Reproductive BioMedicine Online (2016) 33, 149–160

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Higher β-HCG concentrations and higher birthweights ensue from single vitrified embryo transfers

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Dr John Yovich graduated in Medicine at the University of Western Australia in 1970, progressing into specialist Obstetrics and Gynaecology practice in 1976. Thereafter Dr Yovich presented his MD thesis *"Human pregnancies achieved by In-Vitro Fertilisation"* following laboratory research and clinical work undertaken with Professor Ian Craft at the Royal Free Hospital in London (1976–1980). This thesis and more than 200 other refereed publications from the early years can be found online in Research Gate, the scientific online network. He established PIVET Medical Centre in his hometown of Perth in 1981, the first private independent fertility management facility in Australia.

Abstract To examine the effect of cryopreservation on developmental potential of human embryos, this study compared quantitative β-HCG concentrations at pregnancy test after IVF-fresh embryo transfer (IVF-ET) with those arising after frozen embryo transfer (FET). It also tracked outcomes of singleton pregnancies resulting from single-embryo transfers that resulted in singleton live births ($n = 869$; with 417 derived from IVF-ET and 452 from FET). The initial serum β-HCG concentration indicating successful implantation was measured along with the birthweight of the ensuing infants. With testing at equivalent luteal phase lengths, the median pregnancy test β-HCG was significantly higher following FET compared with fresh IVF-ET (844.5 IU/l versus 369 IU/l; $P < 0.001$). Despite no significant difference in the average period of gestation (38 weeks 5 days for both groups), the mean birthweight of infants born following FET was significantly heavier by 161 g (3370 g versus 3209 g; $P < 0.001$). Furthermore, more infants exceeded 4000 g ($P < 0.001$) for FET although there was no significant difference for the macrosomic category (≥4500 g). We concluded that FET programme embryos lead to infants with equivalent (if not better) developmental potential compared with IVF-ET, demonstrated by higher pregnancy β-HCG concentrations and ensuing birthweights.

KEYWORDS: β-HCG, birthweight, FET, IVF-ET, single embryo transfer, vitrification

http://dx.doi.org/10.1016/j.rbmo.2016.04.014

Introduction

Cryopreservation might compromise the cleavage-stage embryo or the trophoblast, thus affecting the ability of the embryo to implant. Any negative effect of frozen embryo transfer (FET) may be due to apoptotic damage to embryos as a result of cryopreservation, the thawing process, or both (Li et al., 2012). However, we have an optimistic view about vitrification (Vajta et al., 2009), and this is in agreement with a recent publication (Sites et al., 2015) that found that vitrification (rather than slow-freezing) had no negative effect on the initial beta-human chorionic gonadotrophin (β-HCG) concentration or developmental potential of embryos cryopreserved.

It is well known that β-HCG plays important roles in the success of implantation and establishment of early pregnancy. Its role in embryo implantation may be exerted through its corresponding receptors on the endometrium. It also stimulates adenylate cyclase and production of progesterone through action on its receptors in trophoblast cells. Additionally, β-HCG induces relaxin secretion by the corpus luteum during the luteal phase and in early pregnancy. Both the relaxin and progesterone produced are important in the maintenance of early pregnancy (Keay et al., 2004). The serum β-HCG concentration could therefore be a good indicator of how successful a pregnancy is going to be, as shown in our earlier report (Lingam and Yovich, 2007).

PIVET Medical Centre has been using the vitrification process since late 2007 (Kuwayama et al., 2005). Anecdotal evidence suggested that the β-HCG pregnancy test in patients receiving FET appeared to be higher than those receiving fresh IVF-embryo transfer (IVF-ET). This raised the question of whether FET patients will continue to have a better outcome than those with fresh transfer, especially with regards to the birthweight. This study was carried out to address this observation.

Materials and methods

Patient selection and embryology

The data for this retrospective report was extracted from the database at PIVET Medical Centre (1 April 2008 until 30 April 2014 inclusive). All single-embryo transfer (SET) procedures following IVF-ET were analysed and their pregnancy outcomes compared with those of single cryopreserved embryo transfer procedures using vitrified-only embryos. No treatment cycles were excluded due to age or patient history, but those cycles utilizing donor oocytes were excluded because of the mixed component of cryopreserved oocyte followed by fresh embryo transfer. Women who were found to carry twins after the SET were invariably monozygotic and were excluded from the study for a possible confounding effect (**Figure 1**). Most embryo transfers at PIVET were either day 3 or day 5 (blastocyst) embryos. Day 3 embryos were graded based on PIVET's clinical protocol, which has been simplified from an earlier version (Yovich and Grudzinskas, 1990). Day 5 embryos were graded using the Gardner blastocyst grading system (Gardner and Schoolcraft, 1999). Embryos graded BC or CB or less would not be cryopreserved. Yovich et al., 2015a has reclassified these into specific groups based on the implantation rates as well as live birth rate.

SET cycles, in keeping with Australian standards (Macaldowie et al., 2015), were selected to avoid any bias in the interpretation of the pregnancy test β-HCG arising from other embryos even if such failed to implant. In addition, analysis was performed only on those with the outcome of singleton live births, hence excluding biochemical (non-continuing) pregnancies, miscarriages or blighted ova, and ectopic pregnancies as well as terminations and stillbirths. No cases included embryos screened for pre-implantation genetic diagnoses so would not be likely to cause bias towards higher implantation rates and better quality pregnancies (**Figure 1**). Pregnant patients who were lost to follow-up were tabulated as "no known outcome" and were also excluded despite having a clinical pregnancy diagnosed at 7 weeks' gestation.

Embryos not transferred during a fresh cycle were cryopreserved by vitrification using the Cryotop method (Kuwayama et al., 2005; Seet et al., 2012), mostly at the blastocyst stage following culture in Sydney IVF blastocyst medium (Cook Medical) applying sequential phases for fertilization, cleavage and blastocyst stages. Follicle stimulation, oocyte recovery, transfer and cryopreservation as well as embryo culture systems have been fully described elsewhere (Stanger et al., 2012; Yovich et al., 2012; Yovich and Stanger, 2010; Yovich et al., 2015b).

Ovarian stimulation for IVF cycles

Patients were stimulated with long down-regulation, flare cycle or antagonist protocols (Yovich et al., 2012; Yovich and Stanger, 2010). The selection of the stimulation protocol was at clinician's discretion, but the antagonist regimen was usually used for younger women with higher antral follicular count (AFC), and the flare regimen for older women with low AFC.

Ovulation triggering and luteal support for IVF

Ovulation triggering was usually initiated with a single dose of recombinant HCG (rHCG; Ovidrel: Merck Serono), two ampoules equating to approximately 13,000 IU rHCG, when there were at least two leading follicles ≥18 mm in diameter. For patients with fewer than four follicles or a previous poor recovery, three ampoules (Ovidrel) approximating to 19,500 IU rHCG, was used as the trigger. In those antagonist cycles with excessive follicle recruitment (>12 follicles over 12 mm), gonadotrophin-releasing hormone agonist (Lucrin: Abbott) trigger 50 IU was used. Oocyte recovery was at 35–37 h post trigger. IVF-ET luteal support was based on the number of oocytes recovered (Yovich et al., 2012), involving rHCG injections (where oocyte numbers were ≤12). Pregnyl 1500 IU s.c. was administered on days 6, 9, 12, and 15 after trigger with or without progesterone pessaries (Wembley Pharmacy compounded for PIVET).

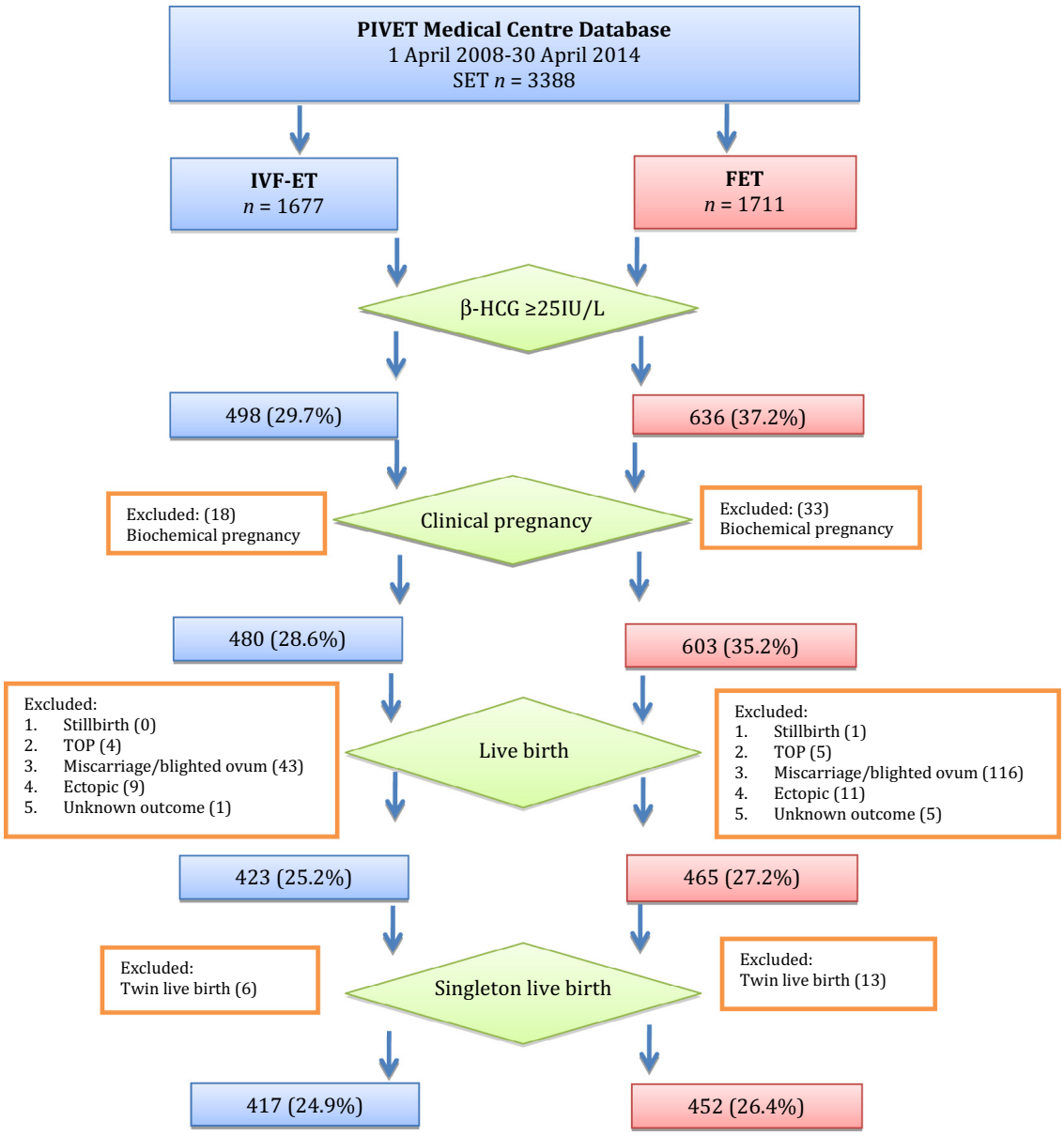

Figure 1 Flow chart of SET treatment cycles selection for pregnancy test β-HCG value and singleton live-birth outcome following IVF-ET versus FET. FET = frozen embryo transfer; HCG = human chorionic gonadotrophin; IVF-ET = IVF fresh embryo transfer; TOP = termination of pregnancy.

Endometrial preparation and luteal support for FET cycles

FET-hormone replacement therapy

FET-hormone replacement therapy (FET-HRT) (Yovich et al., 2015b) was used in 393 (87%) FET cycles. Briefly, oestradiol valerate tablets (Progynova 4 mg t.d.s.; Schering Plough) were administered from day 1 of the cycle followed by oestradiol vaginal pessaries (10/20 mg o.d.) on day 10 and continued for 5 days ±1 (or extended further until endometrial lining was ≥8 mm). Then progesterone pessaries were administered (400 mg b.d. and combo pessary nocte [progesterone 500 mg + oestradiol 2 mg]), which indicated the end of the artificial "follicular" phase and onset of the artificial "luteal" phase (Figure 2). Day 3 embryo transfer was performed on the fourth day of progesterone pessaries, whereas day 5/6 embryos were transferred on the sixth day of progesterone pessaries. The Progynova, progesterone and combo pessaries were continued for luteal support until 10 weeks' gestation and then weaned off over 2 weeks.

FET-low-dose stimulation

FET-low-dose stimulation (FET-LDS) was used in 55 (12%) FET cycles and started on day 3 of the menstrual cycle with daily low dose FSH (50–75 IU) or Tamoxifen 20 mg b.d. from day 2 to day 10. Transvaginal ultrasound scan and serum oestradiol, progesterone and LH were monitored from day 8 every second day until ready for HCG trigger (follicle ≥16 mm and

Luteal phase day	Fresh IVF-ET	FET-LDS/NAT	FET-HRT
0	Trigger day	Trigger day	
1	Day after trigger	Day after trigger = day 14 of pregnancy dating	Progesterone pessaries started = day 14 of pregnancy dating
2	TVOA = day 14 of pregnancy dating		
3			
4		Day 3 ET	Day 3 ET
5	Day 3 ET		
6	rHCG	Day 5/6 ET: rHCG	Day 5/6 ET
7	Day 5 ET		
8			
9	rHCG	rHCG	
10			
11			
12	rHCG	rHCG	
13			
14			
15	rHCG	rHCG	
16			
17			
18			
19	Pregnancy test	Pregnancy test	Pregnancy test

Figure 2 Overview of luteal day estimation and day 14 pregnancy dating for the different treatment cycles, showing days when luteal phase HCG support injections were applied. FET = frozen embryo transfer; HRT = hormone replacement therapy; IVF-ET = IVF fresh embryo transfer; LDS = low-dose stimulation; NAT = natural; rHCG = recombinant human chorionic gonadotrophin; TVOA = transvaginal oocyte aspiration.

endometrial lining ≥8 mm). Four days after trigger (Pregnyl 10,000 IU), a day 3 embryo was transferred, or day 5 or 6 embryos were transferred 6 days after trigger (Figure 2). Luteal supports were achieved by administering Pregnyl 1500 IU on days 6, 9, 12 and 15 after trigger (similar to the IVF-ET regimen).

FET-natural

FET-natural (FET-NAT) was applied in only 4 (0.9%) FET cycles, where the TV ultrasound scan and serum oestradiol, progesterone and LH concentrations were monitored from day 8 every second day until ready for trigger (follicle ≥16 mm and endometrial lining ≥8 mm). Following Trigger (Pregnyl 5000 IU), luteal supports were administered as per FET-LDS.

β-HCG measurement

β-HCG assays (Siemens, Cat# 10634917) were performed according to the manufacturer's instructions, using the ADVIA Centaur XP Immunoassay System (Siemens). In-house coefficients of assay variability were <7% for β-HCG in the range of 2–1000 IU/l. Any higher values were performed in dilution without affecting the coefficients of variability.

Pregnancy detection and luteal day definitions in multiple treatment types

Patients had their blood drawn by venipuncture (07.30–09.30 am) daily Monday to Saturday. For IVF-ET, luteal day

1 was the day after trigger (the first day of detectable progesterone elevation), and transvaginal oocyte aspiration (TVOA) was conducted on luteal day 2, being the second day of significant progesterone elevation. The pregnancy test was performed 17 or 18 days after TVOA (luteal day 19 or 20) (**Figure 2**). The later day was for Monday pregnancy test, being 18 days after TVOA but avoiding a Sunday test. Gestation is traditionally calculated from onset of the last menstrual period (LMP), with the 14th day regarded as the day of ovulation i.e. day 2 of the luteal phase or progesterone elevation. With TVOA referenced as day 0 = "ovulation", all the IVF-ET treatment cycles were referenced to that day as day 14 for gestation determination.

For FET-LDS and FET-NAT, the day after trigger was also considered luteal day 1, which was managed by HCG injections (day 6, 9, 12 and 15) (**Figure 2**). The pregnancy test was performed on day 19, being 4 days after the last HCG on day 15, when β-HCG concentrations are known to be <15 IU/l for non-pregnant cases (**Figure 2**). Concentrations 15–24 IU/l were regarded as equivocal and repeated 3 days later, sometimes resulting in clinical pregnancies and occasionally proceeding to live birth, but mostly resulting in pregnancy failure. Although β-HCG concentrations above 5 IU/l constitute a significant detection, we applied ≥25 IU/l for the diagnosis of pregnancy (to avoid detecting residual rHCG from the trigger or luteal phase support regimens).

For FET-HRT, the first day of progesterone pessaries was considered as day 1 of the artificial luteal phase, and pregnancies were diagnosed on day 19 (or day 18 or day 20 to avoid Sunday). For IVF-ET, the gestational age was calculated by applying the concept that day 14 of an adjusted menstrual cycle occurred on day 2 of the luteal phase. For FET-HRT, the first day of progesterone pessary was considered equivalent to day 14 of pregnancy dating; and for FET-LDS, the day after trigger was taken as day 14 of pregnancy dating (**Figure 2**).

Progression of the pregnancies were further confirmed weekly by β-HCG determination (up until 8 weeks' gestation), and dated at 7 weeks' gestation using transvaginal ultrasound. If an intrauterine gestational sac with fetus and fetal heartbeat was not clearly detected, the diagnosis was pursued by further investigations and categorized as delayed miscarriage, ectopic gestation, pregnancy of unknown location, biochemical or blighted ovum (Yovich and Lower, 1991).

Thereafter, patients were managed until 12 weeks with a review scan as part of first trimester screening prior to referral to their obstetrician. PIVET data tracking ensures all women were contacted within 2 weeks after their expected delivery date to determine pregnancy outcome if not already reported.

Ethical consideration

PIVET is accredited with the National Australian Reproductive Technology Committee and the Reproductive Technology Council of Western Australia. These agencies monitor all activities conducted at PIVET. Reporting of the data was approved under Curtin University Ethics Committee approval no. RD_25–10 general approval for retrospective data analysis 2015.

Statistical analysis

SPSS version 22 (IBM Corp., USA) was used for the statistical analysis. Independent sample t-test or one-way ANOVA were used to compare means and Mann–Whitney *U*-test or Kruskal–Wallis test was used to compare the median. Pearson chi-squared test was used when groupings of data were analysed. Statistical significance was considered when the *P*-values were <0.05.

Results

During the 6-year study period, there were 3388 SET procedures undertaken using vitrified-warmed embryo transfer and IVF-ET protocols (**Figure 1**). This generated a β-HCG concentration of ≥25 IU/l for 1134 women ($n = 636$ [37.2%] FET and $n = 498$ [29.7%] IVF-ET; $P < 0.001$) with 1083 progressing to clinical pregnancies ($n = 603$ [35.2%] FET and $n = 480$ [28.6%] IVF-ET; $P < 0.001$) and produced 888 live-birth deliveries ($n = 465$ FET and $n = 423$ IVF-ET) including monozygotic twins ($n = 13$ FET and $n = 6$ IVF-ET). Although pregnancy rates were higher in the FET group, the live-birth rates were similar (27.2% versus 25.2%; ns). **Figure 1** shows that whilst pre-clinical (biochemical) pregnancy losses were similar (3.6% versus 5.2%), clinical-stage losses (miscarriage and ectopics) were significantly higher from FET than IVF-ET (21.1% versus 10.8%; $P < 0.001$). Twin pregnancies (19 pairs), stillbirth ($n = 1$), pregnancies that were terminated due to fetal abnormalities ($n = 9$), failed pregnancies – either miscarriage/blighted ovum ($n = 159$) or ectopic pregnancies ($n = 20$) – were excluded from analysis. Patients with confirmed clinical pregnancies but were lost to follow-up were also excluded ($n = 6$).

Of the 603 FET clinical pregnancies, 122 (20.2%) were derived from embryos cryopreserved in a freeze-all cycle and 481 (79.8%) were derived from supernumerary embryos from a standard IVF-ET cycle. There were 291 SET cycles from freeze-all embryos with pregnancies arising in 122 cases (41.9%) and 81 resultant live births (27.8% of SET). For the supernumerary cryopreserved embryos, there were 1420 SET procedures resulting in 481 clinical pregnancies (33.9%) and 371 resultant singleton live births (26.1%). Importantly, only single live birth pregnancy outcomes were analysed in this data set (452 FET and 417 IVF-ET) – signifying 26.4% (452/1711) and 24.9% (417/1677) singleton live birth per SET.

Patient demographics show that the mean age of patients at the time of the SET for fresh cycles was significantly lower than at the time of the FET (32.8 versus 34.3 years; $P < 0.001$) (**Table 1**). However, this might not be relevant clinically as the higher success rates in assisted reproductive treatments are related to women aged less than 35 years and this was demonstrated in the mean of both groups. Nonetheless, the FET group had more patients in the ≥40 age groups (13% versus 6%). Furthermore, the mean body mass index (BMI) of both groups at the time of transfer did not differ significantly and therefore should not have any influence on the outcome analysis (**Table 1**). In addition, there was no significant difference in the gender proportion of infants born in IVF-ET or FET cycles (51.1% male versus 48.9% female in both treatment protocols) (**Table 1**). Furthermore, there was a low incidence of gestational diabetes mellitus (GDM) with

Table 1 Demographics for patients with singleton live birth following SET of fresh IVF-ET and FET including infant gender and presence of GDM.

Parameter	*IVF-ET* n = *417 (48%)*	*FET* n = *452 (52%)*	*Total*	P-*value*
Age (years)				
Mean ± SD	32.8 ± 4.4	34.3 ± 4.8		<0.001[a]
<35 years, *n* (%)	267 (64.0)	226 (50.0)	493 (56.7)	<0.001[b]
35–39 years, *n* (%)	125 (30.0)	166 (36.7)	291 (33.5)	<0.05[b]
40–44 years, *n* (%)	25 (6.0)	51 (11.3)	76 (8.7)	<0.01[b]
≥45 years, *n* (%)	0 (0.0)	9 (2.0)	9 (1.0)	<0.01[b]
Total, *n* (%)	417 (100)	452 (100)	869 (100)	
BMI				
Mean ± SD	24.9 ± 4.8	24.8 ± 9.9		NS[a]
<18.5, *n* (%)	17 (4.1)	14 (3.1)	31 (3.6)	NS[b]
18.5–24.9, *n* (%)	232 (55.6)	271 (60.0)	503 (57.9)	NS[b]
25.02–9.9, *n* (%)	97 (23.3)	117 (25.9)	214 (24.6)	NS[b]
≥30.0, *n* (%)	71 (17.0)	50 (11.1)	121 (13.9)	NS[b]
Total, *n* (%)	417 (100)	452(100)	869 (100)	
Live-birth gender				
Male, *n* (%)	213 (51.1)	231 (51.1)	444 (51.1)	NS[b]
Female, *n* (%)	204 (48.9)	221 (48.9)	425 (48.9)	
Total, *n* (%)	417 (100)	452(100)	869 (100)	
Confirmed GDM cases				
Male, *n* (%)	5 (2.3)	7 (3.0)	12 (2.7)	
Female, *n* (%)	4 (2.0)	7 (3.2)	11 (2.6)	NS[b]
Total, *n* (%)	9/417 (2.2)	14/452 (3.1)	23/869 (2.6)	

BMI = body mass index; FET = frozen embryo transfer; GDM = gestational diabetes; IVF-ET = IVF fresh embryo transfer; NS = no significant difference; SET = single-embryo transfer.
[a]Independent t-test.
[b]Pearson chi-squared test.

both treatment regimens: 2.2% and 3.1% for IVF-ET and FET, respectively. When analysed by gender, again there was no significant difference in relation to confirmed GDM for males and females in IVF-ET (2.3 and 2.0%, respectively) and in FET (3.0 and 3.2%, respectively) indicating that gender and GDM had no confounding influence.

There was no significant difference in the serum β-HCG concentration, period of gestation, or birthweight for different IVF ovarian stimulation protocols and FET protocols (Supplementary **Table S1**). Therefore, the different IVF-ET protocol groups can effectively be treated as one whole group when analysing the outcome analysis of the study. The same applies to the different FET protocols.

In addition, the IVF-ET group had an almost equal percentage of cleavage and blastocyst embryo transfers (54.2% and 45.8%) (**Table 2**), whereas the FET group had significantly more blastocyst transfers (79.6% versus 20.4%; $P < 0.001$, **Table 2**). However, when only high-grade embryos were taken into account, an unequivocal result was obtained. Here, there was a significant difference using Pearson chi-squared test ($P = 0.042$), but when an additional continuity correction was applied, there was no significant difference in terms of embryo quality observed between the two groups (**Table 2**). Conversely, following extraction and analysis of only the high-grade embryos, there were significant differences between both groups in relation to serum β-HCG and birthweights ($P < 0.001$ and $P = 0.003$ respectively; **Table 2**). Interestingly, when we investigated whether gender played a role in the different effects observed between IVF-ET and FET, we detected a significant influence (**Figure 3**). There were significant differences in mean β-HCG concentrations between IVF-ET and FET for both male ($P < 0.0001$) and female ($P < 0.0001$) groups (**Figure 3A**). However, birthweights were only significantly different between IVF-ET and FET in female infants (**Figure 3B**; $P < 0.01$). Again significant differences in mean β-HCG concentrations between IVF-ET and FET were shown when cleavage-stage embryos or blastocysts were transferred (**Figure 3C**; $P < 0.0001$), or when high-quality or lower-quality embryos were transferred (**Figure 3E**; $P < 0.0001$ and $P < 0.001$ respectively). These significant differences were not fully replicated for birthweights, although some differences were found (**Figure 3D, F**). FET with high-quality embryos showed higher weights ($P < 0.05$) and FET with lower quality showed higher weights than IVF-ET with high-quality embryos ($P < 0.0001$).

The median of the pregnancy test serum β-HCG values for all transfers in each treatment type (including all types of embryos) is shown in **Table 3**. There was a significantly higher β-HCG value in the FET group compared with the IVF-ET group (844.5 versus 369.0 IU/l; $P < 0.001$). Although the majority of serum β-HCG determinations were performed on day 19 of the luteal phase ($n = 653$; 75.1%) for both transfer groups as expected, some were conducted on other luteal days (day 15–24), and this was due to various reasons including patient convenience, anxiety or increased monitoring (Supplementary **Table S2**). Under the IVF-ET treatment protocol, both day 3

Table 2 Embryo transfer days and embryo quality for fresh IVF-ET and FET groups.

	Total n = *869 (%)*	*IVF-ET* n = *417 (%)*	*FET* n = *452 (%)*	P-*value*
Embryo transfer days				
Day 2/3/4 (cleavage stage)	318 (36.6)	226 (54.2)	92 (20.4)	
Day 5/6 (blastocyst stage)	551 (63.4)	191 (45.8)	360 (79.6)	<0.001[a]
Embryo quality				
High	705 (81.1)	350 (83.9)	355 (78.5)	
Medium	132 (15.2)	49 (11.8)	83 (18.4)	0.019[a]
Low/poor	32 (3.7)	18 (4.3)	14 (3.1)	
Embryo quality				
High	705 (81.1)	350 (83.9)	355 (78.5)	0.042[a]/NS[b]
Not high	164 (18.9)	67 (16.1)	97 (21.5)	
Parameters of high-grade only embryos				
Serum β-HCG (IU/l)				
Median (IQR)	–	378 (341.3)	852 (861.0)	<0.001[c]
Birthweight (g)				
Mean ± SD	–	3193.9 ± 591.7	3334.9 ± 662.2	0.003[d]

FET = frozen embryo transfer; HCG = human chorionic gonadotrophin; IQR = interquartile range; IVF-ET = IVF fresh embryo transfer; NS = not statistically significant.
[a]Pearson chi-squared test.
[b]Continuity corrected chi-squared for 2 × 2 tables (unless specified, *P*-value for a = b).
[c]Mann–Whitney *U*-test.
[d]Independent t-test.

and day 5 embryos were transferred 1 day later than those of the FET group. In order to investigate this difference, a subanalysis of the β-HCG concentration was carried out using β-HCG taken on days 15 to 18 inclusive (for FET), and compared with that taken on days 19 to 24 in the IVF-ET group (**Table 4a**). Although this placed the FET group in an earlier β-HCG pregnancy testing range, the FET group still had a significantly higher median β-HCG compared with the IVF-ET group (733 versus 373 IU/l; $P < 0.001$). However, the majority (75.1%) of the β-HCG tests were actually performed on luteal phase day 19, and strict analysis of all cases at day 19 showed that the FET group had significantly higher β-HCG concentrations (783.0 IU versus 370.0 IU; $P < 0.001$) and a difference in mean birthweight of 163.1 g ($P = 0.001$) regardless of delivery at the same gestational age (**Table 4b**). Deeper day 19 analysis focusing on gender, cleavage/blastocyst transfer and embryo quality is shown in **Figure 4**. Again it was demonstrated that β-HCG concentrations were significantly higher in FET than in IVF-ET when stratified for gender (male $P < 0.0001$, female $P < 0.001$; **Figure 4A**), cleavage/blastocyst transfer (both $P < 0.0001$; **Figure 4C**) and embryo quality (high $P < 0.0001$, low $P < 0.05$; **Figure 4E**). Conversely, there was no difference in birthweights between IVF-ET and FET cycles when the same embryo stage or quality was transferred (**Figure 4D, F**). However, birthweights were significantly increased in female FET infants ($P < 0.01$) compared with IVF-ET females from cycles with day 19 β-HCG analysis, but were not significantly elevated in male infants, which indicated a gender-dependent effect (**Figure 4B**).

Overall, there was a significantly higher mean birthweight in the FET group (3370 g versus 3209 g; $P < 0.001$), but no significant difference in gestation period (Supplementary **Table S3**). Further analysis revealed that the FET group had significantly more birthweights in the ≥4000 g compared with the IVF-ET group (14% versus 6.0%; $P < 0.001$), but no significant difference was observed between the treatment groups for the ≥4500 g birthweight (Supplementary **Table S3**).

Discussion

Vitrification of embryos, with subsequent warming and transfer, has become a successful process for achieving pregnancy in assisted reproductive treatment, but the question as to whether it is equivalent or better than fresh IVF-ET is entirely unknown (Weinerman and Mainigi, 2014). Several investigations have attempted to determine the perinatal outcome following FET and IVF-ET. A large Finnish study (Pelkonnen et al., 2010) found that FET had no adverse effect in relation to prematurity, low birthweight or size for gestational age when compared with fresh IVF-ET. This study utilized a slow freezing protocol and not vitrification techniques. Similarly, a Nordic study (Wennerholm et al., 2013) supported their findings, but this study used both slow-freeze and vitrification techniques for embryo freezing. However, neither study linked the β-HCG pregnancy test and birthweight outcome to FET transfers. Nonetheless, numerous variables require consideration in comparing FET cycles to fresh IVF-ET cycles, making the study rather complex.

The strengths of our study include the large sample size spreading over 6 years, selection of only SET with singleton live births, along with FET cycles using vitrified embryos only (Kuwayama et al., 2005). It also addressed in detail the possible effect of differences in pregnancy β-HCG testing days and subsequent infant gender, as well as both the quality and developmental stage of transferred embryos. Consistently, higher pregnancy β-HCG concentrations were observed with FET transfers in comparison with IVF-ET, even after

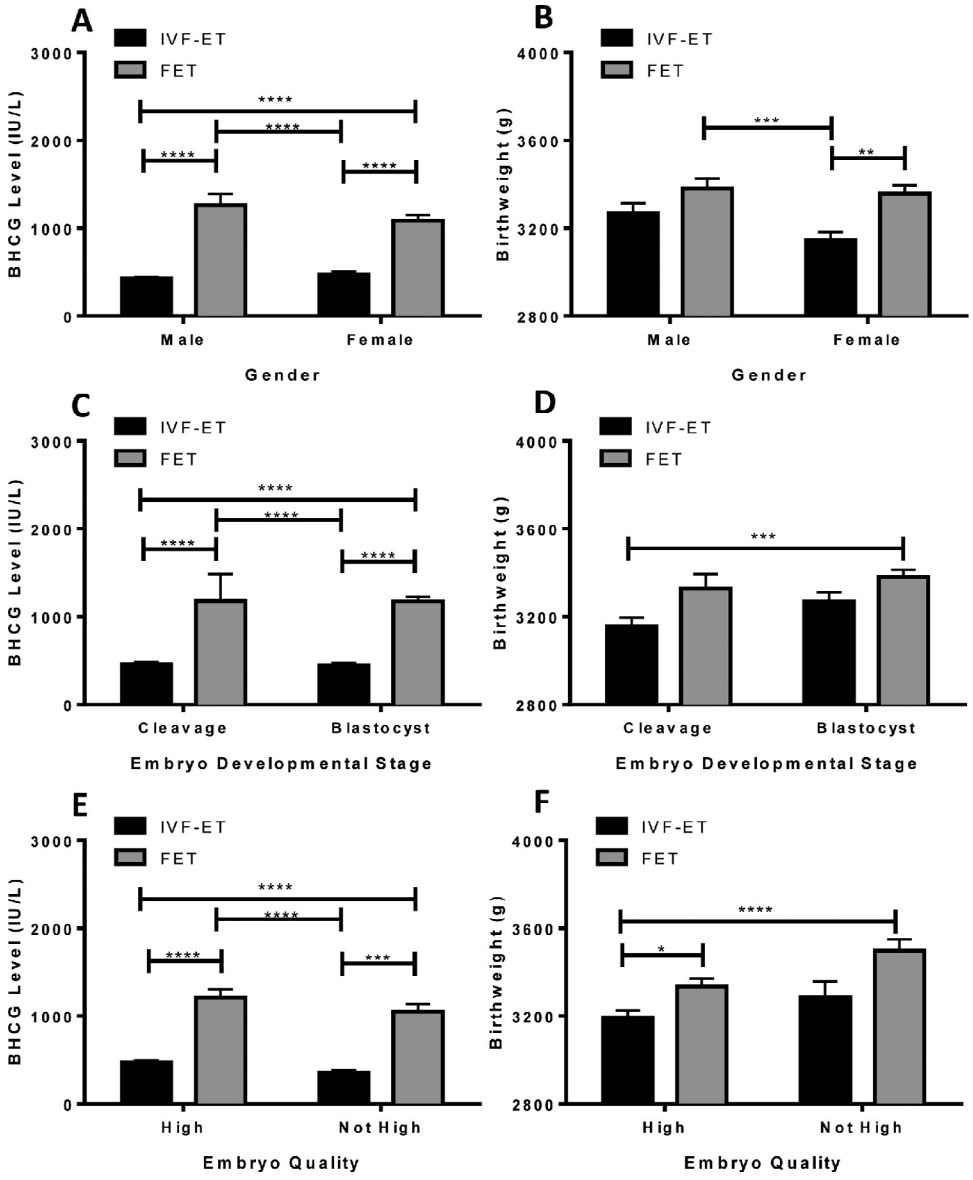

Figure 3 Mean β-HCG concentrations and birthweights for IVF-ET and FET cycles when grouped according to infant gender (A and B), transferred embryo development stage (C and D) and embryo quality (E and F), respectively. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. FET = frozen embryo transfer; HCG = human chorionic gonadotrophin; IVF-ET = IVF fresh embryo transfer.

Table 3 Mean luteal day and β-HCG value for pregnancy test.

Parameter	*IVF-ET* n = *417*	*FET* n = *452*	P-*value*
Day of luteal phase Mean ± SD	19.0 ± 0.4	19.2 ± 0.9	NS[a]
Serum β-HCG (IU/l) Median (IQR)	369 (334.5)	844.5 (891)	<0.001[b]

FET = frozen embryo transfer; HCG = human chorionic gonadotrophin; IQR = interquartile range; IVF-ET = IVF fresh embryo transfer; NS = not statistically significant.
[a]Independent t-test.
[b]Mann–Whitney *U*-test.

accounting for infant gender, embryo quality and developmental stage. In addition, significantly higher pregnancy β-HCG concentrations were still observed even when the FET group was sub-analysed according to the equivalent luteal day for β-HCG testing.

Given the existing problem of traditional clinical gestational dating with its reference to LMP and the "inaccurate" estimation of the day of ovulation, along with the challenge of matching the luteal phases in the absence of ovulation, we have described a system for undertaking those comparisons. Moreover, ovarian stimulation and luteal phase characteristics can potentially impact on the chance of implantation and resulting β-HCG concentrations (Fauser and Devroey, 2003; Humaidan et al., 2012). More specifically, the use of HCG support injections in the luteal phase of most of the IVF-ET cycles (where oocyte numbers collected were ≤12),

Table 4 Pregnancy test β-HCG values at different day of luteal phase by type of SET of the singleton live births following IVF-ET and FET.

	IVF-ET: LP ≥19 n = *408*	*FET: LP ≤18* n = *44*	P-*value*
Analysis involving β-HCG on LP ≤18 for FET *vs* LP ≥19 for IVF-ET (452 cycles)			
Serum β-HCG (IU/l)			
Median (IQR)	373.0 (338.0)	733.0 (549.0)	<0.001[b]
Analysis involving β-HCG on LP 19 only (653 cycles)	IVF-ET *n* = 391	FET *n* = 262	*P*-value
Serum β-HCG (IU/l)			
Median (IQR)	370.0 (321.0)	783.0(795.5)	<0.001[b]
Gestation at delivery (days)			
Mean ± SD	270.6 (15.7) 38w 5d (2.2w)	3208.4 (596.2) 38w 5d (2.2w)	NS[a]
Baby's weight (gram)			
Mean ± SD	3208.4 (596.2)	3371.5(619.0)	*P* = 0.001[b]

d = day; FET = frozen embryo transfer; HCG = human chorionic gonadotrophin; IQR = interquartile range; IVF-ET = IVF fresh embryo transfer; LP = luteal phase day; NS = not statistically significant; SET = single-embryo transfer; w = week.
[a]Independent t-test.
[b]Mann–Whitney *U*-test.

as well as FET-NAT and FET-LDS cycles, could potentially influence the pregnancy test β-HCG concentrations. However, in this study, the minimum period of 4 days between the last HCG injection and the pregnancy test minimized this possibility. On the other hand, if indeed the use of HCG in the luteal phase impacted on the pregnancy test β-HCG concentrations, it could only potentially create an artificial elevation, which while affecting the vast majority of IVF-ET cycles, would only affect <15% of FET cycles in this study (i.e. FET-NAT and FET-LDS). Furthermore, if β-HCG concentrations were artificially elevated in the fresh IVF-ET cohort, then this could only reduce the difference between the IVF-ET and FET β-HCG, and we still observed a statistically significant difference. In addition, we have demonstrated that different stimulation protocols did not alter the β-HCG concentration, gestation period or birthweight (Supplementary **Table S1**).

Our study is similar to a recently reported retrospective study (Ozgur et al., 2015), and both demonstrated higher β-HCG concentrations at the pregnancy test with higher implantation rates and higher infant birthweights for FET cycles. Conversely, we differ in that the difference in β-HCG concentrations and the difference in birthweights was greater in our study. This may be due to several methodological differences including: (i) our fastidious methodology in attempting to precisely match the luteal-phase datings between IVF cycles and the artificial HRT regimen of the FET cycles; and (ii) our unique HRT regimen with higher dosage micronized progesterone pessaries (Yovich et al., 2015b).

We speculate that the higher β-HCG FET values might be contributed to by an improved preparation for endometrial receptivity in FET cycles (Buck et al., 2012; Roque et al., 2013). This window of opportunity might be affected in fresh embryo transfer because of the supraphysiologic concentrations of oestradiol and progesterone during the follicular phase following ovarian stimulation, and may adversely advance the endometrium rather than in natural or FET cycles (Kolibianakis et al., 2002; Roque et al., 2013). Interestingly, our previous study had demonstrated that mid-luteal serum progesterone, rather than oestradiol, was a major influencing factor, along with embryo quality, for optimum implantation rates and subsequent live births (Yovich et al., 2015b). The mid-luteal progesterone concentration maintained within a precise range yielded more desirable outcomes. In the current study, our sub-analysis of just the high-grade embryos transferred in both the IVF-ET and FET groups, showed that significantly higher serum β-HCG concentrations were still observed in FET cycles, although it is not clear why this was not replicated in birthweights.

Overall the data indicated that there was a significant effect of FET on mean birthweight (3370 g versus 3209 g), even though there was no significant difference between the period of gestation at delivery for both transfer groups. The differences in the birthweight might imply that babies born via FET had a more optimized chance for better growth potential. In addition, even with significantly more births in the ≥4000 g group for FET, (14% versus 6%), there was no significant difference in the ≥4500 g (macrosomic) proportion in our study, which contrasts with a previous report of significantly more large for gestational age babies after FET (Pinborg et al., 2014). Consequently, this group might not be more predisposed to instrumental or surgical delivery. However, this point must be taken with caution, as the number of cases involved was very small: five for IVF-ET and 10 for the FET group (1.2% versus 2.2%). Importantly, we did observe some gender-dependent effects in our outcomes. When comparing IVF-ET and FET β-HCG concentrations in males and in females, the concentration was consistently higher in FET cycles for both genders, and this effect was observed in the whole cohort or when luteal day 19 was selected out. Furthermore, the difference appeared to be more pronounced for male infants. However, we found that birthweights were only significantly higher in FET cycles when the subsequent child was female. Again this was consistent when analysing the whole dataset and at luteal day 19. We speculate that, since males

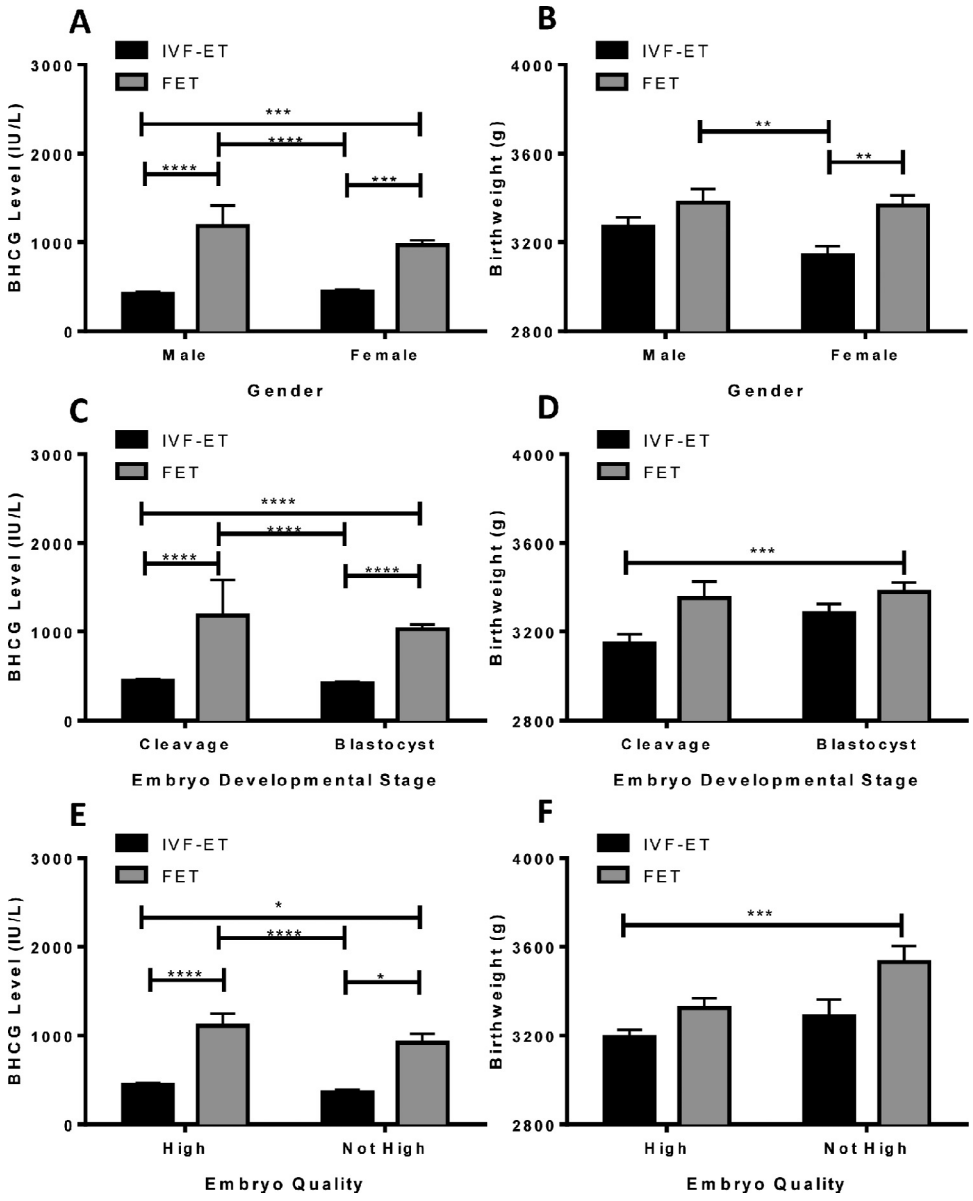

Figure 4 Mean β-HCG concentrations and birthweights for IVF-ET and FET cycles when tested at luteal day 19 only and grouped according to infant gender (A and B), transferred embryo development stage (C and D) and embryo quality (E and F), respectively. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. FET = frozen embryo transfer; HCG = human chorionic gonadotrophin; IVF-ET = IVF fresh embryo transfer.

tend to be larger for gestational age (Wennerholm et al., 2013), the potential influencing effect of FET on birthweight is either lost or diluted (as there is an increasing trend). However, it is definitely more pronounced in female infants, but the potential biological mechanism(s) responsible for this gender-based difference is completely unknown – particularly considering that there was no significant difference in the onset of GDM related to either treatment type or infant gender.

With respect to the derivation of cryopreserved embryos selected for SET, 20% were derived from freeze-all cycles, who tend to be younger with a high AFC. Analysis of this subgroup might expect a higher implantation rate than those cases derived from supernumerary embryos. This appeared the case for clinical pregnancies but not for live birth rates, as there were significantly higher rates of miscarriage and ectopics in the FET group. This may relate to our earlier study (Yovich et al., 2015b), which analysed HRT effects on optimizing implantations including enhancement of lower-quality embryos when mid-luteal progesterone concentrations are optimized at 50–99 nmol/l. This appears a unique observation related to the PIVET regimen of FET/HRT; however, the live-birth rates were also higher, indicating that this feature should be further examined as a specific study.

The fact that mostly blastocysts were transferred in the FET group, as opposed to the IVF-ET group, which utilized almost an equal number of cleavage-stage embryos and blastocysts, may partly explain why FET cycles are usually more

successful than IVF-ET in the modern assisted reproduction era. However, these differences did not significantly influence the β-HCG concentrations or birthweight when the same embryo stage or embryo quality was transferred in the same treatment type cycles (i.e. FET or IVF-ET). This indicated that for β-HCG concentrations particularly, the major driver of different concentrations was FET treatment rather than embryo stage or quality.

Our group has ventured certain ideas on the benefits of short periods of physiological stress improving blastocyst development rates and quality in bovine studies (Vajta et al., 2010). Perhaps vitrification provides a form of positive "sublethal" stress to embryos, and this idea is supported by a recent publication showing that Heat shock protein (Hsp 70) is elevated in gestational diabetes (Garamvölgyi et al., 2015). Such possibilities deserve exploration at a molecular level.

We conclude that embryos following FET can possibly lead to heavier infants, particularly if they are female. In addition, the data indicated that FET cycles will tend to produce higher β-HCG concentrations at pregnancy test on day 19. However, the causative mechanism(s) for this remains completely elusive. These finding may have significant implications for fertility practice in general – in that the β-HCG concentration may be applied as an indicator of potential developmental progress of the embryo.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.rbmo.2016.04.014.

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Declaration: The authors report no financial or commercial conflicts of interest.

Received 8 December 2015; refereed 20 April 2016; accepted 20 April 2016.