Original Investigation

The relationship between serum estradiol and progesterone levels one day before frozen embryo transfer and pregnancy rates in artificially prepared frozen embryo cycles: are there any threshold serum hormone levels to predict pregnancy in luteal support by the vaginal and subcutaneous route combined?

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Abstract

Objective: To investigate the potential influence of serum estradiol (E_2) and progesterone (P_4) levels, measured one day before artificially prepared frozen embryo transfer (FET), on pregnancy rates in women who received combined vaginal and injectable P_4.

Material and Methods: This retrospective cohort study analyzed the association between serum E_2 and P_4 levels on the day before FET in 167 cases prepared with hormone replacement therapy between February 2022 and October 2023. The primary outcomes assessed were the pregnancy and live birth rates. We modeled a cut-off serum value based on luteal support for pregnancy. Luteal support was through a combination of vaginal suppositories and subcutaneous injections. Multivariate logistic regression was used to test relationships between pregnancy outcomes and independent variables. Cut-off values were evaluated using receiver operating characteristic (ROC) analysis and percentile analysis.

Results: No significant relationships were found between serum E_2 or P_4 levels on the day before FET and pregnancy rates. The mean E_2 level was 169.0±51.9 pg/mL for individuals who achieved conception and 177.7±56.9 pg/mL for individuals who did not conceive (p=0.45). The corresponding values for P_4 were 28.1±18.4 ng/mL and 31.2±25.4 ng/mL, respectively (p=0.73). No differences were observed in body mass index (BMI) or endometrial thickness between the groups. Cut-off values for predicting pregnancy using E_2 and P_4 could not be determined using ROCs. However, no one in the lowest 10th percentile of serum P_4 levels conceived (range 10.0-15.6 ng/mL). When multivariate logistic regression was used, this finding lost significance suggesting that low serum levels are related to age, BMI, and/or other factors.

Conclusion: In artificially prepared FET cycles, the serum E_2 and P_4 levels one day before embryo transfer do not significantly affect pregnancy rates in women with serum E_2 levels between 150-300 pg/mL and P_4 between 10-40 ng/mL when ROC was used for evaluation. However, percentile analysis suggests that serum P_4 levels should be more than 15.6 ng/mL when combined injectable and vaginal P_4 is used for programed FET. Although this finding may be due to the confounding effects of age, BMI, and other factors affecting steroid metabolism, when controlled for in the multivariate logistic regression.

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Introduction

Progesterone (P_4) plays a vital role in implantation, as it leads to endometrial differentiation, myometrial quiescence, and immune modulation and possesses anti-inflammatory properties (1). It plays this role in both natural ovulations, fresh *in vitro* fertilization (IVF) cycles, and frozen embryo transfer (FET) cycles. The advantages of embryo vitrification include reduced rates of ovarian hyperstimulation syndrome and possibly higher pregnancy rates than fresh IVF cycles, although this remains controversial (2,3). The high survival rates of vitrified-thawed embryos have made FET very common. In the United States of America (USA), FET is practiced in approximately 70% of cases (4).

During hormone replacement therapy (HRT) cycles for FET, exogenous P_4 is the only means of providing luteal support when the corpus luteum is absent. In natural conceptions, the production of P_4 for implantation during the first trimester is roughly 50-55 mg/day, and serum values during this period typically range between 25-30 ng/mL (5). It is conceivable that these values could serve as the target levels for FET cycles. Nevertheless, this range may vary significantly in supplemented FET cycles because of the diverse characteristics of patients, including body mass index (BMI), vaginal and peripheral blood supply, and age.

For luteal support in HRT cycles, exogenous P_4 can be administered through vaginal, subcutaneous, intramuscular (IM), oral, or rectal routes. Vaginal P_4 exerts a uterine effect by bypassing the first-pass effect of the liver seen in oral and injectable routes. Vaginal P_4 results in lower serum levels but higher concentrations in endometrial tissue when compared to other routes of administration. In prior studies, micronized vaginal P_4, 800 mg/day yielded a high endometrial tissue level of 11.5 ng/mL, however, the serum level was 11.9 ng/mL, which was a sub-physiological level. When IM P_4 was used, 100mg/day the serum level was on average 69.8 ng/mL, while the endometrial tissue level remained at 1.4 ng/mL (6). However, the endometrial biopsies in both the injectable and vaginal groups showed similar levels of secretory transformation, indicating a low threshold for such alteration (7,8). When administered vaginally, the serum P_4 levels consistently remain sub-physiological, ranging from 10 to 15 ng/mL (9,10). In contrast with intra-muscular P_4, uterine P_4 remains in a sub-physiological state even though supra-physiological serum levels are obtained. The administration of P_4 in combination, with both vaginal and intra-muscular or subcutaneous combined, ensures that serum and endometrial levels both remain within physiological ranges. Thus, attaining blood and endometrial levels comparable to a natural pregnancy (11). However, whether this is important for success remains controversial, because in many studies pregnancy rates are excellent with vaginal P_4 supplementation alone (12,13).

Despite extensive research, the most effective route and dosage of P_4 administration for luteal support in FET cycles has not been established. Europe favors vaginal administration, while the USA tends to favor the IM route or IM and vaginal combined (14). Subcutaneous P_4 is not available in North America. Despite studies suggesting a correlation between vaginal P_4 administration and low pregnancy rates (15), the literature contains conflicting results (16,17). These findings would suggest variations in patient response and pregnancy rates when using the same P_4 delivery type and dose. Therefore, the administration of luteal phase support could be customized based on the patient's age, weight, genetic profile, and hormonal metabolism (18,19). Some recent studies have suggested that monitoring serum P_4 levels before or during embryo transfer can provide valuable insights into reproductive outcomes (20). Poor obstetric outcomes have been linked to low pre-transfer P_4 levels, and administering an extra (rescue) dose of P_4 might improve outcomes (21-23).

As combined P_4 administration yields physiological serum P_4 levels in the range of those observed during natural pregnancy, we opted for this protocol in our facility, aiming to attain enhanced physiological P_4 levels by employing a combination of vaginal micronized P_4 ovules and subcutaneous P_4, quantified by measuring serum levels of P_4 and estradiol (E_2) on FET day-1. Our hypothesis proposes that pre-FET serum P_4 and E_2 levels may influence pregnancy outcomes. The threshold values for the administration of combined P_4 during pregnancy have not been adequately researched. Thus our aim was to determine if there is a specific threshold value for E_2 or P_4 in patients who are undergoing combined subcutaneous and vaginal P_4 treatment for luteal phase support.

Material and Methods

All ethics protocols are followed as per the Declaration of Helsinki. This study was approved by the İstanbul Atlas University Non-Interventional Research Ethics Committee (approval number: 03, date: 04.03.2024). All women had signed an informed consent that their data may be used in research studies.

The evaluation focused on FET cycles conducted in a single center from February 2022 to October 2023. In this retrospective cohort study, the predictive value of serum E_2 and P_4 levels on implantation rates in hormone-supplemented FET cycles was evaluated. The present study investigated if specific cut-off values for serum E_2 and serum P_4 could predict pregnancy when measured one day before FET (FET-1). A cohort of 167 patients who underwent FET were studied. A comparison was made between 79 patients who successfully conceived and 88 patients who failed to conceive. This study subsequently investigated the cut-off value of serum P_4 level for predicting pregnancy in 72 patients who did and did not conceive (52 pregnancies), who received combined route (vaginal + subcutaneous) P_4 for

luteal phase support. Natural and modified natural cycles of FET were excluded. Due to the prohibition of gamete donation in our country, all subjects used autonomous gametes.

The vitrification of the embryo was performed using an equilibration solution comprising 7.5% ethylene glycol and 75% dimethyl sulfoxide for 8-12 minutes. They were subsequently exposed to a vitrification solution of 15% ethylene glycol, 15% dimethyl sulfoxide, and 0.5 molar sucrose for 60-90 seconds. The specimens were loaded into a hemi-straw using drops smaller than 1 μ and then submerged in liquid nitrogen. Processing was maintained at room temperature. The embryos underwent a warming process at 37 °C for 1 minute in a 1 mL solution of 1M sucrose, followed by 3 minutes in 1 mL solution of 0.5M sucrose. The embryos were then exposed to a 10-minute incubation in a 1 mL HEPES solution containing 20% human serum albumin. Subsequently, the embryos were placed in a culture solution and kept for 2-4 hours until transfer.

The expanded embryos underwent transfer with FET, the predominant approach for embryo transfers in our center. The FET endometrial preparation protocol we used was a step-up oral E_2 method. Administration of E_2 tablet 2mg orally (Estrofem® tablet 2 mg, Novo Nordisk, Malov, Denmark) twice a day for seven days was started and then was increased to three times a day for six days, commencing on days 2-3 of menstruation. In patients were unable to tolerate E_2 orally, who exhibited insufficient endometrial thickness (ET), or who had a serum E_2 level of less than 150 pg/mL, we implemented the administration of supplementary E_2 via a vaginal or transdermal patch.

This treatment was preceded by transvaginal ultrasonography (TVUSG) (Voluson P8, General Electric Company, WI, USA) to confirm the absence of early selected follicles (>11 mm) or functional ovarian cysts. The ET and ovaries were evaluated using TVUSG. If the ET was ≥7 mm, the serum P_4 level <1.5 ng/mL, and the E_2 level >150 pg/mL after at least 10 days of E_2 use, luteal phase support was started. To achieve this, support was given using a combined route involving vaginal micronized P_4 (4x200 mg) and subcutaneous water-based P_4 (2x25 mg). Other routes and dosages were also used, including vaginal P_4 only. These subjects using other routes were included in the evaluation of serum levels on pregnancy outcomes but excluded from the analysis on factors for prediction of pregnancy that only included the combined vaginal and subcutaneous P_4 group.

Oral P_4 was not used because of technical difficulties measuring its serum levels. Our decision to opt for the combined route was driven by the objective of achieving more consistent physiological blood levels, mitigating potential absorption issues associated with the vaginal route, as well as addressing the challenges associated with IM administration, such as sterile abscesses, and pain.

In patients with a P_4 level below 10 ng/mL, we added additional P_4. Before transfer, we tried maintaining the serum E_2 level between 150-300 pg/mL and P_4 between 10-40 ng/mL. If the P_4 level was low, we employed the IM route for rescue in patients receiving single drugs and in obese patients receiving combined drugs. Post-rescue, we re-evaluated serum hormone levels on the day of transfer and proceeded with embryo transfer in patients who achieved the predetermined target values. We discontinued the cycle when hormone levels reached highs or lows outside of physiologic parameters. PGT-A was not performed except in a single case. If the serum beta-human chorionic gonadotrophin (β-hCG) value exceeded 5mIU/mL within 10-12 days following transfer, we considered this a pregnancy. The hormone tests were performed using chemiluminescence immunoassay (Abbott Alinity Analyzer, Abbott Laboratories, Chicago, IL, USA). The Abbott Alinity P_4 assay has a linear measuring range of 0.5-40.0 ng/mL (1.6-127.2 nmol/L), with intra-assay coefficients of variation (CV%) ranging from 2.7% to 5.6% and inter-assay (within-laboratory) CV% ranging from 3.1% to 6.1%. The Abbott Alinity E_2 assay demonstrated a linear measuring range of 5 to 5,000 pg/mL, with intra-assay CV% ranging from 2.5% to 5.3% and inter-assay CV% ranging from 3.1% to 7.3%.

Statistical analysis

The data analysis was conducted using IBM SPSS, version 23 (SPSS corporation, Chicago IL, USA). The presence of a normal distribution was evaluated through the Kolmogorov-Smirnov and Shapiro-Wilk Tests. In Table 1, Mann Whitney U Test was used for the comparison of age, embryo cryopreservation age (ECA), Gravidity, Parity, BMI (kg/m^2), FET CL (day), E_2 (pg/mL), luteinizing hormone (LH) (mIU/mL), P_4 (ng/mL) variables that did not conform to normal distribution, while Independent Samples t-test was used for the comparison of max ET (mm) and Post-IM P_4 variables that conformed to normal distribution. In Table 2, Pearson chi-square test was used to compare the number of ET and ET day according to the groups. In Table 3, receiver operating characteristic (ROC) Analysis was used to determine cut-off values for P_4 and E_2 variables in predicting pregnancy. Table 4 shows the percentile values of E_2 and P_4 variables. In Table 5, independent variables affecting the biochemical pregnancy probability were analyzed by Binary Logistic Regression Analysis. In Table 6, the independent variables affecting the clinical pregnancy probability were analyzed by binary logistic regression analysis. In Table 7, the independent variables affecting the probability of live birth were analyzed by binary logistic regression analysis. The analysis results are presented as frequency (percentage) for categorical variables, mean ± standard deviation, and median (minimum-maximum) for quantitative variables. The significance level was set at $p<0.05$. Data was divided into percentile groupings to further understand relationships with pregnancy outcomes.

Results

The parameters of the patients who achieved conception following FET (group A, n=79) and those who did not (group B, n=88) are presented in Table 1. The two groups displayed similar

demographic characteristics including age, BMI, gravidity, and parity. No significant differences were observed between the groups regarding the FET cycle duration, maximal ET, ECA, and pre-transfer E_2, LH, and P_4 values.

Interestingly, in cycles where initial P_4 levels were low and rescue supplementation was needed, the serum P_4 level among individuals who achieved conception was 20.5±5.4 ng/mL, while in those who did not conceive it was 46.4±13.7 and this difference was approaching significance (p=0.08).

The pregnancy rates as a function of the number of embryos transferred and the day of development are displayed in Table 2. The pregnancy rate following the transfer of two embryos vs. single (53.2% vs. 46.8%) was higher but not significantly so. Women primarily had blastocyst transfer, with 71% in group A and 92% in group B.

A ROC analysis was conducted to determine the optimal cut-off value for P_4 and E_2 the day before embryo transfer, in predicting pregnancy among patients using combined P_4 for luteal support in FET. The area under the curve was 0.427 for P_4 (Figure 1) and 0.465 for E_2 (Figure 2). There were no significant cut-off values for P_4 and E_2 parameters in predicting pregnancy in combined P_4 users (p=0.166 and p=0.441, respectively) (Table 3). The serum P_4 and E_2 values in FET cycles were similar in those who did and did not conceive, leading to the absence of significant discriminating cut-off values. Of note, the E_2 value was not assessed before FET in seven patients who achieved pregnancy.

The serum E_2 and P_4 levels measured one day before FET were divided into percentiles to further investigate if certain non-binomial distributions could be detected in predicting outcomes and are presented in Table 4. Among 72 patients, the mean E_2 value within the 0-10 percentile range was recorded as 97.7±8.8 pg/mL, with a minimum value of 85.0 and a maximum value of 107.0 pg/mL, within the 11-90 percentile range as 165.9±35.9 pg/mL, with a minimum value of 108.0 and a maximum value of 247.0 pg/mL, and in the 91-100 percentile range as 280.2±42.7 pg/mL, with a minimum value of 251.0 pg/mL and a maximum value of 360.0 pg/mL (Table 5).

Among the 60 patients in the group who experienced pregnancy (11-90^{th} percentile), the median E_2 values were 165.9 pg/mL (129.9-201.9 pg/ml) one day before FET.

The percentiles of the P_4 parameter in pregnant women using combined P4 were examined, with no pregnant women identified within the 0-10 range. The mean P_4 value within the 0-10 percentile range was 12.6±2.4 ng/mL, with a minimum value of 10.0 ng/mL and a maximum value of 15.6 ng/mL. Within the 11-90 percentile group the mean was 29.7±12.0 ng/mL, with a minimum value of 16.7 ng/mL and a maximum value of 65.6 ng/mL. Within the 91-100 percentile group, the P_4 serum value exhibited a mean of 78.0±15.7 ng/mL, with a minimum of 67.5 ng/mL and a maximum of 105.7 ng/mL.

Serum P_4 levels before FET were assessed in a cohort of 53 patients who underwent combined P_4 supplementation and successfully achieved pregnancy. Among this group (11^{th}-90^{th} percentile), the mean P_4 level was 29.3 ng/mL (15.2-43.5 ng/mL) in 49 individuals.

The rate of pregnancies achieved by vaginal P_4 was 41.9%, whereas combined (vaginal + subcutaneous) P_4 users had a similar pregnancy rate of 50.0% (p=0.36).

Independent variables affecting the probability of biochemical pregnancy, clinical pregnancy, and live birth were analyzed by multivariate binary logistic regression analysis when controlling for confounding effects and the data is presented in Tables 5-7. When the model was analyzed, the independent variables lost significance for the probability of a positive pregnancy, clinical pregnancy, or live birth, including E_2 or P_4 levels.

Table 1. Comparison of parameters between the groups

	Group				**p**
	Patients who achieved pregnancy (n= 79)		**Patients without pregnancy (n=88)**		
	Mean ± SD	**Median (min-max)**	**Mean ± SD**	**Median (min-max)**	
Age	31.6±5.56	32 (18-44)	32.25±5.75	32 (21-44)	0.587
ECA	30.19±5.55	30 (18-42)	31.51±5.99	31 (21-44)	0.210
Gravidity	0.68±0.97	0 (0-5)	0.67±1.22	0 (0-7)	0.420
Parity	0.25±0.47	0 (0-2)	0.19±0.54	0 (0-3)	0.147
BMI (kg/m²)	27.82±6.32	26.7 (17-57.6)	26.45±4.99	24.6 (20-37.4)	0.153
FET CL (day)	19.19±2.32	19 (13-28)	19.26±1.47	19 (15-24)	0.193
Max ET (mm)	9.94±1.51	10 (6.9-14)	9.61±1.61	9.83 (6.3-15)	0.180
E2 (pg/mL)	169.75±51.88	162.5 (85-360)	177.68±56.91	168.5 (92-318)	0.449
LH (mIU/mL)	5.15±4.56	3.91 (0.1-21)	5.71±4.79	4.8 (0.1-26.81)	0.332
P4 (ng/mL)	28.07±18.39	23.3 (6.5-105.7)	31.15±25.35	23.05 (2.4-117.6)	0.732
Post-IM P4	20.5±5.43	20 (15-26)	46.4±13.65	46.5 (33-60)	**0.008**

SD: Standard deviation, Min: Minimum, Max: Maximum

Table 2. Distribution of the number of embryo transfers and embryo transfer days according to groups

	Group		Test statistics	p*
	Patients who achieved pregnancy (n=79)	Patients without pregnancy (n=88)		
Number of ETs				
1	37 (46.8%)	48 (54.5%%)	0.990	0.320
2	42 (53.2%)	40 (45.5)		
ET day				
3	6 (7.6%)	7 (8%)	2.256	0.324
4	2 (2.5%)	0 (0%)		
5	71 (89.9%)	81 (92%)		
*Pearson's chi-square test, ET: Embryo transfer				

Table 3. ROC Analysis results for cut-off values for P4 and E2 parameters in determining pregnancy in combined vaginal and subcutaneous progesterone users

Parameter	AUC (% 95 CI)	p
P4	0.421 (0.309-0.533)	0.166
E2	0.456 (0.345-0.568)	0.441
ROC: Receiver operating characteristic, AUC: Area Under the curve, CI: Confidence interval, P4: Progesterone		

Table 4. Percentile distribution of E2 value and progesterone according to groups

	Group		
	Patients who achieved pregnancy (n=79)		Patients without pregnancy (n=88)
Percentile	**E2**	**Progesterone**	**E2**
0-10	10 (11.4%)	0 (0%)	6 (8.3%)
11-90	68 (77.3%)	48 (92.3%)	60 (83.3%)
91-100	10 (11.4%)	4 (7.7%)	6 (8.3%)
E2: Estradiol			

Table 5. Logistic regression analysis of independent variables affecting the probability of pregnancy among those with an embryo transfer

	Biochemical pregnancy		Multiple	
	No biochemical pregnancy (n=88)	Biochemical pregnancy (n=79)	OR (%95 CI)	p
Age	32.25±5.75	31.6±5.56	1.107 (0.885-1.386)	0.372
Embryo freezing age	31.51±5.99	30.19±5.55	0.853 (0.693-1.05)	0.134
Gravidity	0.67±1.22	0.68±0.97	0.789 (0.509-1.223)	0.290
Parity	0.19±0.54	0.25±0.47	1.89 (0.647-5.524)	0.245
BMI	26.45±4.99	27.82±6.32	1.053 (0.984-1.127)	0.137
FET cycle length	19.26±1.47	19.19±2.32	1.105 (0.889-1.374)	0.369
Max endometrial thickness (mm)	9.61±1.61	9.94±1.51	1.113 (0.873-1.42)	0.387
E2 (pg/mL)	177.68±56.91	169.75±51.88	0.998 (0.991-1.005)	0.599
LH (mIU/mL)	5.71±4.79	5.15±4.56	0.976 (0.897-1.061)	0.561
P4 (ng/ml)	31.15±25.35	28.07±18.39	0.991 (0.974-1.008)	0.288
No of ET				
1	48 (56.5%)	37 (43.5%)	Reference	
2	40 (48.8%)	42 (51.2%)	1.557 (0.683-3.552)	0.292

Table 5. Continued

	Biochemical pregnancy		Multiple	
	No biochemical pregnancy (n=88)	Biochemical pregnancy (n=79)	OR (%95 CI)	p
HRT protocols				
E2 + P4 (vaginal + subcutaneous)	52 (50%)	52 (50%)	1.527 (0.616-3.786)	0.361
E2 + P4 (single drug)	36 (57.1%)	27 (42.9%)	Reference	

Mean ± standard deviation; frequency (percentage)
OR: Odds ratio, CI: Confidence interval, E2: Estradiol, LH: Luteinizing hormone, P4: Progesterone, BMI: Body mass index, FET: Frozen embryo transfer, ET: Embryo transfer, HRT: Hormone replacement therapy, Max: Maximum

Table 6. Logistic regression analysis of independent variables affecting the probability of clinical pregnancy among those who conceived

	Clinical pregnancy		Multiple	
	No clinical pregnancy (n=13)	Clinical pregnancy (n=66)	OR (%95 CI)	p
Age	31±6.66	31.71±5.39	1.218 (0.717-2.068)	0.465
Embryo freezing age	30.23±6.08	30.18±5.49	0.844 (0.516-1.382)	0.501
Gravidity	0.62±0.87	0.7±0.99	2.022 (0.479-8.534)	0.338
Parity	0.31±0.48	0.24±0.47	0.099 (0.006-1.757)	0.115
BMI	29.42±10.17	27.51±5.31	0.942 (0.837-1.061)	0.325
FET cycle length	18.46±1.81	19.33±2.39	1.327 (0.79-2.229)	0.284
Max endometrial thickness (mm)	9.72±1.18	9.98±1.57	1.729 (0.873-3.424)	0.116
E2 (pg/mL)	160±50.73	171.9±52.32	1.006 (0.989-1.024)	0.473
LH (mIU/mL)	5.62±4.02	5.05±4.69	1.051 (0.865-1.277)	0.616
P4 (ng/ml)	30.5 ±20.23	27.58±18.14	1.003 (0.959-1.049)	0.899
No of ET				
1	9 (24.3%)	28 (75.7%)	Reference	
2	4 (9.5%)	38 (90.5%)	5.209 (0.633 – 42.862)	0.125
HRT protocols				
E2 + P4 (vaginal + subcutaneous)	10 (19.2%)	42 (80.8%)	0.154 (0.014 – 1.739)	0.130
E2 + P4 (single drug)	3 (11.1%)	24 (88.9%)	Reference	

Mean ± standard deviation; frequency (percentage)
OR: Odds ratio, CI: Confidence interval, E2: Estradiol, LH: Luteinizing hormone, P4: Progesterone, BMI: Body mass index, FET: Frozen embryo transfer, ET: Embryo transfer, HRT: Hormone replacement therapy, Max: Maximum

Table 7. Logistic regression analysis of independent variables affecting the probability of live birth among those who had a clinical pregnancy

	Live birth rate		Multiple	
	No live birth (n=6)	Live birth (n=53)	OR (%95 CI)	p
Age	31±5.4	31.45±5.13	1.099 (0.741-1.629)	0.639
Embryo freezing age	29.17±6.77	30.04±5.19	---	---
Gravidity	0.83±0.75	0.62±1	---	---
Parity	0.33±0.52	0.23±0.42	---	---
BMI	31.37±6.28	26.64±4.83	0.697 (0.481-1.009)	0.056
FET cycle length	18.67±1.63	19.42±2.21	2.424 (0.548-10.72)	0.243
Max endometrial thickness (mm)	9.83±1.27	9.96±1.63	1.615 (0.672-3.881)	0.284
E2 (pg/mL)	145.33±42.6	171.91±46.4	0.998 (0.972-1.025)	0.901
LH (mIU/mL)	6.34±6.49	4.62±3.44	0.812 (0.494-1.334)	0.411

Table 7. Continued

	Live birth rate		Multiple	
	No live birth (n=6)	**Live birth (n=53)**	**OR (%95 CI)**	**p**
P4 (ng/ml)	19.07±9.16	29.18±19.42	1.103 (0.915-1.328)	0.304
No of ET				
1	4 (16.7)	20 (83.3)	Reference	
2	2 (5.7)	33 (94.3)	1.031 (0.045-23.413)	0.985
HRT protocols				
E2 + P4 (vaginal + subcutaneous)	3 (8.3)	33 (91.7)	1.685 (0.097-29.222)	0.720
E2 + P4 (single drug)	3 (13)	20 (87)	Reference	

Mean ± standard deviation; frequency (percentage)
OR: Odds ratio, CI: Confidence interval, E2: Estradiol, LH: Luteinizing hormone, P4: Progesterone, BMI: Body mass index, FET: Frozen embryo transfer, ET: Embryo transfer, HRT: Hormone replacement therapy, Max: Maximum

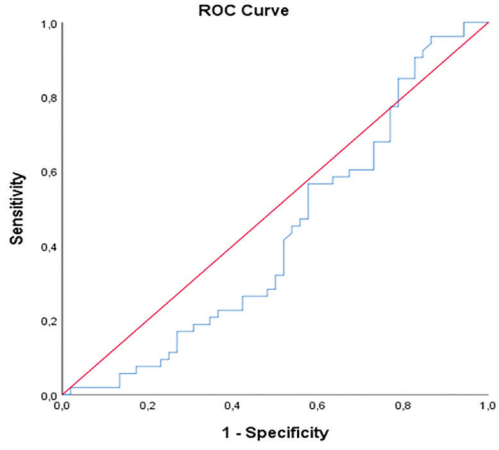

Figure 1. ROC curve for serum progesterone level in determining pregnancy
ROC: Receiver operating characteristic

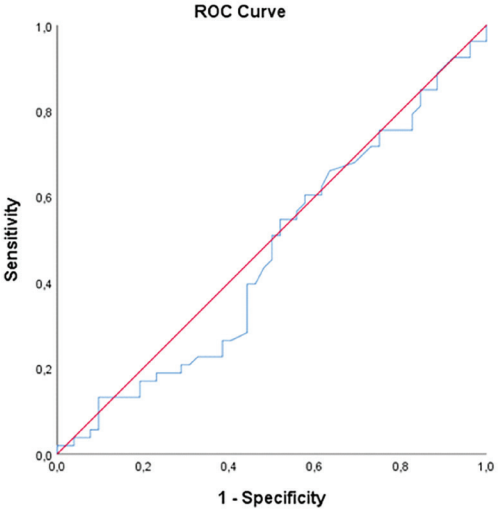

Figure 2. ROC curve for serum estradiol level in determining pregnancy
ROC: Receiver operating characteristic

Discussion

Our findings indicate that serum E_2 and P_4 measurements measured one day before transfer do not serve as predictive factors for pregnancy rates in autologous FET cycles when different routes of E_2 supplementation and combined vaginal and injectable P_4 were used. The only exception to this was the group with a serum P_4 level of less than 15.5 ng/mL, who should likely have the embryo transfer aborted, since no pregnancies were seen in this group. This finding of P_4 cut-off is likely related to different P_4 metabolism due to patient age and BMI, since it was not significant in a multivariate logistic regression analysis controlling for confounding effects.

Similar to our results, previous studies have reported that pre-transfer E_2 monitoring did not affect pregnancy outcomes. Niu et al. (12) investigated the serum E_2 level on the day of P_4 initiation and determined no notable difference in ET and pregnancy rates. They reported E_2 levels at 25, 25-75, and 75-100 percentile as 110, 191, and 299 pg/mL ($p<0.01$) and pregnancy rates of 44%, 40.8% and 41.9% ($p>0.05$ for all comparisons), respectively. In addition, they found that ET did not correlate with serum E_2 level (23). These authors concluded that the serum E_2 level did not predict pregnancy in an E_2 step-up artificial endometrial preparation protocol in the absence of pituitary down-regulation. Comparable findings have been documented in other studies (24,25).

In contrast, Goldman et al. (26) reported that the quartile with the highest serum E_2 levels (mean 528 pg/mL) on the day of P_4 initiation had significantly lower ongoing pregnancy (OP) and live birth rates (LBR) compared to the quartile with the lowest levels (mean 212 pg/mL) (relative risk 0.66 and 0.70 for OP and LBR, respectively). No discrepancies were observed between the groups regarding ET and miscarriage rates. These findings led the researchers to conclude that elevated E_2 levels on the day of P_4 initiation in FET cycles with artificial preparation could be deleterious to implantation and live LBR.

In a randomized controlled study, Racca et al. (27) examined the effects of 7 and 14 days of E_2 priming in the artificial cycle for FET. They reported no significant differences regarding pregnancy, miscarriage, and LBR rates. The study observed similar serum E_2 levels after 7 and 14 days of E_2 priming, measuring 225.0±73.8 pg/mL and 228.0±100.8 pg/mL, respectively (p=0.84). The measurement of comparable serum E_2 levels among individuals who conceived and those who did not conceive in our study, suggests that assessing E_2 before transfer may lack practical utility.

Considering individualized luteal phase support to attain physiological P_4 levels during natural pregnancy is a rational approach to possibly enhance the pregnancy rate in FET (20,21,28). One study reported that the vaginal-only administration of P_4, widely used in Europe, exhibited lower OP rates compared to IM or IM and vaginal administration. For that reason, the vaginal-only arm of the study was prematurely halted (15). A consensus has yet to be reached regarding the optimal serum P_4 level before or during transfer in artificial FET cycles. Melo et al. (29) conducted a thorough multicentre prospective cohort study to investigate the effect of frozen embryo transfer regimen on the association between serum P_4 and live birth. Their study reported serum P_4 levels <7.8 ng/mL were associated with reduced odds of live birth and the mean adjusted probability of live birth increased non-linearly from 37.6% to 45.5% as serum P_4 rose between the 10th (7.8 ng/mL) and 90th (24.0 ng/mL) centiles.

A separate study indicated that administering 40 mg IM P_4 might rescue results if serum P_4 levels were low on the day of FET (<10 ng/mL) (30). In their study, Labarta et al. (22) found that the minimum threshold for rescue was 9.2 ng/mL in patients who received vaginal P_4 alone. Our study primarily evaluated the combined route (vaginal 600 mg/day plus subcutaneous 50 mg/day) for luteal support in FET cycles, while P_4 levels were assessed one day before transfer. Our study showed no significant discrepancy in serum P_4 levels between patients who achieved pregnancy and those who did not. From our finding, we infer that serum P_4 on FET-1 does not independently predict conception when considering other factors. The optimal threshold for combined P_4 administration remains inadequately investigated.

Based on our findings, we conducted a subgroup analysis of patients who achieved pregnancy. Our aim was to ascertain whether there was a predictive threshold for pregnancy when implementing the combined P_4 regimen. No significant threshold value could be identified for pregnancy determination because of the similarity in serum P_4 levels among patients who achieved pregnancy with the combined application. Similarly, an analysis was conducted for the E_2 value, and no threshold value was identified. However, using percentiles suggested that a low P_4 level may affect outcomes, with a level of less than 15.5 ng/mL failing to result in any pregnancies. Although this was a small group the results are interesting and warrant further study. As the debate persists, well-designed prospective studies are necessary. When using multivariate logistic regression to control for confounding effects, the results lost significance, suggesting that the variables used in the analysis may contribute to alterations in P_4 metabolism that caused these low levels and the lack of pregnancies in this group.

Study limitations

While acknowledging the limitations of the study, such as the small number of cases and the retrospective nature, it is worth noting that the study's strength lies in the homogeneity of the patient group's demographics. The study yielded no serum E_2 and P_4 cut-off values to predict pregnancy on FET day 1 when using ROCs. The observation that individuals who conceived displayed serum P_4 levels between 15 and 43 ng/mL suggest potential lower limits for P_4 on FET-1.

Conclusion

Our findings indicate that striving to attain physiologic levels comparable to natural pregnancy through measuring serum E_2 and P_4 levels one day before transfer in autologous artificial FET cycles does not yield noteworthy variations in pregnancy outcomes. The study yielded no serum E_2 and P_4 cut-off values to predict pregnancy on FET day 1 by ROC analysis. In artificially prepared FET cycles, the serum E_2 and P_4 levels one day before embryo transfer did not significantly affect pregnancy rates in women with serum E_2 levels between 150-300 pg/mL and P_4 between 10-40 ng/mL, again using ROC curve analysis. However, the observation that all individuals who conceived had serum P_4 levels above 15.5 ng/mL suggests a lower limit for for P_4 on the day before embryo transfer. Women with P_4 levels less than this value should be considered for cycle cancellation. Of note, multivariate logistic regression analysis suggested that these findings may be due to confounding factors affecting P_4 metabolism. Larger, prospective studies are needed to validate our findings.

Ethic

Ethics Committee Approval: *This study was approved by the İstanbul Atlas University Non-Interventional Research Ethics Committee (approval number: 03, date: 04.03.2024).*

Informed Consent: *All women had signed an informed consent that their data may be used in research studies.*

Footnotes

Author Contributions: *Surgical and Medical Practices: L.D., Concept: L.D., Design: L.D., Data Collection or Processing: L.D., Analysis or Interpretation: L.D., M.H.D., Literature Search: L.D., Writing: L.D., M.H.D.*

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