Higher E-HCG levels and higher birthweights ensue from single vitrified embryo transfers.

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Abstract

The effect of cryopreservation on the developmental potential of the human embryo has not been fully established. This study compares pregnancy outcomes after fresh IVF-ET with those arising after frozen embryo transfer (FET). In this study, only singleton pregnancies resulting from single embryo transfers (SETs) that eventuated in singleton live birth were included (n=869), along with only vitrified frozen embryos. Pregnancies were examined according to their derivation being IVF-ET (n=417) or FET (n=452). The initial level of the serum β-HCG, indicating successful implantation, was measured along with the birthweight of the ensuing infants. The median pregnancy test β-HCG was significantly higher following FET (844.5IU/L; $p<0.001$), compared to fresh IVF-ET (369IU/L). In addition, the mean birthweight of infants born following FET was significantly heavier by 161g (3370g versus 3209g; $p<0.001$). Furthermore, more infants exceeded 4000g ($p<0.001$) for FET, while there was no difference for those in the macrosomic category (≥ 4500g). We conclude that embryos from FET programmes, led to higher β-HCG measurements and higher birthweights.

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

Introduction

Cryopreservation might compromise the cleavage stage embryo or the trophoblast, thus affecting the ability of the embryo to implant. Any negative effect of 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), which found that vitrification (rather than slow-freezing) had no negative effect on the initial β-HCG level 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 level β-HCG 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).

Our clinic - PIVET Medical Centre, has been using the vitrification process since late 2007 (Kuwayama et al., 2005). Anecdotal evidence suggested that the pregnancy test β-HCG in patients receiving FET appeared to be higher than those receiving fresh 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 our database (1 April 2008 until 30 April 2014 inclusive). We analysed all single embryo transfer

(SET) procedures following IVF fresh embryo transfer (IVF-ET) and compared their pregnancy outcomes with single frozen-thawed embryo transfer (FET) procedures using vitrified-only embryos. No treatment cycles were excluded due to age or patient history, but those cycles utilising 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). Embryo graded BC or CB or less would not be cryopreserved. Yovich et al., 2015[a] 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) pregnancy, miscarriage or blighted ovum, ectopic pregnancies as well as terminations and stillbirths. No cases included embryos screened for pre-implantation genetic diagnoses so would not likely 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 (Yovich et

al, 2015[b]; Yovich and Stanger, 2010; Yovich et al., 2012; Stanger et al., 2012).

Ovarian stimulation for IVF cycles

Patients were stimulated with long down-regulation, flare cycle, or antagonist protocols (Yovich and Stanger, 2010; Yovich et al., 2012). 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) ratings, and the flare regimen for older women with low AFC ratings.

Ovulation induction and luteal support for IVF

Ovulation induction was usually initiated with a single dose of rHCG (Ovidrel: Merck Serono), 2 ampoules equating to approximately 13,000IU rHCG, when there were at least 2 leading follicles ≥18mm in diameter. For patients with <4 follicles or a previous poor recovery, 3 ampoules (Ovidrel) approximating to 19,500IU rHCG, was used as the trigger. In those antagonist cycles with excessive follicle recruitment (>12 follicles over 12mm), gonadotrophin-releasing hormone agonist (Lucrin: Abbott) trigger 50IU was used. Oocyte recovery was at 35-37 hours 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 1500IU sc was administered on Days 6, 9, 12, and 15 after trigger with or without progesterone (P4) pessaries (Wembley Pharmacy compounded for PIVET)

Endometrial preparation and luteal support for FET cycles

1. FET- Hormone Replacement Therapy (FET-HRT)

FET-HRT (Yovich et al., 2015[b]) was used in 393 (87%) FET cycles. Briefly, oestradiol valerate tablets (Progynova 4mg TDS; Schering Plough) were administered from Day 1 of the cycle followed by, Oestradiol (E2) vaginal pessaries (10/20mg OD) on Day 10 and continued for 5 days ± 1 (or extended further until endometrial lining was ≥8mm). Then P4 pessaries were administered (400mg BD and combo pessary nocte (P4 500mg + E2 2mg)), 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

4th day of P4 pessaries, whereas Day 5/6 embryos were transferred on the 6th day of P4 pessaries. The Progynova, P4 and combo pessaries were continued for luteal support until 10 weeks gestation and then weaned off over 2 weeks.

2. FET- Low Dose Stimulation (FET-LDS)

FET-LDS was used in 55 (12%) FET cycles and started on day 3 of the menstrual cycle with daily low dose FSH (50-75IU) or Tamoxifen 20mg BD from day 2 to 10. Transvaginal (TV) ultrasound scan and serum E2, P4 and LH were monitored from Day 8 every second day until ready for HCG trigger (follicle ≥16mm and endometrial lining ≥8mm). Four days after trigger (Pregnyl 10,000IU), 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 1500IU on Days 6, 9, 12, and 15 after trigger (similar to the IVF-ET regimen).

3. FET-Natural (FET-NAT)

FET-NAT was applied in only 4 (0.9%) FET cycles, where the TV ultrasound scan and serum E2, P4 and LH levels were monitored from Day 8 every second day until ready for trigger (follicle ≥16mm and endometrial lining ≥8mm). Following Trigger (Pregnyl 5,000IU), luteal supports were administered as per FET-LDS.

β-HCG Measurement

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

Pregnancy Detection & Luteal Day Definitions in Multiple Treatment Types

Patients had their blood drawn by venipuncture (07.30-09.30am) daily Monday to Saturday. For IVF-ET, luteal Day 1 was the day after trigger (the first day of detectable P4 elevation), and transvaginal oocyte aspiration (TVOA) was conducted on luteal Day 2, being the second day of significant P4 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. For gestation determination, it is traditionally, calculated from onset of the last menstrual period (LMP) so that the 14th day was regarded as the day of ovulation i.e. Day 2 of the luteal phase or P4 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 four days after the last HCG on Day 15, when β-HCG levels are known to be <15IU/L for non-pregnant cases (Figure 2). Levels 15-24IU/L were regarded as equivocal and repeated three days later, sometimes they showed delayed elevation into confirmed pregnancy and usually destined for pregnancy failure, but occasionally they resulted in a live birth. Although β-HCG concentrations above 5IU/L constitute a significant detection, we applied ≥25IU/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 P4 pessaries was considered as Day 1 of the artificial luteal phase, and pregnancies were diagnosed on Day 19 (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 P4 pessary was considered equivalent to the 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 TV ultrasound. If an intrauterine gestational sac with fetus and fetal heart beat was not clearly detected, the diagnosis was pursued by further investigations and categorised 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 two 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-22 Software was used for the statistical analysis. Independent sample t-test or one way ANOVA were used to compare means and Mann-Whitney U or Kruskal Wallis Tests were used to compare the median. Pearson F2 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 Frozen-thawed Embryo Transfer (FET) and IVF Fresh Embryo Transfer (IVF-ET) protocols (Figure 1). This generated a β-HCG level of ≥25IU/L for 1134 women (n=636 FET and n=498 IVF-ET) with 1083 progressing to clinical pregnancies (n=603 (35.2%) FET and n=480 (28.6%) IVF-ET) and produced 888 live birth deliveries including monozygotic twins (n=465 FET and n=423 IVF-ET). Although pregnancy rates were higher in the FET group (41.9% vs 33.9%; p=0.01), the livebirth rates were similar (27.8% vs 26.1%; p=0.6). Figure 1 shows that whilst pre-clinical (biochemical) pregnancy losses were similar (3.6% vs 5.2%; p=0.25), clinical-stage losses (miscarriage and ectopics) were significantly higher from FET than IVF-ET (10.8% vs 21.1%; p<0.0001). Twin pregnancies (19 pairs), stillbirth (n=1), pregnancies that were terminated due to fetal abnormalities (n=9), failed pregnancies – either miscarriages/ 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 SETs). 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 analyzed 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 single embryo transfer for fresh cycles was significantly lower than at the time of the FET (32.8 vs 34.3 years) (Table 1). Although this might not be relevant clinically as the higher success rates in ART 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% vs 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 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 level, period of gestation, or birthweight for different IVF ovarian stimulation protocols and FET protocols (Supplemental 1). Therefore, the different IVF-ET protocol groups can effectively be treated as one whole group when analyzing 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 % & 45.8%) (Table 2), whereas the FET group had significantly more blastocyst transfers (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 X^2 ($p=0.042$), but when an additional continuity correction was applied ($p=0.052$), 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 (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 E-HCG levels between IVF-ET and FET for both male and female groups (Figure 3). However, birthweights were only significantly different between IVF-ET and FET in female infants. Again significant differences in mean E-HCG levels between IVF-ET and FET were shown when cleavage stage or blastocysts were transferred, or when high-quality or not high-quality embryos were transferred. However, these significant differences were not replicated for birthweights (Figure 3).

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 to the IVF-ET group (844.5 vs 369.0IU/L; $p \leq 0.001$). 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 (Supplemental 2). Under the IVF-ET treatment protocol, both Day 3 and Day 5 embryos were transferred one day later than those of the FET group. In order to investigate this difference, a sub-analysis of the β-HCG level was carried out using β-HCG taken on Days 15 through to Day 18 inclusive (for FET), and compared to that taken on Days 19 through to Day 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 to the IVF-ET group (733 vs 373IU/L; $p \leq 0.001$). 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 levels and mean birthweight of 163.1g ($p=0.001$) regardless of delivery at the same gestational age (Table 4b). Deeper Day 19 analysis focusing on gender, cleavage/blastocyst transfer and embryo quality again demonstrated that E-HCG levels were significantly higher in FET than in IVF-ET (Figure 4). Conversely, there was no difference in birthweights between IVF-ET and FET cycles when the same embryo stage or quality was transferred (Figure 4). However, birthweights were increased in female FET infants from cycles with Day 19 E-HCG analysis, but were not significantly elevated in male infants, which indicated a gender-dependent effect (Figure 4).

Overall, there was a significantly higher mean birthweight in the FET group (3370g vs 3210g), but no significant difference in gestation period (Supplemental 3). Further analysis revealed that the FET group had significantly more birthweights in the ≥4000g compared to the IVF-ET group (14% vs 6.0%), but no significant difference was observed between the treatment groups in the ≥4500g birthweight (Supplemental 3).

Discussion

Vitrification of embryos, with subsequent thawing and transfer has become a successful process for achieving pregnancy in assisted reproductive technologies, 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 to 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, subsequent infant gender, as well as both the quality and developmental stage of transferred embryos. Consistently, higher pregnancy E-HCG levels were observed with FET transfers in comparison with IVF-ET, even after accounting for infant gender, embryo quality and developmental stage. In addition, significantly higher pregnancy E-HCG levels were still observed even when the FET group was sub-analysed according to the equivalent luteal day for E-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 E-HCG levels (Fauser and Devroey, 2003; Humaiden 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), as well as FET-NAT and FET-LDS cycles, could potentially influence the pregnancy test E-HCG levels. However, in this study, the minimum period of 4 days between the last HCG injection and the pregnancy test minimised this possibility. On the other hand, if indeed the use of HCG in the luteal phase

impacted on the pregnancy test E-HCG levels, 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 E-HCG levels were artificially elevated in the fresh IVF-ET cohort, then this could only reduce the difference between the IVF-ET and FET E-HCG, and we still observed a statistically significant difference. In addition, we have demonstrated that different stimulation protocols did not alter the E-HCG level, gestation period or birthweight (Supplemental 1).

Our study is similar to a very recently reported retrospective study (Ozgur et al., 2015), and both demonstrated higher E-HCG levels at the pregnancy test with higher implantation rates and higher infant birthweights for FET cycles. Conversely, we differ in that the difference in E-HCG levels 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., 2015[b]).

We speculate that the higher E-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 levels of E2 and P4 during the follicular phase following controlled ovarian stimulation (COS), 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 P4 rather than E2, was a major influencing factor, along with embryo quality, for optimum implantation rates and subsequent livebirths (Yovich et al, 2015[b]). The mid-luteal P4 level 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

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 (3370g versus 3210g), 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 optimised chance for better growth potential. In addition, even with significantly more births in the 4000g group for FET, (14% vs 6.2%), there was no significant difference in the ≥4500g (macrosomic) proportion in our study, which has been reported previously (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 were very small, 5 for IVF-ET and 10 for the FET group (1.2% vs 2.2%). Importantly, we did observe some gender-dependent effects in our outcomes. When comparing IVF-ET and FET E-HCG levels in males and in females, the level 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 tend to be larger for gestational age (Wennerholm et al. 2013), that 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 antral follicle count. Analysis of this sub-group 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, 2015[b]) which analysed HRT effects on optimizing implantations including enhancement of lower quality embryos when mid-luteal P4 levels are optimized at 50-99nmol/L. This appears a unique observation related to the PIVET regimen of FET/HRT, however the livebirth rates were also higher indicating 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 utilised 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 ART era. However, these differences did not significantly influence the E-HCG levels 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 E-HCG levels particularly, the major driver of different levels 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 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 E-HCG levels 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

level may be applied as an indicator of potential developmental progress of the embryo.

Author's contributions

KNK, KBM and PH analysed the data. KBM wrote the first draft of the manuscript including tables and figures. KNK, JC and JLY revised the document. All authors have approved and contributed to the final written manuscript.

Declaration

The authors report no financial or commercial conflicts of interest.

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Figure Legends

Figure 1: Flow Chart of SET Treatment Cycles Selection for Pregnancy Test β-HCG value and Singleton Livebirth Outcome Following IVF-ET versus FET.

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.

Figure 3: Mean E-HCG levels and birthweights for IVF-ET and FET cycles when grouped according to infant gender (A & B), transferred embryo development stage (C & D) and embryo quality (E & F), respectively.

Figure 4: Mean E-HCG levels and birthweights for IVF-ET and FET cycles when tested at Luteal Day 19 only and grouped according to infant gender (A & B),

transferred embryo development stage (C & D) and embryo quality (E & F), respectively.

Table 1: Demographics for patients with Singleton live birth following SET of 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	267 (64.0%)	226 (50.0%)	493 (56.7%)	
35-39 years	125 (30.0%)	166 (36.7%)	291 (33.5%)	**<0.001**[b]
40-44 years	25 (6.0%)	51 (11.3%)	76 (8.7%)	
≥ 45 years	0 (0.0%)	9 (2.0%)	9 (1.0%)	
Total	**417 (100%)**	**452 (100%)**	**869 (100%)**	
BMI				
Mean ± SD	24.9 ± 4.8	24.8 ± 9.9		ns[a]
<18.5	17 (4.1%)	14 (3.1%)	31 (3.6%)	
18.5 – 24.9	232 (55.6%)	271 (60.0%)	503 (57.9%)	0.059[b]
25.0-29.9	97 (23.3%)	117 (25.9%)	214 (24.6%)	
≥ 30.0	71 (17.0%)	50 (11.1%)	121 (13.9%)	
Total	**417 (100%)**	**452(100%)**	**869 (100%)**	
Livebirth Gender				
Male	213 (51.1%)	231 (51.1%)	444 (51.1%)	
Female	204 (48.9%)	221 (48.9%)	425 (48.9%)	ns[b]
Total	**417 (100%)**	**452(100%)**	**869 (100%)**	
Confirmed GDM Cases				
Male	5 (2.3%)	7 (3.0%)	12 (2.7%)	
Female	4 (2.0%)	7 (3.2%)	11 (2.6%)	ns[b]
Total	**9/417 (2.2%)**	**14/452 (3.1%)**	**23/869 (2.6%)**	

SD= Standard deviation a=Independent T test b= Pearson χ^2 test

GDM= Gestational Diabetes ns= no significant difference

Table 2R

Table 2: Embryo Transfer days & Embryo Quality for IVF-ET & FET groups

Embryo Transfer days	Total N=869	IVF-ET N=417	FET N=452	P
D 2/3/4 (Cleavage Stage)	318 (36.6%)	226 (54.2%)	92 (20.4%)	
D5/6 (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] / 0.052[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)		378.5 (341.3)	852.0 (861.0)	< 0.001[c]
Birthweight (g)				
Mean ± SD		3193.9 ± 591.7	3334.9 ± 662.2	0.003[d]

a= Pearson X^2 Test b= continuity corrected χ^2 for 2x2 tables (unless specified, P for a=b)
c=Mann- Whitney U Test d= Independent T Test SD=Standard Deviation IQR=interquartile range

Table 3: Mean Luteal Day and E-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**
Serum E-HCG (IU/L)			
Median (IQR)	369 (334.5)	844.5 (891)	**P<0.001[b]**

IQR= Interquartile range SD= Standard deviation a= Independent T Test b= Mann-Whitney U Test

Table 4: Pregnancy test E-HCG values at different Day of Luteal Phase by type of SET of the singleton live births following IVF-ET and FET.

a. Analysis involving β-HCG on Luteal Phase Day ≤18 for FET vs Luteal Phase Day ≥19 for IVF-ET (452 cycles)	IVF-ET :LP ≥19 n= 408	FET: LP ≤18 n= 44	P value
Serum β-HCG value (IU/L)			
Median (IQR)	373.0 (338.0)	733.0 (549.0)	**P<0.001[b]**
b. Analysis involving β-HCG on Luteal Phase Day 19 only (653 cycles)	**IVF-ET n= 391**	**FET n= 262**	**P value**
Serum β -HCG value (IU/L)			
Median (IQR)	370.0 (321.0)	783.0(795.5)	**P<0.001[b]**
Gestation at delivery (days)			
Mean ± SD	270.6 (15.7)	270.7 (15.0)	P= 0.893[a]
	38w 5d (2.2w)	38w 5d (2.2w)	
Baby's weight (gram)			
Mean ± SD	3208.4 (596.2)	3371.5(619.0)	**P= 0.001[b]**

IQR= Interquartile range SD= Standard deviation a= Independent T Test b= Mann-Whitney U Test

Figure 1:
Flow Chart of SET Treatment Cycles Selection for Pregnancy Test β-HCG value and Singleton Livebirth Outcome Following IVF-ET versus FET.

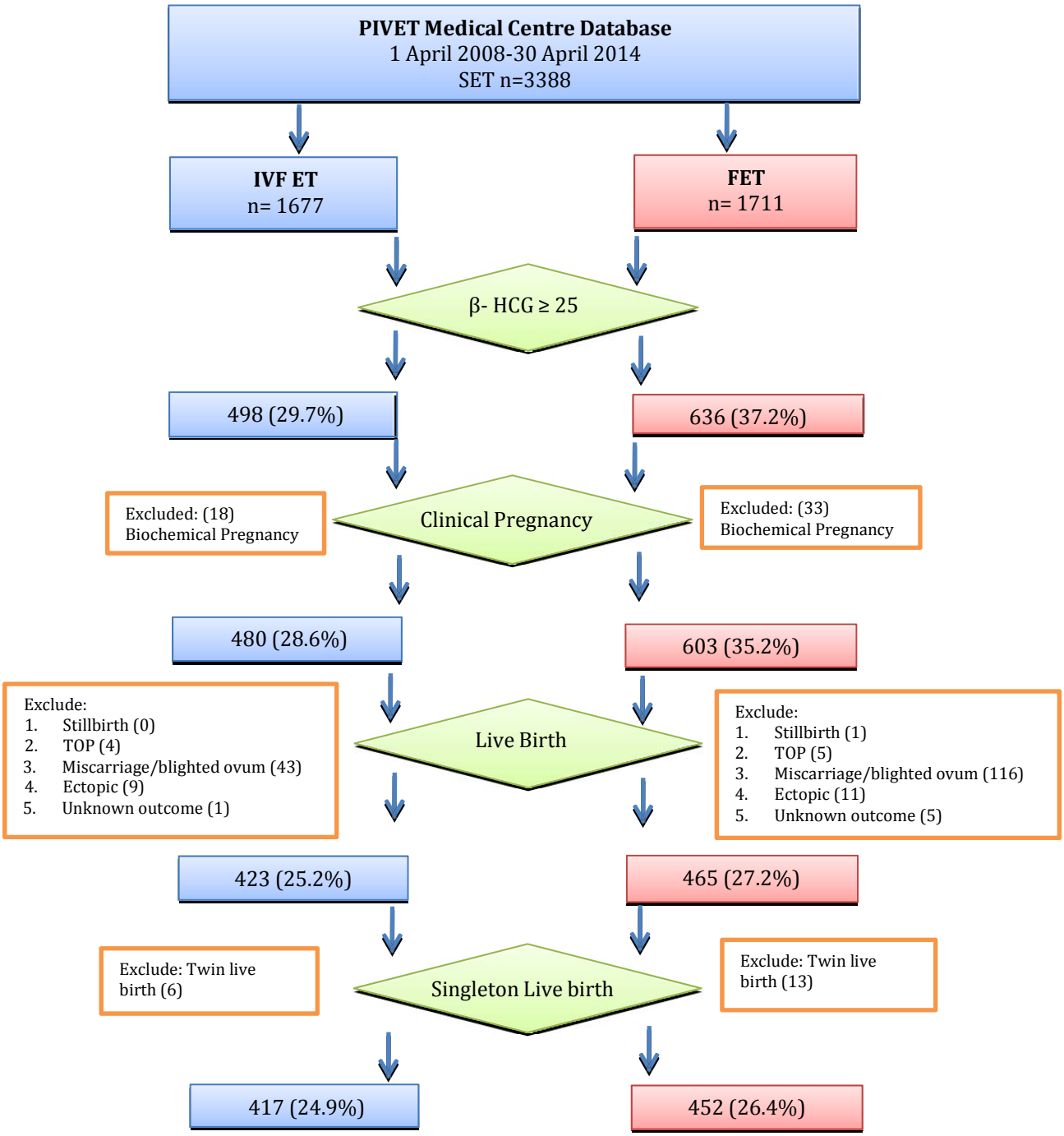


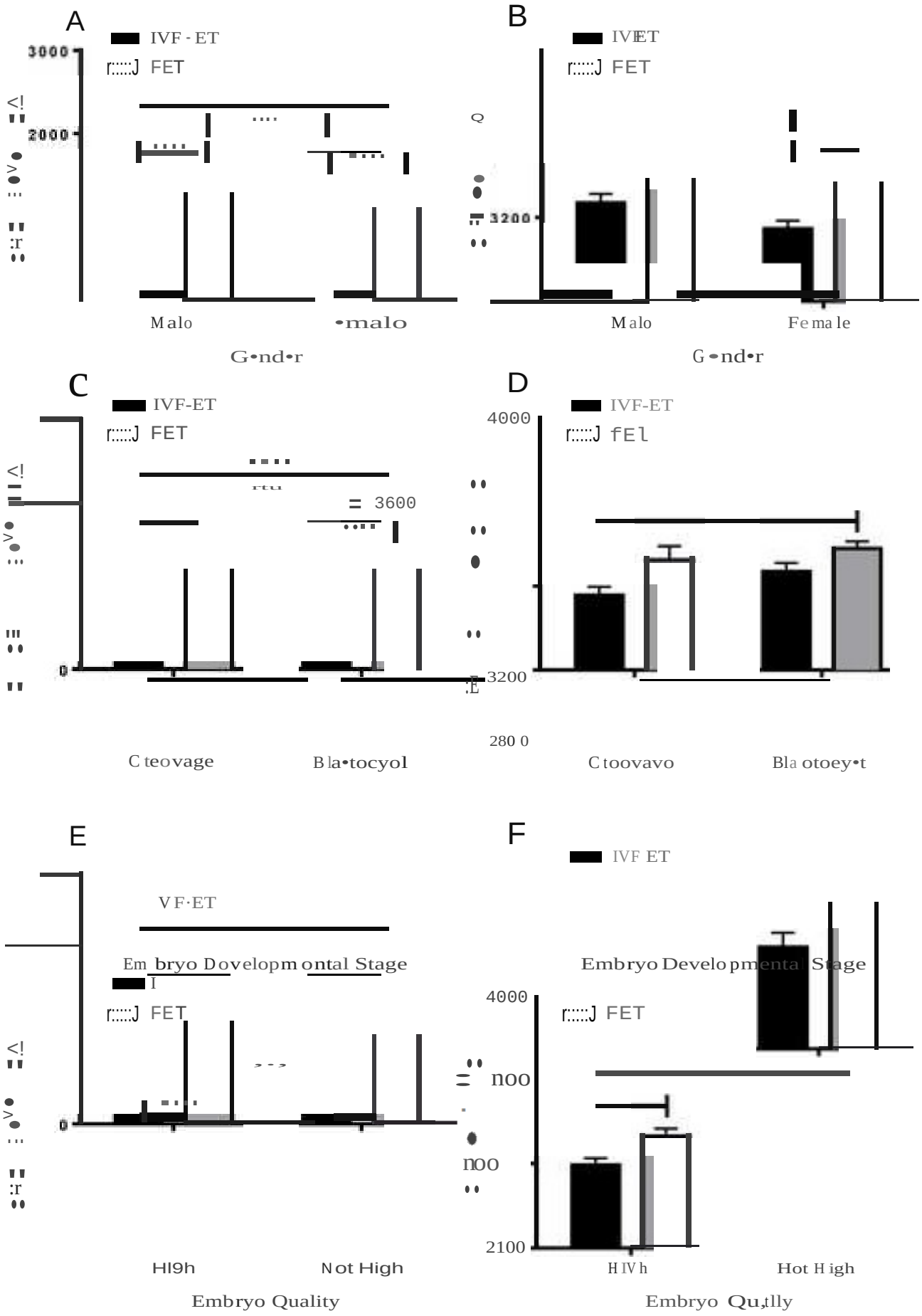
Figure 2R

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.

	IVF-ET	FET-LDS/NAT	FET-HRT
LUTEAL PHASE DAY			
0	Trigger Day	Trigger Day	
1	Day After Trigger	Day After Trigger = Day 14 of pregnancy dating	P4 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 ET: rHCG	Day 5 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

Agure3R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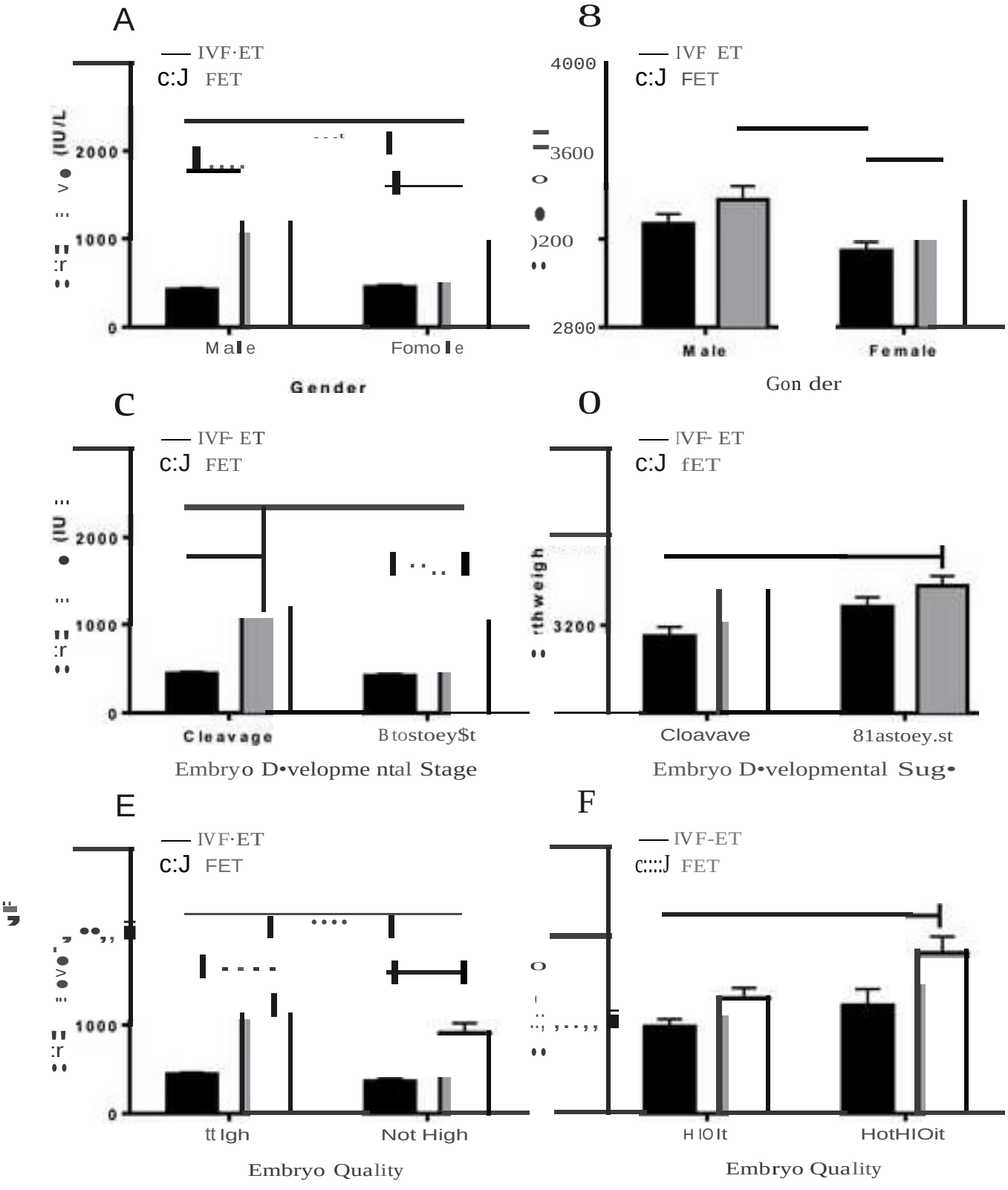
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- Ethical considerations

PIVET is accredited with both the self-regulatory National Australian authority (Reproductive Technology Accreditation Committee) as well as the statutory Western Australian State accreditation body (Reproductive Technology Council of Western Australia established under the Western Australian Human reproductive Technology Act, 1991). These agencies monitor all activities conducted at PIVET and demand oversight by an NHMRC–constituted Institutional Ethics Committee who endorse all clinical and laboratory protocols. PIVET laboratories are also accredited with NATA – the National Australian Testing Authority which requires strict adherence to quality assurance protocols. Specific ethics approval was not required for this study as all procedures and blood tests were embraced by routine approved clinical protocols. However reporting of the data was approved under Curtin University Ethics Committee approval no. RD_25-10 general approval for retrospective data analysis 2011.

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