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ORIGINAL RESEARCH

Association of Midluteal Serum Estradiol Level on Outcome of Hormone Replacement Therapy Frozen Embryo Transfer [HRT-FET] Cycles

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ABSTRACT

Background: Hormone Replacement Therapy-Frozen Embryo Transfer (HRT-FET) cycles are widely used in assisted reproductive technology, yet the optimal midluteal estradiol (E2) level on embryo transfer day remains unclear. **Aim:** To evaluate whether serum E2 levels on the day of embryo transfer influence clinical outcomes in HRT-FET cycles. **Settings and Design:** This prospective observational study was conducted from July to December 2024 at a tertiary reproductive center in India. **Materials and Methods:** Fifty infertile women were allocated to either Day 3 (n=14) & Day 5 (n=36) embryo transfers. After downregulation, endometrial preparation was performed with oral estradiol valerate, followed by daily intramuscular progesterone. Participants were stratified into low (≤187 pg/mL) or high (>187 pg/mL) E2 groups on embryo transfer day. Clinical pregnancy rate (CPR) and implantation rate (IR) were measured. **Statistical Analysis Used:** Data analysis was conducted using SPSS version 29. Groups were compared using independent t-tests, Mann–Whitney U, and chi-square tests. Binary logistic regression assessed associations between E2 levels and other parameters. A p-value <0.05 was considered significant. **Results:** In Day 5 transfers, the high E2 group had higher CPR (46.6% vs. 28.57%) and IR (28% vs. 15%), though differences were not statistically significant. Serum E2 was negatively correlated with BMI (p=0.044) and positively correlated with endometrial thickness (p=0.012). E2 showed a significant association with CPR (p=0.039). **Conclusions:** This study emphasizes that the higher midluteal estradiol levels on embryo transfer day may improve pregnancy outcomes in HRT-FET cycles, especially in Day 5 transfers.

Keywords: Hormone Replacement Therapy, Frozen Embryo Transfer, Estradiol Levels, Clinical Pregnancy Rate, Endometrial Preparation

INTRODUCTION

Frozen Embryo Transfer (FET) cycles have become a cornerstone of assisted reproductive technology (ART), offering significant advantages over fresh embryo transfer cycles [1,2]. Improvements in cryopreservation techniques, such as vitrification, have not only increased embryo survival rates but also enhanced pregnancy and live birth outcomes [3]. The success of FET cycles is primarily influenced by three key factors: embryo quality, endometrial receptivity and embryo transfer technique. [4]. There are various protocols for endometrial preparation in FET cycles, among which Hormone Replacement Treatment cycle using exogenous estradiol and progesterone is most commonly used method, in which exogenous estradiol can be administered as oral tablets, patches or vaginal pessaries however no scientific data favors superiority of one route of administration over another[5,6].

Vyas et al. highlighted a bell-shaped relationship between estradiol (E2) levels and reproductive outcomes, where both very high and very low levels reduce success. While estradiol's role in preparing the endometrium is well known, the optimal serum levels on embryo transfer day for the best clinical outcomes

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remain uncertain[7]. Existing studies have predominantly focused on estradiol levels during the early luteal phase or at the time of progesterone initiation. For instance, Alsbjerg et al. [8] demonstrated a positive association between midluteal estradiol levels and live birth rates, emphasizing estradiol's role in endometrial receptivity.Similarly, Haddad et al. [9] reported higher live birth rates (63.4%) when estradiol levels ranged between 300–500 pg/mL one day before euploid blastocyst transfer cycles.

Despite these findings, there remains a paucity of data specifically addressing estradiol levels on the day of embryo transfer. This gap in knowledge is critical, as serum estradiol levels on the transfer day may better reflect the endometrial environment immediately preceding implantation.

This study aims to evaluate the association between serum estradiol level measured on the day of embryo transfer and clinical outcomes in HRT-FET cycles. By categorizing patients into low and high estradiol groups, we seek to determine whether estradiol levels on the transfer day influence key outcomes such as clinical pregnancy and implantation rates. Understanding this relationship could provide valuable insights into hormonal optimization strategies, ultimately improving the success rates of FET cycles and advancing personalized approaches in ART.

MATERIAL AND METHODS

Study Population

This prospective observational study was conducted at the Department of Reproductive Medicine and Surgery, National Institute of Medical Sciences and Research, Jaipur, and Evaa Hospital (a unit of NIMS) from July 2024 to December 2024. The study duration was six months. Infertile couples attending the outpatient department were recruited after obtaining written informed consent.

Eligibility Criteria

The study included infertile women eligible for Day 3 or Day 5 frozen embryo transfer, where metaphase II (M2) oocytes had been fertilized through intracytoplasmic sperm injection (ICSI). Participants were aged between 21 and 45 years, had at least one high-quality cleavage-stage or blastocyst-stage embryo available for transfer, had a body mass index (BMI) ranging from 19 to 29.9 kg/m², and had undergone counseling for in vitro fertilization (IVF).

Exclusion criteria included the presence of hydrosalpinx, uterine abnormalities likely to impair embryo implantation, persistently thin endometrium (<7 mm) after 18 days of estradiol treatment, difficult embryo transfer, refusal to participate in the study, uncontrolled maternal medical conditions.

Endometrial Preparation

Endometrial preparation followed our hospital protocol. On Day 2 or 3 of the menstrual cycle, after downregulation with intramuscular injection of leuprolide acetate 3.75 mg administered on Day 21 of the previous cycle, baseline transvaginal ultrasonography (TVS) was performed. Endometrial preparation began with oral estradiol valerate 2 mg three times daily. On Day 8 of estradiol administration, endometrial thickness was assessed via TVS. If the thickness was less than 7 mm, the dose of estradiol valerate was increased to 2 mg four times daily.

When the endometrium reached a thickness of ≥7 mm with good vascularity, blood samples were collected for estradiol and progesterone levels, which were analyzed using chemiluminescence immunoassay (CLIA). Subsequently, intramuscular injections of progesterone 100 mg were administered daily.

Embryo Transfer

On Day 4 or 6 of progesterone administration, blood samples were collected again to measure serum estradiol and progesterone levels. Cryopreserved embryos were thawed blastocystswere scored according to theGardner and Schoolcraft grading systemand top-quality blastocysts 3AA, 3AB, 3BA, 4AA, 4AB, 4BA, 5AA, 5AB, and 5BA were transferred under transabdominal ultrasound guidance [10].

According to the Istanbul consensus opinion, the highest-quality Day-3 embryo as having 8 equally sized mononucleated blastomeres with 0-10% fragmentation was transferred under transabdominal ultrasound guidance[11].Post-transfer, oral estradiol valerate was continued alongside intramuscular progesterone 100 mg daily, oral dydrogesterone 10 mg three times daily, and natural micronized progesterone gel (8% w/w, 90 mg) administered vaginally at bedtime. Serum beta-hCG levels were assessed 14 days post-transfer. Women with positive beta-hCG results were followed up with an early pregnancy scan.

Cycle Outcomes

The clinical pregnancy rate was calculated as the number of cycles resulting in clinical pregnancy divided by the total number of embryo transfer cycles, multiplied by 100. The implantation rate was defined as the number of gestational sacs observed on ultrasound divided by the total number of embryos transferred, multiplied by 100.

Data Analysis

Statistical analysis was performed using SPSS version 29 and Microsoft Excel. The Kolmogorov-Smirnov test was used to assess the normality of data distribution. Continuous variables were presented as means with standard deviations (SD) or medians with interquartile ranges (IQR), while categorical variables were expressed as frequencies and percentages. Differences between the low estradiol (E2) and high estradiol (E2) groups were evaluated using

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independent t-tests or Mann-Whitney U tests for continuous variables, and chi-square (χ^2) tests for categorical variables. Binary logistic regression analysis was conducted to identify predictors of clinical pregnancy success in frozen embryo transfer (FET) cycles. A p-value of <0.05 was considered statistically significant.

RESULTS

Baseline characteristics of the study participants

A total of 50 patients were included in this study, comprising 14 (28%) in the Day 3 embryo transfer subgroup and 36 (72%) in the Day 5 embryo transfer subgroup. Patients having serum estradiol level ≤ 187 pg/ml on the day of embryo transfer were categorized in the low estradiol (Low E2) group, and >187 pg/ml were categorized in the high estradiol (High E2) group respectively. In both Day 3 and Day 5 embryo transfer subgroups, no significant differences were observed between the low and high estradiol groups in terms of age, Body mass index, type of infertility and indications for in vitro fertilization, except for the duration of infertility in the Day 3 embryo transfer subgroup **(Table 1)**.

Table 1: Baseline characteristics of the study population

Variable	Low E2 group (N=4)	High E2 group (N=10)	*p*-value
Day 3 embryo subgroup	***total number of cases = 14 (28 %)***		
No. of cases (%)	04 (28.57 %)	10 (71.43 %)	
Age (Years)	33.25 ± 1.89	32.50 ± 1.43	0.433
BMI (kg/m^2)	26.53 ± 0.672	27.46 ± 1.27	0.154
Duration of infertility (years)	9.00 ± 1.15	7.00 ± 1.15	**0.013**
Type of Infertility, n (%)			
Primary	03 (75 %)	0 5 (50 %)	0.411
Secondary	01 (25 %)	0 5 (50 %)	
Indication of IVF, n (%)			
Ovulatory disorders	01 (25 %)	03 (30 %)	
Endometriosis	01 (25 %)	01 (10 %)	
Factor (Male)	00	0 1 (10 %)	0.847
Tubal factor	01 (25 %)	0 1(10 %)	
Unexplained	01 (25 %)	0 4 (40 %)	
Day 5 embryo subgroup	***total number of cases = 36 (72 %)***		
No. of cases (%)	21 (58.33 %)	15 (41.67 %)	
Age (Years)	33.90 ± 5.78	34.73 ± 3.34	0.622
BMI (kg/m^2)	27.09 ± 1.06	26.80 ± 1.48	0.505
Duration of infertility (years)	6.62 ± 2.88	7.80 ± 2.33	0.200
Types of Infertility, n (%)			0.425
Primary	14 (66.67 %)	08 (53.33 %)	
Secondary	07 (33.33 %)	07 (46.67 %)	
Indication of IVF, n (%)			0.620
Ovulatory disorders	09 (42.86 %)	08 (53.33 %)	
Endometriosis	02 (9.52 %)	01 (6.67 %)	
Factor (Male)	02 (9.52 %)	02 (13.33%)	
Tubal factor	01 (4.77 %)	01 (6.67 %)	
Unexplained	07 (33.33 %)	03 (20.00 %)	

BMI – Body mass index, **IVF-** In-vitro fertilization
**p*-value considered statistically significant at < 0.05. The *p-values* of significant variables were bolded.

Endocrinal and Embryological Characteristics

Day 3 Embryo Transfer Subgroup:

The low E2 group had significantly lower serum estradiol levels on Day 0 of progesterone initiation (298.75 ± 142.69 pg/ml vs. 534.3 ± 114.9 pg/ml, p=0.007) and on the day of embryo transfer (154.75 ± 18.46 pg/ml vs. 412.6 ± 93.8 pg/ml, p<0.001). Implantation (9% vs. 16%, p=0.059) and clinical pregnancy rates (25% vs. 40%, p=0.081) were lower in the low E2 group, though not statistically significant **(Table 2).**

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

Variable	Low E2 group (n=4)	High E2 group (n=10)	*p*-value
Serum Estradiol on day 0 of progesterone initiation (pg/ml)	298.75 ± 142.69	534.3 ± 114.9	**0.007**
Serum progesterone on day 0 of	0.69 ± 0.14	0.98 ± 0.47	0.263

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progesterone initiation (ng/ml)			
Endometrial thickness on day 0 progesterone initiation (mm)	9.68 ± 1.59	10.12 ± 0.42	0.600
Serum Estradiol on the day of embryo transfer (pg/ml)	154.75 ± 18.46	412.6 ± 93.8	**<0.001**
Serum Progesterone on the day of embryo transfer (ng/ml)	14.50 ± 3.10	16.69 ± 5.29	0.460
Number of embryos transferred	2.75 ± 0.50	2.50 ± 0.52	0.433
Clinical pregnancy rate	25 %	40 %	0.081
Implantation rate	9 %	16 %	0.059
*p-value considered statistically significant at < 0.05. The *p-values* of significant variables were bolded.			

Day 5 Embryo Transfer Subgroup

Serum estradiol levels were significantly lower in the low E2 group on Day 0 of progesterone initiation (240.47 ± 168.16 pg/ml vs. 3 63.0 ± 114.94 pg/ml, p=0.020) and on the day of embryo transfer (139.19 ± 31.76 pg/ml vs. 326.73 ± 120.7 pg/ml, p=0.001). Implantation (15% vs. 28%, p=0.450) and clinical pregnancy rates (28.57% vs. 46.6%, p=0.320) were higher in the high E2 group but not statistically significant **(Table3)**.

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

Variable	Low E2 group (n=21)	High E2 group (n=15)	*p*-value
Serum Estradiol on Day 0 of progesterone initiation (pg/ml)	240.47 ± 168.16	363.0 ± 114.94	**0.020**
Serum progesterone on day 0 of progesterone initiation (ng/ml)	0.30 ± 0.30	0.40 ± 0.33	0.377
Endometrial thickness on day 0 progesterone initiation (mm)	10.49 ± 0.56	10.87 ± 0.84	0.117
Serum Estradiol on the day of embryo transfer (pg/ml)	139.19 ± 31.76	326.73 ± 120.7	**0.001**
Serum Progesterone on the day of embryo transfer (ng/ml)	17.42 ± 5.02	16.61 ± 3.64	0.599
Number of embryos transferred	1.90 ± 0.30	1.67 ± 0.48	0.079
Clinical pregnancy rate	28.57 %	46.6 %	0.320
Implantation rate	15 %	28 %	0.450
*p-value considered statistically significant at < 0.05. The *p-values* of significant variables were bolded.			

Impact of midluteal E2 levels on HRT-FET outcomes

Regression analysis revealed a significant negative association between BMI and serum estradiol (p=0.044), while endometrial thickness showed a significant positive association (p=0.012). CPR also demonstrated a significant positive association with serum estradiol (p=0.039), whereas IR did not show a significant association (p=0.872). Other variables, including age and infertility duration, were not significantly associated with serum estradiol levels **(Table 4).**

Table4. Association of midluteal serum estradiol level on outcome of hormone replacement therapy frozen embryo transfer

Variable	B	Beta	t	95% CI	p-value
Age (years)	6.165	0.391	1.482	-2.669 to 15.82	0.349
BMI	-20.037	-0.331	-2.104	-39.489 to -0.585	**0.044**
Infertility Duration	5.021	0.288	1.865	-0.478 to 10.519	0.072
Endometrial Thickness	76.041	0.439	2.665	17.778 to 134.305	**0.012**
IR	6.935	0.028	0.162	-79.933 to 93.803	0.872
CPR	95.161	0.382	2.154	4.921 to 185.401	**0.039**
BMI-Body mass index**, IR**-Implantation rate**, CPR**- Clinical pregnancy rate, **CI**-Confidence interval *p-value considered statistically significant at < 0.05. The *p-values* of significant variables were bolded.					

DISCUSSION

This study investigated the impact of midluteal serum estradiol (E2) levels on clinical outcomes in Hormone Replacement Therapy-Frozen Embryo Transfer (HRT-FET) cycles. Participants were divided into two groups based on serum estradiol levels on the day of embryo transfer: low E2 (≤187 pg/mL) and high E2 (>187 pg/mL). The results revealed that the high E2 group had a higher clinical pregnancy rate (46.6%)

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compared to the low E2 group (28.57%) in the Day 5 embryo transfer subgroup.

While limited research directly addresses the relationship between serum estradiol levels on the day of embryo transfer and HRT-FET outcomes, our findings are consistent with previous studies. Alsbjerg et al. [8] demonstrated a positive correlation between elevated midluteal estradiol levels (292–409 pg/mL) and live birth rates (59%) following blastocyst transfer, emphasizing estradiol's role in endometrial preparation for implantation. Similarly, Haddad et al. [9] found that estradiol levels of 300–500 pg/mL one day before euploid blastocyst transfer were associated with a 63.4% live birth rate, further supporting estradiol's critical role in enhancing endometrial receptivity.

Currently, there is no standardized cut-off for serum estradiol levels on the day of embryo transfer. Zhihong Niu et al. [12] reported no significant difference in pregnancy rates (32.2%, 38.4%, and 36.3%) across serum estradiol levels in the 0–25th, 25–75th, and 75–100th percentiles measured on the day of progesterone initiation, suggesting that serum estradiol levels do not predict pregnancy success in HRT-FET cycles. Similarly, Tomar et al. [13] found no significant relationship between clinical pregnancy rates (68.8%, 78.1%, and 75%) or implantation rates (36.98%, 32.03%, and 29.69%) across the 0-25th,25-75th, and more than 75thpercentiles of estradiol levels on the day progesterone was initiated. Our study highlights that while endometrial thickness was comparable between the two groups, estradiol's impact may extend to molecular mechanisms that enhance uterine receptivity.

According to Qing Li et al. [14], estradiol levels on the day of progesterone initiation were notably lower in the ongoing pregnancy/live birth (OP/LB) group compared to the non-OP/LB group during cleavage-stage embryo transfers (214.75 ± 173.47 pg/mL vs. 253.20 ± 203.30 pg/mL; $P = 0.023$). In contrast, for blastocyst-stage embryo transfers, no significant variation in estradiol levels was detected between the OP/LB and non-OP/LB groups.Similarly, in our study, no significant differences were observed in clinical pregnancy rates ($P = 0.320$) and implantation rates ($P = 0.450$) between the low and high estradiol groups in blastocyst-stage embryo transfers.

Conversely, Katherine L et al. [15] observed significantly higher estradiol levels on the day of euploid frozen embryo transfer in nonpregnant patients (610.9 pg/mL) compared to pregnant patients (373.3 pg/mL), suggesting that supraphysiological estradiol levels might reduce the success of euploid single embryo transfers. They also found that endometrial thickness before progesterone initiation was significantly greater in pregnant patients.

Aysen Yuccturk et al. [16] reported that in HRT-FET cycles, serum estradiol levels >204.0 pg/mL on the day of progesterone initiation were associated with clinical pregnancy. However, estradiol levels measured at the start of the menstrual cycle, on the day of embryo transfer, and three days post-transfer showed no significant differences between pregnant and nonpregnant patients. Nicholson et al. [17] found that estradiol level at the 2nd quintile (between 219–316 pg/mL) on the day of progesterone initiation were associated with the highest clinical pregnancy (61.1%) and live birth rates (50%) in patients receiving oral estrogen.

Our findings support the hypothesis that serum estradiol levels on the day of embryo transfer influence clinical pregnancy rates in HRT-FET cycles. While existing evidence highlights the complex role of estradiol in endometrial receptivity, further research is required to establish standardized cut off values and elucidate the molecular pathways involved.This study's strength includes its prospective design, targeted focus on serum estradiol levels on embryo transfer day, comprehensive outcome measures, robust statistical analyses and subgroup comparisons (Day 3 vs. Day 5 transfers) enhance its reliability. Conducted in a real-world clinical setting, the findings are highly relevant to practical ART applications.

Limitations

One of the limitations of our study is the relatively small sample size, especially in the Day 3 embryo transfer subgroup, which may have reduced the statistical power to detect differences in some variables, particularly implantation and clinical pregnancy rate. Future studies should incorporate larger sample sizes and consider additional biomarkers of endometrial receptivity, as well as the molecular mechanisms through which Estradiol affects implantation and pregnancy success.

CONCLUSION

This study highlights the significant role of serum estradiol levels during hormone replacement therapy frozen embryo transfer (HRT-FET) cycles in influencing clinical and embryological outcomes. Higher estradiol levels were associated with improved implantation and clinical pregnancy rates, particularly in the Day 5 embryo transfer subgroup, although not all differences reached statistical significance. These findings underscore the importance of optimizing hormonal parameters to enhance clinical pregnancy outcomes in FET cycles. Further research with larger sample sizes is warranted to validate these results and refine treatment protocols.

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