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Comparison of the duration of estradiol administration and the effect on pregnancy outcome of day 3 vitrified–warmed embryo transfer cycle: a randomized controlled trial

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Research Article

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Summary

Based on the fact that the follicular phase in the menstrual cycle has length variation, it has been assumed that the duration of oestrogen (E2) administration could also be variable; therefore, for the first time, this randomized clinical trial study was conducted to investigate and compare the duration of estradiol administration and the effect on pregnancy outcomes in the cleavage-stage frozen embryo transfer (FET) cycle. We included women aged 20–40 with a normal uterus on hysteroscopy between September and December 2022 and who were divided randomly into three groups: group A [$n = 79$; 8–11 days of oestrogen before progesterone (P4) supplementation], group B ($n = 78$; 12–14 days of oestrogen before P4 supplementation), and group C ($n = 76$; 15–18 days of estrogen before P4 supplementation). Serum levels for E2 on the initial progesterone day and P4 on the transfer day were measured. The effect of the duration of E2 administration on clinical pregnancy and pregnancy loss was investigated. We found no significant differences between the three groups in the clinical pregnancy rate ($P = 0.696$) and clinical abortion rate ($P = 0.925$) according to the duration of the E2. There was no significant difference in the E2, P4 levels, and endometrial thickness in pregnant vs. non-pregnant women. The mean of the E2 and P4 levels was 300.03 ± 22.21 and 25.36 ± 5.78, respectively. Our findings suggest that variation in the length of E2 administration (8–18 days) before progesterone initiation in day 3 FET cycles does not affect pregnancy outcome and transfer time can be flexibly arranged.

Introduction

Embryo transfer is one of the key steps for successful artificial reproductive treatment (ART). More efficient and safe vitrification methods (Loutradi *et al.*, 2008) include an antagonist protocol followed by a freeze-all strategy and embryo transfer in a frozen embryo transfer (FET). This has become a promising option to increase the live birth rate (Blockeel *et al.*, 2016). Recently, the proportions of FET cycles were 35.5% and 77.0% as reported by the European Society of Human Reproduction and Embryology (ESHRE) and the United States nationwide database, respectively [Gaskins *et al.*, 2023; European IVF Monitoring Consortium (EIM) *et al.*, 2022].

Ensuring the synchronization of embryo development and endometrial receptivity is a key step in obtaining pregnancy (Tabibzadeh, 1998). Estradiol causes the proliferation of endometrial cells, expression of progesterone receptors, and ovulation suppression in FET cycle preparation (Press *et al.*, 1986). It seems that it plays an important role in increasing endometrium receptivity (Young, 2013) and early embryo development (Ramathal *et al.*, 2010).

Based on the fact that the follicular phase in the menstrual cycle has variable length therefore, unlike progesterone (P4), the duration of oestrogen (E2) administration before FET can be artificially changed according to the endometrial thickness, the availability of PGT results, or the patient's preference. But some studies have shown that long oestrogen administration (>28 days; Bourdon *et al.*, 2018), and short oestrogen administration (<10 days; Borini *et al.*, 2001b) before FET reduced live birth rate, but some reject this issue and show that endometrial receptivity and ART outcomes are not affected by the duration of E2 administration (Liu *et al.*, 2018; Du *et al.*, 2021; Eid *et al.*, 2021). Therefore, despite the increase in the FET cycle, the optimal endometrium preparation protocol for synchronization between receptive endometrium and the embryo is still a matter of debate.

Most of the studies on the effect of estradiol administration on clinical outcomes have been conducted retrospectively on donor egg cycles (Borini *et al.*, 2001b) to decrease the heterogeneity of cleavage-stage embryos.

This randomized clinical trial study which considered confounder factors was conducted to investigate and compare the duration of estradiol administration and the effect on pregnancy outcomes in the cleavage-stage FET cycle.

Materials and methods

Study design and participants

This prospective, randomized controlled trial study was approved by the Ethics Committee of Shiraz University of Medical Sciences (Ethics Committee Approval Number: IR.SUMS.REC.1401.342) and registered on 28 September 2022 (ID number: IRCT20220906055898N1).

In this study, 320 women who underwent an autologous programmed vitrified–thawed day 3 embryo transfer cycle in Zeinabiyyeh Hospital Infertility clinic were enrolled from September to December 2022. Informed consent was obtained from all of the participants.

We included patients with at least two good-quality embryos for transfer with normal body mass index (BMI), less than 45 years old, and healthy uterine cavity confirmed by hysterosalpingography and hysteroscopy or saline ultrasound endometrial thickness ≤7 mm on 7 days after estradiol administration.

We excluded cycles with oocyte or embryo donation, *in vitro* maturation (IVM), preimplantation genetic diagnosis, repeated implantation failure, poor responder, endometriosis, adenomyosis, severe male factor, uncontrolled chronic disease, or incomplete data.

In this study, the samples were first selected based on inclusion criteria by a convenient sampling method, then samples were divided randomly into three intervention groups: Group A (8–11 days of estrogen before P4 supplementation), group B (12–14 days of oestrogen before P4 supplementation), and group C (15–18 days of oestrogen before P4 supplementation) by randomized block allocation method using random sequence generation software. The randomization process was done by a study methodology consultant.

In this study, patients and physicians were blinded and unaware of the randomization process. Patients did not communicate with each other and were visited at separate times. The type of catheters and physicians that performed the embryo transfers were the same.

The data analyzer was blinded to the type of grouping and group information.

The sample size was calculated as 90 in each group based on the data of similar studies and using GPOWER software [power: 80%, α: 0.05, mean difference: 1.6, loss rate = 20%, and standard deviation (SD): 3.2; Bourdon *et al.*, 2018].

Ovarian stimulation and laboratory procedure

Patients underwent ovarian stimulation with the gonadotropin-releasing hormone antagonist (GnRH-ant) protocol described previously elsewhere (The ESHRE Guideline Group on Ovarian Stimulation *et al.*, 2020). When a minimum of two mature follicles measuring 18 mm were achieved, oocyte maturation was triggered using 10,000 IU recombinant human chorionic gonadotropin (hCG; Darou Pakhsh Pharmaceutical MFG Co., Tehran, Iran) intramuscularly (IM) alone or with 0.5 mg GnRH agonist (CinnaFact®; CinnaGen, Tehran, Iran) in combination with 5000 IU hCG (Darou Pakhsh Pharmaceutical MFG Co., Tehran, Iran).

Vaginal oocyte retrieval was performed under transvaginal ultrasound (TVUS) guided needle aspiration 34–36 h after triggering was administered.

Approximately 4 h after retrieval, oocytes were fertilized by intracytoplasmic sperm injection (ICSI) or conventional insemination (IVF) as indicated, and good embryos were vitrified at the cleavage stage (day 3) using a vitrification method, as previously described elsewhere (Rezazadeh Valojerdi *et al.*, 2009).

Embryo grading and vitrified–warmed embryo protocol

Good-quality embryos were defined as day-3 embryos without multinucleation, with <25% fragmentation, having symmetry of eight or more blastomeres of stage-specific size, and at least 50% of the blastomeres were intact after warming (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011).

Good embryos that were frozen on day 3 post-fertilization by vitrification method were warmed on embryo transfer day (Rezazadeh Valojerdi *et al.*, 2009). Then, after warming the embryos were placed in a 20 µl drop of SAGE 1-StepTM (ORIGIO, Denmark) culture medium, which was pre-incubated and placed under paraffin oil at 37°C with 5% O_2 and 6% CO_2 in the Astec benchtop incubator (Astec, Japan) until embryo transfer.

We used UTM medium (Origio, Denmark) and laser-assisted hatching (Lykose®; Hamilton Thorne, Beverly, MA, USA) for all of the embryo transfer cycles.

Endometrial preparation transfer

In the present study, all patients underwent artificial cycles for FET. A vaginal ultrasound examination was performed on the second or third day of the menstrual cycle for evaluation of ovarian cyst and endometrium thickness (EMT). In total, 6–8 mg of estradiol valerate (Aburaihan Pharmaceutical Co., Tehran Iran) was taken orally daily to stimulate endometrial growth and preventing dominant follicle formation; vaginal ultrasound was performed for EMT examination 7 days later. Patients with EMT > 7 mm were assigned randomly to the study groups (A: 8–11 days; B: 12–14 days; C: 15–18 days).

The drug dose was adjusted according to the EMT (up to 8 mg per day). If the patient's conditions were appropriate (EMT > 7 mm, serum oestrogen level ≥ 150 pg/ml, and serum progesterone < 1.5 ng/ml, to rule out premature ovulation) 100 mg progesterone (Iran Hormone Pharmaceutical Co., Tehran, Iran) IM daily was administrated 3 days before FET.

Two or three good embryos were transferred using an ultrasound guide and Wallace catheter (Cooper Surgical, Denmark). The number of embryos transferred was determined by the patient's age, cause of infertility, previous embryo transfer failure, and embryo quality.

For luteal support the patients received estradiol valerate 6–8 mg/day and progesterone (Femogex-IH) 100 mg/day IM (Iran Hormone Pharmaceutical Co., Tehran, Iran) until 3 days after FET, then estradiol valerate 6–8 mg/day, suppository progesterone (Fertigest®) 800 mg/day (Aburaihan Pharmaceutical Co., Tehran, Iran) was administrated.

Serum human chorionic gonadotropin level was checked 14 and 19 days after embryo transfer (ET). If the chemical pregnancy was positive, vaginal sonography was performed 3 weeks later to identify the numbers of gestational sacs and the presence of heartbeats. Corpus luteum support was continued up to the 12th week of gestation.

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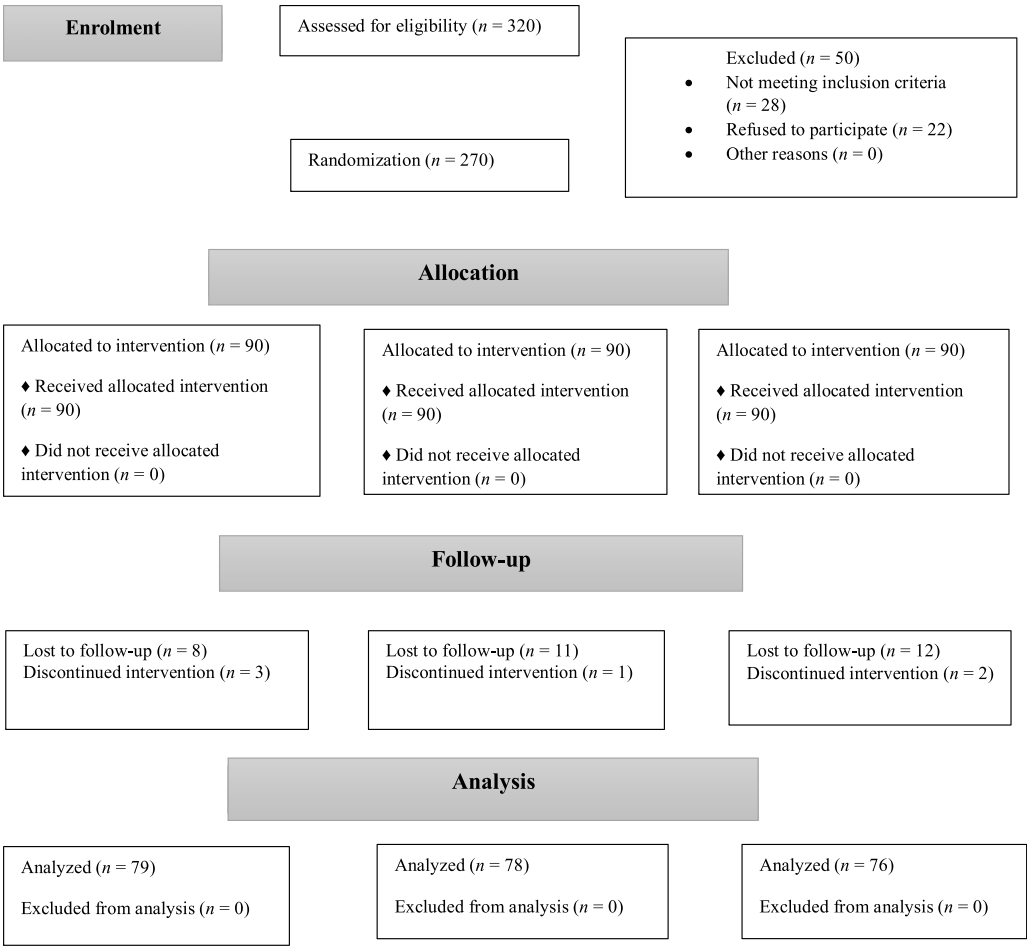

Figure 1. CONSORT flow diagram of participants.

Serum analysis

In total, 230 blood samples were sent to the laboratory for E2 and P4 levels measurements on progesterone administration day and ET day, respectively.

For E2 level detection, an electrochemiluminescence immunoassay kit (Architect Estradiol 7K72; Abbott, USA) with an analytical sensitivity of ≤ 10 pg/ml and functional sensitivity of ≤ 25 pg/ml was used. For P4 level detection, an electrochemiluminescence immunoassay kit (Architect Progesterone 7K77; Abbott, USA) with an analytical sensitivity ≤ 0.1 ng/ml was used.

Outcome measures and definition

The primary outcome of this study was endometrial thickness on the day of progesterone administration, defined as the distance between the two layers of endometrium. The secondary outcomes were biochemical pregnancy, which was defined as cases with serum human chorionic gonadotropin level ≥ 25 IU/L 14 days after ET, and clinical pregnancy defined as the visualization of the gestational sac with heartbeats by TVUS at ≥ 5 weeks of gravidity; clinical abortion was described as pregnancy loss before 20 weeks of gestation.

Statistical analysis

The collected data were analyzed by applying SPSS software, version 22 (SPSS Inc., Chicago, IL, USA).

We expressed the continuous data as means ± SD and categorical variables as frequency (%). The normality of the variables was checked based on the Kolmogorov–Smirnov test. Depending on the normality of the distribution, analysis of variance (ANOVA) or Kruskal- Wallis test was used as suitable. Pregnancy rate and causes of infertility between groups were assessed by chi-squared test.

We used independent sample *t*-tests or Mann–Whitney *U*-tests to compare age, BMI, endometrial thickness, estradiol, follicle-stimulating hormone (FSH), and anti-Müllerian hormone (AMH) between the positive and negative pregnancy groups. A *P*-value of <0.05 was considered a significance level.

Results

Study population

The process of our clinical trial is detailed in Figure 1. First, 320 women FET candidates were selected based on the inclusion criteria, but then 22 women refused to participate, and 28 women had an endometrial thickness of less than 7 mm on the 7th day of estradiol administration and were excluded from the study.

In total, 270 FET cycles were divided into three groups (Figure 1). Overall, 233 cleavage ETs were analyzed in this study. Group A ($n = 78$) 8–11 days, group B ($n = 79$) 12–14 days, and group C ($n = 76$) 15–18 days oestrogen was used for endometrial preparation.

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Table 1. Baseline characteristics, and cycle features of FETs

Variables		Group A ($n = 78$) mean ± SD or n (%)	Group B ($n = 79$) mean ± SD or n (%)	Group C ($n = 76$) Mean ± SD or n (%)	P-value	
Female age at oocyte retrieval (years; mean ± SD)		32.95 ± 5.32	33.73 ± 5.14	33.93 ± 5.80	0.491	
Female age at FET day (years; mean ± SD)		33.64 ± 5.62	34.81 ± 4.72	34.54 ± 6.01	0.521	
Male age at IVF cycle (years; mean ± SD)		37.78 ± 5.76	37.65 ± 6.63	37.84 ± 6.13	0.982	
BMI (kg/m^2; mean ± SD)		25.92 ± 3.72	25.52 ± 3.89	25.02 ± 4.42	0.231	
Basal serum FSH (mIU/ml; mean ± SD)		5.59 ± 1.70	5.48 ± 1.48	4.96 ± 1.29	0.024	
AMH (ng/ml; mean ± SD)		3.12 ± 2.52	3.27 ± 2.31	3.85 ± 2.43	0.147	
Serum oestrogen level before P4 administration day (pg/ml; mean ± SD)		301.21 ± 25.17	302.46 ± 25.45	298.85 ± 22.58	0.649	
Serum progesterone level at transfer day (pg/ml; mean ± SD)		25.36 ± 5.78	25.10 ± 5.98	26.52 ± 6.16	0.301	
Aetiology of infertility tubal factor male factor (N, %)	Male	12 (15.6)	21 (27.3)	15 (20.5)	0.199	
	Decreased ovarian reserve	32 (41.6)	28 (36.4)	20 (27.4)		
	PCO	19 (24.7	13 (16.9)	24 (32.9)		
	Unexplained	14 (18.2)	15 (19.5)	14 (19.2)		
Method of fertilization (N (%))		IVF	11 (14.1)	9 (11.4)	3 (3.9)	0.082
		ICSI	67 (85.9)	70 (88.6)	73 (96.1)	
No of embryos transferred (mean ± SD)			2.12 ± 1.01	2.43 ± 0.89	2.52 ± 0.65	0.754
The endometrial thickness on the first day of progesterone administration (mm; mean ± SD)		8.16 ± 0.49	8.07 ± 0.41	8.00 ± 0.35	0.193	0.193

Group A: low group; B: medium group; C: long group; Data presented as mean ± SD or N (%). SD: standard deviation; N: count.

Baseline characteristics

Patient characteristics are detailed in Table 1. Overall, there were no statistical differences between the three groups in baseline characteristics, except for a statistically lower basic FSH in group C compared with the other groups (4.96 ± 1.29 *vs.* 5.59 ± 1.70, 5.48 ± 1.48, $P = 0.024$).

The average thickness of the endometrium was from 8 to 8.16 and there was no significant difference between the three study groups ($P = 0.193$).

The average serum oestrogen level before P4 administration day and serum progesterone level at transfer day were 300.03 ± 22.21 pg/ml and 25.36 ± 5.78 ng/ml, respectively.

Vitrified–warmed ET outcomes

Clinical outcomes among the study groups are presented in Table 2. We found no significant differences between the three groups in chemical pregnancy rate, clinical pregnancy rate, and clinical abortion rate according to the duration of the E2.

Clinical pregnancy outcomes according to baseline characteristics and FET cycle features are presented in Table 3.

In our study, there was no significant difference between the pregnant and non-pregnant groups in the number of embryos transferred, AMH, FSH, female age, male age, BMI, and endometrial thickness (Table 3).

Table 2. Study groups outcomes

Parameter (unit)	Group A ($n = 78$), n (%)	Group B ($n = 79$), n (%)	Group C ($n = 76$), n (%)	P-value
Chemical pregnancy rate/ cycle (N, %)	21 (26.92)	25 (31.64)	25 (32.89)	0.696
Clinical pregnancy rate/ cycle (N, %)	18 (23.07)	23 (29.11)	23 (32.89)	0.723
Clinical abortion rate (N, %)	5 (6.41%)	4 (5.06%)	4 (5.26%)	0.925

Group A: low; Group B: medium; Group C: long. Data presented as numbers (%).

There was no significant difference between E2 and P4 levels in pregnant vs. non-pregnant women.

The cumulative dose of oral oestrogen ($P = 0.414$) and days of oral oestrogen administration ($P = 0.299$) were similar between pregnant vs. non-pregnant women.

Discussion

To the best of our knowledge, most published data to date on the duration of E2 administration and ART outcomes have a

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Table 3. Clinical pregnancy outcome according to baseline characteristics and FET cycle features

Variables	Clinical pregnancy		*P*-value
	Positive ($n = 71$)	Negative ($n = 162$)	
Female age at oocyte retrieval (years; mean ± SD)	33.41 ± 5.17	33.59 ± 5.54	0.812
Female age at embryo transfer (years; mean ± SD)	34.54 ± 5.95	34.85 ± 5.23	0.892
Male age (years; mean ± SD)	38.24 ± 6.68	37.54 ± 5.92	0.441
BMI (kg/m^2; mean ± SD)	25.09 ± 4.69	25.67 ± 3.69	0.310
Endometrial thickness (mm; mean ± SD)	7.98 ± 0.38	8.12 ± 0.44	0.883
Basic FSH (mIU/ml; mean ± SD)	5.39 ± 2.65	5.32 ± 1.60	0.767
AMH (ng/ml; mean ± SD)	3.39 ± 2.65	3.42 ± 2.35	0.924
Serum oestrogen level before P4 administration day (pg/ml; mean ± SD)	300.03 ± 22.21	301.23 ± 25.35	0.729
The cumulative dose of oral estrogen (mg; median (IQR))	84	82	0.414
Days of oral estrogen administration (Days; mean ± SD)	13.25 ± 2.78	12.85 ± 2.68	0.299
No embryos transferred (mean ± SD)	2.54 ± 0.92	2.41 ± 0.33	0.982

Data presented as: mean ± SD or median (IQR). IQR: interquartile range; SD: standard deviation.

retrospective design, retrospective observational studies have limitations in controlling all the confounding factors, and the present study is the first randomized controlled trial study that has investigated the duration of estradiol administration and pregnancy outcome in day 3 vitrified–warmed ET cycles.

Our prospective study, with a good design, despite previous retrospective studies, made it possible to study the effect of the duration of estradiol administration on pregnancy outcomes in a heterogeneous population and the interpretation of the data can be generalized and applicable to clinical practice.

Furthermore, one baseline characteristic differed between groups: the mean basic FSH was significantly lower for the 15–18 day group than for the 8–11 day and the 12–14 day groups.

In this study the pituitary was not suppressed using a GnRH-a, then it was very important to start oestrogen treatment on day 1 or day 2 of the menstruation cycle.

Endometrial thickness ≥7 mm is considered the cutoff for endometrial receptivity, below which we cancelled an ET (Hofmann *et al.*, 1996; Israel *et al.*, 1996; Weissman *et al.*, 1999; Shapiro *et al.*, 2014; Wu *et al.*, 2014). To try to reduce any measurement bias, two physicians independently measured the endometrial thickness from recorded images whilst being blinded to the pregnancy outcome. All women had an endometrial thickness between 8 – 8.16 mm and this factor was not found as a predictive factor for pregnancy outcome.

We found that variation in the duration of E2 administration before progesterone initiation in cleavage-stage FET cycles did not statistically affect the pregnancy outcomes.

In FET cycles, despite the transfer of good-quality embryos, the pregnancy rate may remain low (Ma *et al.*, 2003; Aflatoonian *et al.*, 2010) because receptivity of the endometrium is dependent on the hormonal status of the endometrium at the time of implantation (Engmann *et al.*, 2008). Steroid receptors are controlled by endometrial gene expression (Critchley *et al.*, 2002). Prolonged E2 administration above 28 days may hurt the endometrial gene expression profile (Chang *et al.*, 2011; Altmäe *et al.*, 2016) endometrial receptivity (Krasnow *et al.*, 1996), and live birth rate (Bourdon *et al.*, 2018). Sekhon *et al.* (2019) showed that the duration of estradiol administration before progesterone initiation did not affect the frozen euploid blastocyst transfer outcomes. Reignier *et al.* (2021) reported the duration of estradiol administration did not affect live birth between the two groups (35 days vs. less than 21 days). Our study showed a better rate of clinical pregnancy and lower clinical abortion in the prolonged E2 administration groups (B and C groups) than the short E2 administration group (8–11 days), but it did not achieve statistical significance. Navot *et al.* (1989) showed lower pregnancy rates in a shortened E2 administration group (5–10 days). A higher abortion rate has been reported using a shorter E2 administration period < 10 days (Bourdon *et al.*, 2018) and > 40 days (Michalas *et al.*, 1996).

Borini *et al.* (2001a) have reported that prolonged oestrogen administration beyond 40 days is associated with a high rate of breakthrough bleeding. In our study, the longest duration of estrogen administration was 18 days, and no breakthrough bleeding was seen in patients.

Younis *et al.* (1992) suggested that an optimal period of E2 administration was between 12 and 19 days, but Michalas *et al.* (1996) reported that the optimal duration of E2 administration was 6–11 days. Our results indicated that the duration of the estradiol administration between 8–18 days could not be a negative effect on clinical pregnancy and clinical abortion. This wide interval allowed flexibility regarding the scheduling of the ET. For clinicians, it requires careful planning and organization to schedule the ET in keeping with the patient's needs whilst preserving optimal pregnancy rates.

When women were divided into positive clinical pregnancy and negative clinical pregnancy groups, no significant differences were found in the endometrial thickness on the first day of progesterone administration, serum progesterone level at transfer day, and, serum oestrogen level before P4 administration day between the two groups.

Embryo quality has a key role in successful embryo implantation. In our study, two or three good cleavage-stage embryos were transferred and there was no significant difference in the number of embryos transferred between intervention groups and positive clinical pregnancy (2.54 ± 0.92) and negative clinical pregnancy (2.41 ± 0.33) groups.

Li *et al.* (2022) analyzed serum E2 levels in cleavage-stage FET cycles and reported high variation in the E2 levels ranging from 2.72 to 1142.78 pg/ml. The reason for the high variation in E2 serum levels among patients in their study was the non-observance of a fixed time in taking blood samples. Therefore, in our study, blood samples for serum E2 measurements were taken at 8–9 a.m.

Li *et al.* (2022) reported avoiding E2 levels exceeding a threshold of 413.6 pg/ml in FET cycles at the cleavage stage. In our study, the mean E2 level was 300.03 ± 22.21 pg/ml.

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Fritz *et al.* (2017) and Li *et al.* (2022) demonstrated that the serum E2 level on the progesterone initiation day was significantly higher in the non-pregnant group, but we showed that the serum E2 level was not significantly different between pregnant and non-pregnant groups ($P = 0.729$).

Du *et al.* (2021) reported that the duration of estrogen administration did not affect the neonatal and perinatal outcomes. Other studies have shown that exposure to supraphysiological doses of estrogen, may hurt perinatal outcomes (Pereira *et al.*, 2017; Zhang *et al.*, 2018, 2022). In our study, we did not investigate obstetrical delivery and neonatal outcomes, for example birth weight. The investigation of live birth rate, obstetrical, perinatal, and neonatal outcomes is suggested in future studies with larger sample sizes.

This prospective clinical trial study provides evidence that in day 3 FET cycles variation in the length of E2 administration, 8–18 days after the second day of menstruation, did not adversely affect the clinical pregnancy rate and clinical abortion rate. Our study implies that FET cycles can be flexibly scheduled without affecting the pregnancy outcome.

Author contribution. Study conception and design: all authors. Data collection: all authors. Data analysis and interpretation: all authors. Drafting of the article: all authors. Critical revision of the article: all authors.

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Declaration of interest. The authors report no financial or commercial conflicts of interest.

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