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Original Article

Are the estrogen levels on the day of frozen-thawed embryo transfer related to the outcomes in hormonal replacement treatment cycles?

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Abstract: Objective: To investigate the impact of estrogen (E_2) levels on the day of embryo transfer on pregnancy outcomes of frozen-thawed embryo transfer (FET) in hormonal replacement treatment (HRT) cycles. Methods: A total 193 HRT cycles was retrospectively identified. Cycles were divided into two groups: high E_2 group (>150 pg/ml) and low E_2 groups (≤ 150 pg/ml). Stimulation and embryological characteristics were compared between the two groups.Result(s): Regarding the E_2 levels on the day of frozen-thawed embryo transfer, no significant differences were found in clinical pregnancy rate between high E_2 groups and low E_2 groups in both Day 3 or Day 5 embryo-transfer subgroups (P>0.05). The logistic regression analysis revealed that E_2 levels on the day of embryo transfer were not independently associated with positive clinical pregnancy (Day 3 embryo-transfer subgroup: OR = 1.004, 95% CI: 0.997-1.011, P = 0.237; Day 5 embryo-transfer subgroup: OR = 1.001, 95% CI: 0.994-1.09, P = 0.761), and the areas under the receiver operating characteristic curve examining were 0.551 in Day 3 embryo-transfer subgroup and were 0.556 in Day 5 embryo-transfer subgroup. Conclusion(s): The concentration of serum E_2 on the day of embryo transfer cannot serve as an indicator to predict the outcomes of artificial FET cycles.

Keywords: Estrogen, frozen-thawed embryo transfer, hormonal replacement treatment cycles, clinical pregnancy rate

Introduction

Due to the improvement of ovarian stimulation protocols and the development of laboratory technologies, infertile patients who received in-vitro fertilization/intracytoplasmic sperm injection (IVF/ICSI) treatment can have surplus healthy embryos to be frozen for later transfer. As an important part of IVF/ICSI techniques, frozen-thawed embryo transfer (FET) has multiple advantages: 1) preventing complications of IVF/ICSI such as ovarian hyper-stimulation syndrome; 2) increasing the cumulative pregnancy rate; 3) acquiring a better endometrium synchronism [1-3].

There are various protocols for endometrial preparation in FET cycles [4], among which hormonal replacement treatment (HRT) cycle using exogenous E_2 and progesterone (P) is the most popular one. However, there is no consensus on which method is better, and many studies have showen that pregnancy rate achieved in artificial cycles is similar to that in natural cycles [5, 6]. Clinicians prefer to use natural cycles for women with regular ovulation because it has no hormonal intervention, but the patients should bare the risk of cancelations due to anovulation. Artificial cycles with exogenous hormonal replacement work for both ovulatory and anovulatory women, which allow clinicians to manage their schedules more effectively [7].

Precise timed synchronization between endometrial development and the implanting embryo is essential for obtaining high embryo implantation rate and pregnancy rate. Endometrial preparation plays an important role in FET cycle. In China, endometrial preparation with exogenous hormones is the most commonly-used method for FET cycles in many centers [8]. So far, most studies about FET cycles have focused on embryological factors and the thickness of endometrium, while little attention has been paid to the serum steroid hormone levels on the day of embryo transfer [9].

In HRT cycles, exogenous E_2 supplement is initiated in early phase of menstrual cycle and P supplementation begins when endometrial thickness has reached 8 mm and over, which is generally deemed as suitable for embryo implantation. Besides endometrial thickness, E_2 levels are also regularly monitored for endometrial receptivity. However, whether serum E_2 levels on the day of embryo transfer can serve as an indicator for clinical pregnancy is doubtful. The effect of serum E_2 levels on the day of embryo transfer is poorly defined in the literature and needs further evaluation [10]. The main purpose of this study was to investigate the impact of serum E_2 levels on day of embryo transfer on pregnancy outcomes for FET in programmed cycles in HRT cycles.

Materials and methods

Study population

Retrospective study was performed in Center for Reproductive Medicine, the Third Affiliated Hospital, SunYat-sen University. Patients undergoing IVF/IVSI with embryo cryopreservation were recruited from December 2015 to December 2016. Patients met the following criteria were studied: (1) patients who underwent frozen-thawed embryos transfer treatment; (2) the endometrial preparation was initiated with oral estradiol valerate; (3) the endometrial thickness was no less than 8 mm on the day when P was administrated; (4) a normal endometrial ultrasound imaging. Exclusion criteria: (1) cases that embryos transfer performed by inexperienced physicians; (2) patients with other forms of estrogen medicine supplementation; (3) cases over 3 embryos transfer; (4) abnormal ultrasonogram of cavity. This retrospective cohort study was approved by the Third Affiliated Hospital of Sun Yat-Sen University Reproductive Medicine Ethic Committee.

Endometrium preparation

Endometrial preparation was initiated on cycle day 2, and oral estradiol valerate (Progynova, Bayer) was taken 4 mg/d from cycle day 2 to day 6, and changed to 6 mg/d from day 7 to day 11. After 10-15 days of estradiol valerate administration, P administration was given to transform the endometrium when the endometrial thickness reached 8 mm or thicker. Cycles were canceled if endometrial thickness failed to reach 8 mm. Estradiol valerate and progesterone were administered for 14 more days after embryos transfer. When pregnancy was achieved, estradiol valerate and progesterone supplementation continued until the 8^{th} to 10^{th} week of gestation. P supplementation varied from oral administration of 10 mg bid (Duphaston; Abbott Biologicals), vaginal administration (1 # qd, Crinone; Merck-Serono) and intramuscular injection (40 mg qd, Progesterone Injection; Shanghai General Pharmaceutical).

Embryo transfer

All embryos were cryopreserved by vitrification. Day 3 embryo transfer was performed three days after P administration and day 5 embryo transfer was performed five days after P administration.

Cycle outcomes

Beta-human chorionic gonadotropin (β-hCG) concentration was tested on the 14^{th} day after embryo transfer. Biochemical pregnancy was defined as a positive urine test for β-hCG or serum β-hCG concentration>25 IU/L. Clinical pregnancy was diagnosed if a pregnant sac was observed in ultrasound examination 5 weeks after FET. Implantation rate was defined as the number of gestational sacs observed by ultrasound divided by the number of embryos transferred.

Statistical analysis

Data were collected for patient demographics and cryo-thawed cycle characteristics and outcomes. In order to obtain reasonable subgroup sample sizes for meaningful statistical analysis, patients were divided into two groups according to the serum levels on the day of embryo transfer: $E_2 \leq 150$ pg/ml and 150 pg/ml $< E_2$. And cases were separated into two subgroups according to different stages of embryos transferred. A comparison of the quantitativevariableswasperformedusingatwo-sample t-test. For a comparison of the categorical variables, χ^2 test was performed.

Enter-method binary logistical regression analysis was used to assess patient's age, serum E_2 levels on the day of P administration, serum E_2 and P levels on the day of embryo transfer, endometrial thickness, the number of embryos transferred, and the depth of embryos transferred. Moreover, the predictive relationship between serum E_2 levels on the day of embryo

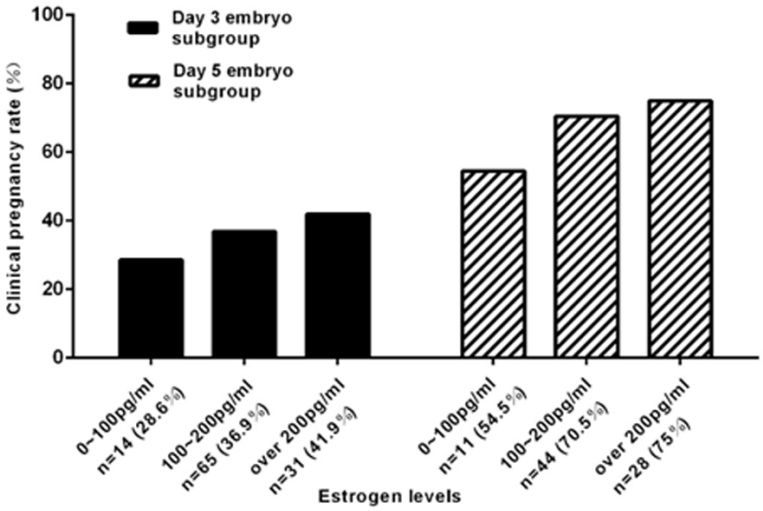

Figure 1. Clinical pregnancy rate according to estrogen levels on the day of embryo transfer. Note: Data are presented as the case number of the class (case number of the class).

Table 1. Baseline characteristics

Variable	Low E_2 group	High E_2 group	*P* value
Day 3 embryo subgroup			
No. of cases (n)	50	60	
Age (y)[a]	35.9±5.8	35.7±6.0	0.87
Body mass index (kg/m^2)[a]	22.3±2.7	21.8±2.7	0.37
Duration of infertility (y)[a]	5.2±4.7	4.2±4.5	0.26
Basal FSH (IU/ml)[a]	7.35±3.07	8.22±3.50	0.18
Day 5 embryo subgroup			
No. of cases (n)	35	48	
Age (y)[a]	30.1±5.4	31.9±4.4	0.10
Body mass index (kg/m^2)[a]	21.1±2.3	21.1±2.1	0.75
Duration of infertility (y)[a]	3.8±2.6	3.7±2.5	0.89
Basal FSH (IU/ml)[a]	7.07±2.23	6.55±1.69	0.23

Note: Values are mean ± SD or number (percentage) E_2 = estrogen.
a. Data are presented as the mean ± SD.

transfer and positive clinical pregnancy was examined by a receiver operator characteristic (ROC) curve to determine its predictive value. IBM SPSS statistics 20 was used for statistical analysis. *P*<0.05 was considered statistically significant.

Results

Patient characteristics

Totally, 193 cycles were involved in this study, including 110 cycles in D3 subgroup (day 3 embryo transfer subgroup) and 83 cycles in D5 subgroup (day 5 embryo transfer subgroup). It seems that there was a trend that the clinical pregnancy rate increased with the increasing E_2 levels on the day of embryo transfer (**Figure 1**). **Table 1** presents the clinical characteristics of all patients. No significant differences were found in age, BMI, duration of infertility and basal FSH between low E_2 group and high E_2 group respectively in different subgroups (*P*>0.05).

Endometrial preparation characteristics and outcomes of frozen-thawed embryos transfer

Endometrial preparation characteristics between low E_2 groups and high E_2 groups were not significantly different regarding E_2 usage duration, total doses of E_2 administration, P levels on P administration day and on the day of embryo transfer, and endometrial thickness. The number of transfer embryos, and embryo transfer distance from the fundus (TDF) were not significantly different. E_2 levels on the P administration day and on the day of embryo transfer were significantly different between the two groups respectively in each subgroup (**Tables 2**, **3**).

The FET outcomes of D3 subgroup were illustrated in Table 2. Patients in the D3 subgroup obtained comparable biochemical pregnancy rate (36.0% vs. 42.2%, *P*>0.05), clinical pregnancy rate (34.0% vs. 40.0%, *P*>0.05) and implantation rate (21.4% vs. 25.2%, *P*>0.05) for different E_2 levels. Better outcomes were achieved in D5 subgroup, and results of intra-group comparison were as follows: biochemical pregnancy rate (71.4% vs. 81.3%, *P*>0.05), clinical pregnancy rate (68.6% vs. 70.8%, *P*>0.05) and implantation rate (59.3% vs. 69.7%, *P*>0.05) (**Table 3**).

Controlling for confounding factors

Binary logistic regression using the enter method was produced to evaluate confounding variables in predicting positive pregnancy. Variables

Table 2. Stimulation and embryological characteristics in day 3 embryo transfer subgroup

Variable	Low E_2 group (n = 50)	High E_2 group (n = 60)	*P* value
Progynova duration (d)[a]	13.6±1.2	13.4±0.9	0.28
Total doses of Progynova (mg)[a]	72.0±8.7	70.9±7.5	0.50
Variation on progesterone administration day			
E_2 (pg/ml)[a]	163.8±49.9	216.9±97.1	0.001
P (ng/ml)[a]	0.46±0.38	0.47±0.37	0.82
Endometrial thickness	9.19±1.27	9.30±1.56	0.69
Variation on the day of embryo transfer			
E_2 (pg/ml)[a]	115.5±25.8	216.4±63.0	<0.001
P (ng/ml)[a]	16.13±8.14	15.67±8.90	0.78
No. of transfer embryos[a]	2.06±0.37	1.98±0.39	0.30
TDF (cm)[a]	1.27±0.34	1.29±0.27	0.82
Biochemical pregnancy rate[b]	36.0% (18/50)	42.2% (27/60)	0.34
Clinical pregnancy rate[b]	34.0% (17/50)	40.0% (24/60)	0.28
Implantation rate[b]	21.4% (22/103)	25.2% (30/119)	0.59

Note: Values are mean ± SD or number (percentage). E_2 = estrogen; P = progesterone; TDF = embryo transfer distance from the fundus. a. Data are presented as the mean ± SD. b. Data are presented as the percentage of the class (case number of the class).

Table 3. Stimulation and embryological characteristics in day 5 embryo transfer subgroup

Variable	Low E_2 group (n = 35)	High E_2 group (n = 48)	*P* value
Progynova duration (d)[a]	14.9±0.6	15.0±0.2	0.59
Total doses of Progynova (mg)[a]	79.7±3.5	80.0±1.2	0.59
Variation on progesterone administration day			
E_2 (pg/ml)[a]	147.7±39.8	221.1±92.8	<0.001
P (ng/ml)[a]	0.44±0.19	0.59±0.55	0.11
Endometrial thickness	9.35±1.39	9.47±1.37	0.71
Variation on the day of embryo transfer			
E_2 (pg/ml)[a]	110.0±26.1	226.0±472.1	<0.001
P (ng/ml)[a]	18.12±15.34	15.63±11.07	0.39
No. of transfer embryos[a]	1.69±0.47	1.57±0.50	0.45
TDF (cm)[a]	1.22±0.28	1.21±0.26	0.90
Biochemical pregnancy rate[b]	71.4% (25/35)	81.3% (39/48)	0.29
Clinical pregnancy rate[b]	68.6% (24/35)	70.8% (34/48)	0.82
Implantation rate[b]	59.3% (35/59)	59.7% (46/77)	0.96

Note: Values are mean ± SD or number (percentage). E_2 = estrogen; P = progesterone; TDF = embryo transfer distance from the fundus. a. Data are presented as the mean ± SD. b. Data are presented as the percentage of the class (case number of the class).

involved were as follows: age, concentrations of E_2 on the day of P administration and on the day of embryos transfer, concentrations of P on the day of embryos transfer, numbers of embryo transferred, TDF and endometrial thickness on the day of P administration. The serum E_2 levels on the day of embryo transfer were found to be not independently associated with positive clinical pregnancy (D3 subgroup: OR = 1.004, 95% CI: 0.997-1.011, *P* = 0.237; D5 subgroup: OR = 1.001, 95% CI: 0.994-1.09, *P* = 0.761). In D3 subgroup, logistic regression analysis revealed that age (OR = 0.836, 95% CI: 0.767-0.911, *P*<0.001) and P levels on the day of embryos transfer (OR = 1.061, 95% CI: 1.002-1.124, *P*<0.044) were independently associated with positive clinical pregnancy. In D5 subgroup, no varia-

Table 4. Binary logistic regression analysis of factors for prediction of a positive clinical pregnancy in FET cycles in two subgroups

Factors to predict a positive clinical pregnancy	B	Exp (B), 95% CI	*P*-value
Day 3 embryo subgroup			
Age	-0.179	0.836, 0.767-0.911	<0.001*
E_2 levels on the day of P administration	-0.001	0.999, 0.993-1.005	0.788
E_2 levels on the day of embryos transfer	0.004	1.004, 0.997-1.011	0.237
P levels the day of embryos transfer	0.059	1.061, 1.002-1.124	0.044*
Numbers of embryo transferred	0.745	2.106, 0.552-8.042	0.276
Embryo transfer distance from the fundus	0.284	1.329, 0.301-5.865	0.707
Endometrial thickness on the day of P administration	0.006	1.006, 0.732-1.382	0.972
Day 5 embryo subgroup			
Age	-0.032	0.969, 0.873-1.075	0.552
E_2 levels on the day of P administration	<0.001	1.000, 0.993-1.008	0.937
E_2 levels on the day of embryos transfer	0.002	1.001, 0.994-1.009	0.761
P levels the day of embryos transfer	-0.012	0.988, 0.951-1.028	0.558
Numbers of embryo transferred	0.533	1.704, 0.599-4.847	0.318
Embryo transfer distance from the fundus	-0.556	0.574, 0.083-3.971	0.573
Endometrial thickness on the day of P administration	0.336	1.399, 0.916-2.136	0.120

Note: *statistically significant. E_2 = estrogen; P = progesterone.

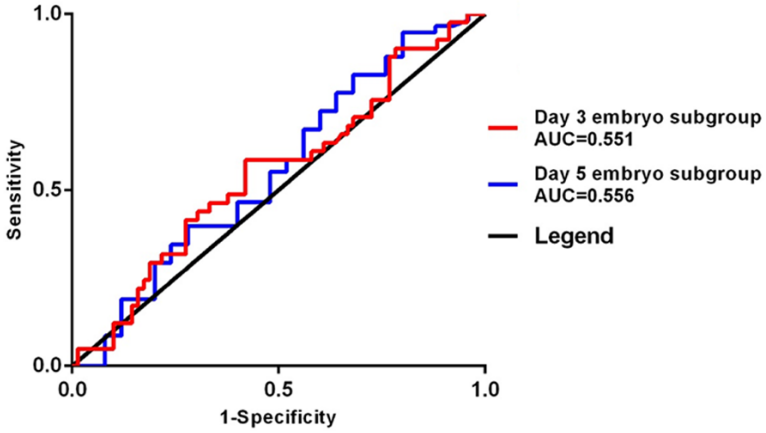

Figure2. Receiver operator characteristic (ROC) curve for the predictive value of serum E_2 levels on the day of embryo transfer for clinical pregnancy. Note: AUC = area under ROC curve.

bles were found to be independently associated with positive clinical pregnancy (**Table 4**).

ROC curves were constructed and the area under the curves can be seen in **Figure 2**. The areas under the curve examining E_2 levels on the day of embryo transfer as a predictor of clinical pregnancy were found to be 0.551 in D3 subgroup and be 0.556 in D5 subgroup. The low values for the AUC of the ROC curves indicated that the E_2 levels on the day of embryo transfer were not a good predictor for positive clinical pregnancy in each subgroup.

Discussion

The present study indicated that there was no correlation between the serum E_2 levels on the day of frozen-thawed embryo transfer and clinical pregnancy rate in HRT cycles. Results showed that low E_2 groups and high E_2 groups had similar biochemical pregnancy rate, clinical pregnancy rate and implantation rate. what's more, the low AUC of the ROC value suggested that E_2 levels on the day of embryo transfer cannot serve as an appropriate indicator for clinical pregnancy.

Thus, our results verified the results of previous studies that low serum E_2 levels were enough to access normal implantation with serum E_2 <50 pg/ml on oocyte donation cycles [11]. There is a strong evidence that the outcomes of FET cycle are closely linked to the synchronization between endometrial maturation and embryo development. Although currently there is no full agreement as to the impact of E_2 levels on the

results of FET cycle, the biochemical monitor of E_2 is routinely carried out on the day of frozen-thawed embryo transfer in HRT cycle [12, 13]. Previous studies reported that pinopode expression, a biological marker to 'locate implantation or nidation window', was affected by E_2 and P [14]. However, unlike P, E_2 is permissive but not essential for endometrial preparation and it regulates cytokines and adhesion molecules through endocrine and/or paracrine signals [15, 16]. Our statistical results accord with the theory and the permissive role played by E_2 may be the reason for the limited impact on the clinical pregnancy in FET cycle.

To our knowledge, there were some studies reported about the impact of serum E_2 on pregnancy rate in FET cycles and in fresh embryo transfer cycles. A recent study analyzed the relation between E_2 (late follicular phase) levels and pregnancy outcomes in HRT cycles. The results showed that late follicular phase serum E_2 levels were not able to predict pregnancy outcomes in HRT cycles [4]. In addition, another study on 1287 cycles demonstrated that serum E_2 levels had no impact on the outcomes of FET cycles when the endometrial thickness was between 7 mm to 15 mm [10]. In line with our data, several studies suggested that serum E_2 levels were an unimportant index to FET outcomes and there existed a wide range of E_2 levels to receive optimal endometrial receptivity [13, 17, 18]. what is noteworthy is that all these findings failed to examine the E_2 levels on the expected time of embryo transfer.

Here, we analyzed a selected IVF/ICSI population that was well-controlled for major variables known to affect outcomes. The endometrial thickness, P transformation and transfer skill were known to play important parts in clinical outcome, and they worked to the advantage of the present study. First, endometrial thickness over 8 mm on P administration day was conducted as one of the inclusion criteria. As a result, the average endometrial thicknesses were 9.31 mm in D3 subgroup and 9.47 mm in D5 subgroup respectively, which had reached the cut-off value of 9 mm obtained in other studies [9, 19, 20]. Second, although the P usage for luteal phase support in FET cycles is more complex and less conclusive, the P administration protocol we used was based on the literatures and our clinical experience [21]. We believed that the endometrium was fully transformed by P administration according to serum P levels (15.7 ng/ml in D3 subgroup and 17.2 ng/ml in D5 subgroup) on the day of embryo transfer in the recommended range of relevant studies [22]. Third, according to the previous researches, the recommended depth of embryo transferred from the fundus was over 1 cm. Here, all transplantations were carried out by experienced physicians and the average depth of embryo transferred was 1.25 cm from the fundus, which reduced the effect caused by the site of embryo transfer [23, 24]. The logistic regression analysis revealed that E_2 levels on the day of embryo transfer were not independently associated with positive clinical pregnancy (Day 3 embryo subgroup: OR = 1.004, 95% CI: 0.997-1.011, P = 0.237; Day 5 embryo subgroup: OR = 1.001, 95% CI: 0.994-1.09, P = 0.761).The result further proves that monitoring the E_2 levels on the day of embryo transfer cannot predict the clinical outcomes in HRT cycles.

Nevertheless, the present study was not without limitations. Besides the shortages of the retrospective study design, one main limitation of our study was the insufficient number of enrolled patients. Results are not generalizable to patients who use other methods of exogenous hormone replacement such as vaginally-delivered steroid hormones. Thus, better quality and larger randomized controlled trials are needed for further investigation.

In conclusion, we suggest that it is not necessary to monitor serum E_2 levels on the day of frozen-thawed embryo transfer neither for day 3 embryo transfer nor for day 5 embryo transfer in HRT cycles.

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Disclosure of conflict of interest

None.

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