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Comparison of serum progesterone levels on the day of frozen embryo transfers according to type of endometrial preparation: a single centre, retrospective study

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Research Article

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Summary

The live birth rate following embryo transfer is comparable between spontaneous, stimulated and artificial cycles. However, the pregnancy loss rate appears elevated with hormonal therapy, possibly due to luteal insufficiency. This study aimed to determine whether the serum progesterone level on transfer day differed according to the endometrial preparation method for frozen embryo transfer (FET). Twenty spontaneous cycles (SC), 27 ovarian stimulation cycles (OS) and 65 artificial cycles (AC) were retrospectively studied from May to December 2019 in a single French hospital. The primary endpoint was the level of serum progesterone on the day of FET between the three endometrial preparation methods. The mean serum progesterone level on transfer day was 29.47 ng/ml in the OS group versus 20.03 ng/ml in the SC group and 14.32 ng/ml AC group ($P < 0.0001$). Progesterone levels remained significantly different after logistic regression on age and anti-Müllerian hormone (AMH) level. There was no significant difference in demographic and hormone characteristics (age, body mass index, embryo stage of embryo, type of infertility, basal follicle stimulating hormone, luteinizing hormone, estradiol and AMH levels), endometrial thickness, number and type of embryos transferred, duration of infertility, pregnancy rate, live birth rate and pregnancy loss rate. No difference was found in serum progesterone levels between clinical pregnancy with fetal heartbeat and no clinical pregnancy (no pregnancy or pregnancy loss, 17.49 ng/ml vs 20.83 ng/ml, respectively, $P = 0.07$). The lower serum progesterone level found on FET day in the AC group should be further investigated to see whether this difference has a clinical effect on the live birth rate.

Introduction

Frozen embryo transfers (FET) are increasingly common (De Geyter *et al*., 2018) due to the modification of clinical protocols favouring freezing in patients at risk of hyperstimulation and from improvements in biological techniques such as cryopreservation and oocyte vitrification. The endometrium is prepared to allow transferred embryo implantation via various endometrial preparation protocols. In a stimulated cycle, endometrial preparation is performed through ovulation induction using follicle stimulating hormone (FSH). Ovulation is triggered with human chorionic gonadotropin (hCG) when there is a main follicle, producing a corpus luteum that secretes progesterone in the luteal phase and can be sustained with exogenous progesterone (Labrosse *et al*., 2020). This method requires regular monitoring. In a natural (spontaneous) cycle, endometrial preparation is not induced; ovulation can be natural or induced by an hCG trigger. This method requires regular monitoring and is complicated when the transfer falls on a weekend. In an artificial cycle (AC), the endometrium is prepared by oral or transdermal estradiol to stimulate the growth of the endometrium, whilst blocking the patient's gonadotropic pathway, and administering progesterone in the luteal phase to differentiate the endometrium. This method is easier to monitor and manage. The choice of protocol depends on the existence or not of an ovulatory cycle, the presence of endometriosis and adenomyosis, and the patient's preference.

There is no consensus on which type of preparation is best, and implantation rates per embryo are comparable between the methods (Peeraer *et al*., 2015). However, results on the rate of pregnancy loss with substituted cycles are contradictory (Ghobara *et al*., 2017;

Mackens *et al.*, 2017; Tomás *et al.*, 2012), (Mackens *et al.*, 2017) there is no apparent difference in the rate of ongoing pregnancies or birth rate according to cycle type (Peigné *et al.*, 2019). However, a recent retrospective study on a large multicentre cohort showed a significant increase in early pregnancy loss when using AC for endometrial preparation before FET (Vinsonneau *et al.*, 2022). Some authors have shown that the AC resulted in fewer clinical pregnancies when the progesterone level on the day of the FET was lower, especially <9.2 ng/ml (Cédrin-Durnerin *et al.*, 2019; Labarta *et al.*, 2017). Another team recently proposed a higher progesterone threshold of 20.6 ng/ml (Boynukalin *et al.*, 2019). Other studies have demonstrated better clinical pregnancy rates if the progesterone level on Day 10 of the transfer was >35 nmol/l (Alsbjerg *et al.*, 2018), >50 nmol/ml on Day 16 (Basnayake *et al.*, 2018), or >7.9 ng/ml in the middle of the luteal phase (Arce *et al.*, 2011). A recent meta-analysis found a cut-off of 10 ng/ml for the luteal serum concentration of progesterone, although there is no consensus on when to measure progesterone or the optimum threshold (Melo *et al.*, 2021).

Our aim was to compare progesterone levels on the day of transfer according to endometrial preparation protocol (spontaneous, stimulated or AC). The secondary objectives were to characterize normal progesterone levels depending on the day of embryo transfer, to observe the rate of pregnancies, live birth and pregnancy loss depending on the preparation protocol, and to determine the progesterone level threshold for clinical pregnancies.

Materials and methods

Study design and participants

This was a retrospective observational study on data from patients who had undergone an FET cycle at Nîmes University Hospital from May 2019 to December 2019. Medical and sociodemographic data were collected using medical JFIV® software. The study was approved by the local Institutional Review Board (IRB no. 20.02.01). Patients had previously agreed to the use of their data in future research. All patients who did not respond to the non-opposition letter were studied. Patients without a progesterone level test on the day of the transfer were excluded from the study.

Embryo freezing, thawing and transfer

Patients were grouped according to endometrial preparation protocol: spontaneous cycle (SC), ovarian stimulation (OS) or artificial cycle (AC). For endometrial preparation protocols, see Methods S1.

Frozen embryos at the cleavage or blastocyst stage were transferred between Days 2–5. The embryos were frozen by vitrification (Vit Kit-Freeze, Irvine Scientific®, Paris, France) according to the manufacturer's procedure (Wirleitner *et al.*, 2013). After thawing, embryos were eligible for FET if >50% of the cells were intact on Day 2 or 3. Blastocyst-stage embryos were eligible for transfer if <25% lysis was observed. The development stage and the number of embryos transferred were determined on a case-by-case basis, up to a maximum of three embryos.

Outcomes

The primary outcome was progesterone level (ng/ml) on the day of embryo transfer. Samples were taken at 10 a.m. The demographic variables analyzed were body mass index (BMI), the type (primary/secondary) and duration of infertility, cause of infertility (tubal, ovulatory, male factor, endometriosis), smoking status and hormone levels on Day 3 [FSH, luteinizing hormone (LH), estradiol and anti-Müllerian hormone (AMH)] and day of transfer (cleavage stage or blastocyst).

The embryonic development stage was assessed at the time of transfer (early transfer on Days 2–3, cleavage or prolonged culture on Days 4–6), the number of embryos transferred, the maternal age at freezing and thawing, the thickness of the endometrium before progesterone treatment, and the levels of LH and estradiol on the day of transfer were also recorded.

Positive pregnancy was considered for a positive hCG blood test (>100 UI/l). Progressive pregnancy with heartbeat activity detected at 7 weeks using ultrasound was classed as 'clinical pregnancy with fetal heartbeat'. A pregnancy documented by positive hCG without fetal heartbeat at 7 weeks of gestational age was classed as 'pregnancy loss'. A 'live birth' was considered as the birth of at least one living baby after 22 weeks of gestation.

We compared, according to the endometrial preparation method, the serum progesterone level at the end of the FET cycle in terms of pregnancy rate per cycle, the rate of pregnancy loss per pregnancy and the rate of clinical pregnancies with fetal heartbeat per cycle (Zegers-Hochschild *et al.*, 2017).

Data sources/measurements

Progesterone, LH and estradiol levels were analyzed via electrochemiluminescence using the Elecsys Progesterone III, Elecsys LH and Elecsys Estroadiol III assays, respectively, performed on a Cobas e801 (Roche Diagnostics) analyzer. The Day 3 hormone tests (FSH, LH, estradiol and AMH) were either performed in our laboratory or in a community laboratory. Endometrial thickness was measured using an S10 ultrasound machine (Voluson, GE Healthcare) and a transvaginal probe.

Bias

To reduce the risk of inclusion bias, we included all patients with known progesterone levels during the inclusion period. We performed a logistic regression analysis to limit the effect of possible differences in the population studied.

Sample size

Based on a previous study in which women received 200 mg vaginally administered progesterone twice a day (versus three times per day here), we estimated a C_{max} progesterone level with a substituted cycle of 13 ± 4 ng/ml 18. The expected average progesterone level at the middle of the luteal phase with an SC was 19.4 ± 6.4 ng/ml. Therefore, the number of subjects necessary to observe a difference of 6 ng/ml with an alpha risk of 5% and a power of 90% in a unilateral test was 19 subjects in each group.

Statistical methods

Quantitative variables are described by mean ± standard deviation or median and interquartile range, whilst qualitative variables are described by percentages. Means were compared by parametric analysis of variance (ANOVA) test or Kruskal–Wallis test, and percentages were compared using the chi-squared test or non-parametric Fisher's exact tests, as appropriate. Patients with missing variables for the primary outcome were not included in the study. If a variable was missing for a secondary outcome measure, the patient was excluded from the analysis.

When the association of the quantitative and qualitative variables (three modalities) was significant, multiple comparisons (2 × 2) were made with alpha risk adjustment using the Benjamini–Hochberg

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Table 1. Baseline patient characteristics

	SC group ($n = 20$)	OS group ($n = 27$)	AC group ($n = 65$)	*P*-value
Early embryo transfers (Days 2–3), *n* (%)	6 (30%)	8 (29.6%)	29 (44.6%)	0.2814
Blastocyst transfers (Days 4–6), *n* (%)	14 (70%)	19 (70.4%)	36 (55.4%)	
Patient age at the time of freezing (years), mean ± standard deviation (SD)	33.04 ± 5.04	31.76 ± 4.6	32.5 ± 4.78	0.6528
Patient age at the time of transfer (years), mean ± SD	33.84 ± 4.53	32.62 ± 4.54	33.37 ± 4.69	0.6512
Body mass index (kg/m^2), median (IQR)	21 (19.5; 24)	22 (19; 28)	24 (21; 28)	0.0600

Table 2. Demographic data of the study population

	SC group ($n = 20$)	OS group ($n = 27$)	AC group ($n = 65$)	*P*-value
Patients with secondary infertility, *n* (%)	3 (15%)	10 (37.04%)	21 (32.31%)	0.2325
Duration of infertility (years), median (IQR)	3.5 (2.5; 6)	3 (3; 4)	4 (3; 6)	0.2789
Type of infertility				
Tubal, *n* (%)	7 (35%)	11 (40.7%)	19 (29.2%)	0.5530
Ovulatory, *n* (%)	4 (20%)	3 (11.1%)	23 (35.4%)	0.0428
Male, *n* (%)	7 (35%)	9 (33.3%)	23 (35.4%)	0.9823
Endometriosis, *n* (%)	0	3 (11.1%)	7 (10.8%)	0.3042
Smoking, *n* (%)	6 (30%)	4 (14.8%)	15 (23.1%)	0.4660
FSH (IU/l) on Day 3, median (IQR)	6.8 (5.85; 7.55)	6.1 (5; 7.4)	6.7 (5.4; 7.55) MD 1	0.6717
LH (IU/l) on Day 3, median (IQR)	6.2 (4.65; 8.1)	5.9 (4.3; 7.9)	6.7 (4.8; 9.27)	0.4266
Oestradiol (ng/ml) on Day 3, median (IQR)	50.5 (34.5; 57.5)	48 (32.75; 69) MD 3	44 (33.5; 54) MD 2	0.6531
AMH (ng/ml), median (IQR)	2.72 (1.62; 5.05)	2.9 (1.3; 6.34)	3.6 (1.7; 6.9)	0.3662
Endometrial thickness (mm) before starting progesterone, median (IQR)	9 (8.5; 10)	9 (7; 9)	9 (8; 10)	0.1526
Number of embryos transferred, *n* (%)				
1	12 (60%)	19 (70.37%)	44 (67.69%)	0.7424
2 or 3	8 (40%)	8 (29.63%)	21 (32.31%)	

AMH: anti-Müllerian hormone; FSH: follicle stimulating hormone; LH: luteinizing hormone; MD: missing data.

method. A multinomial logistic regression model was used to evaluate the primary endpoint and we performed adjustments for age, dysovulation, BMI and AMH, because these factors could affect the choice of preparation protocol (Yu *et al.*, 2015). A second model was made to account for the number of follicles to make sure that the difference in progesterone level was not linked to a difference in the number of follicles, only in the spontaneous versus stimulated groups.

A *P*-value <0.05 was considered to be statistically significant. Calculations were performed using SAS (SAS Institute, Cary, NC, USA) version 9 and R version 3.6.1 (R Development Core Team, 2019) and R® statistical software (version 3.6.1; Foundation for Statistical Computing, Vienna, Austria).

Results

Participants

During the inclusion period, 226 patients underwent FET, of whom 49 underwent a natural cycle (SC group), 48 a stimulated cycle (OS group), and 129 an artificial cycle preparation

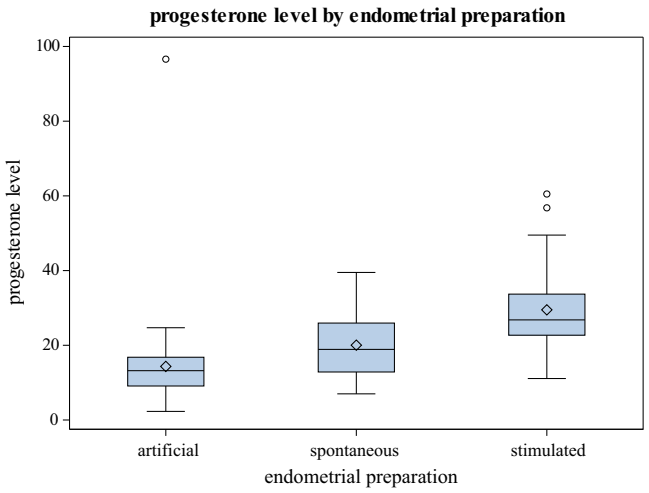

Figure 1. Box plots showing serum progesterone levels (ng/ml) by endometrial preparation protocol in which diamonds denote the mean, horizontal lines denote median, and boxes show the interquartile range (IQR).

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Table 3. Hormone levels on day of transfer and pregnancy outcomes

	SC group ($n = 20$)	OS group ($n = 27$)	AC group ($n = 65$)	*P*-value
Progesterone (ng/ml), median (IQR)	18.9 (12.8; 25.9)	26.8 (22.7; 33.7)	13.2 (9.1; 16.8)	<0.0001
Oestradiol (ng/ml), median (IQR)*	136 (86.0; 202.0)	144 (121.5; 167.5)	249 (170.0; 317.0)	0.0003
LH (IU/ml), median (IQR)*	7.5 (5.2; 9.4)	5.4 (1.0; 6.3)	2.6 (1.0; 4.2)	0.0012
Pregnancy rate per cycle (number), *n* (%)	8 (40.0%)	6 (22.2%)	19 (29.2%)	0.4166
Rate of pregnancy loss/pregnancies, *n* (%)	1/8 (12.5%)	2/6 (30%)	8/19 (42.1%)	0.9027
Rate of clinical pregnancy with fetal heartbeat/cycle (number), *n* (%)	7 (35%)	4 (14.8%)	11 (16.9%)	0.2161
Live birth rate/cycle (number), *n* (%)	7 (35%)	4 (14.8%)	11 (16.9%)	0.2161

*Missing data for nine patients in the SC group, 19 patients in the OS group and 38 patients in the AC group.

Table 4. Progesterone levels according to transfer day and type of endometrial preparation

	Progesterone (ng/ml), median (IQR)		
	Cleavage stage	Morula–Blastocyst	*P*-value
SC group ($n = 20$)	20.9 (9.9–22)	17 (13.2–29.2)	0.6501
OS group ($n = 27$)	23.15 (17.5–29.25)	29 (23.2–38.4)	0.1301
AC group ($n = 65$)	12.3 (9.5–16.6)	13.65 (8.7–17.5)	1

(AC group; Figure S1). Twenty-nine patients in the SC group, 24 patients in the OS group and 64 patients in the AC group were excluded, largely due to missing progesterone concentration levels measured on the day of the transfer due to staffing levels. Therefore, 20 patients were eligible for inclusion in the SC group, 27 patients in the OS group and 65 patients in the AC group.

No difference was seen between the three groups concerning the number of embryos transferred early or at the blastocyst stage, and there was no significant difference in patient age at embryo freezing or transfer (Table 1). BMI also did not differ significantly between groups.

More patients in the AC group had ovulatory infertility (35% vs 11 % in the SC group and 20% in the OS group; $P = 0.04$; Table 2). In contrast, the type of infertility, duration of infertility and the baseline hormone levels, the thickness of the endometrium and the number of embryos transferred were not different between groups.

Primary outcome: progesterone level on the day of transfer according to endometrial preparation protocol

Serum progesterone levels on the day of transfer were significantly higher in the OS group, whilst estradiol was significantly higher in the AC group and LH was highest in the SC group (Figure 1, Table 3). The median progesterone level in the AC group was 13.2 ng/ml (50th percentile), the 30th percentile in our population was for patients with a serum progesterone level of 9.82 ng/ml, and the 10th percentile for patients with a serum progesterone level of 6.38 ng/ml.

Univariate analysis

The difference between the groups was significant in the univariate model ($P < 0.0001$). Increasing the progesterone level by 1 ng in a univariate analysis showed an OR of 1.15 [95% CI (1.08; 1.22), $P < 0.0001$] of the patient being in the stimulated group and 1.09 [95% CI (1.02; 1.15), $P = 0.0073$] of being in the spontaneous group compared with the artificial group.

Progesterone level did not differ according to cleavage stage versus morula–blastocyst stage, regardless of the protocol (Figure 3 and Table 4).

Multivariate analysis

The results did not change significantly after adjustment for AMH and dysovulation and BMI (Table 5). We found an adjusted OR of 1.15 [95% CI (1.08; 1.23), $P < 0.0001$] for the patients in the stimulated group and 1.08 [95% CI (1.01; 1.15), $P = 0.02$] in the spontaneous group compared with the artificial group (Table 5).

Secondary outcomes

We observed no between-group difference in rates of pregnancy (40% SC, 22.2% OS versus 29.8% AC; $P = 0.42$), pregnancy loss (12.5% SC, 30% OS versus 43% AC; $P = 0.9$) or clinical pregnancy with fetal heartbeat (35% SC, 14.8% OS versus 16.4% AC, $P = 0.21$; Table 3). In all patients, no significant difference was found in progesterone levels on the day of transfer between patients with clinical pregnancy with fetal heartbeat versus those experiencing pregnancy loss or no pregnancy (17.49 ng/ml vs 20.83 ng/ml respectively, $P = 0.7$; Figure 2).

Effect of number of follicles on progesterone level and endometrial preparation method

To ensure that the difference in progesterone levels observed between spontaneous and stimulated cycles was not due to a higher number of follicles in the stimulated cycle (and therefore the corpora lutea), we performed a multivariate analysis of progesterone levels according to the number of follicles on the day of induction (for the SC and OS groups). The adjustment did not modify the effect of the group of membership on the progesterone level.

Discussion

Our study shows a significant difference in serum progesterone levels depending on the type of endometrial preparation. Endometrial preparations during a natural or stimulated cycle, with a corpus luteum secreting progesterone, resulted in higher serum progesterone levels. This luteal insufficiency with endometrial preparations with an artificial (substituted) cycle, without a corpus luteum, marked by lower progesterone levels on the day of transfer, might partly explain the higher rate of pregnancy loss

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Table 5. Multivariate analysis

Effect	Group (vs artificial)	Coefficients	Standard error	OR	95% CI	*P*-value
Progesterone level	Stimulated	0.1372	0.0340	1.147	1.073; 1.226	<0.0001
Progesterone level	Spontaneous	0.0739	0.0328	1.077	1.010; 1.148	0.0241
Ovulatory_PCOS	Stimulated	−1.0618	0.8255	0.346	0.069; 1.744	0.1984
Ovulatory_PCOS	Spontaneous	0.0805	0.7084	1.084	0.270; 4.345	0.9095
AMH	Stimulated	0.00725	0.0852	1.007	0.852; 1.190	0.9322
AMH	Spontaneous	−0.1344	0.0968	0.874	0.723; 1.057	0.1650
BMI	Stimulated	0.0364	0.0590	1.037	0.924; 1.164	0.5368
BMI	Spontaneous	−0.1172	0.0727	0.889	0.771; 1.026	0.1070

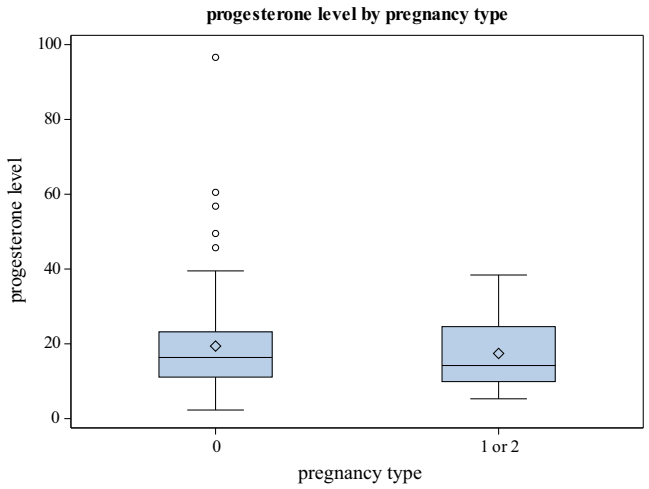

Figure 2. Box plots showing serum progesterone level (ng/ml) by pregnancy outcome: no pregnancy (no clinical pregnancy/pregnancy loss) versus live birth (single/multiple). Diamonds denote the mean, horizontal lines denote median, and boxes show the interquartile range (IQR).

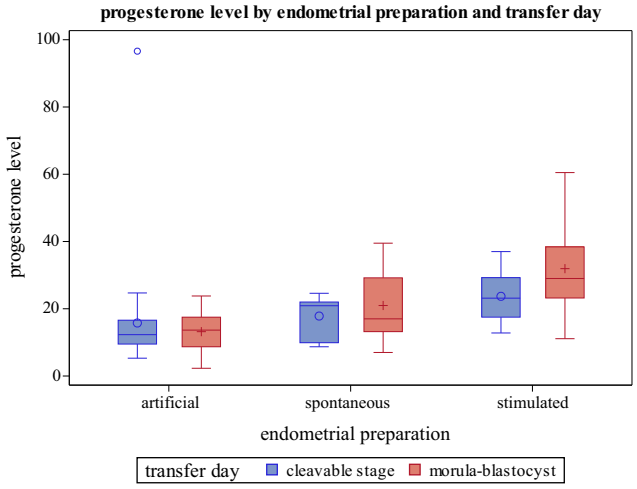

Figure 3. Box plots showing serum progesterone level (ng/ml) according to transfer day and type of endometrial preparation. Circles and crosses denote the mean, horizontal lines denote median, and boxes show the interquartile range (IQR).

observed in these patients in certain studies (Tomás *et al.*, 2012). However, we observed no significant difference in the rate of pregnancy loss or live birth rate.

Following the work of Labarta *et al.* (Labarta *et al.*, 2017; Labarta, 2019), many teams measure progesterone in the luteal phase of ACs in clinical practice (Alsbjerg *et al.*, 2018; Arce *et al.*, 2011; Basnayake *et al.*, 2018). This is controversial. The receiver operating characteristic (ROC) curve of progesterone predictive of ongoing pregnancy in patients with an AC showed a threshold value of 11 ng/ml (Labarta *et al.*, 2017). However, the area under the curve (AUC) was 0.59, a very low score, questioning the clinical significance of this marker and showing a weak discriminating property of the progesterone threshold. The same team recently found a threshold of 8.8 ng/ml, in which 31% of the total population had a progesterone level below the target (Labarta *et al.*, 2021). Therefore, Labarta *et al.* found that 30% of patients had insufficient luteal phase impregnation and would benefit from a modification of progesterone support. In our study, the 30th percentile was 9.82 ng/ml, which is consistent with the work of several teams. Having such a large proportion of the population below the cut-off value is concerning; rather than measuring the progesterone level in clinical practice, another type of endometrial preparation might be more appropriate. Here, we showed that a spontaneous or stimulated cycle produces higher progesterone levels. Alternatively, we could modify our AC protocol to obtain higher serum progesterone levels for the whole population.

Increasing the doses of vaginal progesterone did not noticeably increase the serum progesterone level. Indeed, it is the method of application that affects serum progesterone level, with intramuscular application of subcutaneous progesterone providing a greater effect (Paulson *et al.*, 2014). Preliminary data suggest that when the progesterone level is below 9 ng/ml on the day of transfer, subcutaneous progesterone can achieve a clinical pregnancy rate comparable with patients with a normal level of serum progesterone 20. Indeed, according to Sator and colleagues (Sator *et al.*, 2013), the maximum concentration of progesterone, obtained at 0.92 h after the injection of 25 mg of subcutaneous progesterone, was 57.84 ng/ml. The half life was 13.06 h, which guarantees sufficient progesterone levels with an injection every 24 h.

Dydrogesterone could also be added orally in the luteal phase. Oral dydrogesterone is non-inferior compared with vaginally applied micronized progesterone in *in vitro* fertilization using fresh embryos (Tournaye *et al.*, 2017). Few studies have investigated pregnancy rates with luteal phase support with dydrogesterone, and the results do not appear to differ from luteal phase support, in a modified natural cycle, with vaginal micronized progesterone (Ozer *et al.*, 2021). Furthermore, the application of dydrogesterone does not increase serum progesterone levels, as it is not easy to

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measure in routine care. It is therefore difficult to monitor and confirm its value in the luteal phase.

Whilst most studies show no difference in birth rate irrespective of the endometrial preparation protocol, they are mainly retrospective studies or studies with small numbers pooling ovulating and anovulating patients. One recent study showed an improved birth rate with a SC versus AC in patients without ovulatory infertility (Singh *et al.*, 2020). Finally, the absence of corpus luteum in protocols with substituted cycles could lead to a risk of obstetric complications, especially pre-eclampsia (von Versen-Höynck *et al.*, 2019), postpartum haemorrhage, and caesarean section (Ginström Ernstad *et al.*, 2019). The limitation of our study was that the sample size was too small to detect a difference in the rate of clinical pregnancy, pregnancy loss or live birth rate depending on the protocol.

In conclusion, the serum progesterone level on the day of transfer was significantly lower for an AC preparation compared with a spontaneous or stimulated cycle preparation in FET. Further studies are required to evaluate the effect of a lower progesterone level in the luteal phase at the time of FET and to evaluate whether modifying the protocol with an AC could correct the progesterone level in the luteal phase.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S0967199423000163

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Conflict of interest. The authors declare none.

Ethical standards. The authors assert that all procedures contributing to this work complied with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

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