Outlook

Is ovarian stimulation detrimental to the endometrium?

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Abstract

Ovarian stimulation in assisted reproduction technology produces lower implantation rates per embryo transferred than natural and ovum donation cycles, suggesting suboptimal endometrial development due to the abnormal concentrations of hormones used to recruit more oocytes. After the publication of several studies on the gene expression profile of endometrial receptivity in the natural cycle using microarray technology, researchers have investigated the impact of ovarian stimulation on the gene expression pattern of the endometrium. Ovarian stimulation cycles that use gonadotrophin-releasing hormone (GnRH) agonists and antagonists have been analysed in detail during the window of implantation to establish differences compared with the natural cycle. This paper reviews results obtained in different studies to elucidate the changes induced by the different protocols used in clinics. At the morphological level, no relevant alteration was observed in endometrial development in the early and mid-luteal phases in women undergoing ovarian stimulation following GnRH antagonist treatments. However, the gene expression pattern of the endometrium showed some differences. In addition, the endometrial development after GnRH antagonist mimics the natural endometrium more closely than after GnRH agonist at both the morphological (no relevant differences) and molecular level (only 23 genes dysregulated at high dose). Clinical implications of these differences should be analysed in more detail.

Keywords: *endometrial receptivity, GnRH agonist, GnRH antagonist, microarrays, ovarian stimulation*

Introduction

Human endometrium is a dynamic tissue that undergoes well-defined cycles of proliferation, differentiation, and shedding (menstruation) in response to the prevailing endocrine and paracrine environment to reach the receptive status. In the natural cycle, the ovary releases one oocyte in each standard menstrual cycle, 14 days before the next menstruation. If the oocyte is fertilized, it arrives at the endometrium at the blastocyst stage (day 5–6). This is the same stage at which receptiveness in the endometrium occurs, in a synchronized manner with the development of the embryo.

In assisted reproduction technology, the main goal of ovarian stimulation protocols is to trigger oocyte maturation of an appropriate number of follicles for the treatment of infertility. A higher number of oocytes increases the rate of success. However, lower implantation rates per transferred embryo than those in natural cycles remain a major problem that is compensated by increasing the number of transferred embryos (American Society for Reproductive Medicine, 2002) at the cost of increased numbers of twin and triplet pregnancies. Furthermore, implantation rates obtained in oocyte donation programmes are higher than in women under ovarian stimulation, suggesting that the low rates observed when the ovaries are stimulated are related to a detrimental effect on the endometrium.

Several studies have addressed this issue. In patients who display a high response to gonadotrophins, supraphysiological concentrations of oestradiol on the day of human chorionic

gonadotrophin (HCG) administration are deleterious to embryonic implantation (Simón *et al.*, 1995, 1998, 2003; Pellicer *et al.*, 1996). Furthermore, it has been demonstrated that while low doses of oestradiol maintain the uterus in a receptive state, high doses cause it to become refractory in mice (Ma *et al.*, 2003). Uterine receptivity is diminished during ovarian stimulation compared with natural cycles (Paulson *et al.*, 1990).

A substantial number of alterations have been documented using morphological methods. For example, advancement in the early luteal phase has been described using histology (Seif *et al.*, 1992; Psychoyos, 1994; Kolb and Paulson, 1997; Kolibianakis *et al.*, 2003) and scanning electron microscopy (Nikas *et al.*, 1999; Giudice, 2003).

Others researchers have elucidated biochemical changes in the endometrium induced by ovarian stimulation. In this context, down-regulation of the endometrial oestrogen and progesterone receptors (Develioglu *et al.*, 1999) and biochemical changes in the endometrial fluid (Simón *et al.*, 1996) have been described. As has been mentioned before, this is not surprising, considering that the aim of ovulation induction is to recruit a sufficient number of oocytes, and as a side-effect supraphysiological concentrations of steroid hormones and paracrine mediators are produced, and these molecules target the endometrial cells.

The impact of some specific molecules on the development of endometrial receptivity has been reported from different perspectives, encompassing the genomic impact of progesterone on endometrial stromal cell development *in vitro* (Okada *et al.*, 2003), interleukin (IL)-1β (Rossi *et al.*, 2005) or IL-11 (White *et al.*, 2005). More recently, the in-vitro effects of steroids on freshly isolated endometrial endothelial cells have also been reported (Krikun *et al.*, 2005).

However, the most attractive strategy to investigate the genomic profile of the endometrium and the impact of ovarian stimulation protocols on endometrial receptivity has been the use of microarray technology, which allows studying of the entire gene expression pattern of a tissue in a single experiment.

Functional genomic studies of the human endometrium in natural cycles

Following completion of the human genome sequence, the main goal of researchers has been to identify the genes involved in the physiological and pathological processes of their particular topics. Biotechnology has developed, in parallel, new tools to take the best from the information coming from the Human Genome Project. The success of this project has generated a burst of the '–omics' sciences: genomics is the study of genomes and the complete collection of genes that they contain; functional genomics, also known as transcriptomics, attempts to analyse patterns of gene expression and to relate this to function; metabolomics is a large-scale approach to monitoring as many as possible of the compounds involved in cellular processes in a single assay to derive metabolic profiles; proteomic approaches examine the collection of proteins to determine how, when, and where they are expressed; and bioinformatics, although not graced with the -omics suffix, remains a key element in collection, management and analysis of large-scale data sets that are generated by the approaches described here. These technologies allow the analysis of thousands of molecules in a single experiment that ultimately makes possible a global view of the molecular profile of a biological sample. One of these newly developed tools is microarray technology, initially described in 1995 (Schena *et al.*, 1995).

In the field of human reproduction, most of the studies performed have been functional genomic analyses, directed mainly to deepening the molecular knowledge of human endometrial physiology. Studies with human endometrial cells include cDNA and oligonucleotide analyses of endometrial stromal cell differentiation stimulated by cAMP ± progesterone (Popovici *et al.*, 2000; Brar *et al.*, 2001; Tierney *et al.*, 2002), compared with non-decidualized endometrial stromal cells. Some of these were time-courses, and have resulted in important insights into biochemical pathways participating in the process of endometrial stromal decidualization, with new players being involved.

Much information has been gained, in a whole functional genomic context, by employing endometrial biopsies. Some authors have analysed the gene expression profile of the endometrium throughout the menstrual cycle (Ponnampalam *et al.*, 2004; Talbi *et al.*, 2006). One of them concluded that it is possible to classify endometria precisely according to their transcriptional profile, regardless of the morphological appearance. More importantly, they established the existence of clusters of genes characteristic of the different phases of the cycle, highlighting the potential of gene expression profiling for the development of molecular tools in the evaluation of endometrial status (Ponnampalam *et al.*, 2004). This study has been confirmed and extended by the work of Linda Giudice and her group, who have dissected the molecular phenotyping of human endometrium throughout the menstrual cycle phases underlying its biological processes in normo-ovulatory women (Talbi *et al.*, 2006).

Other studies have focused on the receptive endometrium, and have defined the gene expression profile of this tissue during the window of implantation (Carson *et al.*, 2002; Kao *et al.*, 2002; Borthwick *et al.*, 2003; Riesewijk *et al.*, 2003; Mirkin *et al.*, 2005). Although only one gene, osteopontin, was consistently up-regulated in all five studies, there are several important molecules that have been highlighted by their presence in four of the five papers. Some of them are proteins previously identified in the endometrium, with or without a described function. Genes involved in lipid metabolism (apolipoprotein D), immune response [decay accelerating factor for complement, serine or cysteine proteinase, interleukin (IL)-15], regulation of cell cycle (growth arrest and DNA-damage inducible, alpha), ion binding (annexin IV) or enzymes with different functions in different tissues (monoamine oxidase A). More detailed reviews of these studies can be found in Giudice (2003) and Horcajadas *et al.* (2004).

The results obtained by these laboratories, taken together, have demonstrated that endometrial receptivity is an equilibrated, complex and active process involving hundreds of up- and down-regulated genes. These results also suggest that a key molecule with the capacity to regulate endometrial

receptiveness by itself does not exist. However, these studies have also shown that some molecules are more relevant than others in the development of receptiveness, and that there is a short cluster of 25 genes that are always regulated in some way in natural cycles and dysregulated in non-optimal conditions (Horcajadas *et al.*, 2007).

One of the main drawbacks of the previous studies is the inability to differentiate among the different cell types present in the endometrium. Therefore, the findings described above obtained by microarray technology have to be added to other findings obtained by other methods. Some authors have assigned separately essential candidates to the different phases of embryonic implantation. Mucine (MUC)-1 (also with a role in first attachment), MUC4, MUC6 and MUC16 which form the epithelial glycocalyx are necessary for epithelial polarity, a key characteristic of the epithelial surface. A brief review of the relevance of candidate adhesion systems for the second phase attachment, such as basigin (CD147), CD44, osteopontin, several integrin subfamilies, trophinin and CD9, is given by Aplin (2006). In any case, the role of these genes should be tested by functional analysis in animal or in in-vitro models in the near future.

Functional genomic studies of the human endometrium in natural versus stimulated cycles

Following the studies performed in natural cycles, efforts were centred on the genomic impact of ovarian stimulation protocols on the human endometrium during assisted reproduction treatments. The aim of the first study was to investigate the impact of ovarian stimulation using urinary gonadotrophins in a long protocol with GnRH agonists without progesterone supplementation (similar to the natural cycle) on endometrial gene expression profiles during the window of implantation by comparing the profiles at day HCG+7 of ovarian stimulation versus day LH+7 of a previous natural cycle in the same women. For this purpose, microarray technology by Affymetrix (GeneChip HG_U133A, USA) was used, which contained more than 22,000 genes to be tested simultaneously (Horcajadas *et al.*, 2005). Results were validated by semi-quantitative polymerase chain reaction (PCR) and quantitative PCR experiments.

It was found that more than 558 genes showed a differential expression of more than two-fold when ovarian stimulation and normal cycles were compared at HCG+7 versus LH+7. Analysing this list of genes, a surprisingly high number of genes involved in endometrial receptivity (window of implantation genes) were found to aberrantly expressed in endometria following ovarian stimulation (342 genes) (**Table 1**), showing the expression levels to be more similar to those in a non-receptive endometrium. This clearly showed that endometrial development is hampered and delayed under these conditions, as other authors previously had suggested (Horcajadas *et al.*, 2005).

This study simultaneously re-analysed the LH+2 versus LH+7 endometrial gene expression profiles in previous natural cycles in the same subject using a specific GeneChip, and the results obtained were consistent with previously published results (Riesewijk *et al.*, 2003).

Functional genomic studies of the human endometrium in ovarian stimulation cycles: agonists versus antagonists

In 2004, Mirkin and colleagues compared the gene expression profile in the peri-implantation endometrium in natural versus gonadotrophin-stimulated cycles using recombinant FSH (rFSH), with either GnRH agonist or GnRH antagonist, with or without progesterone supplementation of the luteal phase (Mirkin *et al.*, 2004). Endometrial biopsies were collected in the previous natural cycle 8 days after the LH peak (LH+8) and 9 days after HCG administration (HCG+9) in the next ovarian stimulation cycle. Analysis was performed with high-density oligonucleotide microarrays (GeneChip HG_U95Av2 Array; Affymetrix), containing more than 12,000 gene targets. Other structural and functional features of the endometrium were also investigated. The observations made corroborated the morphological changes previously described by other authors. However, those changes were associated with significant, albeit small, variations in gene expression (18 genes per expressed sequence tag, with a fold change ranging between 1.55 and 3.40) (**Table 2**). Mirkin and co-workers concluded that although ovarian stimulation causes structural and functional changes compared with natural cycles, small changes were found when gene expression patterns were compared, and that ovarian stimulation may therefore not have a major impact on endometrial receptivity. They also concluded that significant changes were found when comparing cycles using GnRH agonist versus GnRH antagonist (13 genes significantly different) (Mirkin *et al.*, 2004).

In the laboratory, a second study was performed to evaluate the impact of standard and high doses of a GnRH antagonist (ganirelix) in stimulated cycles compared with GnRH agonist (buserelin); both protocols were supplemented with progesterone. All the groups were initiated with a fixed dose of rFSH, and endometrial biopsies were collected at HCG+2 and HCG+7 in ovarian stimulation cycles. Endometrial collection at LH+2 and LH+7 from the previous natural cycle was included as a control.

At day HCG+2, endometrial dating, oestrogen and progesterone receptors, and pinopode appearance were comparable in all the groups, including the natural cycle. At HCG+7, endometrial dating, steroid receptors and the presence of pinopodes were comparable in both GnRH antagonist groups and the natural cycles. In the protocol employing a GnRH agonist, however, endometrial dating and pinopode expression suggested an arrested endometrial development compared with the other regimens. Gene expression profiles of the treatment cycles were largely comparable with that of the natural cycle at LH+2. For window of implantation genes, expression patterns were closer to those in the natural cycle following standard (50 genes dysregulated) or high dose ganirelix (23 dysregulated) administration compared with buserelin administration (85 dysregulated) (Simón *et al.*, 2005) (**Table 2**). To reflect clinical practice, progesterone supplementation was given in the luteal phase in all three arms of the study. Under this homogenous condition, in each of the treatment groups, expression of about 100 genes was different from that in the natural cycle. This

Table 1. Functional genomic studies performed in ovarian stimulation cycles using gonadotrophin-releasing hormone agonists versus natural cycles (Horcajadas *et al.*, 2005).

Direction of regulation in ovarian stimulation	*No. of genes*	*No. genes in window of implantation*	
		Typically up-regulated (n = 894)	*Typically down-regulated (n = 504)*
Up	281	9	115
Down	277	227	0

Table 2. Functional genomic studies performed in ovarian stimulation cycles using gonadotrophin-releasing hormone agonists and antagonists at different doses versus natural cycles.

Study	*Direction of regulation in ovarian stimulation*	*No. of genes*	*No. genes in window of implantation genes*	
			Typically up-regulated (n = 894)	*Typically down-regulated (n = 504)*
Mirkin *et al.* (2004)	*Antagonist*			
	Up	6	1	0
	Down	6	2	0
	Agonist			
	Up	5	0	0
	Down	1	0	0
Simón *et al.* (2005)	*Antagonist (low dose)*			
	Up	22	0	4
	Down	69	46	0
	Antagonist (high dose)			
	Up	88	0	7
	Down	24	15	1
	Agonist			
	Up	22	3	4
	Down	100	76	2

suggests that endometrial gene dysregulation under ovarian stimulation is affected in a global manner and as a result, a different endometrial profile arises. The endometrial genomic profile after daily treatment with standard or high dose GnRH antagonist in women undergoing ovarian stimulation mimics more closely the natural cycle as compared with GnRH agonist.

Research has continued with the dissection of the molecular features of endometrial receptivity in natural and ovarian stimulation cycles. In the latest analyses, the gene expression pattern of natural and ovarian stimulation cycles was studied throughout the early to mid-secretory phase after ovulation, with collection of endometrial biopsies every 2 days. Microarray data obtained from the microarray analyses of 50 endometrial biopsies were analysed using different methods such as sample and gene clustering, biological processes or selection of differentially expressed genes, as implemented in several microarray data analysis platforms. It has been found that the endometrium from ovarian stimulation cycles undergoes altered development in reaching the receptive status. These differences could be responsible for the lower implantation rates seen with ovarian stimulation, and need further investigation (Horcajadas *et al.*, 2006).

Conclusions

The fact that morphology and gene expression pattern are altered in ovarian stimulation suggests a shift in time in the differentiation towards a receptive endometrium caused by these treatments, rather than the direct dysregulation of a limited number of genes by the hormones used. Indeed, evidence can be found in the literature that, on the day of oocyte retrieval

(36 h after HCG administration), the endometrium appears morphologically advanced (Seif *et al*., 1992; Psychoyos, 1994; Kolb and Paulson, 1997; Kolibianakis *et al*., 2003), whereas delayed, advanced and in-phase endometrium is described during the window of implantation following ovarian stimulation. Studies performed in the laboratory have shown that for many genes that are regulated during the formation of the window of implantation in natural cycles, the expression levels at the time of implantation in ovarian stimulation cycles are more comparable with those of LH+2 (pre-receptive endometrium) than with LH+7 (receptive endometrium) patterns in GnRH agonist protocols (leuprolide acetate) without progesterone supplementation (Horcajadas *et al*., 2005). This observation has also been confirmed in ovarian stimulation cycles using GnRH agonist and two different doses of antagonist with progesterone supplementation (Simón *et al*., 2005). In that study, when investigating specifically for genes where expression is regulated during the window of implantation, more genes were differentially expressed compared with the natural cycle in the GnRH agonist group than in either the low or the high dose GnRH antagonist groups. This suggests that the expression profile of window of implantation genes is closer to the natural cycle profile in the ganirelix groups than in the buserelin group.

These differences suggest a delay in the regulation of gene expression necessary for the formation of a receptive endometrium due to ovarian stimulation treatment. The altered gene expression profiles strongly suggest that a stimulated endometrium is not optimally prepared for implantation in this case. This could have negative effects on the implantation process, and therefore could be one of the main causes of the lower success rates in ovarian stimulation when using this protocol. Defective implantation can take place at very early implantation stages (apposition or adhesion) or in further phases (invasion). Invasion is a very controlled process that requires a subtle dialogue between the trophoblast cells and the maternal tissue that should be in optimal condition (Norwitz *et al*., 2006).

The differences found in earlier work contrast with those published by other authors, especially with Mirkin *et al*. (2004) who, using a similar approach, analysed the morphology and gene expression pattern of the endometrium after ovarian stimulation using both GnRH agonists and antagonists. Although they reported changes in the endometrial morphology, these changes are not reflected in the gene expression pattern of the endometrial biopsy (Mirkin *et al*., 2004). Only three genes belonging to the window of implantation group were shown to be dysregulated in these endometria. Disagreement between the studies of Mirkin *et al*. (2004) and Simón *et al*. (2005) in the gene expression profile of the endometrium under ovarian stimulation treatment could be attributed to the experimental design: sample collection (samples from different patients versus the same patient) and the day of the endometrial collection (LH+8/HCG+9 versus LH+7/HCG+7). One of the final goals of research on the endometrium should be a preconceptional assessment of endometrial receptivity for correcting and optimizing receptivity prior to embryo transfer. Although several tests that use endometrial biopsies are available commercially, such as the Endometrial Function Test (EFT) (Kliman *et al*., 2006) or the E-tegrity Test (Lessey *et al*., 1995), the complexity of this process has made it impossible to find non-invasive specific and sensitive molecular markers. At present, work is being carried out on the design of a molecular tool for endometrial dating containing 300 genes (data not shown) extracted from previous and new gene expression analyses. Perhaps proteomic testing of the endometrial exudates could be an alternative non-invasive method for determining endometrial receptivity (Scotchie *et al*., 2007), or study of the protein production required for embryo nidation. In any case, after the publication of several papers that show that histological evaluation of the endometrium under Noyes' criteria during the last 5 decades (Noyes *et al*., 1950, 1975) is not useful for dating (Murray *et al*., 2004) or for determining fertility status (Coutifaris *et al*., 2004), new objective tools are required for endometrial study. It is believed that microarray technology will emerge as an objective tool for human endometrial dating and evaluation, in particular to test the behaviour of the endometrium in response to new and milder ovarian stimulation protocols. Furthermore, these studies are very useful to elucidate endometrial physiology further and to study the function of the window of implantation genes in order to determine the actual role of these molecules in the implantation process.

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