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Low serum progesterone on the day of frozen blastocyst transfer is associated with a lower live birth rate in hormonal replacement therapy cycles

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ABSTRACT

Research question: Does the serum progesterone (P) level on the day of frozen embryo transfer (FET) affect the live birth rate (LBR) with hormonal replacement therapy (HRT) cycles?

Design: Observational cohort study at university hospital. Patients undergoing single autologous blastocyst FET under HRT using vaginal micronized P. Women were only included once, between January 2019 and March 2020. The serum P level was measured by a single laboratory on the morning of the FET. The primary endpoint was the LBR. Statistical analyses were conducted using univariate and multivariate logistic regression models.

Results: The study included 915 patients. Median (25^{th}-75^{th} percentile) serum P level on the day of FET was 12.5 ng/mL (9.8-15.3). The LBR was 31.5% (288/915) in the overall population. Despite similar implantation rates (40.7% vs. 44.9%, p>0.05), the LBR was significantly lower in women with a P level ≤25th percentile (≤9.8 ng/ml) (26.1% vs. 33.2%, p=0.045) compared with women with P level over Centile 25, and this correlated with a significantly higher early miscarriage rate (35.9% vs. 21.6%, p=0.005). Even after adjusting for potential confounding factors in multivariate analysis, low serum P levels (≤9.8 ng/ml) remained significantly associated with lower LBR.

Conclusion: This study suggests that a minimum serum P level is needed to optimize reproductive outcomes in HRT-cycles with single autologous blastocyst FET. Further studies are required to evaluate whether modifications of P administration routes and/or the dosage can improve pregnancy rates. This would allow further individualization of the management of patients undergoing HRT-FET cycles.

KEYWORDS

Frozen embryo transfer; Hormonal replacement therapy; Micronized vaginal progesterone;

Live Birth; Serum progesterone level

INTRODUCTION

The use of frozen embryo transfer (FET) in assisted reproductive technology (ART) has increased substantially over the past decade (European IVF-monitoring Consortium (EIM)‡ for the European Society of Human Reproduction and Embryology (ESHRE) et al., 2020) as a result of huge improvements in cryopreservation techniques (Wong et al., 2017) and the expansion of elective single-embryo transfers and freeze-all strategies (Bourdon et al., 2020). Various methods, including hormonal replacement therapy (HRT) cycles and natural cycles, have been used to prepare the endometrium for implantation. None, however, has stood out as being superior to the others in terms of the live birth rate (LBR) (Ghobara et al., 2017; Glujovsky et al., 2020).

The HRT cycle method, which consists of the administration of exogenous estradiol (E2) and progesterone (P), is considered advantageous by many clinicians as it allows the day of the embryo transfer to be scheduled and it reduces monitoring requirements (Groenewoud et al., 2018). In such artificial cycles that lack a *corpus luteum*, the addition of exogenous P is a crucial step to mimic the mid-cycle shift to the secretory phase and to achieve synchrony between the endometrium and the embryo's developmental stage (Groenewoud et al., 2018).

To date, there is little consensus regarding the best dosage and the most suitable route for P administration, which are currently chosen based on the clinician's and the patient's preferences (Shoham et al., 2021). Moreover, the same P supplementation is given to all patients, without tailoring the luteal phase support to the patients' characteristics. In a recent trend towards optimization and individualization of P supplementation, increased attention has been paid to the monitoring of serum P levels before the FET. Indeed, recent

research has highlighted that low serum P levels (< 8.8-10.6 ng/mL) around the time of the frozen blastocyst transfer are associated with a lower LBR (Boynukalin et al., 2019; Cédrin-Durnerin et al., 2019; Gaggiotti-Marre et al., 2019; González-Foruria et al., 2020; Labarta et al., 2020, 2017). Yet, the literature in this regard is very heterogeneous, with many differences and limitations among the various studies: (i) inclusion of oocyte donation cycles (Brady et al., 2014; Labarta et al., 2020, 2017), (ii) a small number of patients (Alsbjerg et al., 2018; Cédrin-Durnerin et al., 2019; Gaggiotti-Marre et al., 2019; Labarta et al., 2017), (iii) use of different administration routes and/or dosages of P (Alsbjerg et al., 2018; Alyasin et al., 2021; Boynukalin et al., 2020; Brady et al., 2014; Kofinas et al., 2015; Labarta et al., 2020), (iv) different timing of serum P measurements (Alsbjerg et al., 2018; Gaggiotti-Marre et al., 2019), (v) different immunoassays for serum P measurements (Alyasin et al., 2021), and (vi) inclusion of cleavage-stage FET (Brady et al., 2014; Cédrin-Durnerin et al., 2019). Indeed, to date, there had not been a large study analyzing serum P levels on the day of autologous blastocyst FET under HRT using micronized vaginal P (600 mg/day). Therefore, we investigated whether the correlation between serum P levels and reproductive outcomes could be extrapolated to a large population of women meeting these criteria.

MATERIALS AND METHODS

Ethical approval

This study was approved for publication by the Ethics Review Committee of Cochin University Hospital (CLEP) (n° AAA-2021-08007) and all of the participants provided written informed consent.

Study population and inclusion criteria

We conducted an observational cohort study that included all single autologous FET performed following HRT between January 2019 and March 2020 at the university-based reproductive medicine center of our institution that complied with the following inclusion criteria: (i) requirement for ART, (ii) age < 43 years at the time of oocyte retrieval, (iii) having a single autologous FET at the blastocyst stage, and (iv) endometrial preparation using HRT with exogenous E2 and micronized vaginal P prior to the FET, irrespective of the ovulatory status. The exclusion criteria were: (i) cancelled embryo transfers, (ii) FET derived from vitrified oocyte procedures or oocyte donation procedures, (iii) embryo donation procedures, (iv) natural or stimulated endometrial preparation, and (v) HRT cycles with parenteral P administration for luteal phase support. Cycles with missing data and women lost to follow-up were secondarily excluded. Patients were only included once in the analysis.

IVF/ICSI cycles: clinical and laboratory procedures

All in vitro fertilization (IVF) / intra-cytoplasmic sperm injection (ICSI) cycles were carried out according to our institutional clinical protocols (Maignien et al., 2020). Embryos were cultured at 37 °C in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2. Blastocysts were scored according to the grading system of Gardner and Schoolcraft (Gardner et al., 2002) and they were considered eligible for cryopreservation on day-5 or day-6 if they qualified as full (B3) or expanded (B4-5) blastocysts with a type A-C inner cell mass and/or a type A-C trophectoderm. Blastocysts with a type “C” inner cell mass and a type “C” trophectoderm were not cryopreserved regardless of their degree of expansion.

The precise vitrification and thawing protocol has been reported in detail previously (Bourdon et al., 2017). Briefly, embryo vitrification was performed using closed Cryo Bio System High Security Vitrification straws in combination with DMSO-EG-S as the cryoprotectants (Irvine Scientific® Freeze Kit). For thawing, the Irvine Scientific® Thaw Kit was used.

Endometrial preparation and embryo transfer

Estrogen treatment commenced on day-1 of menstruation, and it consisted of transdermal (0.2 mg/day, simultaneously through two Vivelledot® 100 systems; Novartis Pharma SA, Rueil-Malmaison, France) or oral (8 mg/day, Provames®; Sanofi-Aventis, Paris, France) E2. After 10-14 days of estrogen treatment, a vaginal two-dimensional ultrasound was performed to measure the endometrial thickness (EMT) and a blood sample was drawn to determine the P level so as to ensure that no spontaneous ovulation had occurred. If the EMT thickness was $\geq$ 6 mm and the serum P level was < 1.5 ng/ml, the FET was scheduled (Bourdon et al., 2018). P supplementation began 5 days before the FET with vaginal progesterone at 600 mg daily (a 200 mg vaginal capsule (Utrogestan®; Besins International, Montrouge, France) three times a day), without any change in P dose during the treatment. The last dose before the FET was administered on the morning of the FET (between 7 am to 9 am). The blastocysts were warmed on the day of transfer, *i.e.,* on the 6^{th} day of P exposure. All of the FETs were performed by senior gynecologists with transabdominal ultrasound guidance. The pregnancy tests were performed 9 days after the FET, in laboratories outside the hospital. The women who became pregnant by these procedures continued with the same dose of P and E2 treatment until 12 weeks of gestation.

Serum hormone measurements

The serum P measurements were taken on the day of the FET, in a specific time range (10 am to 11 am), blinded to the doctor, the embryologist, and the patient. Blood samples were analyzed by an electrochemiluminescence immunoassay (COBAS e801 analyzer, Roche Diagnostics GmbH, Germany) with the Elecsys Progesterone III reagents (Roche Diagnostics). The intra- and inter-assay coefficients of variation for the P determinations were respectively 4.2% and 6.2% for a value of 0.66 ng/mL, 2.7% and 3.3% for a value 3.03 ng/mL, 1.1% and 1.7% for a value of 22.0 ng/mL. The accuracy was around 0.01 ng/mL for the analytical performances and around 0.1 ng/mL for the patients assays.

Data analysis and statistics

The general characteristics of the patients were recorded prospectively during face-to-face interviews prior to the FET. The following data were collected: age at retrieval (years), smoking habits, body mass index (BMI) (weight (kg)/height (m^2)), type of infertility (primary or secondary), number of previous IVF/ICSI cycles, day-3 follicle-stimulating hormone (FSH), antral follicle count (AFC), and anti-Müllerian hormone (AMH), type of treatment (IVF or ICSI), and infertility causes (e.g., ovulation disorder, male factor, tubal factor, endometriosis, idiopathic, diminished ovarian reserve, or more than one etiology).

The primary outcome was the LBR, defined as the delivery of any viable infant at 22 weeks or more of gestation (Zegers-Hochschild et al., 2017). The other pregnancy outcomes studied were: clinical pregnancy, defined as an ultrasonographical visible gestational sac (Zegers-Hochschild et al., 2017); early miscarriage, defined as a spontaneous pregnancy demise at less than 10 weeks of gestational age (Kolte et al., 2015); and ectopic pregnancy, defined as

a gestational sac located outside the uterine cavity (Zegers-Hochschild et al., 2017). The following obstetrical and perinatal outcomes were also analyzed: preterm birth (< 37 weeks of gestation (WG)) (Zegers-Hochschild et al., 2017); low birth weight (LBW) (< 2,500 g) (Zegers-Hochschild et al., 2017); and high birth weight (HBW) (≥ 4,000 g) (Declercq et al., 2015).

All of the data were compiled into a digital database and analyzed using SPSS software. A p-value of < 0.05 was considered to be statistically significant. The continuous variables were expressed as means and the standard deviations (SD), whereas the categorical variables were expressed as percentages. The patients were classified into four groups according to the quartiles of serum P. The LBR was calculated for each group to detect a critical threshold related to the probability of LBR. For univariate statistical analysis, we used the Pearson χ 2 test or Fisher's exact test for the qualitative variables and the Mann-Whitney test for the quantitative variables. To identify potential confounding variables that could be independently associated with the LBR, we performed a logistic regression analysis. Confounding factors were tested by univariate analysis and were added in a multiple logistic regression model. The variables included in the multiple regression model were those that were significant by univariate analysis at $P < 0.10$. The degree of correlation between the variables was tested, and when two variables were highly correlated, we introduced only one of them and suppressed the other in the model, such as for gravidity and parity (Spearman correlation 0.789; $p < 0.001$; the latter being excluded), the AMH and the AFC (Spearman correlation 0.619; $p < 0.001$; the latter being excluded). In case of significant differences, odds ratios (ORs) and their 95% confidence intervals (95% CIs) were calculated.

RESULTS

Study population

Our cohort selection process is detailed in Supplemental Figure 1. Between January 2019 and March 2020, 915 patients underwent a serum P level measurement on the day of a single autologous blastocyst FET, using HRT with micronized vaginal P for endometrial preparation, at our tertiary care center. The mean overall population age was 34.9 ± 4.2 years, the mean BMI was 23.9 ± 4.1, and the mean endometrial thickness after E2 supplementation was 9.1 ± 1.9 mm. The mean P level in the study population was 12.90 ± 4.9 ng/mL. The median P level (25^{th}-75^{th} percentile) was 12.5 ng/mL (9.8-15.3 ng/mL). The overall clinical pregnancy rate and LBR were 43.4% and 31.5 %, respectively.

Prognostic factors of live birth

The results of the univariate analysis comparing the patients who had a live birth versus those who did not are presented in Table 1. Not surprisingly, the patients who had a live birth were younger (< 0.001), had lower gravidities (p = 0.002), higher AMH levels and AFCs (p < 0.001 for both parameters), and a higher proportion of day-5 blastocyst FET (p < 0.001) (Ferreux et al., 2018). Regarding the serum P level on the day of the FET, we noted a critical cut-off of 9.8 ng/mL below which the probability of live birth was lower, corresponding to the 25^{th} percentile (p = 0.045).

Multivariate logistic regression showed that a serum P level below 9.8 ng/mL remained an independent factor for a lower LBR (OR 0.68 95%CI [0.48-0.97], p = 0.035) after adjusting for confounding variables, as shown in Table 2. In addition to P concentrations above 9.8 ng/mL, other statistically significant predictors of live birth on multivariate analysis were

lower age of the woman and lower gravidity, transfer of day-5 versus day-6 blastocysts, and higher AMH levels (Table 2).

Pregnancy outcomes according to the serum P level on the day of the FET

Table 3 and Figure 1 show the clinical outcomes of patients according to the serum P level on the day of the FET. Overall, patients with a serum P level ≤ 9.8 ng/mL had a lower LBR (26.1% vs 33.2%, p = 0.045) and a higher rate of early miscarriage (35.9% vs 21.6%, p = 0.005). Regarding the main obstetrical and neonatal outcomes, no significant differences were found between the two study groups in terms of the birth weights or the terms of delivery.

DISCUSSION

Main findings

This observational cohort study of 915 patients who underwent a single autologous FET at the blastocyst stage, with an endometrial preparation using micronized vaginal P in HRT cycles, underscores that serum P levels below a critical threshold on the day of the FET (≤ 9.8 ng/mL) lead to altered reproductive outcomes after univariate and multivariate analysis. Despite similar implantation rates between the two study groups, the LBR was significantly lower in women with P level below the 25th percentile (26.1% vs 33.2%, p = 0.045), and this correlated with a significantly higher miscarriage rate (35.9% vs 21.6%, p = 0.005).

Strengths and Limitations

The strengths of the study are as follows: i) to the best of our knowledge, this is one of the largest studies to date to examine reproductive outcomes according to the serum P levels on the day of the embryo transfer, in HRT-FET cycles; ii) the study population was very homogeneous, with a focus on single autologous blastocyst FET in HRT cycles using micronized vaginal P so as to minimize confounding factors; iii) the serum P level measurements were performed using a standardized methodology, by a single laboratory using the same equipment for all patients; iv) the clinicians were blinded to the P levels, which were analyzed secondarily, thereby avoiding the risk of modification of the luteal phase support as it is often the case in retrospective studies (Cédrin-Durnerin et al., 2019; Volovsky et al., 2020).

The main limitation of this study is its retrospective design, and although the effect of most confounders was controlled for in the multivariable analysis, the presence of residual bias cannot be ruled out. Another limitation is that only autologous blastocyst FET were included in the analysis, which precludes generalization of our results to cleavage-stage FET or oocyte donation cycle procedures. Yet, several studies involving oocyte donation recipients (Labarta et al., 2020, 2017) or day-2/3 embryo transfers (Cédrin-Durnerin et al., 2019) have found a quite similar serum P level cut-off, which reinforces the external validity of our findings. Moreover, the time interval between last P administration and P measurement ranged between 1 and 4 hours, which could have biased the comparison of P concentrations as levels are not steady during the day (von Eye Corleta et al., 2004). However, the time range being pretty narrow, we believe our findings are still worth taking into account as the variability of P levels appears to be low for a time interval of 4 hours (González-Foruria et al., 2020). Finally, only HRT-FET cycles using micronized vaginal P were

analyzed, thereby preventing conclusions from being extrapolated to patients receiving P by other routes, as injectable P has a different bioavailability compared with vaginal P.

Interpretation

The findings of our study confirm previous results reported in HRT-FET cycles with micronized vaginal P for luteal phase support, showing that a minimal P threshold of approximately 10 ng/mL needs to be reached to optimize pregnancy outcomes (Alsbjerg et al., 2018; Cédrin-Durnerin et al., 2019; Gaggiotti-Marre et al., 2019; González-Foruria et al., 2020; Labarta et al., 2020, 2017). For instance, in a prospective cohort study, Labarta et al. found that serum P levels < 8.8 ng/mL on the day of FET were significantly associated with lower ongoing pregnancy rates in both own and donated oocyte cycles, with or without preimplantation genetic testing for aneuploidy (PGT-A) (Labarta et al., 2020). The slight variations in the P cut-off levels among the studies to date may be explained by several factors such as the differences between the study populations, the use of various immunoassays for serum P measurements (Patton et al., 2014), a variability in the time interval between the last vaginal dose administration and the blood testing (González-Foruria et al., 2020), or the use of a different P dosage (Alsbjerg et al., 2018; Alyasin et al., 2021; Labarta et al., 2020, 2017). In any case, the lower P threshold significantly associated with pregnancy outcomes appears to be approximately 10 ng/mL, which is close to the value reported as an adequate level of P production by the corpus luteum in the mid-luteal phase of natural cycles (Gaggiotti-Marre et al., 2020; Hull et al., 1982), which is crucial for achieving the secretory transformation of the endometrium in preparation for embryo implantation.

Conversely, in HRT-FET cycles using parenteral administration of P, the minimal P cut-off levels that have been reported are higher, reaching 13.6 ng/mL (Boynukalin et al., 2019) or even 20 ng/mL (Brady et al., 2014). These discrepancies compared with studies focusing on vaginal P can be explained by the differences in pharmacokinetics (PK) and pharmacodynamics (PD) between the distinct routes for P administration. Indeed, vaginal P yields lower circulating levels but higher intrauterine levels due to the uterine first-pass effect (Cicinelli et al., 2000; Miles et al., 1994; Paulson et al., 2014). Therefore, the choice of P threshold correlated with improved live birth chances in HRT-FET cycles depends on the route of P administration, and it is not a direct reflection of the endometrial tissue P concentrations. The fact that the implantation rates were similar between the two study groups raises the question of a systemic effect of P, in addition to its direct uterine effect. Indeed, one could hypothesize that low serum P levels are sufficient to allow for implantation, but that adequate serum P concentrations are required to achieve the maintenance of the early stages of pregnancy, possibly through the immunomodulatory role of P in favoring embryo tolerance (La Rocca et al., 2014; Shah et al., 2018). This mechanism could participate in explaining the association between the serum P levels and the miscarriage rates found in our study, as well as in previous works (Gaggiotti-Marre et al., 2019; Labarta et al., 2020). This is also in line with recent findings that have shown that P supplementation during the first trimester of pregnancy can reduce the risk of recurrent miscarriages in patients with early pregnancy bleeding (Coomarasamy et al., 2020, 2019; Devall et al., 2021).

In the current study, a wide range of serum P values were observed on the day of FET after use of the same dose and with the same route of administration, which is consistent with the findings of previous studies, for which the percentages of low serum P

concentrations were estimated to be between 25% and 37% (Álvarez et al., 2021; Cédrin-Durnerin et al., 2019; Labarta et al., 2020, 2017). Similar conclusions have also been reached after parenteral administration of P (Brady et al., 2014; Kofinas et al., 2015). The mechanisms accounting for this broad variability in serum P levels remains to be determined. Variations in P metabolism between patients could be a determining factor, as suggested by the lower serum P levels in obese women reported in several studies (Brady et al., 2014; González-Foruria et al., 2020; Labarta et al., 2020), since body weight is a well-known parameter influencing drug absorption, distribution, metabolism, and clearance. Sexual intercourse has also been shown to reduce the absorption of vaginal P (Merriam et al., 2015). Further studies are urgently needed to identify determinants associated with P concentrations in HRT-FET cycles in order to help identify patients who are at risk of insufficient luteal phase support and a compromised LBR.

How to individualize P supplementation in this subset of patients is particularly relevant. Increasing the dosage of P could be a valuable option, although the findings in the literature in this regard are still controversial. For instance, Cédrin et al. reported that doubling the vaginal P dosage from the day of FET did not improve reproductive outcomes in patients with low serum P levels (Cédrin-Durnerin et al., 2019). Likewise, Brady et al. concluded that an increase in the intramuscular P dose after FET was insufficient to rescue pregnancy rates (Brady et al., 2014). By contrast, in a retrospective study of 346 FET cycles over two consecutive time periods, Alsbjerg et al. found that doubling the vaginal P gel supplementation from the beginning of the FET cycle significantly increased the LBR. These discrepancies may be due to the difference in the timing of the increase in P dosage, with a likely ineffectiveness of late therapeutic adaptations. Moreover, as previously shown by a PK/PD study focusing on three different vaginal P dosages, an increase in the P dose does

not proportionally increase serum and uterine P concentrations, which may reflect limited and variable individual P absorption (Paulson et al., 2014). Additional P administration using the same route may not affect the amount of P absorbed, and may therefore be insufficient to rescue pregnancy chances. Consequently, a key to this problem may be the addition of another route of administration, which was recently studied by Alvarez et al. In their prospective study including 574 HRT-FET cycles, patients with low serum P levels the day before FET received additional subcutaneous P injections and they achieved similar pregnancy outcomes compared with those who had adequate initial P levels (Álvarez et al., 2021). The addition of oral P may be another clue to overcome inter-individual variations in vaginal P absorption. The use of oral P for luteal phase support was initially discontinued because of mediocre intestinal absorption with high inter-subject variability and first-pass hepatic effect leading to poor ART outcomes (Licciardi et al., 1999), in addition to badly tolerated hypnotic side-effects. However, a renewed interest has been given to dydrogesterone, an oral progestin with good bioavailability and tolerability profiles. For instance, the results of a recent prospective cohort study including 1364 patients in FET cycles, highlighted higher LBR in the group receiving a combination of vaginal P and dydrogesterone compared with women receiving vaginal P alone (Vuong et al., 2021). Clearly, more studies are needed to confirm these conclusions particularly focusing on the evoked teratogenic potential of dydrogesterone (Zaqout et al., 2015), as well as to consider other therapeutic options. In any case, these results argue for early monitoring of serum P concentrations before FET in order to adapt the luteal phase support strategy.

Conclusion

This is one of the largest studies to date highlighting a link between serum P levels on the day of single autologous blastocyst HRT-FET using micronized vaginal P and LBR. A minimal serum P level threshold of 9.8 ng/mL appears to be necessary to optimize reproductive outcomes, especially to minimize the risk of early miscarriage. Further studies are needed to evaluate whether modification of the P administration route and/or the dosage can improve live birth rates, with the aim of individualizing the management of patients undergoing FET cycles.

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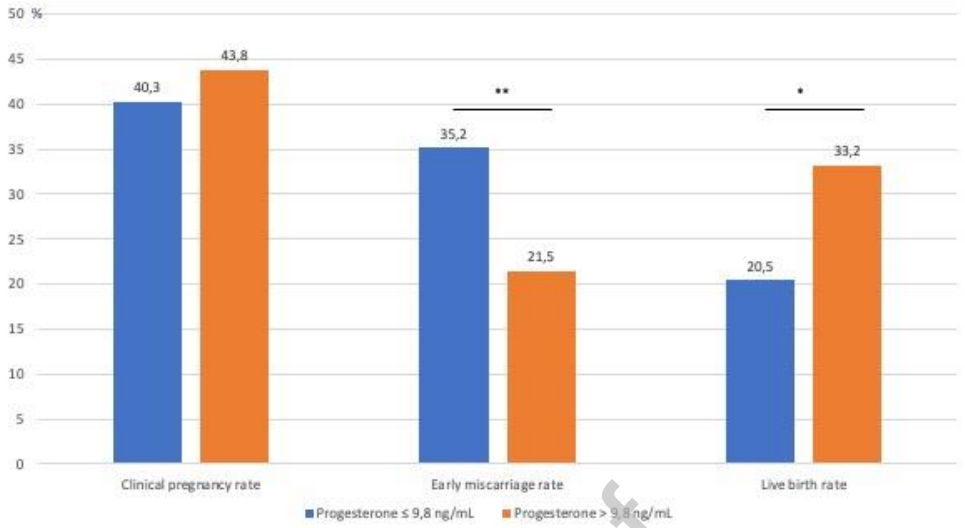

FIGURE 1. Pregnancy outcomes according to the serum progesterone levels on the day of a single autologous frozen blastocyst transfer

*Note: The early miscarriage rate was calculated based on the total number of clinical pregnancies; *, $p < 0.05$; **, $p < 0.01$*

TABLE 1. Characteristics of the study population according to the presence of a live birth

Characteristics [a]	**Live Birth –** (n=627)	**Live Birth +** (n=288)	**p-value**
Age	35.4 ± 4.2	33.9 ± 4.1	< 0.001[u]
BMI (kg/m^2)	23.9 ± 4.1	24.0 ± 4.2	0.857[u]
Smoking habits	64 (10.2%)	29 (10.1%)	0.915[k]
Gravidity	0.8 ± 1.2	0.5 ± 0.8	0.003[u]
Parity	0.4 ± 0.7	0.3 ± 0.6	0.002[u]
Type of infertility			0.074[k]
Primary	455 (72.6%)	225 (78.1%)	
Secondary	172 (27.4%)	63 (21.9%)	
Main infertility cause			0.346[k]
Male infertility	195 (31.1%)	94 (32.6%)	
Endometriosis	216 (34.4%)	90 (31.3%)	
PCOS	35 (5.6%)	26 (9.0%)	
Tubal infertility	100 (15.9%)	43 (14.9%)	
Diminished ovarian reserve	34 (5.4%)	11 (3.8%)	
Unexplained infertility	47 (7.5%)	24 (8.3%)	
Duration of prior infertility (months)	63.1 ± 34.7	62.0 ± 34.8	0.543[u]
Patient's ovarian reserve			
AMH (ng/mL)	3.1 ± 2.8	4.2 ± 4.7	< 0.001[u]

AFC	18.2 ± 11.1	21.4 ± 11.9	< 0.001[u]
Sperm concentration (million sperm/mL)	45.3 ± 41.3	45.6 ± 38.7	0.784[u]
IVF/ICSI rank	1.6 ± 1.4	1.5 ± 1.4	0.205[u]
Type of estradiol treatment for endometrial preparation			0.521[k]
Transdermal	589 (93.9%)	270 (93.8%)	
Oral	25 (4.0%)	9 (3.1%)	
Both	13 (2.0%)	9 (3.2%)	
Endometrial thickness	9.1 ± 2.0	9.1 ± 2.0	0.767[u]
Progesterone level on the day of the FET	12.8 ± 5.1	13.1 ± 4.3	0.272[u]
Progesterone level ≤ 25th percentile (≤ 9.8 ng/mL) on the day of the FET	167 (26.6%)	59 (20.5%)	0.045[k]
Progesterone level ≤ 50th percentile (≤ 12.5 ng/mL) on the day of the FET	322 (51.3%)	135 (46.9%)	0.209[k]
Type of embryo transferred			< 0.001[k]
Day-5 blastocyst	521 (83.1%)	266 (92.4%)	
Day-6 blastocyst	106 (16.9%)	22 (7.6%)	

Note: BMI = body mass index; PCOS = polycystic ovarian syndrome; AMH = anti-Müllerian hormone; AFC = antral follicle count; IVF = in vitro fertilization; ICSI = intra-cytoplasmic sperm injection; FET = frozen embryo transfer

[a] *The continuous data are presented as means ± the standard deviation; the categorical data are presented as numbers (percentages).*

[k] *Pearson's Chi-square test*

[u] *Mann-Whitney U test*

TABLE 2. Variables significantly associated with the live birth rate after a single autologous frozen blastocyst transfer in hormonal replacement therapy cycles: multiple logistic regression analysis

Parameters [a]	**OR [95% CI]**	**Adjusted OR [95%CI]**
Age at retrieval	0.92 [0.89-0.95]	0.93 [0.89-0.97]
Transfer of a Day-6 blastocyst (versus Day-5 blastocyst)	0.41 [0.25-0.66]	0.47 [0.28-0.77]
Progesterone level ≤25th p (9.8ng/ml) on the day of the FET (versus > 25th p)	0.71 [0.50-0.99]	0.68 [0.48-0.97]
AMH level	1.09 [1.04-1.14]	1.07 [1.02-1.12]
Gravidity	0.77 [0.66-0.90]	0.83 [0.69-0.98]

Note: OR = odds ratio; CI = confidence interval; FET = frozen embryo transfer; p = percentile; AMH = anti-Müllerian hormone

[a] *Variables included in the model: Women's age; Gravidity; AMH level; IVF/ICSI rank; Progesterone level the day of the FET (≤ 25th percentile versus > 25th); Type of infertility (primary versus secondary) and type of blastocyst transfer (Day-6 blastocyst versus Day-5 blastocyst)*

TABLE 3. Pregnancy outcomes according to the serum progesterone levels on the day of the single autologous frozen blastocyst transfer

Parameters	**Progesterone level ≤ 25th p (≤ 9.8 ng/mL)**	**Progesterone level > 25th p (> 9.8 ng/mL)**	**p-value**
Implantation rate[1]	92/226 (40.7%)	310/689 (44.9%)	0.260 [k]
Ectopic pregnancy rate[2]	0/92 (0.0%)	5/310 (1.6%)	0.271 [k]
Clinical pregnancy rate per embryo transfer	92/226 (40.7%)	305/689 (44.3%)	0.348 [k]
Early miscarriage rate[3]	33/92 (35.9%)	66/305 (21.6%)	0.005 [k]
Live birth rate per embryo transfer	59/226 (26.1%)	229[*]/689 (33.2%)	0.045 [k]
Gestational age at delivery (WG)	39.1 ± 2.8	39.4 ± 2.6	0.446 [u]
Preterm delivery (< 37 WG)[4]	8/59 (13.6%)	20/229 (8.7%)	0.265 [k]
Post term delivery (> 41 WG)[4]	15/59 (25.4%)	53/229 (23.1%)	0.713 [k]
Birth weight (grams)	3313.1 ± 625.7	3309.0 ± 624.1	0.994 [u]
Term LBW[5]	0/51 (0.0%)	3/209 (1.4%)	0.389 [k]
Term HBW[5]	0/51 (0.0%)	3/209 (1.4%)	0.389 [k]

Note: p = percentile; WG = weeks of gestation; LBW = low birth weight; HBW = high birth weight

[1] *Implantation rate = number of gestational sacs/number of embryos transferred*

[2] *Ectopic pregnancy rate = number of ectopic gestational sacs/total number of gestational sacs*

[3] *Early miscarriage rate = number of early miscarriages/number of clinical pregnancies*

[*] *Of the 302 clinical pregnancies, 5 were late miscarriages, 4 were medical terminations of pregnancy, and 1 was an intra-uterine fetal death.*

[4] *Calculated based on the total number of live births (n=288)*

[5] *Calculated based on the total number of live births at term (≥ 37WG) (n=260)*

[a] *The continuous data are presented as means ± the standard deviation; the categorical data are presented as numbers (percentages)*

[k] *Pearson's Chi-square test*

[u] *Mann-Whitney U test*

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KEY MESSAGE

Serum progesterone levels below a critical threshold (≤ 9.8 ng/mL) on the day of frozen blastocyst transfer led to significantly lower live birth rates, in hormonal replacement therapy cycles.