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Hatching status before embryo transfer is not correlated with implantation rate in chromosomally screened blastocysts

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STUDY QUESTION: Do the reproductive outcomes from the transfer of fully hatched (FH) blastocysts differ from those of not fully hatched (NFH) blastocysts?

SUMMARY ANSWER: Biochemical pregnancy rate (BPR), implantation rate (IR), live birth rate (LBR) and early pregnancy loss (EPL) rate are similar in FH and NFH single euploid blastocyst embryo transfers.

WHAT IS KNOWN ALREADY : The use of extended culture and PGS often leads to transfer of an embryo that is well developed and frequently FH from the zona pellucida. Without the protection of the zona, an FH embryo could be vulnerable to trauma during the transfer procedure. To date, no other study has evaluated the reproductive competence of an FH blastocyst transfer.

STUDY DESIGN, SIZE, DURATION: The retrospective study included 808 patients who underwent 808 cycles performed between September 2013 and July 2015 at a private academic IVF center. Of these, 436 cycles entailed transfer of a NFH blastocyst ($n = 123$ fresh transfer, $n = 313$ frozen/thawed embryo transfer (FET)) and 372 cycles entailed transfer of an FH blastocyst ($n = 132$ fresh, 240 FET). Fresh and FET cycles and associated clinical outcomes were considered separately. LBR was defined as the delivery of a live infant after 24 weeks of gestation.

PARTICIPANTS/MATERIALS, SETTING, METHOD: Trophectoderm biopsies were performed on Day 5 (d5) or 6 (d6) for embryos meeting morphology eligibility criteria (set at ≥3BC). Morphologic grading was determined using a modified Gardner–Schoolcraft scale prior to transfer. A single euploid embryo was selected for transfer per cycle on either the morning of d6, for fresh transfers or 5 days after progesterone supplementation for patients with transfer in an FET cycle. Embryos were classified as NFH (expansion Grade 3, 4 or 5) or FH (expansion Grade 6) cohorts. The main outcome measure was IR.

MAIN RESULTS AND THE ROLE OF CHANCE: In the fresh transfer group, IR was similar between NFH and FH cycles (53.7% versus 55.3%, $P = 0.99$, odds ratio (OR) 0.9; 95% confidence interval (CI) 0.6–1.5). Secondary outcomes were also statistically similar between groups: BPR (65.9% versus 66.7%, OR 1.0; 95% CI: 0.6–1.6), LBR (43.1% versus 47.7%, $P = 0.45$, OR 1.2; 95% CI: 0.7–1.9) and EPL rate (22.8% versus 18.2%, OR 1.3; 95% CI: 0.7–2.4). After adjusting for age, BMI, endometrial thickness at the LH surge and oocytes retrieved in a logistic regression (LR) model, the hatching status remained not associated with IR ($P > 0.05$). In the FET cycles, IR was similar between NFH and FH cycles (62.6% versus 61.7%, OR 1.0; 95% CI: 0.7–1.5). Secondary outcomes were similar between groups: BPR (74.1% versus 72.9%, respectively, OR 1.1; 95% CI: 0.7–1.6), LBR (55.0% versus 50.0%, OR 0.8; 95% CI: 0.6–1.1) and EPL rate (18.9% versus 22.9%, respectively, OR 0.8; 95% CI: 0.5–1.2). After adjusting for age, BMI, endometrial thickness at the LH surge and oocytes retrieved in an LR model, the hatching status was not shown to be associated with implantation ($P > 0.05$).

LIMITATIONS, REASONS FOR CAUTION: Limitations include the retrospective design and data from a single institution. Additionally, the study was limited to patients that developed high-quality blastocysts suitable for biopsy.

WIDER IMPLICATIONS OF THE FINDINGS: The results suggest that FH embryos are not more fragile or less likely to implant when compared to NFH counterparts. We found no evidence of altered IR or other clinical outcomes in the transfer of FH euploid embryos.

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Key words: IVF / blastocyst hatching / comprehensive chromosomal screening / implantation / trophectoderm biopsy / preimplantation genetic screening

Introduction

Over the past two decades, a greater understanding of human oocyte and embryo development (Fragouli *et al.*, 2013; Franasiak *et al.*, 2014) has enhanced infertility treatment outcomes (Sunderam *et al.*, 2012). Several techniques have been implemented to improve implantation rates (IRs) (e.g. assisted hatching (Cohen *et al.*, 1992), gamete/embryo cryopreservation (Cobo *et al.*, 2012), extended culture media (ECM) (Gardner *et al.*, 1998) and PGS (Scott *et al.*, 2013). However, the use of ECM and PGS often mandates the transfer of a more developed, fully hatched (FH) embryo. Of key interest is the fragility of expanded, chromosomally screened embryos and the impact on reproductive outcome.

Advancements in ECM (ASRM, 2013) have successfully enabled the development of embryos to Day 5 (d5) or 6 (d6) after vaginal oocyte retrieval (VOR) (Thomas *et al.*, 2010). This permits the identification of blastocysts with little or no implantation potential (Glujovsky *et al.*, 2012). While ECM studies raise questions of zona pellucida hardening and the possible inability of an embryo to hatch (Cohen *et al.*, 1992), little attention has been given to the impact of the transfer of an FH embryo.

Embryo screening techniques have benefited from ECM. Ploidy assessment via trophectoderm biopsy (Werner *et al.*, 2015) can assist clinicians to objectively select a high-quality embryo (Scott *et al.*, 2013). However, the use of ECM and PGS often leads to transfer of a more developed, and frequently FH, embryo (Hardarson *et al.*, 2012). Additionally, laser-assisted biopsy techniques increase the likelihood of hatching (Jones *et al.*, 2006). Without zona protection, an FH embryo is presumed to be vulnerable to trauma during transfer. The hatching process can occur before or at transfer in both fresh and frozen embryo transfer (FET) cycles, with embryos vitrified at expansion Grade 4 or 5 (hereafter referred to as not-fully hatched (NFH)) often observed FH after rewarming.

Hatching effect on clinical outcomes remains unknown. Concerns that the high pressure to which blastocysts are exposed during pipetting (Hiraoka *et al.*, 2004) might rupture the trophectoderm and induce blastocoelic fluid leakage have been raised. Although no study has adequately assessed this opinion, an FH euploid embryo is expected to be more fragile and less likely to implant than a NFH euploid embryo. With increasing utilization of ECM and PGS as part of a freeze-all strategy, an investigation of the impact of the transfer of an FH embryo on reproductive outcomes is particularly pressing.

Material and methods

Study design and patient population

A single-center, retrospective cohort analysis of patients identified from an electronic medical records database who completed an IVF cycle with quantitative-PCR (qPCR)–based PGS from September 2013 to July 2015 was performed. Transferred embryos were classified as either NFH (expansion Grades 3–5) or FH (expansion Grade 6) according to their status right before transfer and to a center-modified Gardner and Schoolcraft scale (Gardner and Schoolcraft, 1999), which includes a D category for inner cell mass (ICM) (few cells, disorganized) and trophectoderm (very few cells). Embryos scored at <3BC were excluded as they were ineligible for biopsy. One euploid embryo was selected for transfer per cycle on either the morning of d6 for fresh transfers or, for patients undergoing an FET, 5 days after progesterone supplementation. Included patients required a normal endometrial cavity and a basal Day 3 FSH level of ≤13 mIU/ml. If patients had multiple cycles, only the first cycle from either cohort was included. Donor egg cycles were excluded.

Stimulation protocol

Patients underwent controlled ovarian hyperstimulation (COH) for IVF as described previously (Rodriguez-Purata *et al.*, 2016). Oocyte maturation was induced with recombinant hCG alone (Ovidrel®, EMD Serono, Rockland, MA, USA) or with 40 IU leuprolide acetate (Lupron®, AbbVie Laboratories, Chicago, IL, USA) concomitant with 1000 IU hCG (Novarel®, Ferring Pharmaceuticals, Parsippany, NJ, USA) in patients at risk of ovarian hyperstimulation syndrome. Patients underwent VOR under ultrasound guidance 36 hours post-surge and were inseminated by ICSI because of the possibility of genetic testing of the embryos.

Laboratory procedures

Embryo culture technique

Embryos were cultured to the blastocyst stage as previously described (Rodriguez-Purata *et al.*, 2016). On Day 3 (d3) of embryo development, all embryos underwent 'assisted hatching' (not to be confused with hatching/hatched status by the Gardner–Schoolcraft scale) by the creation of a 25–30 μm opening in the zona pellucida with a 200–300 μs pulse from a ZILOS-tk Laser (Hamilton Thorne Biosciences, Beverly, MA, USA) to boost trophectoderm herniation.

Embryo biopsy technique

Blastocyst trophectoderm biopsies were performed on d5 and/or d6, contingent upon morphological eligibility (embryos ≥3BC). Embryo biopsy was carried out as described previously (Rodriguez-Purata *et al.*, 2016). Two to nine trophectoderm cells were analyzed by qPCR (Treff *et al.*,

2012). Since variability persists in hatching rate, not all d5 embryos are eligible for biopsy. Biopsy samples were placed in hypotonic wash buffer and submitted for immediate analysis in embryos biopsied on d5 and for later analysis in those embryos biopsied on d6. Day 5 samples were available for processing before 10:00 p.m., with results available by d6. Embryos biopsied on d6 were vitrified after biopsy, and the results were available within 2 weeks. Patients were encouraged to undergo freeze-all cycles; a strategy that allows for the availability of the genetic results of all embryos prior to transfer selection. Biopsied embryos received a genetic interpretation of euploid or aneuploid.

Cryopreservation–rewarming technique

The cryopreservation and rewarming technique has been described previously (Rodriguez-Purata *et al.*, 2016). After rewarming, embryo survival was determined according to the appearance of the blastomeres, zona pellucida and the ability of the blastocoel to re-expand. Degenerated embryos were cataloged as non-surviving.

Study groups

Fresh and FET cycles and associated clinical outcomes were considered separately.

Fresh transfer cycles

Expanded embryos on d5 were biopsied, and results were received the morning on d6. Embryos were classified as NFH or FH at transfer (Fig. 1A). Because d6 embryos were checked twice (first: for biopsy decision on the afternoon of d5; second: for selection of the morphologically best embryo among euploid embryos), a sub-analysis that segregated cycles by the specific moment the embryo was observed FH was performed: (i) FH before biopsy on d5, therefore FH before transfer, (ii) NFH before biopsy on d5 but FH before transfer and (iii) NFH before biopsy on d5, NFH before transfer (control group) (Fig. 1B). Luteal phase support was accomplished with micronized progesterone vaginally (Endometrin®, Ferring Pharmaceuticals Inc., Parsippany, NJ; or Crinone®, Actavis Pharma,

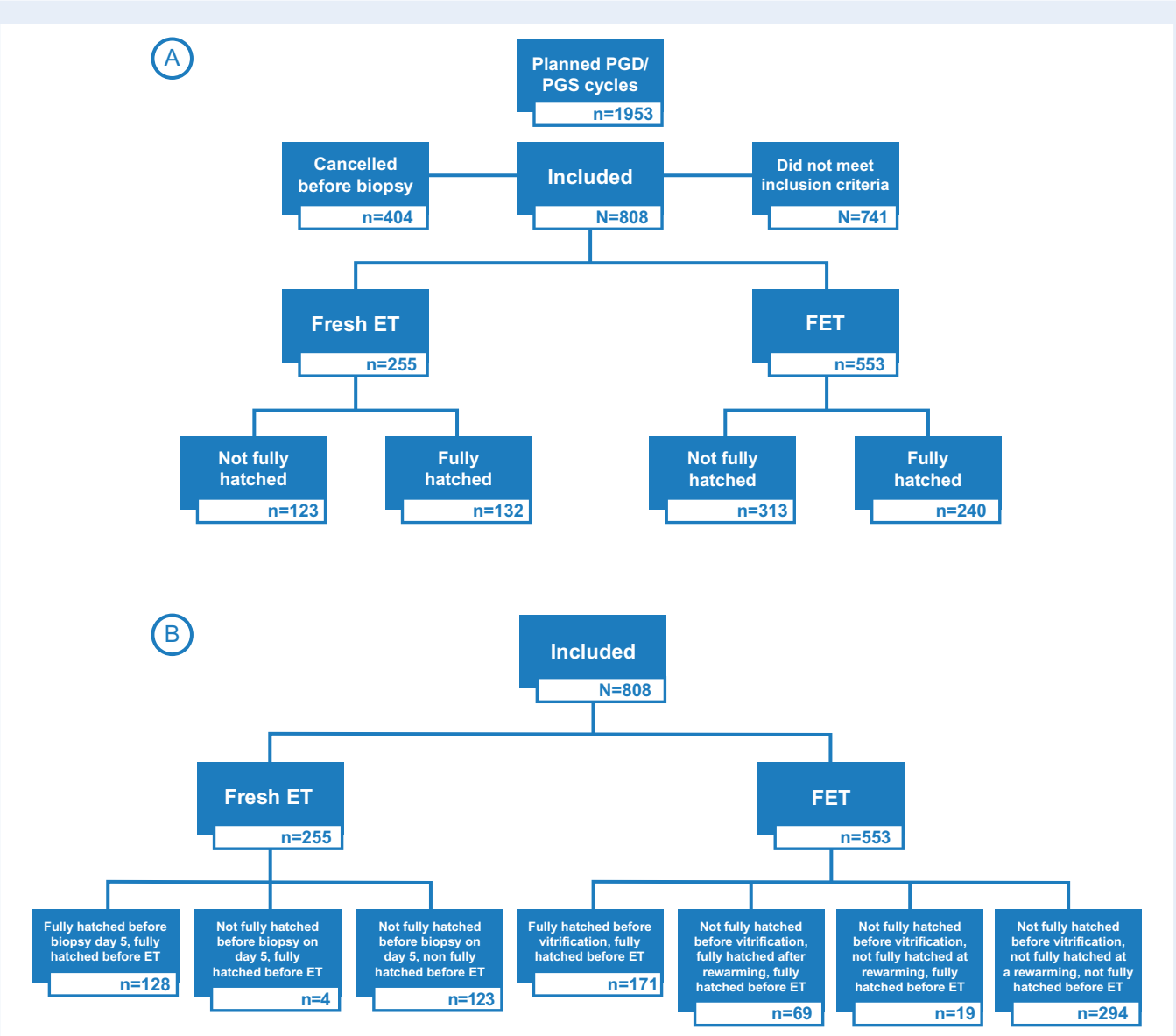

Figure 1 Flow diagram of the study. (**A**) Main analysis; (**B**) secondary analysis. ET: embryo transfer; FET: frozen embryo transfer.

Parsippany, NJ, USA) and orally (Prometrium®, AbbVie Inc., North Chicago, IL, USA) beginning the day after VOR.

FET cycles

The transfer was performed under a synthetically prepared endometrium. Embryos used for an FET cycle were available after one of the following scenarios: supernumerary euploid embryos biopsied on d5 and vitrified on d6 after a morphologically superior embryo were chosen for the fresh transfer; supernumerary embryos biopsied on the morning of d6 of the fresh cycle, therefore with a pending genetic result or after a freeze-all cycle in which all biopsied embryos were cryopreserved. Following menses, patients began oral estradiol (Estrace®; Teva Pharmaceuticals, Sellersville, PA, USA) 2 mg twice daily for 1 week, then 2 mg three times daily. Endometrial thickness was assessed weekly until a thickness of ≥7 mm was observed. Immediately thereafter, 50 mg of intramuscular progesterone daily (Progesterone injection®; Watson Pharma Inc., Parsippany, NJ, USA) was added. Thawing and transferring of the embryo was performed after 5 days of progesterone supplementation. Embryos were classified as NFH or FH at transfer (Fig. 1A). Because embryos from FET cycles were checked three times before transfer (first: before vitrification; second: after rewarming; third: before FET), a sub-analysis was performed; according to the particular moment the embryo was observed FH: (i) FH before vitrification, therefore FH after rewarming and before transfer, (ii) NFH before vitrification but FH after rewarming, therefore FH before transfer, (iii) NFH before vitrification, NFH at rewarming but FH before transfer and (iv) NFH before vitrification, NFH at rewarming and NFH before transfer (control group) (Fig. 1B).

Outcome measures

IR was considered the most temporally-related event to test the study's null hypothesis. It was calculated as a ratio of the number of gestational sacs (GS) (determined by ultrasound ~9 days following a positive pregnancy test) to the number of transferred euploid embryos. Monozygotic twins were characterized as one sac in this analysis. Secondary outcomes were biochemical pregnancy rate (BPR), live birth rate (LBR) and early pregnancy loss (EPL) rate. A pregnancy was defined as the detection of β-hCG ≥5 mIU/mL 9 days after the transfer. A live birth was defined as the delivery of a live infant after 24 weeks of gestation. EPL was defined as a loss following a positive pregnancy test and/or detectable GS.

Statistical methods

Statistical analysis was performed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). Descriptive data were compared by unpaired two-sided *t*-test with significance at $P < 0.05$; results are expressed as mean and standard deviation with 95% confidence intervals (CI). Distributions between outcomes were assessed by chi square test (or Fisher exact test for samples <10) with significance established at $P < 0.05$. The Clopper–Pearson interval was used to calculate binomial CI for all reported proportions. Adjusted odds ratios (ORs) and their 95% CI for BPR, IR, LBR and EPL rate were calculated to evaluate the relative odds of each event compared with the reference group of NFH cycles. Study was designed with 85% power to detect a difference of 20% in IR between NFH and FH embryos with a reference proportion of 50% and a two-tailed 5% significance level. The required sample size was computed to be 107 per group.

Univariate logistic regression (LR) analyses were performed to identify candidate factors that were associated with implantation and would also be included in the multivariate regression. Candidate variables included age, anti-Müllerian hormone, FSH, BMI, endometrial thickness at surge, peak estradiol at surge, number of oocytes retrieved, number of embryos biopsied and number of embryos vitrified. We also constructed a multivariate LR model including potential predictors of implantation regardless of their association within a univariate model: age, BMI, endometrial thickness at surge and number of oocytes retrieved. The likelihood of implantation after IVF is presented as an OR with SE and 95% CI.

Regulatory approval

This retrospective study was approved by the Western Institutional Review Board. Informed consent from patients was not obtained, but patient information was anonymized and de-identified prior to analysis.

Results

There were 1953 planned PGS cycles during the study period, of which 20.7% ($n = 404$) were canceled before biopsy, and 41.4% ($n = 808$ cycles/808 patients) met the inclusion criteria (trophectoderm biopsy, single embryo transfer) (Fig. 1A). Overall, 54.0% ($n = 436$) of the patients had a NFH embryo transferred, while 46.0% ($n = 372$) received an FH embryo. When segregated by cycle type, 31.6% ($n = 255$) were transferred fresh (fresh transfer group), of which 48.3% ($n = 123$) of the embryos were NFH and 51.7% ($n = 132$) were FH (Table I). In the FET Group (68.4%, $n = 553$), 56.5% ($n = 313$) of the embryos were NFH and 47.5% ($n = 240$) FH (Table II). Similar patient characteristics and baseline hormone levels were observed in all study cohorts for all cycle types. Demographic characteristics and embryological data are shown in Tables I and II.

Overall, euploidy rate was similar between NFH and FH embryos (58.8% versus 62.5%, respectively, $P > 0.05$) (Fig. 2, Tables I and II). Embryos were biopsied on d5 (57.8% ($n = 2633$)) and on d6 (42.2% ($n = 1926$)) (Table III). Of the 60.5% ($n = 2758$) of embryos reported as euploid, 57.7% ($n = 1591$) were biopsied on d5. Of the 38.3% ($n = 1745$) of embryos reported as aneuploid, 46.9% ($n = 818$) were biopsied on d5. Of the 1.2% ($n = 56$) embryos reported as undetermined (i.e. non concurrent), 42.9% ($n = 24$) were biopsied on d5 (Table III).

Main analysis

Fresh transfer cycles

Fewer embryos reached d3, d5, d6 and were vitrified in the cycles in which NFH embryos were used for transfer compared with the cycles in which FH embryos were used (all $P < 0.05$). Fewer embryos were biopsied on d5, and a smaller proportion of embryos were euploid in the NFH compared FH group ($P < 0.05$). The rates of embryo retention in the transfer catheter were comparable between groups (Table I).

IR was similar between NFH and FH transfer cycles, respectively (53.7% versus 55.3%, $P = 0.99$, OR 0.9 (95% CI: 0.6–1.5)). Secondary outcomes were also similar between study groups: BPR (65.9% versus 66.7%, OR 1.0 (95% CI: 0.6–1.6)), LBR (43.1% versus 47.7%, $P = 0.45$, OR 1.2 (95% CI: 0.7–1.9)) and EPL rate (22.8% versus 18.2%, OR 1.3 (95% CI: 0.7–2.4)) (Fig. 3, Table IV).

After adjusting for age, BMI, endometrial thickness at surge and oocytes retrieved in an LR model, the hatching status remained not associated with implantation ($P > 0.05$) (Table V).

Table I Demographic characteristics and embryological data for fresh embryo transfer cycles.

Group	NFH	FH	P	
Cycles (*n*)	123	132		
Age (years)	36.9 ± 3.8 (95% CI: 36.2–37.6)	36.5 ± 4.2 (95% CI: 35.8–37.2)	NS	
BMI (kg/m^2) at IVF cycle	23.1 ± 4.2 (95% CI: 22.7–24.3)	23.2 ± 4.0 (95% CI: 22.6–24.0)	NS	
Day 3 FSH (mUI/ml)	6.0 ± 2.8 (95% CI: 5.5–6.5)	6.1 ± 3.2 (95% CI: 5.5–6.6)	NS	
Day AMH (ng/ml)	3.0 ± 2.9 (95% CI: 2.3–3.8)	3.5 ± 2.7 (95% CI: 2.8–4.2)	NS	
Day 3 AFC (*n*)	13.1 ± 7.3 (95% CI: 11.8–14.5)	13.2 ± 6.4 (95% CI: 12.1–14.4)	NS	
Endometrial thickness (mm) at LH surge	9.8 ± 1.9 (95% CI: 9.5–10.2)	9.9 ± 2.2 (95% CI: 9.5–10.3)	NS	
Follicles >14 mm at LH surge	12.8 ± 6.1 (95% CI: 11.7–13.9)	13.9 ± 6.5 (95% CI: 12.8–15.0)	NS	
E_2 (pg/ml) level at LH surge	2338.7 ± 1080.7 (95% CI: 2145.8–2531.6)	2485.5 ± 1177.2 (95% CI: 2282.0–2689.0)	NS	
Oocytes retrieved (*n*)	16.9 ± 9.1 (95% CI: 15.3–18.5)	18.0 ± 9.8 (95% CI: 16.4–19.7)	NS	
Oocytes inseminated (*n*)	13.0 ± 8.0 (95% CI: 11.6–14.4)	14.1 ± 8.1 (95% CI: 12.7–15.5)	NS	
Embryos ongoing Day 1 (*n*)	10.4 ± 6.6 (95% CI: 9.2–11.5)	12.1 ± 7.9 (95% CI: 10.8–13.5)	NS	
Embryos ongoing Day 3 (*n*)	10.0 ± 6.4 (95% CI: 8.8–11.1)	11.7 ± 7.7 (95% CI: 10.4–13.0)	0.05	
Embryos ongoing Day 5 (*n*)	7.3 ± 5.0 (95% CI: 6.3–81)	9.1 ± 6.4 (95% CI: 8.0–10.2)	0.05	
Embryos ongoing Day 6 (*n*)	6.9 ± 5.0 (95% CI: 6.0–7.7)	8.4 ± 5.9 (95% CI: 7.4–9.4)	0.05	
Morphology				
Expansion				
4	14.6% (18/123)	NA		
5	85.4% (105/123)	NA		
6	NA	100% (132/132)		
Inner cell mass				
A	77.2% (95/123)	84.8% (112/132)		
B	22.8% (28/123)	15.2% (20/132)		
Trophectoderm				
A	43.1% (53/123)	76.5% (101/132)		
B	66.9% (70/123)	23.5% (31/132)		
Embryos biopsied (n)				
Total	5.5 ± 3.8 (95% CI: 4.9–6.2)	6.8 ± 5.0 (95% CI: 6.0–7.6)	<0.05	
Day 5	3.5 ± 2.5 (95% CI: 3.1–4.0)	4.4 ± 3.6 (95% CI: 3.8–5.1)	<0.05	
Day 6	2.0 ± 1.9 (95% CI: 1.7–2.4)	2.4 ± 2.6 (95% CI: 1.9–2.8)	NS	
Euploidy rate	55.8% (394/706) 95% CI: 0.52–0.60	64.1% (553/863) 95% CI: 0.61–0.67	0.05	OR 1.4 (95% CI: 1.2–1.7)
Embryos vitrified (n)	3.0 ± 3.1 (95% CI: 2.5–3.6)	4.3 ± 4.2 (95% CI: 3.6–5.1)	0.05	
*Retained embryos at transfer	2.4% (3/123) 95% CI: 0.5–7.0	3.8% (5/132) 95% CI: 1.2–8.6	NS	OR 0.64 (95% CI: 0.1–2.7)

Results are expressed as mean ± SD with 95% confidence intervals (CI). Significance established at $P < 0.05$. NS: non-significant; FSH kit: Siemens Immulite® 2000 FSH Kit; AMH: anti-Müllerian hormone (Roche Elecsys® 2010); AFC: antral follicle count; E2: estradiol (Siemens Immulite® 2000 estradiol Kit); OR: odds ratio; FH: fully hatched; NFH: not fully hatched. Retained embryos: embryos being retained in the transfer catheter. * retained in the transfer catheter.

FET cycles

In the FET group ($n = 553$), 83% ($n = 459$), cycles were intended as freeze-all cycles, 14.1% ($n = 78$) utilized a supernumerary embryo biopsied on d5 and cryopreserved on d6, and 2.9% ($n = 16$) utilized a supernumerary embryo biopsied and vitrified on d6. No statistically significant difference was observed in the demographic variables analyzed between groups. An identical rewarming survival rate was observed in the NFH and FH groups. No differences were detected in the rate of embryo retention in the transfer catheter between the NFH and FH groups (Table II).

A similar IR in NFH and FH transfer cycles was observed (62.6% versus 61.7%, OR 1.0 (95% CI: 0.7–1.5)). Secondary outcomes were also similar between groups, respectively: BPR 74.1% versus 72.9%, OR 1.1 (95% CI: 0.7–1.6), LBR 55.0% versus 50.0%, OR 0.8 (95% CI: 0.6–1.1) and EPL rate 18.9% versus 22.9%, OR 0.8 (95% CI: 0.5–1.2) (Fig. 3, Table IV).

After adjusting for age, BMI, endometrial thickness at surge and oocytes retrieved in an LR model, the hatching status was not shown to associate with implantation ($P > 0.05$) (Table V).

Table II Demographic characteristics and embryological data for frozen embryo transfer (FET) cycles.

Group	NFH	FH		
Cycles (*n*)	313	240		
Age (years)	36.1 ± 4.3 (95% CI: 35.6–36.5)	36.4 ± 3.9 (95% CI: 36.0–36.8)	NS	
BMI (kg/m^2) at IVF cycle*	36.5 ± 4.3 (95% CI: 36.0–36.9)	36.7 ± 3.8 (95% CI: 36.2–37.2)	NS	
Day 3 FSH (mUI/ml)*	23.0 ± 4.0 (95% CI: 22.5–23.5)	23.2 ± 4.0 (95% CI: 22.7–23.7)	NS	
Day 3 AMH (ng/ml)*	23.1 ± 4.0 (95% CI: 22.7–23.6)	23.2 ± 4.0 (95% CI: 22.7–23.7)	NS	
Day 3 AFC (n)*	6.0 ± 2.9 (95% CI: 5.6–6.3)	6.0 ± 2.6 (95% CI: 5.7–6.4)	NS	
Endometrial thickness (mm) at LH surge*	4.0 ± 6.4 (95% CI: 3.0–5.0)	3.5 ± 4.2 (95% CI: 2.8–4.2)	NS	
Follicles >14 mm at LH surge*	13.3 ± 7.2 (95% CI: 12.4–14.2)	13.6 ± 8.0 (95% CI: 12.5–14.6)	NS	
E_2 (pg/mL) level at LH surge*	9.8 ± 1.9 (95% CI: 9.6–10.0)	9.9 ± 2.2 (95% CI: 9.6–10.2)	NS	
Oocytes retrieved (*n*)*	9.0 ± 1.6 (95% CI: 8.7–9.3)	8.9 ± 1.4 (95% CI: 8.6–9.2)	NS	
Oocytes inseminated (*n*)*	13.1 ± 6.8 (95% CI: 12.3 13.9)	12.8 ± 6.0 (95% CI: 12.0–13.6)	NS	
Embryos ongoing Day 1 (*n*)*	2506.8 ± 1189.3 (95% CI: 2371.2–2642.4)	2367.1 ± 1084.0 (95% CI: 2226.5–2507.6)	NS	
Embryos ongoing Day 3 (*n*)*	388.0 ± 242.6	383.4 ± 206.5	NS	
Embryos ongoing Day 5 (*n*)*	17.4 ± 11.5 (95% CI: 16.1–18.7)	16.4 ± 9.4 (95% CI: 15.1–17.6)	NS	
Embryos ongoing Day 6 (*n*)*	13.6 ± 9.6 (95% CI: 12.5–14.7)	12.5 ± 8.0 (95% CI: 11.5–13.5)	NS	
Morphology*				
Expansion	10.9 ± 7.8 (95% CI: 10.0–11.8)	10.3 ± 6.9 (95% CI: 9.4–11.2)	NS	
4	10.5 ± 7.4 (95% CI: 9.7–11.3)	10.0 ± 6.7 (95% CI: 9.1–10.8)	NS	
5	6.7 ± 5.3 (95% CI: 6.1–7.3)	6.8 ± 5.3 (95% CI: 6.1–7.5)	NS	
6	5.1 ± 4.2 (95% CI: 4.6–5.6)	5.7 ± 4.8 (95% CI: 5.1–6.3)	NS	
Inner cell mass				
A	51.4% (161/313)	NA		
B	48.6% (152/313)	NA		
Trophectoderm				
A	NA	100% (240/240)		
B	63.9% (200/313)	70.0% (168/240)		
Embryos biopsied (*n*)				
Total	36.1% (113/313)	30.0% (72/240)		
Day 5	26.8% (84/313)	29.6% (71/240)		
Day 6	53.4% (167/313)	51.3% (123/240)		
Euploidy rate	19.8% (62/313)	19.1% (46/240)		
Embryos vitrified	5.3 ± 4.0 (95% CI: 4.8–5.7)	5.0 ± 3.9 (95% CI: 4.5–5.5)	NS	
Survival rate	96.6% (339/351) 95% CI: 94.1–98.2	96.6% (259/268) 95% CI: 93.7–98.5	NS	OR 0.98 (95% CI: 0.4–2.4)
Retained embryos at transfer	0.6% (2/313) 95% CI: 0.1–2.3	1.3% (3/240) 95% CI: 0.3–3.6	NS	OR 0.5 (95% CI: 0.1–3.0)

*Stimulation variables data corresponds to the fresh IVF cycle. Results are expressed as mean ± SD with 95% CI. Significance established at $P < 0.05$.

Subanalysis

Clinical outcomes of all cycles were analyzed according the first moment that the transferred embryo was observed FH in order to identify if the timing of embryo hatching was correlated with IR (Fig. 4, Table VI).

Fresh transfer cycles

FH before biopsy (therefore FH before transfer) versus NFH before biopsy, NFH before transfer (control group). BPR (65.6% versus 65.9%, $P > 0.05$, OR 1.0 [0.59–1.67]); IR (55.5% versus 54.5%, $P > 0.05$, OR 1.0 [0.63–1.71]); LBR (47.7 versus 43.1%, $P > 0.05$, OR 0.8 [0.5–1.4]); and EPL rate (18.0% versus 20.3%, $P > 0.05$, OR 0.9 [0.46–1.61]) of embryos observed FH before biopsy, and therefore at transfer, were similar to those that remained NFH (Fig. 4, Table VI).

NFH before biopsy but FH before transfer versus control group. BPR (75.0% versus 53.7%, $P < 0.05$, 1.6 OR [0.2–15.4]), IR (50.0% versus 65.9%, $P < 0.05$, 0.8 OR [0.11–6.13]), LBR (50.0% versus 43.1%, $P > 0.05$, OR 0.8 [0.1–5.5]) and EPL rate (25.0% versus 20.3%, $P < 0.05$, 1.3 OR [0.1–13.1] of embryos NFH at biopsy but FH at transfer were no different from those that remained NFH (Fig. 4, Table VI).

FET cycles

FH before vitrification, therefore FH after rewarming and before transfer versus NFH before vitrification, NFH after rewarming and NFH before transfer (control group). BPR (75.4% versus 73.8%, $P > 0.05$, OR 1.1 [0.7–1.7]), IR (66.1% versus 63.3%, $P > 0.05$, OR 1.1 [95% CI: 0.8–1.7]), LBR (55.0% versus 55.1%, $P > 0.05$, OR 1.0 [0.7–1.5]) and EPL rate (20.5% versus 18.4%, $P > 0.05$, OR 1.1 [0.7–1.8]) of embryos transferred under an FET cycle observed FH before vitrification were no different from those NFH at any point (Fig. 4, Table VI).

NFH before vitrification but FH after rewarming, therefore FH before transfer versus control group. BPR (66.7% versus 73.8%, $P > 0.05$, OR 0.7 [0.4–1.2]), IR (50.7% versus 63.3%, $P > 0.05$, OR 0.6 [0.4–1.0]) and EPL rate (29.0% versus 18.4%, $P < 0.05$, OR 1.8 [0.9–3.3]) of embryos observed NFH before vitrification, but FH after rewarming were no different from those NFH at any point, although a trend towards poorer results was observed. However, LBR was significantly decreased (37.7% versus 55.1%, $P < 0.05$, OR 0.5 [0.3–0.8]) when the embryo transitioned from NFH to FH after vitrification, with 50% less probability of achieving a live birth (Fig. 4, Table VI).

NFH before vitrification, NFH after rewarming but FH before transfer versus control group. BPR (68.4% versus 73.8%, $P = 0.60$, 0.8 OR [0.28–2.09]), IR (57.9% versus 63.3%, $P = 0.6$, 0.8 OR [0.31–2.0]), LBR (52.6% versus 55.1%, $P > 0.05$, OR 1.0 [0.5–2.3]) and EPL rate

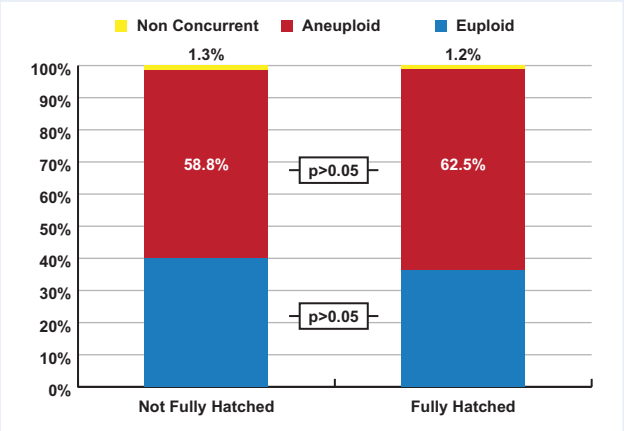

Figure 2 Ploidy rate by blastocyst hatching status (fresh and FET cycles considered together).

Table III Ploidy status and hatching status per day of human embryo biopsy.

	Day 5	Day 6	*P*
Total	57.8% ($n = 2633$)	42.2% (1926)	
NFH	56.2% ($n = 1389$)	43.8% ($n = 1083$)	<0.05
FH	59.6% ($n = 1244$)	40.4% ($n = 843$)	<0.05
Euploid	57.7% ($n = 1591$)	42.3% ($n = 1167$)	<0.05
Aneuploid	46.9% ($n = 818$)	53.1% ($n = 927$)	<0.05
Non-concurrent	42.9% ($n = 24$)	57.1% ($n = 32$)	<0.05

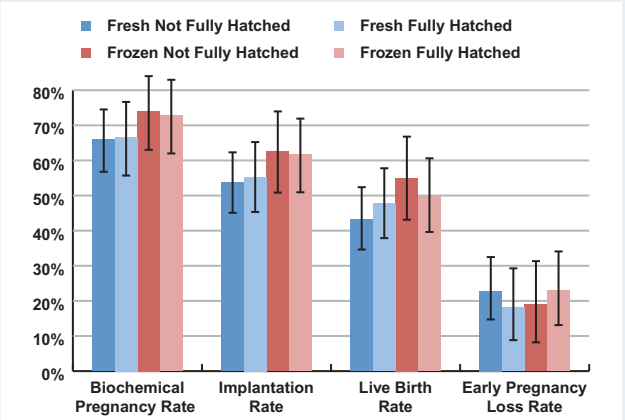

Figure 3 Clinical outcomes of not fully hatched versus fully hatched blastocysts in fresh and FET groups. Error bars represent 95% confidence interval (CI). * $P < 0.05$.

Table IV Clinical outcomes for FH versus NFH embryos.

	Fresh				Frozen			
	NFH	FH	*P*	OR	NFH	FH	*P*	OR
Cycles (*n*)	123	132			313	240		
Biochemical pregnancy rate	65.9% (81/123) 95% CI: 56.8–74.2	66.7% (88/132) 95% CI: 57.9–74.6	NS	OR 0.96 (95% CI: 0.6–1.6)	74.1% (232/313) 95% CI: 68.9–78.9	72.9% (175/240) 95% CI: 66.8–78.4	NS	OR 1.06 (95% CI: 0.7–1.6)
Implantation rate	53.7% (66/123) 95% CI: 44.4–62.7	55.3% (73/132) 95% CI: 46.4–64.0	NS	OR 0.94 (95% CI: 0.6–1.5)	62.6% (196/313) 95% CI: 57.0–68.0	61.7% (148/240) 95% CI: 55.2–67.8	NS	OR 1.04 (95% CI: 0.7–1.5)
Live birth rate	43.1% (53/123) 95% CI: 34.3–52.3	47.7% (63/132) 95% CI: 39.0–56.6	NS	OR 1.2 (95% CI: 0.7–1.9)	55.0% (172/313) 95% CI: 49.3–60.6	50.0% (120/240) 95% CI: 43.5–56.5	NS	OR 0.8 (95% CI: 0.6–1.1)
Early pregnancy loss rate	22.8% (28/123) 95% CI: 15.7–31.2	18.2% (24/132) 95% CI: 12–25.8	NS	OR 1.32 (95% CI: 0.7–2.4)	18.9% (59/313) 95% CI: 14.7–23.6	22.9% (55/240) 95% CI: 17.8–28.8	NS	OR 0.78 (95% CI: 0.5–1.2)

Fresh and frozen cycles are analyzed separately. Binomial CIs for all reported proportions. Adjusted OR and their 95% CI.

Table V Analysis of maximum likelihood estimates of implantation.

Parameter	Odds ratio estimates	95% Wald confidence limits		$P > \chi^2$
Fresh cycles				
Age (years) at IVF cycle	1.034	0.962	1.111	0.3657
BMI (kg/m^2) at transfer cycle	1.006	0.945	1.071	0.8519
Endometrial thickness (mm at LH surge) at transfer cycle	1.065	0.941	1.205	0.3180
Oocytes retrieved at IVF cycle	1.010	0.982	1.039	0.4878
FET cycles				
Age (years) at IVF cycle	0.970	0.891	1.055	0.4742
BMI (kg/m^2) at transfer cycle	0.980	0.905	1.061	0.6203
Endometrial thickness (mm at LH surge) at transfer cycle	1.189	0.970	1.459	0.0961
Oocytes retrieved at IVF cycle	1.011	0.982	1.040	0.4680

Multivariate logistic regression model including potential predictors of implantation: age, BMI, endometrial thickness at surge and number of oocytes retrieved. Fresh and FET cycles considered separately. The likelihood of implantation after IVF is presented as an OR with SE and 95% CI.

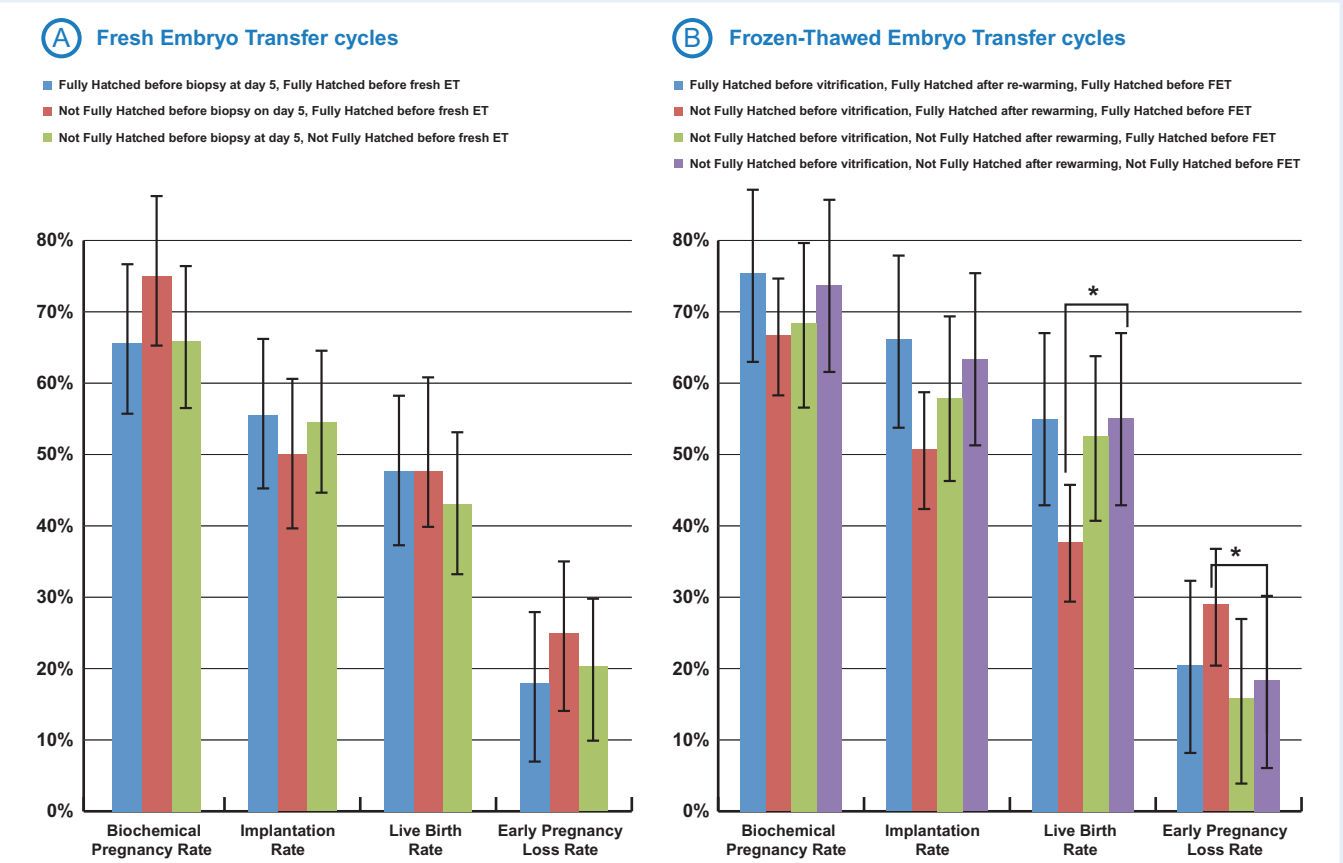

Figure 4 Clinical outcomes segregated according to the time the transferred embryo hatched. (**A**) FET cycles; (**B**) frozen ET cycles. Error bars represent 95% CI. *$P < 0.05$.

(15.8% versus 18.4%, $P = 0.8$, 0.83 OR [0.23–2.96]) of embryos observed FH at transfer were no different from those that remained NFH (Fig. 4, Table VI).

Fresh versus FET cycles

The reproductive outcome of NFH and FH transferred embryos in fresh versus FETs was evaluated (Fig. 5).

Table VI Clinical outcomes of all cycles stratified by the point of hatching.

	Fresh			Frozen			
	FH before biopsy Day 5, FH before ET	NFH before biopsy on Day 5, FH before ET	NFH before biopsy on Day 5, NFH before ET	FH before vitrification, FH after rewarming FH before ET	NFH before vitrification, FH after rewarming, FH before ET	NFH before vitrification, NFH at rewarming, FH before ET	NFH before vitrification, NFH at rewarming, NFH before ET
Cycles (*n*)	128	4	123	171	69	19	294
Biochemical pregnancy rate	65.6% (84/128) (95% CI: 0.57–0.74)	75% (3/4) (95% CI: 0.19–0.99)	65.9% (81/123) (95% CI: 0.57–0.74)	75.4% (129/171) (95% CI: 68.3–81.7)	66.7% (46/69) (95% CI: 54.3–77.6)	68.4% (13/19) (95% CI: 0.43–87)	73.8% (217/294) (95% CI: 0.68–0.79)
Implantation rate	55.5% (71/128) (95% CI: 0.46–0.64)	50.0% (2/4) (95% CI: 0.07–0.93)	54.5% (67/123) (95% CI: 0.45–0.63)	66.1% (113/171) (95% CI: 58.5–73.1)	50.7% (35/69) (95% CI: 38.4–63.0)	57.9% (11/19) (95% CI: 0.33–0.8)	63.6% (186/294) (95% CI: 0.57–0.69)
Live birth rate	47.7% (61/128) (95% CI: 0.38–0.56)	50.0% (2/4) (95% CI: 0.07–0.93)	43.1% (53/123) (95% CI: 0.34–0.52)	55.0% (94/171) (95% CI: 47.2–62.6)	37.7% (26/69) (95% CI: 26.3–50.2)	52.6% (10/19) (95% CI: 28.9–75.6)	55.1% (162/294) (95% CI: 49.2–60.9)
Early pregnancy loss rate	18.0% (23/128) (95% CI: 0.12–0.26)	25% (1/4) (95% CI: 0.01–0.81)	20.3% (25/123) (95% CI: 0.14–0.29)	20.5% (35/171) (95% CI: 14.7–27.3)	29.0% (20/69) (95% CI: 18.7–41.2)	15.8% (3/19) (95% CI: 0.03–0.4)	18.4% (54/294) (95% CI: 0.14–0.23)

Fresh and frozen cycles are analyzed separately. Binomial CIs for all reported proportions. Adjusted OR and their 95% CI. ET: embryo transfer.

When analyzing only NFH embryos, BPR (65.9% versus 74.1%, OR 1.5, 95% CI: 0.9–2.4), IR (53.7% versus 62.6%, OR 1.5, 95% CI: 0.9–2.2) and EPL rate (22.8% versus 18.8%, OR 1.3, 95% CI: 0.8–2.2) were similar between groups with a trend towards more optimal results in FET cycles. LBR after an FET of a NFH embryo was significantly higher compared with fresh transfer: 55.0% versus 43.1%, $P < 0.05$, being 1.6 times more likely to achieve a live birth after an FET cycle (OR 1.6 (95% CI: 1.1–2.4) $P < 0.05$).

When comparing only FH embryos, BPR (66.7% versus 72.9%, OR 1.4, 95% CI: 0.9–2.1), IR (55.3% versus 61.7%, OR 1.3, 95% CI: 0.9–2.0), LBR (47.7% versus 50.0%, OR 0.9, 95% CI: 0.6–1.4) and EPL rate (18.2% versus 22.9%, OR 1.3, 95% CI: 0.8–2.9) were similar although a trend towards superior outcomes was observed in the FET group.

Implantation versus no implantation

Comparison of fresh cycles that did and did not result in implantation revealed significant differences in the average number of antral follicles (14.1 ± 7.8 versus 12.1 ± 5.4, respectively), estradiol level at surge (2279.9 ± 1072.4 versus 2571.9 ± 1182.6) and proportion of embryos with an ICM graded A (86.9% versus 74.6%) or trophectoderm graded B (35% versus 47.5%) (Table VII). After comparison of FET cycles that did and did not result in implantation, significant differences were observed in the number of follicles >14 mm at surge in the stimulation cycle (13.5 ± 6.6 versus 12.2 ± 6.2, respectively) and proportion of embryos with an ICM graded A (73.2% versus 50.7%), trophectoderm graded A (29.1% versus 20.7%) and trophectoderm graded C (20.6% versus 29.1%) (Table VII).

Clinical outcomes in non-PGD cases

Table VIII shows the clinical outcomes in NFH versus FH of all fresh and FET cycles (single embryo transferred, same study period). No statistically significant differences were observed.

Discussion

ECM and PGS have become established procedures in reproductive medicine, widely used to aid in selection of optimal embryos prior to transfer. Adoption of these strategies has increased the prevalence of FH embryos, particularly at the time of transfer. This study was carried out to understand if the transfer of an FH blastocyst was correlated with lower reproductive outcomes when compared to blastocysts transferred at expansion Grade 4 or 5. The findings from this analysis suggest that the presumed uncertainties of embryo vulnerability to damage or adverse outcomes if handled improperly are not well founded, and the deleterious effect of full blastocoelic expansion and zona pellucida loss in euploid embryos is, at best, limited. The results from this study suggest that FH embryos are not more fragile or less likely to implant when compared to NFH counterparts.

Blastocyst hatching is a critical step in the sequence of physiologic events that culminate in implantation. Although the current knowledge base on spontaneous hatching is mainly derived from nonhuman studies performed *in vitro* (Cole, 1967; Wright *et al.*, 1976; Massip *et al.*, 1982; Gonzales and Bavister, 1995; Montag *et al.*, 2000; Niimura *et al.*, 2010), failure to hatch may be one of the many factors limiting human reproductive efficiency (ASRM, 2014). ECM, d3 laser-assisted hatching and trophectoderm biopsy help bypass this potential barrier and

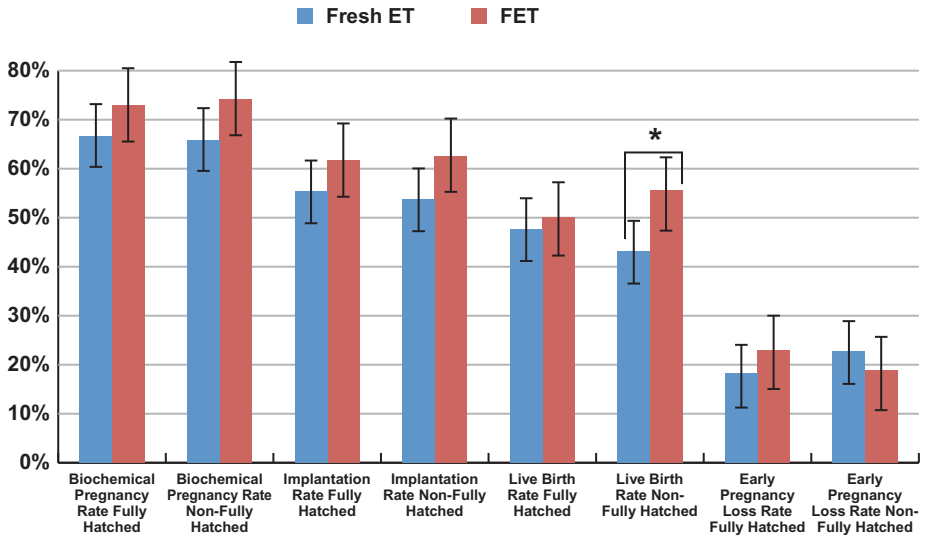

Figure 5 Clinical outcomes of not fully hatched and fully hatched blastocysts in fresh versus frozen cycles. Error bars represent 95% CI. *$P < 0.05$.

facilitate early hatching, thus increasing the potential for an embryo to be biopsied on d5 and/or d6. Nevertheless, new procedures in the laboratory could carry potential concerns. Though there are no previous reports defining it, FH embryos are traditionally suspected to be more sensitive to mechanical damage, particularly at the interface between dividing cells or via splitting of trophectoderm cells where the intercellular contacts may weaker than normal. To our knowledge, this is the first study to evaluate the potential vulnerability to tissue damage that an FH, chromosomally screened embryo may be prone to during handling before a fresh transfer and/or secondary to vitrification-rewarming and subsequent FET. The clinical results do not suggest any detrimental effect after the transfer of zona-free blastocysts, as reflected by similar BPRs, IRs, LBRs and EPL rates between groups, regardless of hatching status or time of hatching.

Delayed blastocoelic expansion has been postulated to be a marker of delayed blastocyst growth and hence poses a barrier to implantation (Luna *et al.*, 2008). Previous impressions have inferred aneuploidy to be a restrictive agent on embryo hatching (Vega *et al.*, 2014), which, when combined with embryo–endometrium asynchrony (Shapiro *et al.*, 2008), renders suboptimal implantation potential. In this study, euploidy rates were comparable between NFH and FH embryos (58.8% versus 62.5%, $P > 0.05$) (Fig. 2, Tables I and II), suggesting that euploidy rates are comparable regardless of hatching status. This finding may pivot clinicians away from any ambiguity (Kroener et al., 2012; Cervantes *et al.*, 2013) over the view that spontaneously hatched blastocysts should be initially selected before less expanded equivalents when ploidy screening is not used, especially in FET cycles. Prospective studies of euploid embryo hatching would better address these concerns.

Although traditionally embryos are biopsied on d5, a growing number are conducted on d6. In this study, 57.8% of the embryos were biopsied on d5 and 42.2% on d6 (Table III). Additionally, even though developmentally delayed embryos biopsied on d6 were shown to have an increased aneuploidy rate, it appears to be advantageous to culture embryos one extra day to allow for blastulation, as still a 42.3% euploidy rate is obtained from d6 embryos.

Concerns regarding survival of FH blastocysts at the time of transfer have been extrapolated from mouse studies in which hatched blastocysts were more likely to bind to the inner surface of the cryo straw (Zhu *et al.*, 1996). However, the authors did not observe such findings in human blastocysts nor for adhesion to the inner surface of the embryo replacement catheter or to culture dishes. To our knowledge, this is the first time that the proportion of retained embryos (retained in the transfer catheter) in a large cohort is reported, both on the basis of the hatching status and on the cycle type. This study did not find any correlation between hatching status and embryo retention.

Another concern regarding expanded blastocysts is the suspected disadvantage to survival rate after vitrification (Cho *et al.*, 2002). Expanded blastocysts have more blastocoelic fluid, in which ice crystals may form during cooling (Cobo *et al.*, 2012). However, with recent advances in cryopreservation methods (Cobo *et al.*, 2012), the quality of frozen embryos and their reproductive potential in general is at least similar (Son *et al.*, 2003), if not possibly better than fresh embryos (Rodriguez-Purata *et al.*, 2016). In this study, a similar survival rate in the NFH and the FH transferred embryos was observed (Table II). This can most likely be linked to the embryo collapsing and leaking fluid immediately after biopsy; a state in which it remains prior to vitrification. The rewarming protocol in this study did not differ between embryos observed NFH or FH prior to vitrification. Our results suggest that embryologists can confidently handle FH embryos similarly to NFH counterparts. Additionally, embryos vitrified at expansion Grade 4 or 5 are sometimes observed FH after the rewarming procedure or at the moment of the transfer (Edgar and Gook, 2012). This study included some embryos that underwent this transition. Although BPR, IR and EPL rate from embryos observed FH after rewarming were similar, a trend toward poorer results was observed. Furthermore, LBR was statistically lower in such cycles (Fig. 4, Table VI). This scenario is most probably explained by the sudden non-physiologic loss of the zona's protection, preventing proper blastocyst apposition for embryo implantation.

Table VII Demographic characteristics and embryological data for fresh and FET cycles that did or did not result in implantation.

Group	Fresh			Frozen		
	Implantation	No implantation	*P*	Implantation	No implantation	*P*
Cycles (*n*)	137	118		340	213	
Age (years) at IVF cycle	36.7 ± 4.2	36.6 ± 3.8	NS	36.1 ± 4.1	36.4 ± 4.1	NS
Age (years) at ET cycle	36.7 ± 4.2	36.6 ± 3.8	NS	36.4 ± 4.1	36.9 ± 4.1	NS
BMI (kg/m^2) at IVF cycle	23.5 ± 4.3	23.4 ± 4.1	NS	22.9 ± 3.9	23.4 ± 4.1	NS
BMI (kg/m^2) at ET cycle	23.5 ± 4.3	23.4 ± 4.1	NS	23.0 ± 3.9	23.4 ± 4.1	NS
Day 3 FSH (mUI/ml) at IVF cycle	6.0 ± 3.0	6.0 ± 3.0	NS	6.0 ± 2.8	5.9 ± 2.8	NS
Day 3 AMH (ng/ml) at IVF cycle	3.4 ± 2.9	3.1 ± 2.8	NS	3.6 ± 4.4	4.0 ± 6.7	
Day 3 AFC (*n*)	14.1 ± 7.8	12.1 ± 5.4	<0.05	13.4 ± 7.2	13.4 ± 8.1	NS
Endometrial thickness (mm, at LH surge) at IVF cycle	10.0 ± 2.1	9.7 ± 2.1	NS	10.0 ± 2.1	9.7 ± 1.9	NS
Endometrial thickness (mm, at LH surge) at transfer cycle	10.0 ± 2.1	9.7 ± 2.1	NS	9.1 ± 1.6	8.7 ± 1.3	NS
Follicles >14 mm at LH surge	13.4 ± 6.4	13.3 ± 6.1	NS	13.5 ± 6.6	12.2 ± 6.2	<0.05
E_2(pg/ml) at LH surge at IVF cycle	2279.9 ± 1072.4	2571.9 ± 1182.6	<0.05	2522.7 ± 1153.4	2324.2 ± 1125.3	NS
E_2 at surge (ET cycle)	2279.9 ± 1072.4	2571.9 ± 1182.6	<0.05	431.4 ± 475.6	388.0 ± 242.6	NS
Oocytes retrieved (*n*)	17.7 ± 9.6	17.2 ± 9.3	NS	17.4 ± 10.7	16.2 ± 10.6	NS
Oocytes inseminated (*n*)	13.7 ± 8.5	13.4 ± 7.6	NS	13.5 ± 9.0	12.5 ± 8.8	NS
Embryos ongoing Day 1 (*n*)	11.1 ± 7.0	11.0 ± 7.4	NS	11.1 ± 7.5	10.0 ± 7.2	NS
Embryos ongoing Day 3 (n)	10.8 ± 6.9	10.9 ± 6.8	NS	10.6 ± 7.2	9.7 ± 6.9	NS
Embryos ongoing Day 5 (n)	8.1 ± 5.7	8.4 ± 5.9	NS	7.0 ± 5.5	6.3 ± 5.0	NS
Embryos ongoing Day 6 (n)	7.6 ± 5.4	7.8 ± 5.7	NS	5.5 ± 4.5	5.2 ± 4.3	NS
Morphology						
Expansion						
4	6.6% (9/137)	7.6% (9/118)	NS	27.9% (95/340)	31.5% (67/213)	NS
5	40.9% (56/137)	41.5% (49/118)	NS	29.1% (99/340)	23.9% (51/213)	NS
6	52.6% (72/137)	50.8% (60/118)	NS	42.6% (145/340)	44.6% (95/213)	NS
Inner cell mass						
A	86.9% (119/137)	74.6% (88/118)	0.05	73.2% (249/340)	50.7% (108/213)	<0.05
B	13.1% (18/137)	25.4% (30/118)	0.05	26.8% (91/340)	49.3% (105/213)	<0.05
Trophectoderm						
A	65.0% (89/137)	53.4% (63/118)	NS	29.1% (99/340)	20.7% (44/213)	<0.05
B	35% (48/137)	47.5% (56/118)	0.05	50.3% (171/340)	50.2% (107/213)	NS
C				20.6% (70/340)	29.1% (62/213)	<0.05
Embryos biopsied (*n*)						
Total	6.1 ± 4.3	6.3 ± 4.7	NS	5.7 ± 4.2	5.4 ± 4.0	NS
Day 5	3.9 ± 2.9	4.1 ± 3.3	NS	3.2 ± 3.5	2.7 ± 3.1	NS
Day 6	2.2 ± 2.3	2.2 ± 2.3	NS	2.5 ± 2.0	2.6 ± 2.1	NS
Euploidy rate	60.9% (500/821)	59.7% (447/748)	NS	60.6% (1141/1884)	57.4% (638/1111)	NS
Embryos vitrified (*n*)	3.6 ± 3.7	3.8 ± 3.9	NS	5.3 ± 4.1	4.9 ± 3.8	NS
Survival rate	NA	NA		96.6% (372/385)	96.6% (226/234)	NS
Retained embryos at transfer	1.5% (2/137)	5.1% (6/118)	NS	0.6% (2/340)	1.4% (3/213)	NS

Results are expressed as mean ± SD. Significance established at $P < 0.05$.

Interestingly, we observed high EPL rates across all groups studied (fresh cycles: 20.3% versus 18.2%, $P = 0.66$; FETs cycles: 18.4% versus 22.1%, $P = 0.9$). Although a significant number of women will suffer a spontaneous abortion without any identifiable abnormality (Saravelos and Regan, 2014), there are other non-genetic etiologies of EPL (Christiansen *et al.*, 2015). This study's finding may be explained by the presence of polyploidic abnormalities undetected by current PGS technology (Werner *et al.*, 2014), embryos with normal chromosomal

Table VIII Clinical outcomes in non-PGD cases (only single ET cycles).

	Fresh			Frozen		
	NFH	FH	P	NFH	FH	P
Cycles (*n*)	1676	3		602	86	
Biochemical pregnancy rate	37.8% (634/1676) (95% CI: 0.35–0.40)	33.3% (1/3) (95% CI: 0.01–0.91)	0.87	50.7% (305/602) (95% CI: 0.47–0.55)	53.5% (46/86) (95% CI: 0.42–0.64)	0.62
Clinical pregnancy rate	28.8% (483/1676) (95% CI: 0.27–0.31)	0% (0/3)	0.27	37.2% (222/602) (95% CI: 0.33–0.41)	34.9% (30/86) (95% CI: 0.25–0.46)	0.71
Early pregnancy loss rate	14.9% (250/1676) (95% CI: 0.13–17)	33.3% (1/3) (95% CI: 0.01–0.91)	0.37	21.9% (132/602) (95% CI: 0.19–0.25)	27.9% (24/86) (95% CI: 0.19–0.39)	0.21

Binomial CIs for all reported proportions. Adjusted OR and their 95% CIs. Significance established at $P < 0.05$.

segregation but not with enough cytoplasmic quality to result in a fully capable embryo or chromosomal mosaicism (Taylor *et al.*, 2014) and all these in conjunction with conservative interpretations of genetic results.

Fresh cycles in which FH embryos were selected for transfer were more likely to originate from cycles with a higher average number of ongoing embryos on d3, d5 and d6 compared with cycles in which NFH embryos are selected (Table I). This was not observed in the planned freeze-all/FET cycle group. Because all embryos undergo the same handling until embryo disposition decision (ET or vitrification), the authors do not believe this variable impacted results although it suggests that there may be an unstated preference among some embryologists for selection of an FH embryo for fresh transfer when the option is available.

In spite of the numerous efforts taken to avoid biases in the study, some limitations have persisted. First, the retrospective nature of the study creates a selection bias. Second, although most of the patients who underwent an IVF/PGS cycle were characteristically 'normal' or 'good' responders, this study was not limited to them. Third, we recognize that not all patients, regardless of COH response, develop high-quality blastocysts; therefore, this approach is not suitable for every patient. Specifically during the period of this study, there were 1953 planned PGS/PGD cycles, of which 20.7% ($n = 404$) were canceled before biopsy. Only 41.4% ($n = 808$) of the cycles were eligible for this study. Fourth, the impetus to use PGS has evolved in 'Fresh' and 'FET' groups. PGS in patients undergoing a fresh transfer is primarily employed to circumvent the age-related increase in aneuploidy and minimize the interval to a successful pregnancy. However, the majority of patients undergoing freeze-all cycles have been affected by a genetic disease (i.e. a single gene disorder) and encounter a lag period before obtaining genetic interpretation, thus preventing their ability to pursue a fresh transfer. While increasingly more patients are being steered toward a freeze-all approach given the potential of increased PRs, persistent differences in PGS indication could impact aneuploidy and/or cancellation rates. Nevertheless, pregnancy outcomes would not be expected to significantly change, as all study patients had at least one euploid embryo available for transfer. Finally, while the study was appropriately powered to detect substantial differences in IR between NFH versus FH embryos, it was not powered to detect significance when analyzing the specific moment that the transferred embryo was observed FH. Findings within the small number of patients included in each subgroup denied us the ability to draw definite conclusions, although the results find no support for an impact of the timing of embryo hatching on IRs.

As the use of ART continues to increase worldwide, it remains of the utmost importance to maintain the safety of techniques used. These manipulations include ECM, ICSI, assisted hatching, PGS and vitrification. In combination with all these strategies, oocyte and embryo handling is also a delicate part of the IVF process that continues to improve. This study suggests that these interventions have no detectable negative impact on clinical outcomes. Nevertheless, the fact that a pregnancy is established does not preclude the presence of underlying anomalies. Therefore, gene expression studies that evaluate intervention versus nonintervention are needed to subsequently determine any potential non-genomic effects. While the results of this study cannot currently lead to any definitive clinical recommendations, we found no evidence of altered IR or clinical outcomes with the transfer of FH euploid embryos.

Authors' roles

J.R.P.: drafted the manuscript, analyzed and interpreted the data; J.G.: writing and statistical support; J.A.L.: editing; M.C.W.: query design; R.S. and C.B.J. embryologist involved in PGS cases, editing; A.B.C.: revision of intellectual content and final version of the manuscript; B.S.: intellectual design and final version of the manuscript.

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Conflict of interest

None declared.

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