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PHYSIOLOGY

Optimization of endometrial preparation results in a normal endometrial function test® (EFT®) and good reproductive outcome in donor ovum recipients

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Abstract *Purpose*: Numerous studies have investigated potential markers of endometrial receptivity as predictors of successful implantation. Cyclin E and p27 have recently been studied using the endometrial function test (EFT). Our objective is to determine the correlation between the expression of cyclin E and p27 and the adequacy of uterine preparation of recipients using donor oocytes. *Methods*: Twenty recipients undergoing preparatory cycles with leuprolide acetate, estrogen, and progesterone. Endometrial biopsies were obtained 10–12 days after progesterone supplementation following the course of estrogen. The tissue was prepared for histological analysis and immunohistochemical staining for cyclin E assessment. The outcome of their subsequent ovum donation cycle was blinded to the reviewer of the EFT. *Results*: All recipients showed normal luteal transformation. Nineteen (95%) of the recipients had a normal EFT. This is significantly higher than what we demonstrated, previously, in unexplained infertility patients, where only 40% of such patients had a normal EFT. Thirteen recipients with a normal EFT had a clinical pregnancy, while 6 did not become pregnant in their subsequent transfer cycles. The sole patient with an abnormal EFT did not conceive on 2 subsequent cycles. *Conclusions*: While a normal EFT does not guarantee a successful pregnancy, an abnormal EFT appears to be associated with pregnancy failure. This may be useful in identifying women who need adjustments to their stimulation protocols prior to progressing to a physically, emotionally, and financially costly cycle.

Keywords Cyclin E · Donor ovum recipients · Endometrial function test · Implantation · Markers of receptivity · P27

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Introduction

Endometrial histology varies throughout the normal menstrual cycle and is influenced by estradiol and progesterone. Estradiol induces proliferation of the basalis and functionalis endometrial layers and progesterone promotes glandular development and secretion, and initiates the necessary changes for allowing embryonic implantation to take place. The synchronization of both hormones appears essential for the process of normal maturation, and an implantation window of 6–8 days after ovulation, has been defined in the human [1, 2].

Current tools for endometrial evaluation are limited. Endometrial assessments are generally based exclusively on the histologic appearance using eight morphologic markers originally proposed by Noyes, Rock and Hertig in 1950 [3]. However, this classification has been shown to have limitations, with high inter- and intra-observer variation [4–7].

Over time, incremental improvements and modifications have been performed on this classification on endometrial assessment. In addition to these modifications, the use of markers has been investigated as a way to better assess development and receptivity.

Numerous studies have investigated potential markers of endometrial receptivity as predictors of successful implantation and, in doing so, have helped to define the cellular and molecular mechanisms by which implantation occurs.

Cell adhesion molecules (integrins, mucins and trophinin) [8], pinopodes [9], cytokines [10], L-selectin ligands [11], homeobox (HOX) genes [12], growth factors [13], MUC1 [14], leukemia inhibitory factor [15], IL-1 receptor type I [16], colony stimulating factor (CSF) [17], MAG (mouse ascites Golgi) mucin [18], glycodelin A [19], matrix metalloproteinases [20], and their inhibitors, as well as estrogen and progesterone [21] receptors have been shown to be involved in apposition and attachment of the blastocyst to the endometrium.

Recently, Dubowy et al., in 2003 [22], described two additional markers of endometrial development, known as, cyclin E and p27. A cell's progression through the mitotic cycle is controlled by cyclins, cyclin-dependent kinases, and their inhibitors. Cyclin E has been shown to be the rate-limiting activator of the mitotic G1 to S phase transition, whereas the cyclin-dependent kinase inhibitor p27 prevents this cell cycle progression. The activity of these markers depends on their interactions and on their subcellular localization. For example, p27 is only active when present in the nucleus. There is evidence that estrogen positively regulates cyclin E while progesterone induces the transition to a p27 dominated state [22].

Cyclin E expression in a midproliferative gland is limited to the cytoplasm of the epithelial cells, without being present in the nucleus. On cycle day 16, cyclin E becomes more prominent in the basal aspect of the glandular cells with only minimal apical or lateral expression. By cycle day 16–17, cyclin E is found exclusively in the basal portion of the cells, however, the nuclei continue to remain negative. By cycle day 17 and 18, there is a progressive loss of cytoplasmic cyclin E expression with a concomitant increase in nuclear involvement. Maximal glandular nuclear reactivity is reached on cycle day 19. After cycle day 19, there is a rapid loss of cyclin E reactivity (Figs. 1–4) [22].

The EFT consists of immunohistochemically staining endometrial biopsies utilizing antibodies against cyclin E, the key regulator of the mitotic cycle's G1/S transition. As previously published, normal expression patterns of cyclin E have been associated with implantation success while abnormal patterns with implantation failure [22].

We tested the proposal that functional analysis of the endometrium using the EFT will correlate with adequacy of uterine preparation for patients undergoing ART using donor oocytes and identify those women who may need adjustments in their stimulation protocols. These results were compared to previous findings in which biopsy samples were examined from women seeking infertility treatment and from volunteers that were fertile.

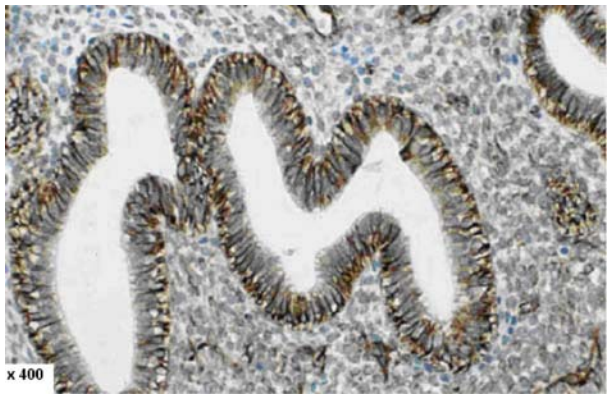

Fig. 1 On cycle day 16, cyclin E staining is predominant in the basal aspect of the epithelial cells with minimal apical or lateral expression (×400)

Materials and methods

The study was a prospective blinded institutional review board approved analysis of endometrial function test (EFT) results performed on endometrial biopsy specimens from preparatory cycles. We set out to evaluate the presence of cyclin E in patients undergoing endometrial synthetic preparatory or mock cycles to determine whether patients are candidates for ovum donation.

Preparatory cycles from 20 recipients were carried out with leuprolide acetate, estrogen, and progesterone. Leuprolide acetate was begun in the mid-luteal phase of the cycle prior to the cycle to be stimulated, at a dose of 1 mg subcutaneously daily and after 10 days, the leuprolide dosage was decreased to 0.5 mg daily. If the serum estrogen and progesterone levels confirmed down regulation, estrogen was begun using estradiol pills at 1 mg orally twice a day for four days, and then increased to 2 mg orally twice a day for four days, and finally to 2 mg orally three times a day for four days. Upon achievement of endometrial thickness of

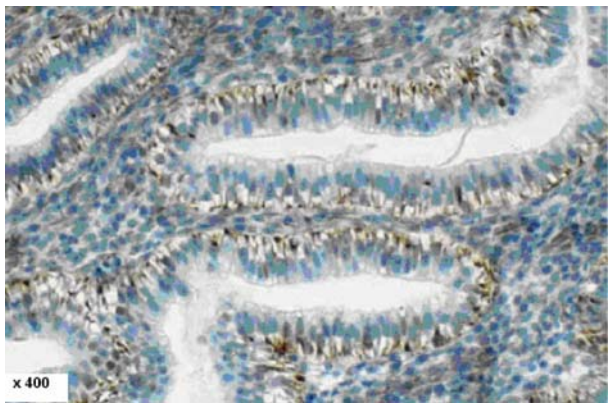

Fig. 2 On cycle day 16–17, cyclin E is expressed only in the basal portion of the epithelial cells with negative nuclei staining and negative apical or lateral reactivity (×400)

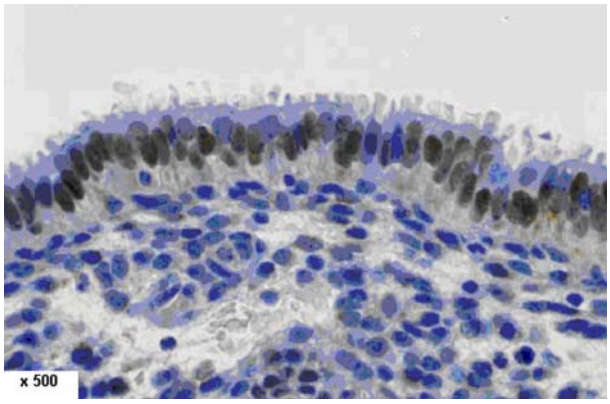

Fig. 3 On cycle day 17–18, cyclin E staining is less evident in the cytoplasma of epithelial cells and predominates within the nuclei (×500)

>7 mm, progesterone was administered as vaginal 200 mg Prometrium® (progesterone, USP) four times a day.

Endometrial biopsies were obtained using an endometrial sampling device (Pipelle, Cooper Surgical, Shelton, Connecticut, USA) from the uterine fundus on day 10–12 of progesterone supplementation following the course of estrogen. The tissue was fixed immediately in 10% neutral phosphate-buffered formalin for at least 24 h, and then embedded in paraffin and prepared for histological analysis utilizing conventional methods. The outcome of their subsequent ovum donation cycle was blinded to the reviewer of the EFT.

These results were analyzed and compared to previously described data by Dubowy et al. [22]. Endometrial biopsies were performed in a separate population of infertile patients and in an additional control group of fertile volunteers, and then, analyzed.

Spearman correlation was used to correlate EFT results with outcome. Chi-square test was used for analyzing rates and proportions. Non-parametric data was analyzed with the Mann Whitney test for analyzing two categories and the Kruskall-Wallis test for more categories, while *t*-test was used for parametric analysis of continuous variables. Significance was considered with a p value < 0.05.

Immunohistochemistry (IHC)

Formalin fixed, paraffin embedded biopsy samples were immunohistochemically stained using diaminobenzidine (DAB) (Sigma-Aldrich, St. Louis, MO) as the chromagen. After deparafinization in xylene and rehydration through graded concentrations of alcohol, antigen retrieval was achieved by heating each section in a 750 watt microwave at 60% power or in a hot water bath maintained at 95–99°C in 0.01 M citrate buffer (pH 6.0). The slides were allowed to heat for 5 min with occasional fluid replacement for evaporation losses, followed by cooling at room temperature for 1 h. Anti-cyclin E (clone HE12) type IgG1, purchased from Neo-Markers (distributed by Lab Vision Corporation, Fremont, CA) was used at a dilution of 1:100. Non-immune mouse ascites (NMA; Sigma-Aldrich) was used as primary antibody for negative controls. The sections were counterstained with hematoxylin.

Interpretation of immunohistochemical (ICH) staining

The specific staining of cyclin E was assessed by the presence of DAB precipitates in the nucleus and/or cytoplasm of the glandular epithelial cells. Biopsies that were shown to be strongly positive were utilized as positive controls for subsequent studies. Endothelial cells within each section were found to act as an internal positive control. The percentage of the glandular epithelial cells that stained, the nuclear and cytoplasmic staining intensity (ranging from 0 for no staining to 3+ for the strongest staining), and the distribution within the glandular epithelium and stroma were evaluated for each specimen. Due to the variability in the amount of surface present in the biopsies, surface expression was not included in our analysis.

We have shown previously that normal fertile controls have little to no nuclear cyclin E expression beyond cycle day 20 [22]. ROC analysis has revealed that the upper limit of normal is 20% nuclear cyclin E beyond cycle day 20. Since all of our samples were collected on or near cycle day 24, we

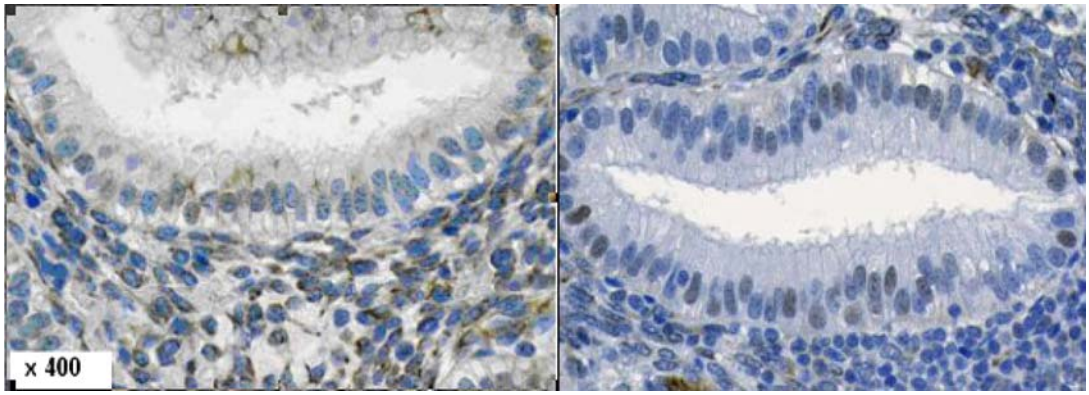

Fig. 4 After cycle day 19, a rapid loss of cyclin E staining is evident (×400)

defined normal cyclin E as being between 0 and 20% nuclear staining and abnormal cyclin E as being any sample with more than 20% nuclear staining in the endometrial glands.

Results

A total of 20 recipients underwent preparatory cycles. The mean age of these patients was 44.4 years. The mean endometrial thickness was 8.6 mm. All of the patients showed normal luteal transformation, determined by the endometrial pattern observed through a transvaginal ultrasound following progesterone treatment. Nineteen (95%) of the 20 ovum recipients had Endometrial Function Tests that were read as normal. This is significantly higher ($p = 0.000006$) than what we demonstrated in unexplained infertility patients where only 30 (40%) of 75 such patients had a normal EFT. All patients in this study subsequently underwent ovum donation cycles. Of the 19 patients that had a normal EFT, 13 became pregnant and had a detectable fetal heart while 6 did not become pregnant in their subsequent transfer cycles. The sole patient with an abnormal EFT did not conceive on 2 subsequent OD cycles.

In a control population of fertile patients, previously described [22], forty-seven of the 48 biopsies examined from the controls had known LH surges. Of these, 36 (77%) exhibited histologic cycle dates within 2 days of the cycle day determined for the LH surge. Twenty of the normal fertile control biopsy samples were dated as cycle day >20 based on stromal characteristics. Four (20%) of these biopsies showed glandular stroma dyssynchrony (GSD, defined as >30% of the glands appearing to be at cycle day < 20 with stroma appearing to be at cycle day >20) (Fig. 5).

In a separate population of infertile patients (unexplained) described by Dubowy et al. [22], in which, one hundred and thirty biopsies were analyzed from 83 naturally cycling women who had sought treatment for infertility. 106 biopsies had known LH surges. Of these, 92 (87%) exhibited histologic cycle dates within 2 days of the cycle day determined as the LH surge. Seventy-five of the biopsies were dated as cycle day >20 by stromal characteristics, with 23 of these (31%) demonstrating glands characteristics of cycle day < 20 consistent with glandular stromal dyssynchrony (GSD).

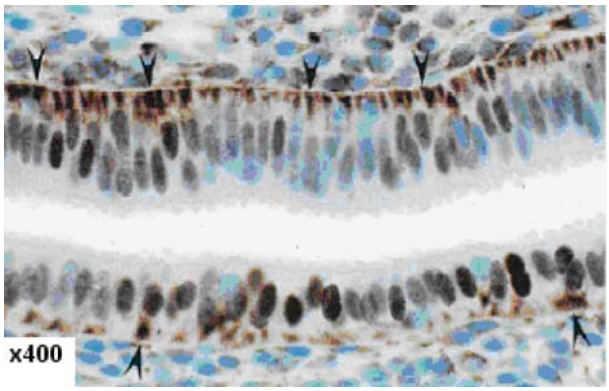

Fig. 5 Abnormal cyclin E patterns in a biopsy sample from a fertile control. The biopsy revealed a stroma with a cycle day of 24, but >90% of the glands were consistent with cycle days 17–18, consistent with glandular stromal dyssynchrony (GSD). Fifty percent of the glandular cells had basal cytoplasmic staining and 60% of the nuclei were positive, consistent with glandular developmental arrest (GDA) at cycle day 18

Discussion

Age related changes in fertility have been largely correlated with oocyte quality rather than sperm or uterine deterioration. Ovum donation, therefore, has shown to be an option that bypasses poor egg quality in patients seeking a pregnancy with a high degree of success. However, other factors are required in order to make this alternative the most effective for these patients. The need for an optimal endometrium is critical in allowing implantation to take place. The evaluation of endometrial development in these patients has been limited and the role of preparatory cycles has been questioned. We have shown for the first times that the protocol used in our preparatory cycles stimulates the natural endometrial cycle and can be used as an adequate marker for uterine preparation.

Traditional markers of uterine adequacy have concentrated on endometrial thickness and pattern [23], generally based exclusively on the histologic appearance of hematoxylin and eosin (H&E) stained tissue. The histologic evaluation has been modified but is still limited. Newer markers have focused on biological and physiological markers, which are predominantly products of the differentiated cell. The only marker commercially available is the $a_v ß_3$ integrin. In addition, the clinical utility of $a_v ß_3$ integrin has recently been questioned [17, 18]. Given the importance of assessing the endometrium and the limitations of the currently available methods, a marker of endometrial development remains a clinically worthy goal.

Unlike patients with unexplained infertility, these donor recipients had a remarkably high incidence of normal endometrial development as assessed by the EFT. This is not unexpected since this group is enriched in women with ovarian infertility as compared to women with implantation failure. The stimulation protocol utilized in this study appears to mimic the natural cycle since 95% of these women had normal endometrial development, which is comparable to the results seen in fertile control patients.

While a normal EFT does not guarantee a successful pregnancy, an abnormal EFT appears to be associated with pregnancy failure. Thus, an abnormal EFT may be useful to identify the select women who need to have their stimulation protocols adjusted prior to progressing to a physically, emotionally, and financially costly ovum donation cycle.

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