy or child birth or having a positive serology for HIV (in the case of IVF and ICSI) and Hepatitis B or C (ICSI). Embryo quality in previous cycles was not regarded as a criterion to determine if a participant was eligible for inclusion.

For fresh cleavage stage embryo transfer cycles, ovarian hyperstimulation, oocyte retrieval, laboratory procedures, embryo selection for transfer and cryopreservation, the transfer itself, and luteal support were performed according to local protocols and were identical in both the intervention and control arm of the study with the exception of the assisted hatching procedure which was only performed in the intervention group. Fresh embryo transfer was performed in the cleavage stage 3 days after ovum pick up, supernumerary embryos were, according to local protocols, cryopreserved 3 or 4 days after ovum pick up in the cleavage or morula stage and transfer of frozen and thawed embryos took place 1 day following thawing in the cleavage, morula, or blastocyst stage.

Block randomization in blocks of 20 participants was applied using Research Manager (Cloud9 Software, The Netherlands) which is fully GCP compliant. Research Manager also served as an electronic case report form for the entry of clinical and laboratory data. Research Manager was password protected and could only be accessed by authorized research personnel, i.e. the staff responsible for inclusion, randomization and entering data in the electronic case report form (eCRF). The code to the study procedure, i.e. intervention or control, was only known to the research staff involved in performing the procedure. Treatment allocation was blinded to subfertile couples, treating physicians and physicians performing the embryo transfer, technicians assisting in the embryo transfer procedure and the statistician performing the statistical analysis. Treatment allocation was only revealed to participants, physicians, laboratory staff, and statistician after statistical analysis was completed.

No sooner than 1 h before embryo transfer, the embryo or embryos destined for transfer, whether fresh or post thawing in the case of frozen embryos, underwent the study procedure. In all embryos selected for transfer in the intervention arm, laser-assisted hatching was performed. Using either a ZILOS-tk (Zona Infrared Laser Optical System, Hamilton Thorne Biosciences Inc., USA) or an OCTAX laser Shot (MTG Medical Technology Vertriebs GmbH, Germany), one-eighth of the circumference of the zona pellucida was breached in case there was sufficient perivitelline space available, or thinned in the case there was insufficient perivitelline space available. Lasers were used in adherence to the instructions for use supplied by the manufacturer in order to minimize the risk of thermal damage to the blastomeres. The ZILOS-tk Laser Shot, an infrared solid state laser diode emitting at a wavelength of 1.46 μm, was used with a power in focus of 140 mW and a pulse length of 0.2–0.4 ms. The OCTAX Laser Shot, an infrared laser diode system emitting at a wavelength of 1.48 μm, was used with a power in focus of 100–150 mW and a pulse length of 3–6 ms.

In the absence of menses, a urinary pregnancy test was performed 14 to 15 days following embryo transfer. Clinical and ongoing pregnancies were confirmed by ultrasound at 8 and 12 weeks of gestational age, respectively. In the case of an ongoing pregnancy, participants received a questionnaire to follow-up on pregnancy and child birth. If no pregnancy and live birth was achieved, participants were eligible for frozen embryo transfers, if applicable. Per subfertile couple, treatment allocation was maintained throughout the entire study period, including all frozen embryo cycles.

Participation in the study started with the onset of ovarian hyperstimulation and ended: (i) with treatment cancellation before oocyte retrieval, (ii) if no embryo transfer was performed (e.g. total fertilization failure) and no embryos were cryopreserved, (iii) if no live birth was achieved and no frozen embryos remained, (iv) if a live birth was achieved, and (v) 1 year after the end of the inclusion period, regardless of whether there were still cryopreserved embryos present.

The primary outcome measure was the cumulative live birth rate per started treatment cycle, including the transfer of frozen and thawed embryos. Live birth is defined as the birth of at least one live born neonate beyond 24 weeks of gestational age.

Secondary outcome measures were the pregnancy rate (cumulative and fresh transfer), ongoing pregnancy rate (cumulative and fresh transfer), and live birth rate (fresh transfer only) per started cycle, per oocyte retrieval, and per embryo transfer. Pregnancy was defined as by ultrasound confirmed foetal heartbeat at 8 weeks gestational age, ongoing pregnancy was defined as by ultrasound confirmed foetal heartbeat at 12 weeks gestational age.

Other secondary outcome measures were the miscarriage rate where a miscarriage was defined as the loss of a intrauterine pregnancy prior to 12 weeks of gestation, the monozygotic twinning rate, which is suspected to be a complication of the assisted hatching technique (Martins *et al.*, 2011; Carney *et al.*, 2012; Hviid *et al.*, 2018) and the incidence of major and minor malformations in the children born as assessed at birth.

Sample size calculation

The required sample size in this superiority trial was calculated using SamplePower 2.0 (SPSS Inc., Chicago, IL, USA). Analysis was based on a two-sample proportion (pregnant/non-pregnant), with an alpha of 0.05 (two-tailed) and a beta of 20%. An absolute effect size of 10% was considered clinically relevant and was in line with the reported effect in the previously mentioned meta-analyses (Martins *et al.*, 2011; Carney *et al.*, 2012). Since robust data on live birth rate per started cycle are not available, the ongoing pregnancy rate was used as a proxy for the live birth rate. The average ongoing pregnancy rate per started cycle in The Netherlands in 2009 and in 2010 was 20% (Smeenk, 2015), thereby taking dropout as a result of, for instance, cancellation of stimulation before oocyte retrieval and total fertilization failure into account. It was calculated that in order to demonstrate a statistically significant increase in live birth rate after assisted hatching from 20% to 30%, 294 participants needed to be included per treatment arm, being a total of 588 subfertile couples.

Statistical analysis

Descriptive statistics were used to examine the differences between the intervention and the control group. The variables included age of the female participant and partner, primary or secondary subfertility, duration of subfertility, medical indication for treatment, female participant BMI, type of treatment (IVF or ICSI), number of retrieved oocytes, and number of embryos being transferred. The normality of data was visualized using the Q–Q plot and tested using the Kolmogorov–Smirnov and the

Shapiro–Wilk normality test. Continuous variables were described as mean and SD when a normal distribution was confirmed or as median and interquartile range when a normal distribution was not confirmed.

Univariate analyses were performed to examine the difference in live birth rate between the intervention and control group. To determine whether laser-assisted hatching increases the secondary outcomes, stratified analyses were performed per treatment cycle started (N = 592), per oocyte retrieval performed (N = 559), and per embryo transfer (N = 535). Differences between the intervention and control group were compared using χ^2 statistics or the Fisher exact test for categorical variables and the independent *T* or Mann–Whitney *U* tests for continuous variables. All tests were two-sided and $P < 0.05$ was considered statistically significant. IBM SPSS Statistics for Windows version 24.0 was used for the analysis. Because of the low number of missing data and since the missing data were unrelated to any information in the dataset, no imputation or adjustments for missing data were performed.

Results

In total, 600 subfertile couples were included and randomized. The flow of participants throughout the study is shown in Fig. 1. A total of 8 participants were excluded after randomization due to protocol violations such as not meeting the inclusion criteria or double entry into the study database resulting in 297 subfertile couples being allocated to the assisted hatching group and 295 to the control group. Follow-up until an endpoint of the trial was complete for nearly all participants, in the assisted hatching group, one subfertile couple was lost to follow-up after the transfer of a frozen/thawed embryo, in the control group, one ongoing pregnancy was lost to follow-up.

Baseline characteristics are shown in Table 1. No statistically significant differences were found in any of the parameters analysed thus demonstrating that both groups were equally distributed following randomization.

The primary and secondary outcome measures, i.e. the cumulative live birth, pregnancy, and ongoing pregnancy rates, are presented in Table 2. With regard to the primary outcome measure, the cumulative live birth rate per started cycle including the transfer of fresh and subsequent frozen/thawed embryos if applicable, there were 77 live births in the assisted hatching group and 68 live births in the control group. This resulted in a cumulative live birth rate of 25.9% versus 23.1% respectively, which proved to be statistically not significantly different (relative risk (RR) 1.125, 95% CI 0.847 to 1.494, $P = 0.416$) The cumulative secondary outcome measures, pregnancy, and ongoing pregnancy rate also were statistically not significant different.

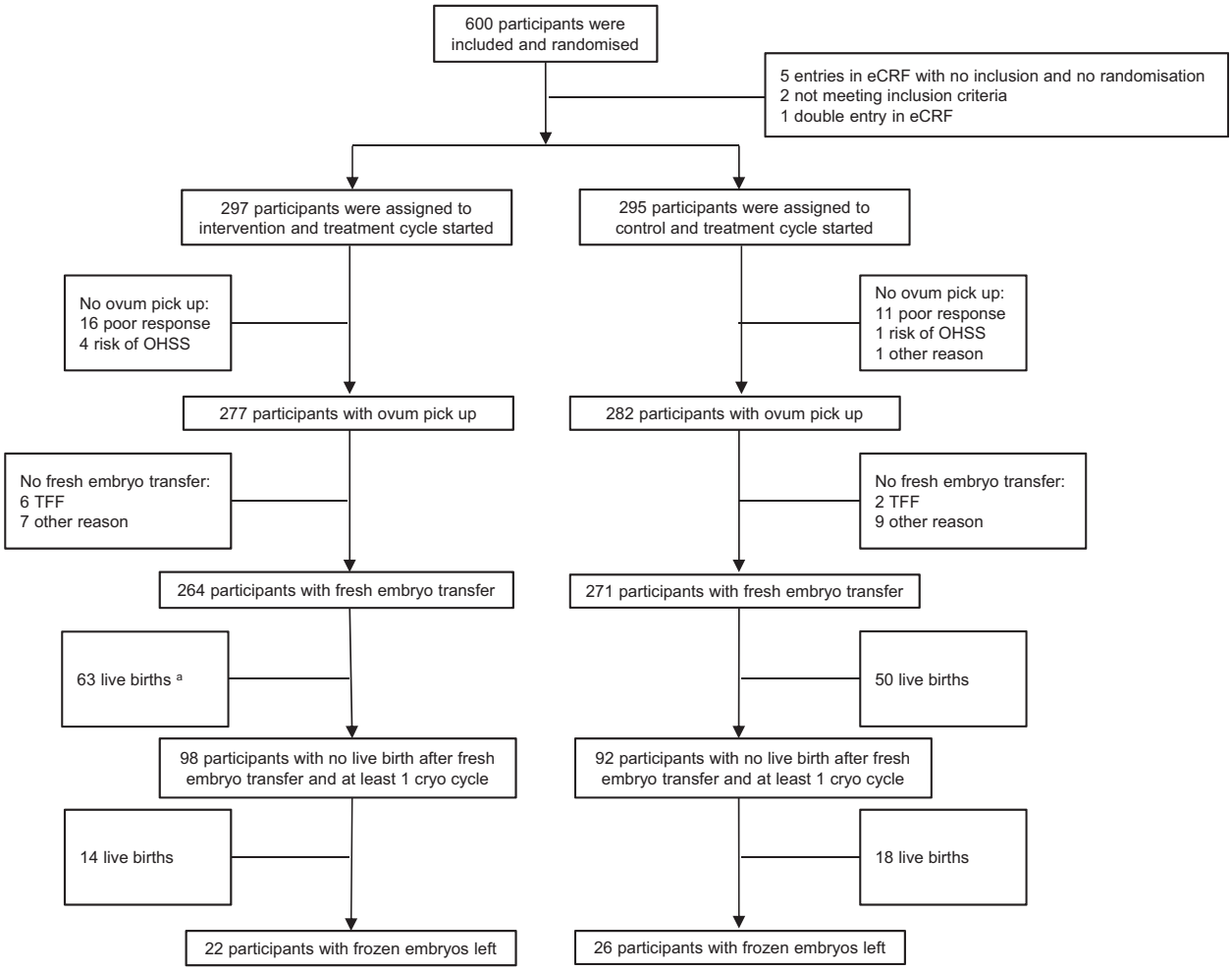

Figure 1. Participant flowchart. Flow of participants with repeated implantation failure through the multicentre double-blinded randomized controlled trial comparing live birth rates after laser-assisted hatching versus controls. eCRF: electronic case report form; OHSS: ovarian hyperstimulation syndrome; TFF: total fertilization failure. [a]Of which one spontaneously conceived after the fresh embryo transfer did not result in a live birth.

Table 1. Baseline characteristics of participants included in the study.

	Assisted hatching	Control	Missing
Total	n = 297	n = 295	
Female age in years (mean ± SD)	34.2 ± 4.3	34.3 ± 4.4	
Partners age in years (mean ± SD)	37.6 ± 5.9	36.9 ± 5.8	n = 5
Subfertility			n = 1
Primary, n (%)	163 (54.9%)	170 (57.8%)	
Secondary, n (%)	134 (45.1%)	124 (42.2%)	
Duration of subfertility in months			n = 1
Median (IQR)	40.0 (30.0)	39.5 (25.3)	
Indication for treatment			n = 4
Unexplained, n (%)	81 (27.5%)	64 (21.8%)	
Male subfertility, n (%)	148 (50.2%)	148 (50.5%)	
Tubal factor, n (%)	27 (9.2%)	36 (12.3%)	
Anovulation/PCOS, n (%)	12 (4.1%)	15 (5.1%)	
Other	27 (9.2%)	30 (10.2%)	
Female body mass index (kg/m^2)			n = 2
Median (IQR)	23.4 (5.8)	23.7 (6.3)	
Treatment			
IVF	41.8%	39.7%	
ICSI	58.2%	60.3%	
Number of oocytes retrieved			n = 1
Median (IQR)	8.0 (7.0)	9.0 (7.0)	
Number of embryos transferred			n = 2
1	36.6%	32.7%	
2	63.4%	67.3%	
Embryo utilization rate,* n (%)	913/1432 (63.8%)	981/1549 (63.3%)	
Quality of embryo(s) transferred			
Fresh transfer, good, n (%)	239/424 (56.4%)	251/449 (55.9%)	
Fresh transfer, good + fair, n (%)	353/424 (83.3%)	383/449 (85.3%)	
Cumulative, fresh + frozen, good, n (%)	292/592 (49.3%)	310/636 (48.7%)	
Cumulative, fresh + frozen, good + fair, n (%)	466/592 (78.7%)	505/636 (79.4%)	
Number included per centre			
A, n (%)	161 (54.2%)	160 (54.2%)	
B, n (%)	43 (14.6%)	43 (14.6%)	
C, n (%)	38 (12.8%)	42 (14.2%)	
D, n (%)	36 (12.1%)	34 (11.5%)	
E, n (%)	19 (6.4%)	16 (5,4%)	

IQR: interquartile range.
* Embryo utilization rate: number of embryos transferred plus frozen divided by the number of zygotes with two pronuclei.

Table 2. Cumulative live birth rate, ongoing pregnancy rate, and pregnancy rate per started cycle.

Outcomes	Assisted hatching n (%)	Control n (%)	Relative risk (95% CI)	*P*-value
Treatment cycles started	297	295		
Primary outcome				
Cumulative live births per couple randomized	77 (25.9%)	68 (23.1%)	1.125 (0.847 to 1.494)	0.416
Secondary outcomes				
Cumulative pregnancies per couple randomized	99 (33.3%)	100 (33.9%)	0.983 (0.784 to 1.233)	0.884
Cumulative ongoing pregnancies per couple randomized	82 (27.6%)	69 (23.4%)	1.180 (0.895 to 1.557)	0.240
Number of couples with frozen embryos left at the end of the trial	22	26		

The secondary outcomes with regard to the fresh transfer only pregnancy rate, ongoing pregnancy rate, and live birth rate, whether per started cycle, oocyte retrieval, or embryo transfer, showed no statistically significant differences between the assisted hatching group and the control group (Table 3).

Results with regard to pregnancy and child outcomes are shown in Table 4. Again, no statistically significant differences were found for the multiple gestation rate and malformation rate in the children born as assessed neonatally. Subanalyses with regard to both minor malformations (such as haemangioma, nevus simplex, nevus flammeus, accessory auricle, and mild cases of ankyloglossia) and major malformations (considered to be invalidating and/or requiring surgery) revealed no statistically significant differences. In the assisted hatching group, two chromosomal abnormal foetuses were found (trisomy 13 and 18, respectively, which resulted in the termination of the pregnancy at 16 and 13 weeks, respectively) and in the control group, one chromosomal abnormal foetus was detected (the pregnancy was terminated at 16 weeks after the diagnosis of trisomy 21 plus XXY). The miscarriage rate after fresh transfer however was lower in the assisted hatching group (17.3% versus 34.6% in the control group, RR 0.532, 95% CI 0.296 to 0.992, $P = 0.047$). Consistency across the laboratory sites of both primary and secondary outcome measures was confirmed after stratification per participating centre (data not shown).

Subanalyses were performed on the cumulative live births per couple randomized, live births fresh transfer only, and the miscarriage rate in couples with a female age of 34 years and younger

Table 3. Live birth rate, ongoing pregnancy rate, and pregnancy rate, fresh transfer only.

Secondary outcomes	Assisted hatching n (%)	Control n (%)	Relative risk (95% CI)	P-value
Per couple randomized	297	295		
Pregnancies	81 (27.3%)	78 (26.4%)	1.031 (0.791 to 1.346)	0.819
Ongoing pregnancies	67 (22.6%)	51 (17.3%)	1.305 (0.941 to 1.809)	0.110
Live births	63 (21.2%)	50 (16.9%)	1.252 (0.896 to 1.749)	0.189
Per oocyte retrieval	277	282		
Pregnancies	81 (29.2%)	78 (27.7%)	1.057 (0.813 to 1.375)	0.679
Ongoing pregnancies	67 (24.2%)	51 (18.1%)	1.337 (0.967 to 1.850)	0.079
Live births	63 (22.7%)	50 (17.7%)	1.283 (0.920 to 1.788)	0.142
Per embryo transfer	264	271		
Pregnancies	81 (30.7%)	78 (28.8%)	1.066 (0.821 to 1.384)	0.631
Ongoing pregnancies	67 (25.4%)	51 (18.8%)	1.349 (0.977 to 1.862)	0.069
Live births	63 (23.9%)	50 (18.5%)	1.293 (0.930 to 1.800)	0.127

Table 4. Pregnancy outcome and malformation rate of the children born.

Secondary outcomes	Assisted hatching n (%)	Control n (%)	Relative risk (95% CI)	P-value
Number of pregnancies	81	78		
Miscarriages per couple randomized	14 (17.3%)	27 (34.6%)	0.532 (0.296 to 0.992)	0.047
Number of cumulative ongoing pregnancies	82	69		
Multiple gestations	10 (12.2%)	11 (15.9%)	0.765 (0.346 to 1.693)	0.509
Number of cumulative ongoing pregnancies	82	69		
Total number of malformations	17 (20.7%)	19 (27.5%)	0.715 (0.408 to 1.254)	0.242
Children with minor malformations	12 (14.6%)	14 (20.3%)	0.721 (0.358 to 1.455)	0.361
Children with major malformations	3 (3.7%)	4 (5.8%)	0.631 (0.146 to 2.724)	0.537
Children with numerical chromosomal aberration	2 (2.4%)	1 (1.4%)	1.683 (0.160 to 18.167)	0.668

versus older than 34 years and couples with one previous fresh embryo transfer cycle and at least two frozen embryo transfers versus participants with two fresh embryo transfer cycles or more (Table 5). Results show a statistically significant decrease in the miscarriage rate in couples with a female age of 34 years and younger (14.9% versus 34.0% in the control group, RR 0.425 (0.184 to 0.985), $P = 0.046$) and two or more previous fresh embryo transfers (13.0% versus 45.9% in the control group, RR 0.391 (0.157 to 0.971), $P = 0.043$).

From our results, it cannot be concluded that assisted hatching results in an increased risk in monozygotic twinning, although the numbers are too low to be meaningful for statistical analysis. In both the assisted hatching group and the control group, one monozygotic pregnancy was observed. In both cases, it concerned a dichorionic triamniotic triplet after the transfer of two embryos in a fresh embryo transfer cycle. None of the twins born were monozygotic in origin as confirmed by ultrasound at gestational age of 12 weeks. None of the pregnancies developed into an ectopic pregnancy.

Embryo damage such as degeneration or loss of at least one blastomere as a result of the study procedures, was considered an adverse event. No adverse events were registered.

Discussion

Our study design was prospectively registered and executed in accordance with the study protocol. Baseline characteristics showed that the assisted hatching group and the control group were well-balanced, and sufficient participants were included and randomized to reach the intended power. Since outcomes were statistically not significantly different, no multivariate analysis to adjust for baseline characteristics was considered necessary.

The study was powered to detect an absolute effect size of 10% with regard to the primary outcome, the cumulative live birth rate, including the transfer of frozen/thawed embryos, on an intention to treat basis. A statistically non-significant absolute increase of 2.8% after assisted hatching was found with a 95% CI ranging from −4.1% to 9.8%, meaning that the true difference, with 95% confidence, is in the range of decreasing the cumulative live birth by slightly over 4% to increasing the cumulative live birth by nearly 10%. It can therefore be concluded that based on this study, we cannot provide evidence for the efficacy of assisted hatching by increasing live birth rates, however, we can neither exclude the possibility that assisted hatching decreases the live birth rates. Therefore, we suggest that assisted hatching should only be offered if information on the absence of evidence of effect and the risk of harm is provided, at no extra costs and preferably only in the setting of a clinical trial taking cost-effectiveness into account.

Two systematic reviews and meta-analyses reported on assisted hatching. Only Lacey *et al.* (2021) reported on the live birth rate, Martins *et al.* (2011) combined the live birth rate with the ongoing pregnancy rate. Lacey *et al.* (2021) found no statistically significant difference between the assisted hatching and the control group in an unselected population, nor in the heterogeneous poor prognosis subpopulation consisting of women with increased age, previous IVF failure, high FSH, or the use of frozen embryos. This was also the case for previous failed IVF and ICSI attempts, although the number of participants in both groups was low. When the meta-analysis is confined to studies using laser-assisted hatching, no statistical significant in live birth rate was demonstrated in a large number of participants (n = 2473). Only one paper reported in a relatively small number of participants (n = 150) on the live birth rate in women with repeated implantation failure after laser-assisted hatching was applied. This

Table 5. Subanalyses with regard to female age and previous fresh embryo transfers.

Outcomes	Assisted hatching n (%)	Control n (%)	Relative risk (95% CI)	P-value
Cumulative live births per couple randomized				
Female age ≤34	48 (32.4%)	45 (29.8%)	1.088 (0.776 to 1.525)	0.623
Female age >34	29 (19.5%)	23 (16.0%)	1.219 (0.741 to 2.003)	0.436
One previous fresh embryo transfer	28 (36.8%)	19 (32.8%)	1.125 (0.706 to 1.803)	0.626
Two or more previous fresh embryo transfers	49 (22.2%)	49 (20.7%)	1.072 (0.755 to 1.523)	0.696
Live births fresh transfer only				
Female age ≤34	40 (27.0%)	34 (22.5%)	1.200 (0.807 to 1.785)	0.367
Female age >34	23 (15.4%)	16 (11.1%)	1.389 (0.766 to 2.520	0.279
One previous fresh embryo transfer	20 (16.8%)	12 (12.5%)	1.345 (0.693 to 2.610)	0.382
Two or more previous fresh embryo transfers	43 (24.2%)	38 (19.1%)	1.214 (0.817 to 1.803)	0.338
Miscarriages per couple randomized				
Female age ≤34	7 (14.9%)	18 (34.0%)	0.425 (0.184 to 0.985)	0.046
Female age >34	7 (20.6%)	9 (36.0%)	0.760 (0.292 to 1.982)	0.575
One previous fresh embryo transfer	8 (22.9%)	10 (24.4%)	0.937 (0.317 to 2.660)	0.876
Two or more previous fresh embryo transfers	6 (13.0%)	17 (45.9%)	0.391 (0.157 to 0.971)	0.043

article could not demonstrate a statistical significant difference (Petersen et al., 2005).

Our results are fully in line with these findings, making the conclusion of the meta-analyses more robust by adding a substantial number of participants. In the most recent Cochrane meta-analysis (Lacey et al., 2021) only one study was included that reported on live births after previous failed IVF attempts (Petersen et al., 2005). We updated the meta-analysis by combining our results with the data from this most recent meta-analysis. The two combined studies showed no evidence of heterogeneity ($P = 0.919$) and resulted in an odds ratio (OR) 1.35 (0.62 to 2.08).

With regard to our secondary outcomes, the pregnancy rate, ongoing pregnancy rate, and live birth rate per started fresh cycle, per oocyte retrieval, and per embryo transfer, both cumulatively including frozen embryos (pregnancy and ongoing pregnancy rate) and fresh cycle only (pregnancy, ongoing, and live birth rate), no statistically significant differences were found. Meta-analyses (Martins et al., 2011; Lacey et al., 2021) reported a small statistically significant effect of assisted hatching on the pregnancy rate, as defined by the demonstration of a foetal heartbeat by ultrasound. However, heterogeneity in the included studies was found. When the analysis was confined to those studies also reporting on live births, a significant effect could no longer be demonstrated (Lacey et al., 2021). In the subgroup of participants with previous failed IVF or ICSI attempts, assisted hatching significantly improved the pregnancy rate. When the analysis only included studies that reported on allocation concealment, a significant difference could no longer be demonstrated (Lacey et al., 2021). In our study, observation bias was negligibly small due to double-blinding for treating physician, the physician and laboratory staff involved in the embryo transfer and the subfertile couple.

In this study, a statistically significant decrease of the miscarriage rate after assisted hatching was found. As shown in the subanalyses, the miscarriage rate after assisted hatching was decreased in couples with a female age of 34 years and younger and couples with repeated implantation failures after two or more fresh embryo transfers. Since our study was neither designed nor powered for the secondary outcomes, caution should be exerted in the interpretation of these results since these findings may be based on chance. Both previously mentioned meta-analyses (Martins et al., 2011; Lacey et al., 2021) reported no difference between the assisted hatching and control groups concerning miscarriage rate both in the unselected group as in the subgroup of participants with or without previous failed IVF or ICSI attempts. Evidence was reported that implantation beyond the endometrial receptivity window resulted in increased miscarriage rates (Wilcox et al., 1999). It is therefore hypothesized that assisted hatching may provide a more optimal synchronization between endometrial receptivity and implantation. Since we demonstrated absence of evidence that assisted hatching results in an increased cumulative live birth rate, the decreased miscarriage rate after assisted hatching is most likely based on chance. We conclude that prevention of miscarriages should not be used as an indication for assisted hatching.

Concerning the safety of the assisted hatching procedure, the monozygotic twinning rate and congenital malformation rate were reported in this study. Increased monozygotic twinning as a risk of assisted hatching is reviewed in two meta-analyses (Martins et al., 2011; Lacey et al., 2021) and was reported in several retrospective studies (Knopman et al., 2014; Luke et al., 2014; Ikemoto et al., 2018) even though another retrospective study found no increased monozygotic twinning after assisted hatching (Mateizel et al., 2016). The evidence is thus to be considered inconclusive. In this study, in both the assisted hatching group and the control group, one dichorionic triamniotic triplet after the transfer of two embryos was found. The study was however not powered to detect differences in the monozygotic twinning rate and the numbers found are too low to base any conclusions on. If a future trial is to be performed, the monozygotic twinning rate should be registered, analysed, and reported. Likewise, the malformation rate for both major and minor malformations, numerical chromosomal aberrations, and total rate for the three subcategories combined, was comparable between the assisted hatching group and the control group. Also in the case of the malformation rate, the numbers were too low to draw any firm conclusions. Nonetheless, the results are in line with a 2015 retrospective study reporting no increase in the risk of major congenital anomalies after assisted hatching (Jwa et al., 2015).

The current study has several potential weaknesses. First, there was a small cohort of subfertile couples that after not achieving an ongoing pregnancy, still had cryopreserved embryos in storage at the endpoint of the trial, i.e. 1 year after the last randomization. Since no statistically significant difference between the intervention versus the control arm of the study with regard to the live birth rate after the transfer of cryopreserved and thawed embryos was demonstrated (14 live births after the transfer of 168 thawed embryos in the intervention arm and 18 live births after transferring 187 thawed embryos in the control arm,

RR 0.657, 95% CI 0.353 to 1.223), it can be assumed that the use of these remaining embryos will not change the main findings. A large retrospective study on data from the United States' Society for Assisted Reproductive Technology (SART) database confirms this finding, assisted hatching on frozen and thawed embryos in the first frozen embryo cycle only slightly decreased the live birth rate (Knudtson *et al.*, 2017). Secondly, at the start of this study, there was no international consensus on the definition of repeated implantation failure. It should be noted, however, that in the meta-analyses on assisted hatching, repeated implantation failure was equally not concisely defined (Martins *et al.*, 2011; Lacey *et al.*, 2021). Even now, as recently reviewed, a universally accepted definition is lacking (Coughlan *et al.*, 2014; Li, 2014; Shaulov *et al.*, 2020; Busnelli *et al.*, 2021). In this study, repeated implantation failure was defined as not having achieved a pregnancy after one of the following conditions was met: (i) by having had at least two consecutive fresh IVF or ICSI embryo transfers, regardless of the number of frozen embryo transfers or (ii) by having had at least one fresh IVF or ICSI transfer and at least two frozen embryo transfers with embryos originating from that fresh cycle. We can therefore not exclude the possibility that assisted hatching might be effective in higher order repeated implantation failures. And finally, mimicking daily practice, not all embryos had sufficient perivitelline space to allow for breaching the zona pellucida because of the risk of applying thermal damage to the blastomeres. Therefore, in part of the embryo, the zona was breached, whereas in others the zona was thinned. The meta-analysis of Lacey *et al.* (2021), however, found no statistically significant difference whether breaching or zona thinning was applied (OR 1.15, 95% CI 0.87 to 1.51 and OR 1.06, 95% CI 0.86 to 1.30, respectively).

In conclusion, we failed to deliver evidence of effect for assisted hatching as an add-on in subfertile couples with repeated implantation failure, as defined by the criteria in this study, in improving live birth rates. Neither can we exclude the possibility that assisted hatching decreases the live birth rates. Since women with repeated implantation failure are considered to benefit most from assisted hatching, we suggest that assisted hatching should only be offered if information on the absence of evidence of effect is provided as well as that the risk of harm cannot be excluded, at no extra costs and preferably only in the setting of a clinical trial taking cost-effectiveness into account.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author and after approval of a written proposal taking into account Dutch legislation and Good Practice guidelines.

Authors' roles

M.H.J.M.C. conceptualized and drafted the study protocol; M.H.J.M.C., B.J.C., and C.C.A.W.v.B. were involved in designing the study; M.H.J.M.C., E.J.S., J.G.D., G.W.v.d.H., E.B.B., and C.C.A.W.v.B. were responsible for the execution of the laboratory procedures and data collection; B.J.C., D.C.S., R.J.T.v.G., J.M.J.S., and V.E.E.G.R. were responsible for the inclusion of participants and clinical procedures; R.M.B. performed the statistical analysis; M.H.J.M.C. and R.M.B. accessed and verified the data; M.H.J.M.C. drafted the manuscript. All authors reviewed the manuscript and approved the final version to be submitted for publication.

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Conflict of interest

None.

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