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ASSISTED REPRODUCTION TECHNOLOGIES

Impact of serum estradiol levels on the implantation rate of cleavage stage cryopreserved-thawed embryos transferred in programmed cycles with exogenous hormonal replacement

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Abstract
Purpose To investigate the impact of late follicular phase serum estradiol (E_2) levels on implantation and pregnancy outcomes of cleavage stage cryopreserved/thawed embryos transferred in programmed cycles with exogenous hormonal replacement.
Methods Retrospective cohort analysis of IVF patients with transfer of cryopreserved-thawed day-3 embryos in E_2 and progesterone (P4) supplemented cycles (n=208 cycles). Main outcome measures: implantation and pregnancy rates according to late follicular phase serum E_2 levels and early secretory phase E_2/P4 ratios.
Results Logistic regression performed for embryo implantation and for pregnancy outcome in relation to E_2 (day 15), P4 (day 15 and 16), before (crude analysis) and after adjustment (adjusted analysis) for baseline characteristics (including age, BMI, serum basal cycle day 3 FSH levels, embryo quality, endometrial lining thickness) showed no significant association. Similarly, ROC analysis showed no impact of cycle day 16 E_2/P4 ratio.
Conclusions Neither late follicular phase serum E_2 nor the early E_2/P4 ratio were able to predict implantation or pregnancy outcome of day-3 cryopreserved-thawed embryos transferred in artificially programmed cycles.

Capsule This retrospective cohort study showed that neither late follicular phase serum E_2 nor the early E_2/P4 ratio were able to predict implantation or pregnancy outcome of day-3 cryopreserved-thawed embryos transferred in artificially programmed cycles.

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Introduction

Embryo cryopreservation represents a remarkable achievement in the IVF setting. It provides multiple management advantages [i] limiting the number of embryos transferred to reduce the incidence of multiple pregnancy; [ii] enhancing couples' chances of pregnancy by allowing multiple transfers originating from a single stimulated cycle (thereby optimizing their total reproductive potential); and [iii] aiding in the clinical management of OHSS [1–3].

There are various clinical protocols for the transfer of frozen-thawed embryos [4]. However, in spite of the successful use of embryo cryopreservation for over two decades there is little consensus on the most effective method of endometrial preparation for the transfer cycle. A recent meta-analysis compared results of natural (spontaneous cycles with or without progesterone [P4] supplementation) versus artificial (estrogen and progesterone hormone supplementation) cycles, and also with/without adjuvant utilization with a GnRH agonist, and reported no differences in the clinical pregnancy, ongoing pregnancy or live birth rates for any treatment group [5].

Our program has published similar outcomes in natural versus artificial cycles for the transfer of frozen/thawed pronuclear and cleavage-stage embryos [2, 6–8]. In presumably ovulatory women, the natural cycle is appealing because of no hormonal intervention, but requires thorough monitoring of peri-ovulatory events, and there are occasional cancelations due to lack of ovulation. On the other hand, the use of programmed cycles with exogenous hormonal replacement provides a more efficient scheduling alternative and is efficient for both ovulatory and anovulatory women, with the

disadvantage of using additional medication, and sporadic cancelations due to unwanted ovulation.

There is also much debate about the impact of hyperestrogenism on endometrial development and implantation, particularly when controlled ovarian hyperstimulation is used in conjunction with fresh embryo transfers [9–11]. Data from our program has provided evidence that high levels of E_2 and also a high cumulative E_2 exposure during the entire follicular phase of gonadotropin-stimulated IVF cycles may have detrimental effects on implantation [12, 13].

Here, the main objective of this study was to investigate the impact of variable serum E_2 levels achieved in the late follicular phase on implantation and pregnancy outcomes of cleavage stage cryopreserved/thawed embryos transferred in programmed cycles with exogenous hormonal replacement.

Materials and methods

We evaluated consecutive transfer cycles after embryo cryopreservation/thawing performed during a 5-year period in our program between 2008 and 2012. We examined the first cryopreservation/thawing cycle after the fresh IVF attempt, which occurred within a 4-year period. Previous data from our laboratories has shown no impact of time of embryo storage on clinical outcomes [14]. A total of 208 consecutive transfer cycles that met the following criteria were included for analysis: women under 43 years of age, a normal uterus (i.e., no fibroids or other structural anomalies) with a normal cavity (as determined by hysterosalpingography, saline infusion sonogram [2D and/or 3D] and/or hysteroscopy), a trilaminar endometrial pattern on cycle day 15 [15], and having had a transfer of 2 good quality day-3 embryos after thawing as determined by number of blastomeres ($n \geq 6$) and morphology score (≥ 3). These inclusion criteria were established in order to control for uterine (normality) and embryonic (number and quality of transferred embryos) factors, in order to optimize the analysis of the impact of variable individual serum steroids on clinical outcomes.

During the time of this study it was the policy of our program to cryopreserve surplus embryos on day 3 (the day of fresh transfer) for future use, and to transfer a maximum of 2 embryos per attempt [16]. All fresh IVF cycles were performed using gonadotropin stimulation with GnRH analogue adjuvant therapy as described elsewhere [3, 17]. The institutional review board of Eastern Virginia Medical School approved this study.

Cryopreservation of embryos was performed at the cleaving (day-3) stage according to a slow-freeze, slow-thaw protocol with a programmed cell freezer (Planer Kryo 10–1.7; T.S. Scientific, Perkasie, PA) using 1.5 mol/L 1,2 propanediol as the cryoprotectant with the addition of sucrose (0.2 mol/L) [3, 13, 14, 18]. Specimens were kept in cryovials containing 0.3 mL of cryoprotective medium and maintained at −196 °C under liquid nitrogen in storage tanks (35 VHC liquid nitrogen storage tank; Taylor-Warton Cryogenic Equipment, Indianapolis, IN). Slow thawing of the embryos to room temperature was performed in a 37 °C water bath for 1 min, followed by 5 min at room temperature followed by dilution of the cryoprotectant and transfer of the specimen to equilibrated culture medium and incubation at 37 °C under 5 % CO_2 in air until cleavage was established. Post-thaw morphological survival of day-3 embryos was defined as surviving of >50 % intact blastomeres and identification of zona pellucida intactness.

No oral contraceptive pills or down regulation with a GnRH agonist were used. Artificial cycle preparation was based on our initial publications [6, 8, 19] and subsequent modifications [2, 3, 14]. Patients were prepared for embryo transfer in all cycles with a fixed protocol as follows. Transdermal 17β-E_2 patches were used (Vivelle Dot; Novogyne Pharmaceuticals, Miami, FL; each patch delivering 0.1 mg/day of E_2) and replaced every other day. On day 1, two patches were applied, and then E_2 administration was gradually increased on cycle days 7 (to three patches every other day) and on cycle day 11 (to four patches every other day). In addition, from cycle day 12 and every other day, 1 mg 17β-E_2 (Estrace; Warner Chilcot, Rockaway, NJ) was started vaginally and continued with the same dose every other alternate day to the estrogen patch. From cycle day 15, the transdermal E_2 dose was decreased (to two patches every other day) alternating with the vaginal Estrace pills (at the same dose). From cycle day 15 P4 was initiated with either vaginal micronized P4 (200 mg/tid, Prometrium; Solvay Pharmaceutical, Baudette, MN) or IM P4 (50 mg per day in oil) according to physicians' preference. Embryos were thawed in the afternoon of day 17 and transferred on day 18. The steroid regimen was continued until week 9 of pregnancy.

On the day of embryo transfer, embryo quality (cleavage and morphology) was re-examined assigning the best quality embryos by a score of 5 and poorest quality embryos by 1, a modification of the criteria of Veeck [20]. An individual embryo quality score was calculated by multiplying the number of blastomeres times the morphology grade. A cumulative embryo score per transfer was calculated by adding the scores of all individual embryos and was then divided by the number of embryos to obtain a "mean score of transferred embryos" (MSTE) [13].

Patients' serum E_2 and P4 levels were measured in the morning of day 15 (late follicular phase, the day of afternoon P4 initiation) and 16 (first day of secretory phase) with a solid-phase enzyme-labeled chemiluminescent competitive immunoassay (Immulite 1000, Siemens Healthcare Diagnostics, Malvern, PA). The intra-assay coefficients of variation were 6.3 % and 5.5 % for E_2 and P4, respectively. The inter-assay

coefficients of variation were 6.4 % for both E_2 and P4. The lower limits of sensitivity were as follows: E_2>20 pg/mL, and P4=0.2 ng/mL respectively.

The endometrial lining was measured in the morning of cycle day 15 using a transvaginal approach and lining thickness (in mm) and pattern (trilaminar in all cases) were determined [15]. In all cases two embryos were transferred to the uterus under transabdominal ultrasonography using a soft pass catheter (Softpass Embryo Transfer Catheter, Cook Ob/Gyn, Spencer, Indiana, USA) [21]. The implantation rate was defined as the number of gestational sacs divided by the number of embryos transferred. A clinical miscarriage was established as a pregnancy loss after identification of an intrauterine gestational sac. A pregnancy was defined as a term delivery.

Statistical analysis

This was a retrospective cohort analysis. IVF data are routinely stored in a database accessed by one dedicated operator, including embryology data and steroid hormone levels. Laboratory values are directly reported into the database, and verified in the electronic medical records as appropriate. Patients identified following inclusion/exclusion criteria were sorted and their data analyzed by an independent biostatistician (HB).

Statistical analyses were performed using SAS version 9.2 (SAS Institute, Cary, NC). An initial power analysis was performed based on serum estradiol levels and pregnancy rate to determine the sample size needed to achieve a beta power of 80 %. Data were described using mean and standard deviation for continuous variables as well as frequencies and percentages for categorical variables. Bivariate associations were examined using Pearson's Chi-square test, independent samples *t*-test, Wilcoxon's rank-sum test or Kruskal-Wallis test, as appropriate. Ordinal logistic regression models were constructed to estimated odds ratios (OR) and 95 % confidence intervals (CI) for the hypothesized relationships, after controlling for confounders. Receiver operating characteristics (ROC) curves were calculated as appropriate. All statistical tests were two-sided and P value<0.05 was considered statistically significant. Data are presented as mean ± standard deviation.

Results

Demographic data for patients divided into 86 pregnant (74 ongoing pregnancies and 12 miscarriages) and non-pregnant (n=122) groups are shown in Table 1. There were no statistical differences among groups regarding age, BMI, basal cycle day 3 serum FSH levels, day 15 endometrial lining thickness, cycle days 15 and 16 E_2 and P4 serum levels, MSET, and number of transferred embryos. The overall implantation rate (208 transfer cycles) was 41 %, the clinical pregnancy rate was 41.4 %, and the delivery rate was 35.6 %. For cycle day 15, E_2 levels were 1001.6±529.1 pg/ml (range 177–2788) and 949.4±517.8 pg/ml (range 88–2663) for pregnant and non-pregnant groups, respectively (not significant). For cycle day 16, P4 levels were 15.1±11.3 ng/ml (range 5.9–104.0) and 15.9±7.0 ng/ml (range 5.1–48.2), for pregnant and non-pregnant groups, respectively (not significant).

Ordinal logistic regression was performed for embryo implantation (0, 1 or 2 embryos implanted) and for pregnancy outcome (yes, no, miscarriage) in relation to E_2 (day 15 and 16) and P4 (day 15 and 16), before (crude analysis) and after adjustment (adjusted analysis) for baseline characteristics including age, BMI, serum basal cycle day 3 FSH levels, embryo quality, endometrial lining thickness (full model with proportional odds assumption met). Odd s ratios and 95 % CI results showed no significant association between E_2 levels in late follicular phase (day 15), or between day 16 serum E_2 or P4 levels or E2/P4 ratio, and implantation, miscarriage or pregnancy outcomes (Table 2).

There were no significant differences in endometrial thickness according to implantation or pregnancy status. There was no correlation between E_2 levels on day 15 and endometrial thickness. There was no minimal endometrial lining thickness (mm) that could be statistically used as a cut-off for successful implantation or pregnancy. Implantation and ongoing pregnancies were successfully established with lowest E_2 levels on cycle day 15 of 177 pg/ml and also with a minimal endometrial thickness of 6 mm.

A sub-analysis performed according to the type of P4 preparation used (vaginal versus IM) revealed significant differences in serum levels on cycle day 16 (vaginal, n=108, 13±10 [range = 5–104 ng/ml] versus IM, n=102, 17.7±5.6 [range 5.6–48 ng/ml], P<0.0001). Furthermore, there were a significant difference in the likelihood of achieving a pregnancy when P4 delivery types were compared (vaginal vs. IM: 51.9 % vs. 66.0 %; OR=1.80, 95 % CI: 1.03–3.16).

Discussion

Here we analyzed consecutive transfer cycles of cryopreserved-thawed day-3 embryos and controlled for major variables affecting implantation, i.e., uterine and embryonic factors, in order to determine the impact of variable serum late follicular E_2 levels and early E_2/P4 ratios on implantation. Results demonstrated that using a fixed follicular phase supplementation E_2 regimen (with E_2 delivered transdermally and vaginally), a wide range of achieved E_2 levels were not associated with statistical changes in implantation or pregnancy rates. Neither were early secretory phase E_2/P4 ratios. In addition, there was no correlation of serum E_2 levels

Table 1 Patients' demographics by pregnancy status

	Pregnant (n=86)	Not-pregnant (n=122)	P-value
Age (years)			
Mean ± SD	31.3±5.5	31.9±5.5	0.11
< 30 (%)	46.5	40.2	0.66
30–39 (%)	43.0	48.4	
≥ 40 (%)	10.5	11.5	
BMI (kg/m^2)	24.6±4.7	25.8±5.1	0.10
< 25 (%)	65.1	59.8	0.09
25–29 (%)	27.9	22.9	
≥ 30 (%)	6.9	17.2	
Basal day 3 FSH levels (mIU/ml)	6.4±2.4	2.8±2.4	0.33
Cycle day 15 E_2 levels (pg/ml)	1001.6±529.1	949.4±517.8	0.48
Cycle day 15 P_4 levels (ng/ml)	1.13±0.99	1.09±0.58	0.76
Cycle day 16 E_2 levels (pg/ml)	557.1±423.8	560.6±433.2	0.78
Cycle day 16 P_4 levels (ng/ml)	15.1±11.3	15.9±7.0	0.58
Endometrial lining (mm)	9.9±2.3	9.5±2.1	0.29
Embryo score (MSET)	29.2±5.4	27.9±5.2	0.09

and endometrial lining thickness, or between endometrial lining and implantation (all patients having a trilaminar endometrial pattern as per study design).

Thus our results confirmed and extended previous data published on an unselected population of recipients of egg donation undergoing transfer of fresh embryos with a different hormonal supplementation regimen of estrogen and P4, where neither E_2 levels nor endometrial lining thickness were associated with pregnancy outcome [22]. Here we analyzed a selected IVF population that was well-controlled for major variables known to affect outcomes. However we acknowledge some limitations of the study. This was a retrospective cohort analysis. Results are not generalizable to patients using other methods of follicular and luteal support. Also in women receiving vaginal steroids, serum E_2 and P4 concentrations do not reflect the in situ endometrial concentration from vaginally-delivered steroid hormones due to the first uterine pass effect [23]. Moreover, serum steroid levels were not examined during the expected time of implantation, and for obvious reasons no histological correlations could be obtained.

Table 2 Ordinal logistic regression for pregnancy outcome (miscarriage, not pregnant, pregnant) in relation to E_2 (Day 15), P4 (Day 15) and P4 (Day 16), before and after adjustment for baseline characteristics

	Continuous outcome		≥ Median vs. < Median	
	OR	95 % CI	OR	95 % CI
Crude Analysis:				
E2 (Day 15)	1.00	0.99–1.00	1.27	0.74–2.18
P4 (Day 15)	1.12	0.78–1.60	1.15	0.67–1.98
P4 (Day 16)	0.99	0.97–1.03	1.26	0.73–2.17
Adjusted Analyses[a]:				
E2 (Day 15)	1.00	0.99–1.00	1.49	0.82–2.72
P4 (Day 15)	1.05	0.73–1.49	0.95	0.53–1.69
P4 (Day 16)	1.00	0.97–1.04	1.45	0.80–2.61

[a] Adjusted for age (years), BMI (kg/m^2) and embryo score

OR odds ratio, 95 % *CI* confidence interval

There is strong evidence that a temporal window of maximal endometrial receptivity exists. Although there is still not full agreement as to the exact timing of embryo implantation in the human, clinical studies suggest that the window is temporally confined to days 20–24 of a normal, ovulatory cycle [24] Data from the assisted reproduction setting have demonstrated that the optimal time for embryo transfer to the uterus is ≤3 days, the so-called 'window of receptivity' [25]. The endometrial receptive state depends on a strict temporal sequence of E_2 priming that induces endometrial proliferation followed by P4-induced differentiation, resulting in the establishment of a 'window of implantation' [26]. Donor oocyte cycles typically achieve the highest implantation rates of all assisted reproduction approaches [27], suggesting that in addition to high quality oocytes the hormonal preparation leading to recipients' endometrial receptivity has been well optimized [28]. Protocols used in artificial programmed cycles consist of a variety of estrogen and P4 preparations and delivery modes, and are typically similar in recipients of egg donation and in IVF patients transferring cyropreserved-thawed embryos. It is agreed that there is no difference in

outcome between vaginal and IM P4 administration [9, 28]. Our data showed an odds ratio (OR=1.8) slightly higher for positive pregnancy outcome when using IM versus vaginal P4 supplementation, but these data need to be validated in a prospective and randomized fashion.

The sequential actions of E_2 and P4 are sufficient to drive a highly receptive endometrium in humans [9, 29]. The mechanisms by which estrogen and P4 act are highly complex and involve multiple nuclear receptors as well as recently described membrane receptors. It is agreed that controlled ovarian hyperstimulation as performed for IVF leads to histopathologic changes and variations in gene expression profiles of the endometrium when compared to natural cycles [11, 30, 31]. The effect of markedly supraphysiologic levels of E_2 and various degrees of endometrial histological advancement have been well characterized [11, 12]. However, the enigma is still present: what is the true impact of variable degrees of embryo-endometrium developmental asynchrony derived from IVF, hyperestrogenism and early luteal progesterone support in the presence of published high clinical embryo implantation rates? [32]. In fact, "high performance" IVF programs report excellent pregnancy rates typically achieving high E_2 levels associated with robust ovarian stimulation protocols [33].

On the other hand, using the artificial cycles described herein for endometrial preparation it appears that there is a wide E_2 range which allows for development of a receptive endometrium. We have performed endometrial biopsies timed to the onset of the window of implantation (day 21) in "mock cycles" of recipients and IVF patients to receive frozen-thawed embryos, and found the endometrium to be histologically in phase in >95 % of cases using the supplementation protocol described here (unpublished data). However it is agreed that histological changes may not truly reflect functional endometrial changes and hopefully novel tests may be on the horizon to predict the receptive state of the uterus after follicular stimulation (and perhaps natural cycles?) [34]. Furthermore in our current study pregnancies were established within a wide range of E_2 levels, with low levels of 177 ng/mL (known to support receptivity) but also with high levels >2000 pg/mL, challenging the concept that "unphysiological moderate" hyperstrogenism can affect implantation. We agree with previous reports that neither serum E_2 nor endometrial thickness are able to predict implantation, and that neither can be clinically employed to cancel a cycle because of insufficient endometrial preparation [22], provided a trilaminar endometrial pattern is present.

Recently the challenging concept of 'freeze all' cycles has been suggested in order to transfer embryos in a more "physiological" environment and not during the hyperstimulated IVF fresh cycle [35]. In fact, others have presented evidence suggestive of impaired endometrial receptivity after ovarian stimulation for IVF in a prospective randomized trial comparing fresh and frozen-thawed blastocyst transfer in normal responders [36]. This information combined with reported very high survival rates following vitrification/warming [37, 38], may provide an alternative to the routine IVF approach [39]. Notwithstanding the introduction or not of these potential practice changes, more data are needed to determine optimal endometrial preparation in artificial cycles resulting in adequate E_2 levels and E2/P4 ratios for achievement of highest receptivity and implantation. It also remains to be determined whether such optimized protocol will fit all patients or whether individual tailoring might be more appropriate.

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