Chapter 6
Endometrial Receptivity by Endometrial Receptivity Analysis (ERA) for Infertility

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Introduction

Reproduction is one of the main basic functions in life, so the inability of having offspring has been one of the greatest concerns of the human being from the beginning of our history. Along the time, the number of couples with infertility problems has been increasing due to relevant changes in our lifestyle, and science has been involved in solving it. One of the main milestones happened in 1978 when Patrick Steptoe and Robert Edwards developed in vitro fertilization (IVF) [1], an assisted reproduction technique (ART) that has helped millions of couples with fertility disorders. Nevertheless, despite the dramatic evolution of reproductive medicine developing therapeutic interventions and new diagnostic tests, the number of IVF newborns is not as high as expected.

Starting from the first steps of life, the implantation process requires three critical players: a viable embryo, a receptive maternal environment, and a successful endometrial–embryo communication. However, from the beginning of ART, all the researches were focused on the embryo, whose development and quality assessment were thought to be the only issues to care about. Consequently, the

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endometrium has been left aside and not been considered susceptible to be treated in a personalized way. During many decades, unless there was an evident uterine pathology or anomaly, all the women were treated equally at the endometrial level. Fortunately, this approach is changing during the last years to understand better the receptive phenotype of the endometrium and to apply personalized medicine to its assessment.

In this chapter, we present the current knowledge regarding the biological mechanisms underlying endometrial receptivity, how can it be evaluated by the endometrial receptivity analysis (ERA), and the clinical relevance of personalizing the embryo transfer.

Endometrial Receptivity

The hormonal regulation of the endometrium leads to cyclical morphologic and functional changes turning it into a plastic organ. Briefly, once menstruation ends, the estrogen level rises, leading to the proliferation of endometrial cell and increasing the endometrial thickness. Then, ovulation will take place, leaving the corpus luteum which will begin to secrete progesterone. This hormone stimulates the endometrial epithelial differentiation and maturation. In case of no pregnancy, hormonal levels decrease leading to the vasoconstriction of the spiral arteries and tissue breakdown, and then regeneration takes place.

During most of the menstrual cycle, the endometrium is refractory to the embryo and only for a few hours acquires the ability to be adhesive. This short period takes place during the mid-secretory phase, and it is known as window of implantation (WOI). This concept was first suggested in 1956 by Hertig and Rock [2] and subsequently demonstrated by many other authors such as Navot [3] and Lessey [4]. The WOI has been widely studied in the last century from different points of view to understand what happens and how it is achieved. Classically, it has been considered that this ideal condition for the embryo implantation occurs between days 19 and 20 of the cycle in all women.

One of the most relevant changes that the endometrium suffers during the acquisition of receptivity includes plasma membrane transformation from a nonadhesive to adhesive surface encompassing remodeling of the endometrial barrier function and resulting in the replacement of the microvilli in the apical membrane with ectoplasmic projections called pinopodes [5].

In the meanwhile, the developing embryo will go through the fallopian tube to enter the uterine cavity. Once there, it starts to produce several molecules that will mediate implantation through interactions with the endometrium. However, as the endometrium is non-receptive during most of the menstrual cycle, synchronization between the embryo development and the endometrial maturation is needed for the implantation. If both players are properly synchronized, the implantation process will begin sequentially its four stages: apposition, adhesion, penetration, and invasion [6].

There are a fair number of molecules implied in the achievement of the receptive stage:

- Several studies have shown that an inhibition of prostaglandin (PG) production is related to implantation failure [7–9]. In fact, PGE2 and PGF2α exhibit a specific lipidomic signature in endometrial fluid, which was shown by Vilella et al. [10], to distinguish between fertile and infertile women as a preliminary screening.
- Studies by Genbacev et al. [11] and Nejatbakhsh et al. [12] identified the relevance of the selection adhesion system in embryonic implantation. However, their role in receptivity remains unclear.
- Integrins are a group of transmembrane cell adhesion molecules that contribute to endometrial receptivity, and they are proposed as markers to detect the WOI [13], but there is still a debate regarding the utility of integrins as molecular receptivity biomarkers [14–18].
- Mucins provide the endometrium with a physical barrier to implantation to prevent embryo attachment that must be overcome. As an example, the blastocyst induces a local clearance of MUC1 during adhesion to enable its implantation at that site [19].
- Cadherins are responsible for calcium-dependent cell-to-cell adhesion, but conflicting results have been reported especially regarding E-cadherin mRNA expression and protein levels [20–24].
- Cytokines facilitate communication among endometrial cells as well as between the endometrium and embryo. The most relevant cytokines involved in receptivity are:
  - LIF: regulates the proportions and amounts of immune cells in the endometrium at the time of implantation [25], mediates interactions between decidual leukocytes and invading trophoblast [26], controls the status of the endometrium through its receptor signaling, and forms pinopodes [25, 27].
  - IL-6: this cytokine may play a paracrine or autocrine role in the periimplantation period since its receptors exist in both endometrium and blastocysts. Furthermore, IL-6 may be a valid predictor of blastocyst quality [28, 29].
  - IL-11 contributes to the decidualization process and stimulates the production of LIF by the endometrium [25, 27, 30].

Besides, some biological processes as the immune response are involved in this phenomenon. Immune cells not only protect the organism against invaders but also allow invasion and maintenance of the fetal semi-allograft in the endometrium. In the human endometrium, several different leukocyte subpopulations exist: uterine natural killer (uNK) cells, macrophages, dendritic cells, and T cells [31]. Further, these cells can contribute to reproductive problems, such as recurrent miscarriage (RM), infertility, and implantation failure, but unfortunately it has been difficult to associate these cells with reproductive failure in women or to develop them as clinical targets, and hence the prognostic value of measuring immune cell parameters remains uncertain [32–34].

How to Evaluate the Receptive Phenotype: The ERA Development

From the moment when the WOI concept was established, many investigations have been focused on finding a receptivity marker that may identify a receptive endometrium. Passing from histological to molecular techniques, transcriptomics has proven its clinical applicability [35].

This transcriptomic approach has allowed identifying different mRNA expression patterns in the endometrium during the whole menstrual cycle, revealing a specific signature for each endometrial stage [35]. In fact, during the acquisition of the receptive transcriptomic profile, an up/downregulation of different genes has been observed. Some of the molecules regulated are implied in the immune response, such as CXCL14, which acts as a major recruitment stimulus for immune cells during the receptive period [36] and as chemotaxis of natural killer cells to cluster around epithelial glands [37]; glycodelin, which is implied in the decreasing of the maternal immune response during implantation [38]; and IL-15, involved in uNK cell proliferation and differentiation [39] from peripheral blood CD16 (−) NK cells [40]. Also, some other molecules are related to the protection of the embryo and endometrium, such as the metallothioneins and glutathione peroxidases (GPXs) (antioxidants), which protect against heavy metals, free radicals, and oxidative damage [41].

Based on endometrial transcriptomics, our group developed a molecular tool known as endometrial receptivity analysis (ERA) [42]. The goal was to translate more than 10 years of research in transcriptomics into the clinical practice in order to evaluate the endometrial receptivity objectively, highlighting the relevance of the maternal contribution in the implantation process.

Even though the ERA protocol was developed using microarray technologies, it is based nowadays in next-generation sequencing (NGS) techniques. RNA extracted from an endometrial biopsy is analyzed in order to obtain the expression pattern of 248 selected genes [43]. This information is analyzed by a computational predictor which classifies the endometrium in one of the different endometrial stages regardless of its histological appearance: proliferative, pre-receptive, receptive, or post-receptive, for the specific day in which the biopsy was taken.

The transition to NGS was accompanied by the development of a new ERA predictor based on machine learning algorithms improved by the acquired know-how after more than 20,000 endometrial biopsies analyzed. For this updating, several supervised machine learning methods were compared (random forest, classification tree, support vector machine, and K-nearest neighbor), resulting in the random forest as the one with the best performance in terms of accuracy (0.88), sensitivity (0.90), and specificity (0.97). This study also evaluated the success rate of the ERA prediction, according to the result obtained in a second endometrial biopsy. This technique has been refined and improved such that the predictor potency provides more detailed insights into the use of gene signature profiles for patient stratification, so the new ERA predictor defines a shorter, optimal WOI frame.

The ERA accuracy was evaluated by comparing its results with those obtained based on Noyes histological criteria, the classical method to date the endometrium [44]. To this aim, a dating set comprising 49 endometrial biopsies was analyzed using ERA by two independent pathologists. The concordance between each method was statistically analyzed by the quadratic weighted Kappa index, and results showed a high concordance for ERA (0.922) against the two pathologists (0.685 and 0.618).

The most important contribution of the ERA has been the objective diagnosis of the WOI, leading to the creation of the concept of personalized embryo transfer (pET). The basis of the test implies that pET must be performed after a receptive result in a subsequent cycle (or even several cycles later) under the same conditions (day and type of cycle) as the original endometrial biopsy.

Displaced Window of Implantation and its Assessment Under Different Clinical Conditions

For many years, it has been thought that the acquisition of endometrial receptivity was common for all women. In that way, the blastocyst transfer was performed routinely after 5 full days of progesterone administration in hormone replacement therapy (HRT) cycles or 7 days after the LH surge in natural cycles. However, the ERA test revealed that there is a proportion of women in whom, after the standard endometrial preparation protocols, their endometrium remains non-receptive [45].

For those patients with a pre-receptive profile, the endometrial receptivity will be reached later than when the biopsy was taken, needing more time of progesterone exposure. On the other hand, those cases with a post-receptive profile have already passed the receptive stage, requiring fewer days of progesterone exposure [45]. This mismatch implies a displacement of their WOI, revealing that it is not open at the same time for all the women.

A WOI displacement is highly relevant in ART since it results in an asynchrony between the embryo and the endometrium. Some patients could be more susceptible to suffer a WOI displacement, which leads to recurrent implantation failure (RIF). This term refers to a situation when repeatedly good-quality embryos are transferred without achieving pregnancy. Due to its dependence on multiple factors, such as general laboratory quality, embryonic and uterine/endometrial factors, etc. [46], there is no international consensus on the definition of RIF. One suggestion, found beyond others, defines RIF as the failure of pregnancy after a total of three cycles with reasonably good-quality embryos being transferred [46]. More strict criteria would speak of the failure of implantation in at least three consecutive IVF attempts, in which one to two embryos of high-grade quality are transferred in each cycle [47].

It is assumed that the endometrial factor contributes to 1/3 of RIF cases. According to a prospective multicenter trial published in 2013 [45], the WOI was delayed or advanced in one out of four RIF patients with more than three previous failed IVF cycles. In these cases, an embryo that is transferred on the “standard”

WOI will find a pre- or post-receptive endometrium, being too early or too late for successful implantation. This study showed that 84% of patients with displaced WOI were pre-receptive at the time when previous embryo transfer had failed, while the remaining 16% were post-receptive. These results were subsequently validated with a second ERA test performed at the time indicated by the first ERA.

Obese patients constitute another risk group for displaced WOI since this condition is associated with infertility. The poor outcome of patients with an elevated body mass index (BMI) could be provoked by the egg and/or embryo quality, the endometrium, or a combination of them. A retrospective study performed in 2014 evaluated the role of the endometrial receptivity in infertile obese women (ref). In this study, three study groups were established based on the BMI; women were classified as normal (BMI 19–24.9; $n = 163$), overweight (BMI 25–30; $n = 47$), or obese (BMI >30; $n = 11$), and the ERA test was performed. The analysis of endometrial receptivity showed that there were no statistically significant differences in overweight women compared to normal weight controls in terms of WOI timing. However, obese patients showed a slight increase in the non-receptive status during the expected WOI compared to normal or overweight patients [48]. In line with this, Comstock et al. published a prospective study in 2017 establishing the same categories of patients based on their BMI and found that the transcriptomic profile of the endometrium during the WOI was altered in obese patients. Interestingly, the more the BMI increased in the subjects, the more pronounced was the displacement of endometrial receptivity and the dysregulation of endometrial gene expression. This data evidence that altered endometrial gene expression in obese patients may contribute to their increased risk of infertility [49].

The influence of endometrial thickness to predict a positive result of IVF treatment and its possible association with receptivity has also been extensively studied. Most authors agree that an endometrium that reaches a thickness of at least 6 mm measured before the administration of exogenous progesterone in the HRT cycles indicates a receptive endometrium that can lead to pregnancy. To demonstrate this using a reliable molecular method, the ERA test was retrospectively analyzed in endometrial samples of HRT cycles classified into three groups according to their thickness: atrophic endometrium (<6 mm), normal endometrium (6–12 mm), and hypertrophic endometrium (>12 mm). The findings showed that samples from normal and increased endometrial thickness maintained a normal ratio of receptive versus non-receptive results. However, it was found that endometrial atrophic samples revealed a significantly higher percentage of non-receptive profiles, with more than 50% of the samples analyzed. These results suggest that the WOI of these patients could be displaced due to the insufficient growth of their endometrium [50].

On the other hand, there are other conditions which are not directly related to a WOI displacement, as is the case of endometriosis. This pathological condition is associated with infertility and refers to the growth of ectopic endometrial tissue outside the uterine cavity. Currently, there is a lack of consensus on the functional mechanisms of this disorder and how many levels does it affect IVF patients. In order to clarify its relationship with endometrial receptivity, a prospective study was designed to assess the endometrial receptivity gene signature in patients with

different stages of endometriosis using the ERA test. The authors observed that there were not differential regulation for the ERA transcriptomics profiles in any of the different stages of the disease compared to healthy women controls, concluding that endometriosis does not increase the risk of having a displaced WOI [51].

The ERA not only reveals if a given patient has a displaced WOI but also predicts when that patient will reach receptivity, indicating specifically how much time of progesterone exposure is needed for her endometrium to acquire this phenotype. So, a receptive profile is divided into three sub-signatures: (1) an optimal receptive profile indicating that the embryo has a high chance of implantation if the transfer is performed under the same conditions in which the biopsy was obtained, (2) an early receptive endometrium indicating that the endometrium needs 12 hours more of progesterone exposure to achieve an optimally receptive profile, and (3) a late receptive profile indicating that 12 hours less of progesterone exposure are needed. On the other hand, in case that the endometrium results pre-receptive at the standard WOI (120 hours progesterone), it would imply that the endometrium needs 1 or 2 more days of progesterone exposure to reach receptivity, while a post-receptive result will imply that it required 1 or 2 days less with progesterone exposure.

Clinical Results of Personalized Embryo Transfer

Implantation failure is critical in reproductive medicine since, beyond monetary considerations, it is an important cause of psychological stress and drop-out factor [52]. In order to minimize the negative impact of repeated implantation failure on infertility patients, it is crucial to optimize conditions before the next embryo transfers. As previously mentioned, it is assumed that the endometrial factor contributes to 1/3 of RIF cases. When it is related to a morphological anomaly or to pathology, it could be treated by a specific intervention or treatment. However, in those cases in which the underlying problem is a displacement of the WOI, it could be reverted by personalizing the embryo transfer according to the individual WOI of each woman.

pET has been widely used around the world from 2010 when ERA was used at clinical level for the first time [45, 53, 54]. First clinical data obtained following pET were published in Ruiz-Alonso et al. [45]. It was a prospective, interventional, multicenter clinical trial composed of 85 RIF patients (with at least 3 previous failed embryo transfer cycles) and 25 control patients (1 or no failed ET cycle) [45]. Results showed that a lower receptivity rate for RIF patients (75% vs. 88%) is one possible reason for their RIF condition. For all the cases with displaced WOI, pET was performed guided by ERA, leading to an embryo transfer at a different day than the standard. Even though the RIF group had several previous failed cycles, once pET was performed, pregnancy rate (PR) and implantation rate (IR) rose to 50% and 38.5%, respectively. This outcome was similar to that of patients who had a receptive result at their first biopsy (PR: 51.7% and IR: 33.9%). Thus, RIF patients

related to endometrial factor can normalize their reproductive outcome through pET guided by ERA after identifying their individual WOI, since otherwise the following embryo transfers would always be performed on the same day regardless of their endometrial receptivity status.

To check the difference between transfer at a non-receptive endometrium versus doing it during the personalized WOI (pWOI), several retrospective studies have been published. First of all, a pilot study showed the comparison within the same patient, comparing previous embryo transfer in a non-receptive endometrium prior to doing the ERA test, and then the outcome once her pWOI was detected. The 17 RIF patients had an ovum donation transfer for both prior and after the ERA and when the day of the embryo transfer was changed in a personalized manner. PR was increased from 19% to 60% and IR from 11% to 40% [54].

Clinical data after analyzing more than 55,000 patients worldwide from more than 1500 IVF clinics indicate that around 70% of RIF patients are receptive on the day in which the biopsy was performed. Hence, 30% are non-receptive, of which 0.5% are proliferative, 10.5% post-receptive, and 89% pre-receptive (non-published data).

Data achieved by different investigators in the last years show similar findings. In an Indian population study [55], a displaced WOI was found in 27.5% of RIF patients. Although not significant, after pET, an ongoing pregnancy rate (OPR) of 42.4% and an IR of 33% were reported, which were equal to their IVF results over 1 year [55]. Similar findings were reported by a retrospective analysis among 50 RIF patients in Japan. After personalizing the embryo transfer, clinical pregnancy rates were 35.3% (12/34) per first pET in the receptive group and 50.0% (5/10) per first pET in the non-receptive group (after correcting their WOI). All the pregnant cases in the non-receptive group achieved pregnancy in their first pET. The IR was 32.8% (20/61) in the receptive group and 31.6% (6/19) in the non-receptive group [56]. On the other hand, in a Canadian study with euploid blastocysts transferred, although not significant, IR and OPR were reported higher (73.7% vs. 54.2% and 63.2% vs. 41.7%, respectively) after pET when compared to patients without pET. The authors concluded that a significant proportion of patients with a history of implantation failure of a euploid embryo have a displaced WOI as detected by the ERA. For these patients, pET using a modified progesterone protocol may improve the outcomes of subsequent euploid frozen embryo transfers [57].

On the other hand, the ERA has also been evaluated for its ability to improve outcomes on patients without previous implantation failures. This was the first prospective, randomized controlled study where patients were randomized using a computational system to three arms: fresh embryo transfer (FET), deferred embryo transfer with cryopreserved embryos (DET), and pET guided by ERA with cryopreserved embryos. Preliminary outcome showed significant differences between PR for pET arm (85.7%) versus FET (61.7%) and DET (60.8%). Although not yet significant, there were also differences in IR (47.8% for pET, 35.3% for FET and 41.4% for DET) and in OPR per embryo transfer (55.1% for pET, 43.3% for FET and 44.6% for DET) [58]. This study included 28 clinics worldwide recruiting patients younger than 37 years, with body mass index (BMI) between 18.5 and 30

and with a normal ovarian reserve. Exclusion criteria were recurrent pregnancy loss and/or severe male factor. PGS was neither an inclusion nor an exclusion criterion.

Finally, the improvement of the new ERA predictor based on NGS has been evaluated by the clinical outcome obtained after pET. For that, the reproductive outcome of 512 patients (from 10 different clinics around the world) was evaluated. These results showed 57.2% IR, 72.9% PR, and 56.3% OPR. When compared with previous publications based on the ERA microarray diagnosis, a significant increase of 17% in IR (95% confidence interval (CI) [0.12, 0.22], p-value <0.0001), 14.9% in PR (95% CI [0.09, 0.21], p-value<0.0001), and 10.6% in OPR (95% CI [0.04, 0.17], p-value = 0.0013) was observed in favor of the new ERA predictor [43].

All these data show how the ERA has enabled clinical assessment of the endometrial factor, helping thousands of patients worldwide by identifying their pWOI. All the published results highlight the relevance of personalized medicine, also at the endometrial level, by synchronizing embryonic development and endometrial receptivity.

Conclusions: What the Future Will Bring?

The complexity of the implantation process has presented a challenge in reproductive medicine, being widely studied to improve the success rate of the IVF clinics. Now, the development of new technologies is allowing the researchers to make strides in understanding the cross talk between embryo and endometrium.

Currently, the ERA test is being applied widely to determine the endometrial receptivity based on transcriptomics. This test has introduced the concept of pET by personalizing the moment in which the transfer should be done, according to the specific timing of the endometrium. Future studies about the endometrial receptivity will have to clarify the role of epigenetics, microRNA, and genetic variants, among others, in those patients with failed cycles despite having a receptive endometrium.

On the other hand, the improvement of RNA and DNA sequencing techniques coupled with the evolution of bioinformatics analysis will provide more and better information about the molecular events taking place during the period of optimal receptivity. Also, it remains a challenge to the possibility of assessing the endometrial factor in a noninvasive way, even in the same transfer cycle, influencing the economic and emotional implications for the patient.

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