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ARTICLE

Mid-luteal serum progesterone concentrations govern implantation rates for cryopreserved embryo transfers conducted under hormone replacement

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Dr John L Yovich, MBBS, MD, FRANZCOG, FRCOG, CREI presented his PhD thesis 'Human Pregnancies Achieved by In-Vitro Fertilisation' following research and clinical work undertaken with Professor Ian Craft at the Royal Free Hospital and University of London (1976–1980), thereafter at the University of Western Australia Department of Obstetrics & Gynaecology based at King Edward Memorial Hospital in hometown Perth. Dr Yovich established the PIVET Medical Centre in 1980, the first private independent comprehensive fertility management facility in Australia. Current research activities are conducted with the Department of Biomedical Sciences at Curtin University with a shared Research Fellow.

Abstract This study explores the relevance of mid-luteal serum hormonal concentrations in cryopreserved embryo transfer cycles conducted under hormone replacement therapy (HRT) control and which involved single-embryo transfer (SET) of 529 vitrified blastocysts. Widely ranging mid-luteal oestradiol and progesterone concentrations ensued from the unique HRT regimen. Oestradiol had no influence on clinical pregnancy or live birth rates, but an optimal progesterone range between 70 and 99 nmol/l ($P < 0.005$) was identified in this study. Concentrations of progesterone below 50 nmol/l and above 99 nmol/l were associated with decreased implantation rates. There was no clear interaction between oestradiol and progesterone concentrations but embryo quality grading did show a significant influence on outcomes ($P < 0.001$ and $P = 0.002$ for clinical pregnancy and live birth rates, respectively). Multiple comparison analysis showed that the progesterone effect was influential regardless of embryo grading, body mass index or the woman's age, either at vitrification or at cryopreserved embryo transfer. The results support the argument that careful monitoring of serum progesterone concentrations in HRT-cryopreserved embryo transfer is warranted and that further studies should explore pessary adjustments to optimize concentrations for individual women to enhance implantation rates.

KEYWORDS: blastocyst grading, clinical pregnancy rate, HRT, IVF, live birth rate, progesterone pessaries

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Introduction

Cryopreserved embryos have become an important source of pregnancies arising from IVF, particularly in current programmes, which increasingly involve elective single-embryo transfer (eSET). Early frozen embryo transfer programmes showed implantation rates much lower than those for fresh embryos, probably related to several reasons, including treatment cycle regimen, which could be a natural, stimulated or hormone replacement (HRT) cycle. Furthermore, the cryopreservation technique (slow-freeze versus vitrification) may also influence outcome. Nonetheless, there is no doubt that cryopreserved embryo transfer will have an increasingly important role in future years, mainly due to increasing interest in eSET, which has the benefit of significantly increasing the cumulative IVF productivity rate (Stanger and Yovich, 2013).

Data from the latest Australia and New Zealand Assisted Reproduction Database (ANZARD) confirms the importance of cryopreserved embryo transfer cycles (Macaldowie et al., 2014). Highlighting both safety and efficiency issues as major considerations, the report shows that the vast majority of IVF cycles are now single-embryo transfers, being 76.3% in 2012 (Macaldowie et al., 2014). This means that more embryos become cryopreserved and the resultant live birth rates per initiated cycles for cryopreserved embryo transfers has improved steadily over the preceding 5 year period (from 16.3 to 20.3%), which, for autologous cycles equates with those from fresh embryos, and exceeds fresh for donation cycles. Australia and New Zealand now share one of the lowest rates of multiple births in the world, at 6.5%. ANZARD **Table 19** reports the quartile ranges from the 78 contributing IVF units and shows that the highest live birth rate per initiated cycle for autologous cryopreserved embryo transfers is 32.0% overall and 42.5% for women under 35 years, data that derives from our PIVET facility (Yovich et al., 2015a).

There are several factors that may contribute to PIVET's favourable results, but much of the methodology is shared by other infertility units. The only absolute difference relates to our specific HRT regimen, being focused on a unique combined oral tablet and vaginal pessary schedule, with monitoring of the mid-luteal concentrations of oestradiol and progesterone. A recent review (Yding Andersen and Vilbour Andersen, 2014) describes an optimal serum mid-luteal progesterone concentration in the range of 80–100 nmol/l for stimulated cycles, although those authors indicated that this had not yet been defined for cryopreserved embryo transfer cycles undertaken under HRT. The current report addresses this consideration.

Materials and methods

Patient selection

This is a retrospective study examining pregnancy outcomes of 529 cycles in women with an age range of 22–49 years, over a 6-year period from 1 January 2008 to 31 December 2013. Patients were selected from those who underwent a cryopreserved embryo transfer cycle with the transfer of a single blastocyst of defined grade, whilst using an HRT regimen. At PIVET, all supernumerary/surplus embryos that reach a minimum grade of 3BB according to the Gardner grading system (Gardner and Schoolcraft, 1999) are cryopreserved; by vitrification since late 2007. During the study period 2016 women had cryopreserved embryo transfer procedures, with 1716 being performed under an HRT regimen (85%). Autologous day 3 embryo transfers were undertaken on 671 cases and day 5/6 blastocyst transfers were performed on 1006; a further 39 transfers involving donor embryos. Of the autologous blastocyst transfers, 529 cycles were single blastocysts that had been cryopreserved by vitrification.

Embryo features

The blastocysts selected for the cryopreserved embryo transfer procedures had post-warm grading of at least 3BB or higher according to the Gardner classification. This alphanumeric code designates the number to signify the stage of blastocoele cavity expansion through to full (4), partial hatching (5) and completely hatched (6); and the letters to signify qualitative stages of development for the inner cell mass and trophectoderm, respectively, from A (high grade) to C (poorest grade). Hence the top grading embryos according to the Gardner classification were scored 4AA and 5AA. Only six of the transferred blastocyst embryos were fully hatched. Graded blastocysts were categorized into groups according to the implantation and live birth rates, namely Top, High, Medium, Modest and Low groups.

Clinical management

The HRT regimen commenced with onset of menses (day 1) and used oestradiol valerate tablets (Progynova 2 mg three times daily (t.d.s.); Schering Plough, Australia). On day 10 of the cycle, serum oestradiol was checked along with transvaginal ultrasound scan (3D ultrasound, Voluson, General Electric) for endometrial thickness, uterine volume and sagittal area. Thereafter, 10 mg oestradiol vaginal pessaries were given for 5 ± 1 days prior to the commencement of the progesterone pessary regimen, which designated the end of the artificial 'follicular' phase and onset of the artificial 'luteal' phase. This day was adjusted to avoid embryo transfer (ET) procedures on Sundays and holidays. The luteal pessary regimen was 400 mg progesterone t.d.s., with the evening pessary also containing oestradiol 2 mg.

Variations included doubling the oestradiol valerate dose to 4 mg t.d.s. times daily for oestradiol concentrations <1000 pmol/l, increasing the vaginal pessaries to 20 mg oestradiol and prolonging the artificial 'follicular phase' to reach endometrial thickness ≥7.5 mm (and ideally with uterine fundal sagittal area ≥15 cm^2 and uterine volume ≥25 cm^3).

All women had mid-luteal oestradiol and progesterone concentrations checked on day 8 or 9 of the progesterone pessary administration, a time classified as the mid-luteal point matching the post-ovulatory stage of a natural or stimulated ovarian cycle.

Vaginal pessaries

All pessaries (PIVET Pessaries) were developed by a single consulting pharmacist to a specific PIVET formulation containing 10 mg and 20 mg micronized 17β-oestradiol, 400 mg micronized progesterone alone or in combination with 2 mg oestradiol (combo pessary); all formulations were set in a standard fatty acid base. The progesterone and 17β-oestradiol are both supplied by PCCA, Professional Compounding Chemists of Australia, Matraville, NSW, Australia.The 'fattibase' was locally prepared from highly refined, hydrogenated and deodorized vegetable oil, predominantly palm and palm kernel oils (Wembley Pharmacy, Perth, Western Australia). The pessaries were subjected to sperm toxicity testing by PIVET's internal sperm survival assay (requiring >85% maintenance of progressive motility over 48 h; De Jonge et al., 2003).

Mid-luteal definition

The luteal phase of the HRT cycle was dated from the start of administration of the progesterone pessaries on the understanding that the progesterone rise in a natural ovulatory cycle occurs with the onset of the LH surge, i.e. 1 day prior to ovulation. The changeover from oestradiol to progesterone pessaries always occurred in the morning. All blastocyst embryos in this study were transferred on the 6th day of progesterone pessaries. The blood test undertaken in this study was therefore 2 days after embryo transfer (Mon–Sat) or sometimes 3 days to avoid Sunday testing. The blood tests for serum assays were undertaken 2–4 h after vaginal pessary insertion.

Pre-cryopreserved embryo transfer assessment and body mass index records

All women were reviewed in the week prior to commencing the cryopreserved embryo transfer cycle as a 'day-21' check. This involved a clinical review and discussion about cycle management. All cases had a transvaginal pelvic scan reviewed to exclude detectable pathologies including ovarian cysts. Even corpus luteal cysts >3 cm would require review or hormonal suppression before proceeding to a cryopreserved embryo transfer cycle. All women also had height and weight measured at this visit, with body mass index (BMI) automatically calculated by computer (Filemaker Pro, Filemaker Inc., USA), which stored all the data for this study. The BMI values were specifically recorded and also categorized as follows: < 20.0, 20.0 to 25.0, 25.1 to 30.0, 30.1 to 35.0 and >35.0 kg/m^2. Cases with BMI over 35 were deferred for weight reduction management, but four cases were accepted following acceptable weight reductions, the highest BMI being 37.72 kg/m^2.

Laboratory handling of embryos

PIVET commenced the Cryotop Vitrification procedure for oocyte/blastocyst cryopreservation (Kuwayama et al., 2005) in October 2007 after an initial transition period, so that 2008 was the first full year for vitrification with a complete changeover. This used in-house media based on the Kuwayama formulations for day 3, day 5 or day 6 embryos post-egg retrieval. The warming procedure was also according to the Kuwayama protocol. All blastocysts vitrified either on day 5 or day 6 were warmed and transferred on the same day of warming (day 6 of progesterone pessaries).

Embryos were cultured in 10 μl of blastocyst media (Sage Biopharma, Gytech, Australia) under mineral oil at 37°C in MINC incubators (COOK Australia) with 6% CO_2, 5% O_2 and nitrogen balance. Prior to transfer, the selected blastocyst was placed in a transfer solution containing blastocyst culture media enhanced with 10% human serum albumin (HSA; Sage Media, Gytech, Australia) before transfer in 10–20 μl of culture fluid.

Embryo transfer procedures

Embryo transfers were conducted in the lithotomy position with moderate Trendelenburg (head down) tilt under ultrasound guidance with non-empty bladder. Depending upon uterine position (anteverted, axial or retroverted) the bladder is allowed to fill to the degree enabling a satisfactory transvesical ultrasonic view of the endometrial cavity, ideally with minimal cervico-uterine angulation. The single blastocyst was transferred using either the K-JETS catheter system (K-Jets-7019-SIVF; COOK Australia Pty Ltd) or the Wallace Classic Catheter (Gytech Pty Ltd, Australia for Smiths Medical, UK) and a clear mid-fundal flash was identified on ultrasound signifying an appropriately conducted embryo transfer procedure.

Serum assays

Hormone analysis was performed in-house using the ADVIA Centaur XP Immunoassay System (Siemans, Erlangen, Germany). The oestradiol (Cat# 10491445 –oestradiol 500 test) and progesteron (Cat# 10491445 – progesterone 250 test) assays were performed according to the manufacturer's instructions. The in-house coefficients of assay variability were <2% for oestradiol in the range 1000 to 10,000 pmol/l and <5% for progesterone in the range 20–200 nmol/l; testing random samples five times. Hormonal concentration data are presented as means ± SE, with SE bars shown.

Pregnancies

Designating the first day of progesterone pessaries as day 1 of the artificial luteal phase, pregnancies are diagnosed on day 19 of progesterone pessary use (or day 20 to avoid Sunday). Although beta human chorionic gonadotrophin (βHCG) concentrations above 5 IU/l constitute a significant detection, longstanding tradition at PIVET requires concentrations >25 IU/l for pregnancy diagnosis. In equivocal cases, women are advised to continue their HRT regimen until clarification 3 days later. Once pregnancy is diagnosed women are advised to continue the regimen until gestational week 10 when a weaning process commences and is completed by week 12 when all exogenous hormonal supports are ceased. The

gestational weeks are calculated from the commencement of progesterone pessaries signifying week 2 (i.e. day 1 of progesterone pessary = day 14 for pregnancy dating). Progesterone and βHCG concentrations are checked each week until week 7.

Clinical pregnancy in this study is defined as the detection of rising βHCG concentrations to week 7, at which stage a transvaginal ultrasound (Voluson 730 Expert, General Electric Australia) was performed. The detection of an intrauterine gestational sac defines clinical pregnancy. A measurable crown rump length and fetal heart beat detection at gestational week 7 defines a viable clinical pregnancy. Those pregnancies fading before this stage or diagnosed as pregnancy of unknown location (including possible ectopic gestations) are excluded. Clinical pregnancy loss refers only to those pregnancies showing an identifiable intrauterine gestational sac but which do not proceed beyond 20 weeks gestation; mostly designated as spontaneous miscarriage or delayed miscarriage and submitted to curettage.

All clinical pregnancies received a further ultrasound between 10–12 weeks for first trimester screening and thereafter referred to an obstetrician who routinely conducted a fetal anatomy ultrasound scan at 18–19 weeks and later reported on pregnancy outcome. A live birth was diagnosed when delivery of a live infant occurred after 20 weeks gestation and cases of monozygotic twins were classified as a single live birth.

Statistical analysis

Data recording included the age of the woman at cryopreserved embryo transfer procedure as well as her age at the time of embryo cryopreservation, the blastocyst score at transfer, blastocyst grading groups (five ratings), mid-luteal oestradiol and grouping (four categories), and mid-luteal progesterone and groupings (four categories).

Statistical analysis of the data was undertaken on GraphPad Prism (v6.0, GraphPad Software Inc., USA), where each of the variables was analysed against clinical pregnancy rate and live birth outcomes. All data with two groups, for example progesterone and outcome, were compared using an unpaired *t*-test and applying the Mann–Whitney analysis. For groups with three or more data sets, an unpaired one-way ANOVA utilizing Tukey's test for comparisons was used, for instance blastocyst gradings and outcome. Finally, the influence of age (at embryo vitrification or at later cryopreserved embryo transfer), BMI and blastocyst group on progesterone concentrations and outcome was determined using an unpaired two-way ANOVA and applying the Sidak test.

Ethical considerations

PIVET is accredited with both the self-regulatory National Australian authority Reproductive Technology Accreditation Committee (RTAC) as well as the statutory Western Australian State accreditation body RTC (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 the National Australian Testing Authority (NATA) 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.

Results

Over the 6-year study period, 529 SET procedures of vitrified blastocysts were undertaken in women aged from 21 to 47 years using an HRT regimen. This generated 271 clinical pregnancies (51.2%) resulting in 205 live birth deliveries (38.8% of cryopreserved embryo transfers; 75.6% of pregnancies) across the age range (21–46 years). There were five monozygotic twin pregnancies in the series (1.8% of pregnancies), all proceeding to live births (nine infants as one fetus of a twin gestation died in the late first trimester). Hence, the total infants were 209, but live births were analysed as single pregnancy outcomes in this data set, i.e. 205. The pregnancies were generated from a broad range of blastocyst gradings and in association with wide ranges of both oestradiol and progesterone concentrations.

Mid-luteal progesterone levels

The mid-luteal serum concentrations of progesterone ranged from 13 nmol/l to 237 nmol/l and clinical pregnancies with live births were recorded across this full range. The mean concentration was 61 nmol/l and the Q1–Q3 range was 44–80 nmol/l (**Figure 1a** and **b**). Although the median concentrations of progesterone were higher among those conceiving (**Figure 1a**; 60 versus 50 nmol/l) the confidence interval was wide and not significant for live births (**Figure 1b**). In the case of clinical pregnancy with the lowest recorded progesterone concentration of 13 nmol/l, the woman may have forgotten to take her pessary that morning, but the regimen was increased to an oestradiol/progesterone combo pessary twice daily (b.d.) along with progesterone t.d.s. The resultant pregnancy resulted in a successful live birth. Pregnancies ensued across all the progesterone groups but there was a distinct and significant rising rate peaking in the range 70–99 nmol/l ($P = 0.005$; **Figure 1c**), thereafter falling to significantly lower rates when progesterone was ≥100 nmol/l ($P = 0.0047$). The live birth rate also peaked significantly in the 70–99 pmol/l group ($P = 0.031$), although the apparent reduction for progesterone ≥100 nmol/l was no longer significant (**Figure 1d**). Pregnancy losses were similar amongst the groups, ranging from 18.8% (progesterone ≥100 nmol/l) to 24.7% (progesterone <50 nmol/l).

Mid-luteal oestradiol levels

The serum oestradiol concentrations showed a wide range, from 320 pmol/l up to 9700 pmol/l, with clinical pregnancies

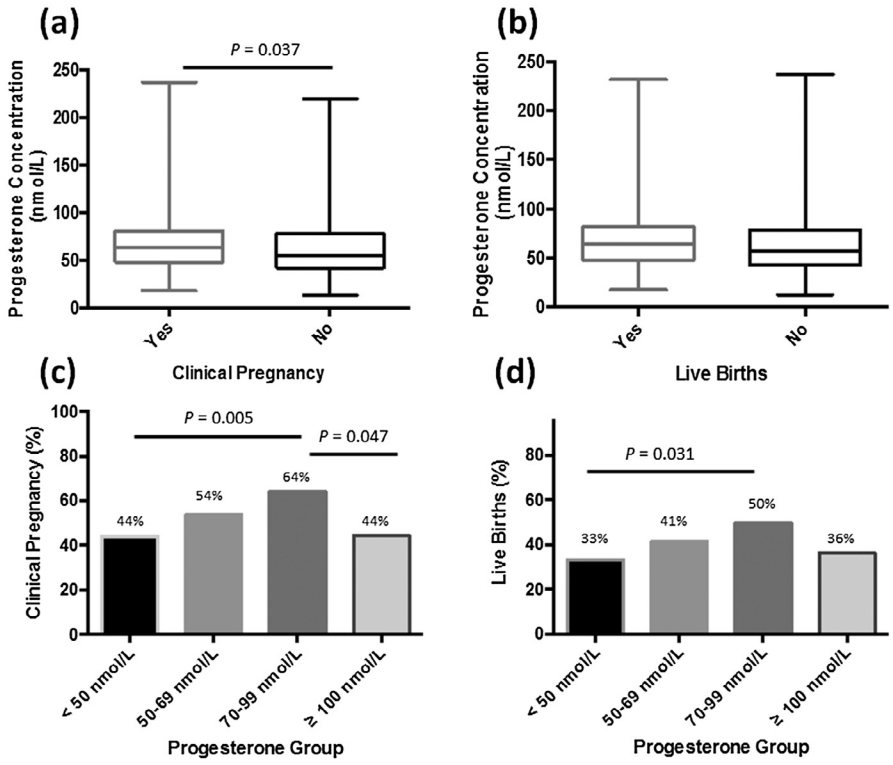

Figure 1 Range of mid-luteal serum concentrations of progesterone in patients for clinical pregnancy (a) and live birth (b) outcomes. Progesterone concentrations were significantly higher in patients achieving clinical pregnancy (a). Bar charts represent the clinical pregnancy (c) and the live birth (d) outcomes for progesterone concentrations when stratified into different groups. Significantly higher numbers of clinical pregnancies and live births were observed when progesterone concentrations were optimal, in the range of 70–99 nmol/l (c and d).

recorded across this entire range. The mean concentration was 2400 pmol/l with the Q1–Q3 quartiles ranging from 1700 pmol/l to 3200 pmol/l (**Figure 2a** and **b**). The lowest oestradiol case corresponded with the lowest progesterone (see above) and live birth was achieved after increasing the pessary regimen for both oestradiol as well as progesterone from the mid-luteal phase. However, unlike progesterone concentrations, there was no relationship between oestradiol concentration or oestradiol group and the chance of clinical pregnancy (**Figure 2c**) or live birth (**Figure 2d**).

Oestradiol/progesterone ratios and BMI

The question of a correlation between oestradiol and progesterone concentrations or any interaction between the two hormones was analysed with respect to the chance of clinical pregnancy and live birth (**Figure 3a** and **b**). When progesterone concentrations were stratified according to oestradiol grouping, mean progesterone concentrations were higher in the maximum oestradiol group of ≥3201 pmol/l (**Figure 3a** and **b**). However, there was no significant difference in mean progesterone concentration in the three lower categories of oestradiol (≤1600–3200 pmol/l). Furthermore, progesterone concentrations were not related to outcome, i.e. whether or not there was a clinical pregnancy or live birth (**Figure 3a** and **b**). In addition, the hormonal data were also adjusted to BMI for possible influence of body mass on serum hormone concentrations. No significant differences were revealed for progesterone concentrations and outcome when adjusted to BMI groupings (**Figure 3c** and **d**). However, oestradiol concentrations appeared to be significantly higher ($P < 0.05$) in patients with the lowest BMI (<20.0 kg/m^2) in comparison with those with mid ranges of BMI (20.1–35.0 kg/m^2), although this did not influence clinical pregnancy outcome (**Figure 3e**). In terms of the live birth subpopulation, BMI grouping was not related to serum oestradiol concentrations and live birth outcome (**Figure 3f**).

Blastocyst gradings

The Top category of blastocyst gradings (4AA and 5AA) generated clinical pregnancy rates of 64% and live birth rates of 50% per transfer. The High category of blastocyst gradings (4AB, 4BA and 3AB) generated clinical pregnancy rates of 54% and live birth rates of 41% per transfer (**Figure 4a** and **b**). The Medium category of blastocyst gradings (5AB, 5BA, 3AA, 3BA and 6AA) generated clinical pregnancy rates of 46% and live birth rates of 38% per transfer. The Modest group of blastocyst gradings (5BB, 4BB and 3BB) generated clinical pregnancy rates of 33% and live birth rates of 21% per transfer (**Figure 4a** and **b**). Of the hatched blastocysts, two pregnancies

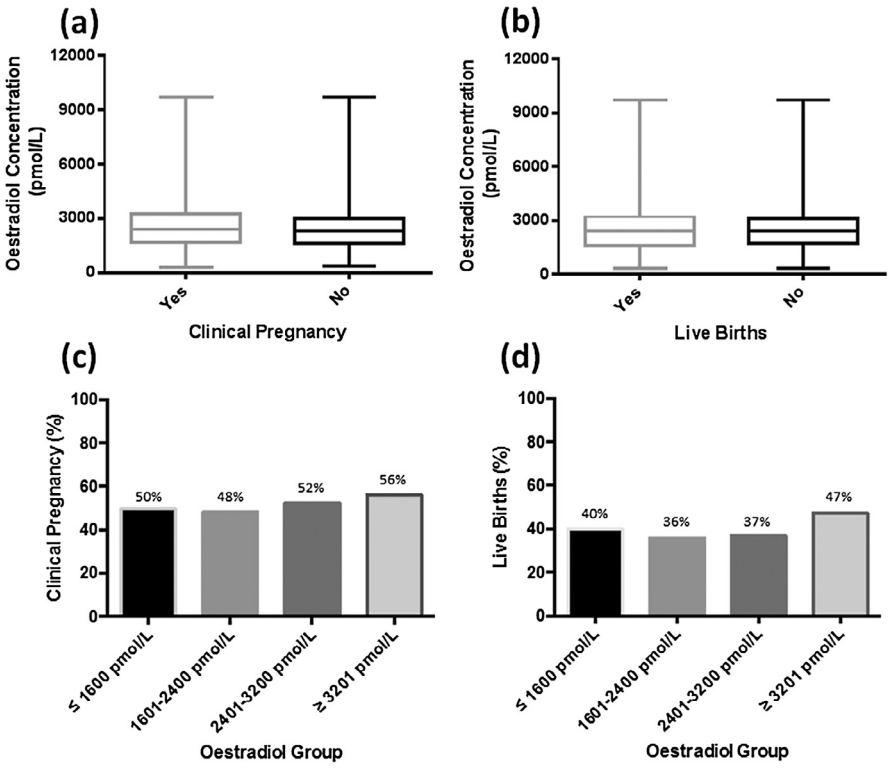

Figure 2 Range of mid-luteal serum concentrations of oestradiol in patients for clinical pregnancy (a) and live birth (b) outcomes. Bar charts represent the clinical pregnancy (c) and live birth (d) outcomes for oestradiol concentrations when stratified into different groups. No statistically significant observations were detected.

arose from the transfer of five hatched blastocysts where the inner cell mass and trophectoderm were both classified high-grade (6AA) and one of these proceeded to a normal live birth; however, no pregnancies were generated from the six hatched blastocysts categorized in the Low group (6AB, 6BA and 6BB).

There were significant differences in both clinical pregnancy and live birth outcomes in relation to the described blastocyst gradings (**Figure 4a** and **4b**); Top grade was better than Medium ($P = 0.042$) and Modest ($P < 0.001$) gradings; and High grade was better than Modest grade ($P < 0.017$). Live birth rates were also significantly higher for Top versus Modest blastocysts ($P < 0.001$) and High versus Modest grades ($P < 0.022$). With respect to blastocyst groupings (**Figure 4c** and **d**), the combined High and Top groupings had a significantly higher pregnancy rate compared with the lower Modest and Medium groupings ($P < 0.001$) as well as live birth rate ($P = 0.002$). Pregnancy losses were significantly higher for the Modest and Medium groupings (41.2%) compared with the Top and High groupings (18.7%, $P < 0.01$, data not shown).

Figure 4e and **f** shows the trend for optimum pregnancy rates and live birth rates for the serum Progesterone range 50–99 nm/l at both Blastocyst Groupings (High and Top as well as Modest and Medium, but these data do not reach statistical significance. The optimal High and Top blastocyst grading showed significantly higher rates for live births ($P < 0.011$) and clinical pregnancies when compared with Modest and Medium grades in the progesterone range <50 nm/l ($P < 0.002$) and 50–99 nm/l ($P < 0.036$).

Influence of age

The influence of age on clinical pregnancy and live birth outcomes is shown with respect to the age of the woman at embryo vitrification (**Figure 5a** and **b**) and the later age at cryopreserved embryo transfer (**Figure 5c** and **d**). Although the data show reducing clinical pregnancy rates with advancing age at cryopreserved embryo transfer, the effect was insignificant up to age 40 years (**Figure 5c**). Beyond age 40 years both pregnancy and particularly the live birth rate were significantly reduced compared with those <35 years ($P < 0.02$ and $P < 0.05$, respectively), with 10 of 22 pregnancies failing to progress beyond 20 weeks (**Figure 5c** and **d**).

Interactions of progesterone and age on outcome

In order to exclude the possibility of bias from the influence of patient age at cryopreserved embryo transfer, and at vitrification on progesterone concentrations and subsequent pregnancy outcome, two-way ANOVA was performed. Here, progesterone concentrations did not vary significantly between age groups at vitrification (**Figure 6a** and **b**), or at cryopreserved embryo transfer (**Figure 6c** and **d**). In addition, when adjusted for age at vitrification and cryopreserved embryo transfer, concentrations did not influence clinical pregnancy (**Figure 6a** and **c**, respectively) or live births (**Figure 6b** and **d**, respectively).

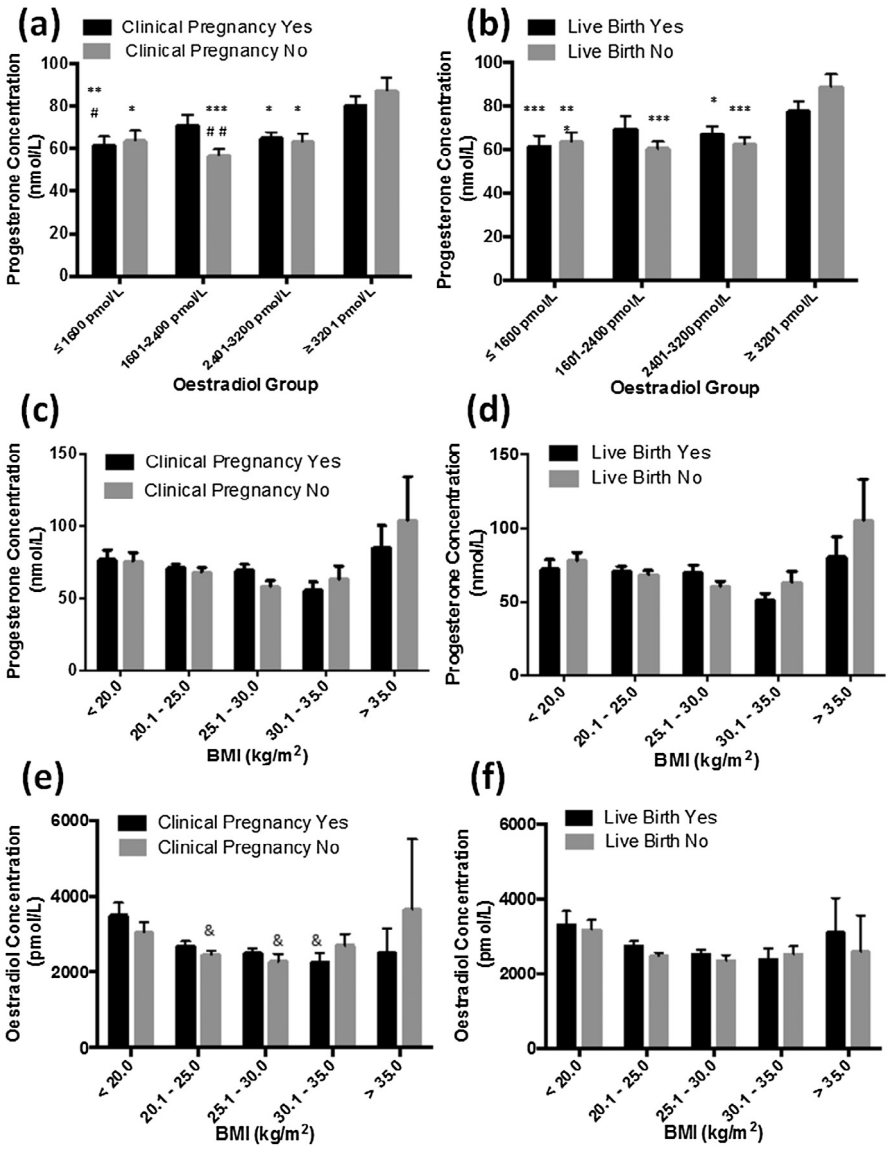

Figure 3 The relationship between progesterone, oestradiol concentrations (mean ± SE) and body mass index (BMI) was analysed with respect to clinical pregnancy and live birth outcomes. No significant difference was observed between progesterone concentrations and outcome when adjusted for oestradiol grouping (a and b). The first three oestradiol groupings are compared with the fourth group (≥3201 pmol/l), in which progesterone concentrations are highest and significance levels shown as described, but this did not influence clinical pregnancy or live birth rates (a and b, respectively). When progesterone concentrations and outcome were adjusted for BMI group, no significant interaction was observed (c and d). Similarly, BMI did not influence oestradiol concentrations and live births (f). However, oestradiol concentrations in patients with lower BMI (<20.0 kb/m^2) were significantly higher ($P < 0.05$) than those with mid-range BMI (e), but this did not influence clinical pregnancy outcome. *'indicates statistical significance from oestradiol ≥3201 pmol/l group negative for clinical pregnancy or live birth (grey bars, $P < 0.05$); # indicates statistical significance from ≥3201 pmol/l group positive for clinical pregnancy (black bars, $P < 0.05$); ' and ' indicates statistical significance for BMI < 20.0 kg/m^2 positive for clinical pregnancy (black bar, $P < 0.05$). Single, double and triple symbols designate $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

Discussion

Applying a linear regression model, a strong correlation between individual blastocyst gradings and the likelihood of both clinical pregnancy (R^2 0.9715) and live birth (R^2 0.9711) has been shown previously (Yovich et al., 2015b).

This study demonstrates that the implantation process is also sensitive to the variability in mid-luteal serum progesterone, but not in oestradiol concentrations in SET procedures undertaken in HRT cycles. The positive relationship between optimal progesterone concentrations (70–99 nmol/l) and implantation rates was very strong and highly specific. In addition, when progesterone concentrations were adjusted for groupings of blastocyst quality, similar outcomes were observed for the wider mid-progesterone range of 50–99 nmol/l with respect to both Modest and Medium

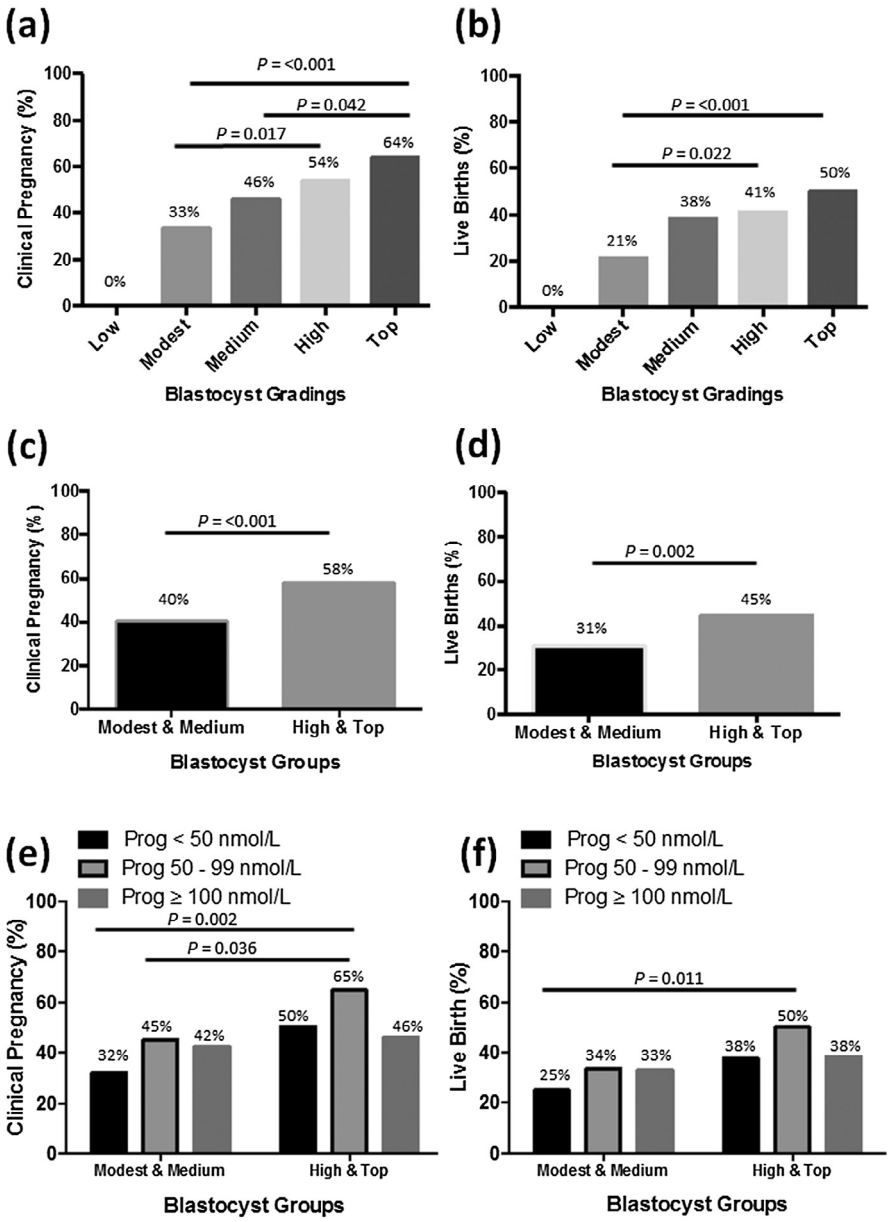

Figure 4 Both clinical pregnancy (a) and live birth (b) rates show a significant rise in relationship to blastocyst gradings ($P = 0.042$ to $P < 0.001$). The two main blastocyst groupings (Modest and Medium versus High and Top) showed highly significant differences in clinical pregnancy ($P < 0.001$) (c) and live birth rates ($P = 0.002$) (d). When progesterone concentrations and outcome were adjusted for blastocyst quality (e and f), similar trends were observed for the mid-progesterone range of 50–99 nmol/l in both Modest and Medium quality blastocysts (45% clinical pregnancy and 34% live births) versus High and Top quality blastocysts (65% clinical pregnancy and 50% live births). The optimal High and Top blastocyst grading showed significantly higher rates for live births ($P = 0.011$) and clinical pregnancies when compared with Modest and Medium grades in the progesterone range <50 nm/l ($P < 0.002$) and 50–99 nm/l ($P < 0.036$).

blastocysts (45% clinical pregnancy and 34% live births) versus High and Top quality blastocysts (65% clinical pregnancy and 50% live births). These rates were significantly higher than those observed with either low progesterone (<50 nmol/l) or excess progesterone (≥100 nmol/l). These data suggested that mid-range progesterone concentrations promoted positive outcomes, independent of blastocyst grading. However, when progesterone concentrations were adjusted for oestradiol concentration, BMI and age at vitrification or age at cryopreserved embryo transfer, no statistically significant interactions were observed in terms of likelihood of clinical pregnancy or live birth.

Interestingly, when progesterone reached the optimal concentration of 70–99 nmol/l, the clinical pregnancy rate reached 64%, even when more older women were included in this group, indicating that the effect of progesterone concentration is more potent than that of age. Obviously, there are several determinant factors of implantation – mid-luteal progesterone concentration, age of patients and quality of embryos – and all will have their individual influence on

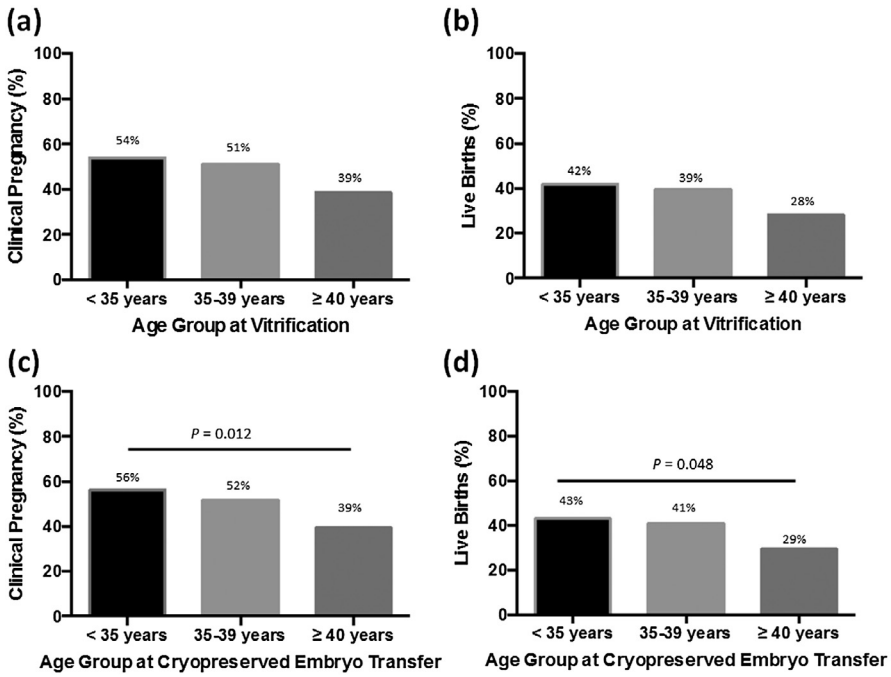

Figure 5 The influence of age on clinical pregnancy and live birth outcomes is shown with respect to age at vitrification (a and b, respectively) and the later age at cryopreserved embryo transfer (c and d, respectively). Clinical pregnancy and live birth rates appear to reduce with advancing age at both vitrification and cryopreserved embryo transfer, but the effect was only statistically significant for age at cryopreserved embryo transfer beyond age 40 years compared with <35 years for both clinical pregnancies ($P < 0.012$) and live births ($P < 0.048$, c and d).

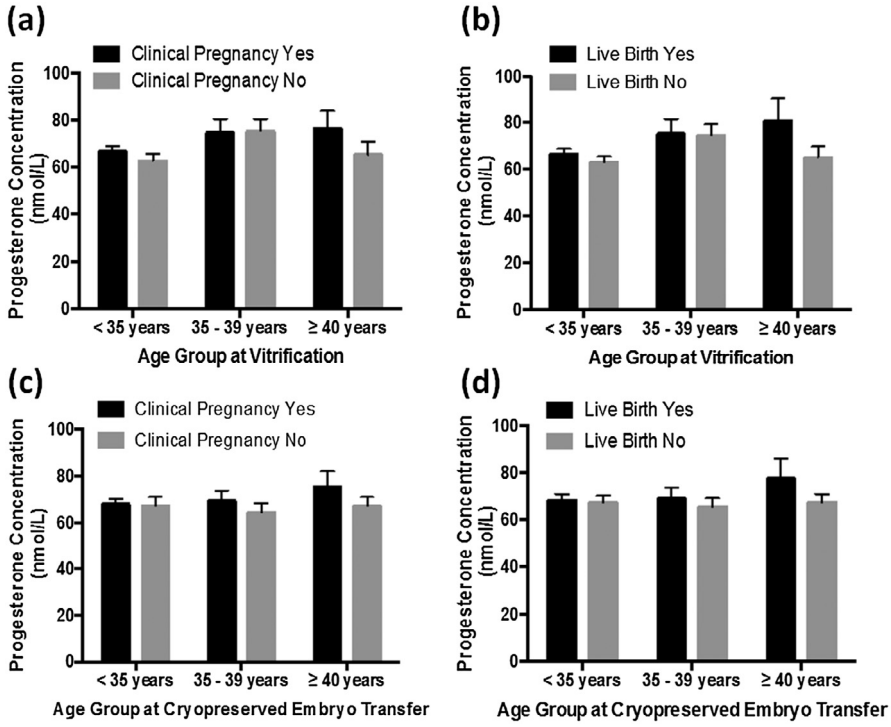

Figure 6 The potential influence of patient age at vitrification and at cryopreserved embryo transfer, on progesterone concentrations and subsequent treatment outcomes was analysed. Progesterone concentrations did not vary significantly between age groups at vitrification (a and b), or at cryopreserved embryo transfer (c and d). When adjusted for age at vitrification, progesterone concentrations did not influence clinical pregnancy (a) or live births (b). Similarly, adjustment of age at cryopreserved embryo transfer did not reveal any significant influence of progesterone concentrations on clinical pregnancy (c) or live births (d).

implantation independently with specific patterns of effect. The positive influence of embryo quality was simply based on the Gardner grading scale, which is uniquely validated in this study (allowing for some subjectivity in grading trophectoderm and inner cell mass features in the middle grades). There was high consistency grading 4AA and 5AA embryos (Top grade) as well as 3BB, 4BB and 5BB (lower Modest grade) (Yovich et al., 2015b).

The optimal range of progesterone (70–99 nmol/l) indicates a narrow window at which to achieve the best implantation rate. However, in reality these factors interact together in any given case and time, and therefore should not be examined separately. This study shows for the first time that monitoring the concentration of progesterone at the mid-luteal phase is an important practice toward achieving implantation, partly because its optimal concentrations could possibly minimize the negative effects of advanced age or lower quality embryos. This finding could explain the recent observations that increasing the pessary dosage schedule in cryopreserved embryo transfer cycles under HRT control significantly improved the pregnancy rate (Alsbjerg et al., 2013). Clearly further research could focus on the relevance of adjusting pessary dosage or route of administration to optimize progesterone concentrations with a view to raising implantation rates.

Interestingly, there was a large variation in steroid concentrations despite all women receiving the same HRT regimen. Clearly further research is required to consider factors that might personalize dosage and optimal route of administration for individual patients. Some studies in this respect have been reported but have not shown any therapeutic effects of the steroid administration method, e.g. between vaginal gels and tablets (Lan et al., 2008; Simunic et al., 2007), although patients may prefer gel over tablets. In the above studies, the actual variation in serum concentrations with each formulation has not been documented. The present results show that even though vaginal administration methods may be preferred, it may also suffer from considerable variation among women in the uptake, absorption and metabolism of each hormone. However, there has been a viewpoint projected that measuring serum progesterone for adequacy of pessary dosage and effect is irrelevant, as pessaries placed in the vagina somehow act directly on the endometrium, initially described as the first uterine pass effect (Bulletti et al., 1997), where radioactive (tritiated) progesterone was shown to travel from vagina to uterus in cadaveric specimens. Consequently, several clinical groups view vaginal administration of progesterone as superior to other routes (Ho et al., 2008; Manno et al., 2005; Tavaniotou et al., 2000). Conversely, a recent investigation has demonstrated that vaginal gel and intramuscular injection of progesterone showed comparable implantation and pregnancy rates in IVF patients receiving vitrified blastocysts (Shapiro et al., 2014). Therefore, we are not aware of any utero-vaginal portal venous system that might enable a true first pass effect for progesterone, nor do we believe that vaginal pessaries saturate the pelvic tissues to any significant degree for direct absorption. Our view, totally supported by the data in this study, is that the endometrium will be completely dependent upon arterial blood carrying progesterone from whatever source (intramuscular, subcutaneous, corpus luteum, vaginal pessary or rectal pessary). This means the endometrium receives progesterone from vaginal pessaries as a standard mechanism, having travelled protein-bound one entire circuit of the vasculature. The blood samples from the ante-cubital fossa simply reflect the concentration of progesterone within this circuit. However, it must be noted that the large variations in serum progesterone from pessaries among women will also cause variation in uterine exposure to progesterone.

With respect to higher mid-luteal progesterone concentrations (≥100 nmol/l) associating with reduced implantation rates, we considered three possibilities, firstly a statistical aberration; secondly underlying break-through ovulation causing premature endogenous progesterone effects on endometrial synchrony as proposed by El-Toukhy et al. (2004); and thirdly the simple effect of 'too much' progesterone accelerating the implantation synchrony window. With respect to the first possibility, it appears unlikely to be a statistical aberration as our observation of this effect has been consistent over the 6-year study period, when examined on an annual, biennial or the total period basis. With respect to the second possibility, the HRT regimen in this study did not include any GnRH analogues to down-regulate the hypothalamic-pituitary–ovarian axis, as advised by El-Toukhy et al. (2004). In consideration of the possibility of underlying ovulation affecting luteal progesterone concentrations and endometrial synchrony, PIVET has reported that evidence shows this to be infrequent (<1%; Yovich et al., 2015a), hence we do not support the idea that GnRH analogues should be applied to prevent background ovulation. Nonetheless it would appear that some personalized adjustment of the pessary regimen may be required, as too much progesterone in the early luteal phase may be detrimental, probably by accelerating secretory changes in the endometrium, thereby affecting embryo–endometrial synchronization. However, once pregnancy is established it appears that the higher the progesterone concentration is, the more likely that pregnancy is protected with an apparently decreased pregnancy loss.

Mechanistically, precise synchronization between endometrial maturation and embryo age during the implantation window period is required to achieve a successful IVF or cryopreserved embryo transfer treatment cycle (Cohen et al., 1988; Prapas et al., 1998). In cryopreserved embryo transfer cycles, Prapas et al. (1998) reported that the clinical pregnancy rate was greatest when day 2 embryos were transferred on the third or fourth day of progesterone rise and less on the second or fifth day. This relates to the current study, in which day 5 embryos were transferred on the sixth day of progesterone rise. This study lends support for the existence of a narrow implantation window, as proposed by Psychoyos (Psychoyos and Mantel, 1985) and the idea that the window is time-sensitive since the pregnancy rates were lower both with low and high progesterone concentrations. One interpretation of this observation is that low progesterone may delay or impede, while excess progesterone concentrations may accelerate, development of endometrium, and consequently either delay or advance the implantation window, both scenarios creating dyssynchronous matching with the embryo.

The role of oestradiol and progesterone in the structural and functional development of endometrium in the preparation for implantation and maintenance of pregnancy is well understood. However, whether a temporal correlation exists between the serum concentrations of these hormones and implantation opportunity remains largely debatable. In addition,

hormone serum concentrations in HRT cycles may not reflect endometrial activity, specifically it has been shown that the oestradiol concentrations is not important for pregnancy, suggesting that its role in implantation is only permissive (DeZiegler, 1995; Edgar, 1995; Ghosh and Sengupta, 1995; Ghosh et al., 1994). This view is supported by the present data, which show no significant relevance of oestradiol across the range 342–8600 pmol/l; neither was there any interactive effect demonstrable with progesterone concentrations. Since oestradiol concentrations at <100 pg/ml (i.e. < 367 pmol/l) were able to induce changes sufficient enough to sustain normal implantation (Remohi et al., 1997), it appears that in the present study the physiological concentration required for a maximum oestradiol action has already been covered and any further increase in oestradiol concentrations will not induce additional benefit (Banz et al., 2002; Niu et al., 2008). The view that increasing progesterone counters oestradiol function by suppressing the production of oestrogen receptors (Speroff and Fritz, 2004) could be a consideration when progesterone concentrations were higher than 100 nmol/l, but this could not be properly examined in this study.

Previous reports recommended that in natural menstrual cycles, the concentration of progesterone in the mid-luteal phase should be between 30 and 40 nmol/l (Abdulla et al., 1983; Li et al., 2008; Van Zonneveld et al., 1994). The current study has shown that women with progesterone concentrations ≥100 nmol/l or <50 nmol/l have a significantly lower pregnancy rate. Since other reviews have not observed a detrimental effect of elevated progesterone (Nawroth and Ludwig, 2005), whether our observations reflect specificity regarding the timing of the blastocyst transfer or a unique consequence of our hormonal regimen remains to be determined. Regardless, we believe that more consideration is required when planning a progesterone supplement during assisted reproductive procedures and clinics may need to review their own regimen and synchronize the day of transfer to serum progesterone concentrations.

Finally, the serum measurement of oestradiol and progesterone concentrations was performed at the mid-luteal phase, 2–3 days after transfer in a defined HRT transfer environment, presumably coinciding with the time of implantation or soon after implantation. Such a temporal approach may prove important, as although it reflects the implantation period, as a therapeutic marker for embryo transfer it may be suboptimal, i.e. too late. Some unpublished data from our group on adjusting the pessary regimen at this stage implied benefit, but suggested that the adjustment may need to be explored at an earlier stage, e.g. 2 or 3 days prior to enable correction by the time of implantation. However, whether the serum steroid concentrations can be sufficiently and effectively modified by changes in pessary management remains to be investigated.

In conclusion, the results from this study have shown that the chance of pregnancy in cryopreserved embryo transfer cycles under hormonal control is highly dependent upon the circulating concentration of progesterone, with an optimal progesterone concentration of 70–99 nmol/l. Embryo quality also dictates the chance of successful implantation but even genetically screened blastocysts implant only 64% (Fiorentino et al., 2014). The present data show that optimal progesterone concentrations can enhance implantation, particularly of lower-graded blastocysts, and the combination of optimal progesterone with high-grade blastocyst largely overcomes the effects of age. These data indicate that PIVET's specific HRT regimen may be an important contributor to the favourable cryopreserved embryo transfer results and monitoring of the mid-luteal progesterone concentrations may be a relevant guide for future studies to adjust the HRT regimen.

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