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Review Article

Ovarian stimulation for in vitro fertilization alters the protein profile expression in endometrial secretion

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Abstract: Failure of the embryo to implant now constitutes the major limiting step in IVF treatment. Successful implantation requires a vital embryo and an effective molecular dialogue with a 'receptive' endometrium. However, what precisely constitutes a receptive human endometrium remains poorly defined. Several observations have indicated that ovarian stimulation for IVF may impair endometrial receptivity. The histological approach to monitor endometrial maturation requires an invasive biopsy that excludes its use during the luteal phase of cycles in which implantation is the end-point objective as in IVF. In recent years, several studies have been reported that the removal of endometrial secretions immediately prior to embryo transfer provides sufficient material for analysis of markers of receptivity without disrupting embryo implantation. Therefore, analysis of protein patterns in endometrial secretion fluid may offer a relatively non-invasive means of assessing endometrial receptivity during fertility treatment cycles. Several studies have shown that protein profile expression in endometrial secretions undergo cyclical changes, and demonstrated significant differences between the natural cycle and stimulated cycle. These findings suggest that endometrial secretion analysis provide a novel means of investigating the effect of ovarian stimulation on the intrauterine environment at the time of embryo transfer, which may help to develop less disruptive ovarian stimulation protocols for IVF in the future.

Keywords: Ovarian stimulation, endometrial secretion, endometrial receptivity, cytokine, in vitro fertilization

Introduction

Despite many advances in assisted reproduction techniques, implantation rates are still low after in vitro fertilization (IVF) and controlled ovarian stimulation [1]. Embryo implantation failure remains the major rate-limiting step in IVF success [2]. Although the quality of the embryo is considered to be the principle determinant of successful implantation, appropriate endometrial maturation and receptivity are necessary. Successful implantation and pregnancy require a vital embryo and an effective molecular dialogue with a 'receptive' endometrium [3]. However, what precisely constitutes a receptive human endometrium remains poorly defined.

Human implantation is a complicated process which is dependent on multiple, successive interactions between the embryo and the endometrium. It is only successful when it occurs during a brief period of the secretory phase of the menstrual cycle [4], usually referred to as the 'window of implantation' or 'window of receptivity' [5]. The duration of this putative 'window of implantation' is primarily determined by sex steroids [6], which regulate the expression of locally acting growth factors, transcription factors, cytokines and chemokines [3, 6].

Ovarian stimulation is used in IVF programs to collect multiple oocytes and to produce multiple embryos per cycle of treatment. Although this procedure enables selection of high quality embryos for transfer, ovarian stimulation also results in supraphysiological levels of P and E2. These elevated sex steroid concentrations may impair endometrial receptivity [6].

Many approaches to assessing endometrial maturation and receptivity have been described

[8]. Still the most widely used method remains that introduced by Noyes *et al*. [9], who defined maturation in terms of histological criteria. However, the histological approach to monitor endometrial maturation requires an invasive biopsy that excludes its use during the luteal phase of cycles in which implantation is the end-point objective as in IVF [10]. In recent years, less invasive techniques have become available to study endometrial maturation. Aspiration and flushing of the endometrial cavity in the peri-implantation period of menstrual cycles has been performed without detrimental effect on pregnancy rates [10-12]. Analysis of protein patterns in endometrial secretion fluid may offer a relatively non-invasive means of assessing endometrial receptivity during fertility treatment cycles. This technique therefore offers a useful alternative to histological evaluation of endometrial biopsies. Several studies have indicated that protein profile expression in endometrial secretions undergo cyclical changes, and demonstrated significant differences between the natural cycle and stimulated cycle [12-14]. Endometrial secretion analysis may open a new 'window' on endometrial receptivity, implantation and the factors which modulate this complex and elusive process. These may provide diagnostic markers of endometrial receptivity, critical for improving current assisted reproductive technologies. In this review, we summarize current knowledge concerning changes in the protein expression of endometrial secretion in the natural cycle and stimulated cycle.

Endometrial secretion in the natural cycle

The viscous fluid secreted by the endometrium, which reflects endometrial function as well as the embryo-endometrial dialogue prior to implantation, is important compartment in the assessment of endometrial maturation and differentiation. The cross-talk that occurs between the embryo and endometrium prior to and during the process of implantation results in production and release of molecules into endometrial secretion. The expression of these molecules is temporally related to the phase of endometrial development [10]. During the period of the secretory phase of the menstrual cycle, the secretions are rich in carbohydrates, glycoproteins, lipids, binding and nutrient transport proteins, ions, glucose, cytokines, enzymes, hormones, growth factors, proteases and their inhibitors and other substances [15, 16]. Therefore they may provide an important source of nutrients for energy and elements for anabolic pathways within the feto-placental unit, regulate placental development, and also modulate maternal immunological responses to the placental tissues [15]. In particular, these secreted proteins are believed to play broader roles in regulating endometrial receptivity. Endometrial secretion has been shown to undergo significant changes in protein content in the transition from the proliferative into the secretory phase [17]. Endometrial secretion composition varies during the menstrual cycle as a result of changes in ovarian steroid serum concentration [18].

The importance of uterine secretions is emphasized in a sheep model, where uterine gland formation is inhibited and pregnancy cannot be established [19]. Deficient glandular activity, usually described as a 'secretory phase defect', has been hypothesized to be an underlying cause of early pregnancy failure in humans [20]. Disrupted secretion of individual soluble factors including cytokines and growth factors into the uterine lumen has been correlated with infertility [12, 21, 22].

Previous studies have shown that expression of glycodelin A (GdA) [23], leukaemia inhibitory factor (LIF) [24-26], macrophage colony-stimulating factor (M-CSF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) [27], interleukins [28], prolactin, insulin-like growth factor binding protein-1 (IGFBP-1) and human chorionic gonadotrophin (HCG) [29] were detected in the endometrial secretion fluid, obtained during the luteal phase. In recent years, an approach has been described whereby endometrial secretion directly aspirated from uterine cavity can be analyzed for the protein expression. Several studies have demonstrated quantitative and qualitative changes of the protein patterns of endometrial secretion during the menstrual cycle. Particular attention has been paid to LIF and GdA, which have been shown to be crucial for successful implantation. These proteins vary during the menstrual cycle [13, 25, 30, 31], and appear to be differentially expressed between fertile and subfertile women [13, 25, 32-34]. Macklon and colleagues have reported detectable expression of LIF in aspirated endometrial secretions in women with endometrial tissue dating of 2

days or later after ovulation [13]. This is consistent with previous studies which have shown that LIF appears in endometrial flushings in the early luteal phase from postovulatory day 2 and onwards [25, 33]. However, Macklon *et al* showed no correlation between LIF levels in endometrial secretion samples with the progesterone serum concentration in the early luteal phase, as well as, no significant correlation between LIF and endometrial maturation. This may be because of the large range of the LIF concentrations observed in endometrial secretions [11, 25, 26, 33]. Therefore, the LIF content of endometrial secretion fluids does not appear to reflect endometrial maturity [13]. However, LIF concentrations in flushings from women with unexplained infertility were significantly lower than those in flushings from normal fertile women, suggesting that LIF play an important role in human embryo implantation. Further studies are needed to clarify this discrepancy.

Global gene profiling studies revealed a significant increase of GdA expression during the window of implantation [35]. Furthermore, endometrial gene expression studies suggest that sex steroids play an important role in regulating endometrial GdA expression [36]. GdA was detected in endometrial secretions when the endometrium was dated as postovulatory day 2 and onwards, and GdA expression increased together with the endometrial maturation detected at the tissue level [13]. This is consistent with a study showing a positive correlation between GdA levels in endometrial flushings and endometrial maturation [30]. Moreover, fertile patients showed higher levels of GdA in uterine flushings compared with the subfertile controls [37], suggesting that an increase of GdA might facilitate implantation. These findings support a possible role for endometrial secretion GdA level as a marker of endometrial maturation [13].

Hannan and colleagues assessed the proteome of endometrial secretions using 2D-differential in gel electrophoresis (2D-DiGE) [38]. Comparison of protein abundances between either different cycle stages, or fertile and infertile women, revealed that several proteins are present at altered levels dependent on cycle stage or fertility status. These proteins were identified by mass spectrometry, including antithrombin III and alpha-2-macroglobulin, whose production was confirmed in endometrial epithelium. Their staining pattern suggests roles during embryo implantation. Assessment of the human endometrial secretome has identified differences in the protein content of endometrial secretions with respect to receptivity and fertility. However, no cytokines or growth factors were identified in this study. This is likely to reflect the limited sensitivity of the 2D-DiGE approach, rather than their absence, particularly for proteins of lower molecular mass. Further studies including fractionation of the lower molecular weight range and specific multiplex analysis of cytokines and growth factors in the endometrial secretions are needed to identify such regulatory proteins, and whether their abundance changes in endometrial secretions during the menstrual cycle.

In a study, Elortza described a comprehensive catalogue of proteins of the endometrial secretions during the secretory phase of the menstrual cycle [39]. Three different but complementary strategies were used. The combination of the three strategies led to the successful identification of 803 different proteins in the International Protein Index (IPI) human database. An extensive description of the endometrial secretions proteome will help provide the basis for a better understanding of a number of diseases and processes, including endometriosis, endometrial cancer and embryo implantation. Therefore, the thorough catalogue of proteins presented can serve as a valuable reference for the study of embryo implantation and for future biomarker discovery involved in pathologic alterations of endometrial function.

Endometrial secretion in the stimulated cycle

It has been indicated that the removal of endometrial secretions immediately prior to embryo transfer provides sufficient material for analysis of markers of receptivity without disrupting embryo implantation. This approach may overcome one of the barriers to the in-vivo investigation of endometrial receptivity in conception cycles: disruption of the process of implantation. No discomfort or side effects of the aspiration were reported by any of the patients [10]. With this technique, factors that are involved in endometrial differentiation and receptivity can be directly related to the outcomes of embryo transfer. Previous studies employing Noyes' criteria to date endometrium at the time of oocyte

retrieval have shown ovarian stimulation to be associated with advancement of endometrial maturation [40-43]. However, it is uncertain to what extent data derived from the peri-ovulatory period can be extrapolated to the peri-implantation phase. Recently, the impact of ovarian stimulation on endometrial maturation during the luteal phase has been studied in both endometrial biopsies and in endometrial secretions. It has been demonstrated that ovarian stimuialion has little impact on tissue-derived markers of endometrial maturation or on LIF, GdA, and progesterone concentrations in the uterine secretion during the window of implantation [44]. Another study also showed no difference in LIF concentrations in endometrial secretions after IVF stimulation compared with the group without stimulation [11].

Boomsma and colleagues have elucidated the impact of ovarian stimulation on the levels of key regulatory cytokines, chemokines, and growth factors in endometrial secretion at the time of embryo transfer [12, 14, 45]. Endometrial secretions aspirated in the spontaneous cycle 6 days after the LH surge or prior to embryo transfer were analyzed by a multiplex immunoassay. The profile of 17 soluble mediators included IL-1β, IL-5, IL-6, IL-10, IL-12, IL-15, IL-17, IL-18, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), macrophage migration inhibitory factor (MIF), eotaxin, interferon-γ inducible protein-10 (IP-10), monocyte chemotactic protein-1 (MCP-1), Dickkopf homolog-1 (Dkk-1), heparin-binding epidermal growth factor (HbEGF) and vascular endothelial growth factor (VEGF) in endometrial secretions have been characterized. A number of mediators were excluded from the panel, either because appropriate antibodies were not available (glycodelin), or because of problems arising from cross-interference (IL-11, LIF and M-CSF). It has been shown that ten mediators (IL-1β, IL-6, IL-12, IL-18, TNF-α, MIF, eotaxin, MCP-1, IP-10, VEGF) were detectable in 90-100% of the samples. HbEGF, IL-5, IL-17, IL-10, Dkk-1 and IL-15 were detected in 23-76%, whereas IFN-γ was not detectable in any of the samples. The endometrial cytokine profile differed significantly from cervical mucus [12]. Moreover, significantly higher concentrations of IL-1β, IL-5, IL-10, IL-12, IL-17, TNF-α, eotaxin, Dkk-1, and heparin-binding EGF were present in endometrial secretions obtained in stimulated compared with natural cycles. The IL-6, MIF, and VEGF-A were also differentially expressed after ovarian stimulation, although not significantly after correction for multiple testing. The VEGF-A was the only mediator that demonstrated a reduced concentration after ovarian stimulation [14]. An explanation for the increase in the concentrations of most mediators after ovarian stimulation may be an increased cytokine expression by endometrial stromal and epithelial cells under the influence of high E2 and P levels. However, it may also be caused by an increased number of uterine natural killer (uNK) cells present in the endometrium under the influence of supraphysiological levels of E [46] and after ovarian stimulation [47]. This is unconsistent with previous studies which have shown that secretion levels of LIF and glycodelin A are investigated by ELISA and no significant differences are observed when comparing natural with stimulated cycles. However, this may be attributed to the low number of patients included in previous studies (n=8) [48, 49].

Furthermore, Boomsma *et al* explored whether a cytokine profile predictive of implantation and clinical pregnancy can be identified in endometrial secretions. It has been indicated significant associations (negative and positive association, respectively) between MCP-1 and IP-10 levels and implantation, and between IL-1β and TNF-α levels and clinical pregnancy. The predictive value for pregnancy of IL-1β and TNF-α was observed to be equivalent and additive to that of embryo quality [45]. MCP-1 has been shown to be a potent attractant and activator of uterine natural killer (uNK) cells [50]. High numbers of uNK cells have been related to miscarriage and infertility [51], which may be consistent with lower secretion levels of MCP-1 being associated with higher implantation rates. IP-10 was demonstrated to stimulate the expression of integrin subunits in trophoblast cells, and recombinant IP-10 promoted adhesion of trophoblast cells to endometrial epithelial cells [52]. The observation that the levels of IL-1β and TNF-α are significantly related to achieving a clinical pregnancy and not embryo implantation suggests that these mediators may have a more important role in later stages of implantation rather than initial apposition and adhesion of the embryo. These results also suggest that the ratio of TNF-α and IL-1β may serve as an indicator of endometrial receptivity, rather than individual absolute values of these mediators, since both coefficients are fairly

similar in magnitude but opposite in direction. Although the predictive value of the markers selected in these studies is too low to be clinically useful, it is noteworthy that the predictive values of the cytokine secretion profile and embryo quality were 'additive', indicating that the endometrium is not simply facilitatory in the implantation process, but is also itself an independent determinant of outcome. In the same study, Boomsma also further confirmed that pregnancy rates were not reduced in women undergoing this procedure prior to embryo transfer in a larger cohort of patients.

Bacterial vaginosis (BV) has been reported to be associated with a significant increased risk of miscarriage in the first trimester in women undergoing IVF, independent of other risk factors [53, 54]. The mechanism by which BV may diminish IVF outcome is unclear. A study has been undertaken to elucidate whether BV is associated with a proinfammatory endometrial secretion cytokine profile and whether there is a relationship between BV and the concentrations of a number of key regulatory cytokines, chemokines and growth factors. It has been indicated that BV is associated with higher concentrations of IL-1β in endometrial secretions compared with women without BV. However, no significant differences were found in the ratios of distinct pro- and anti-inflammatory cytokines in endometrial secretions from women with or without BV [55]. It has been able to show that BV does not significantly affect the intrauterine cytokine profile, since only marginal differences towards a pro-inflammatory milieu were observed. Therefore, it can be concluded that the impact of BV on endometrial receptivity is likely to be minor. Screening for BV in women with implantation failure is not recommended on the basis of these results.

In conclusion, endometrial secretion analysis provides a novel means of investigating the effect of ovarian stimulation on the intrauterine environment at the time of embryo transfer, which may help to develop less disruptive ovarian stimulation protocols for IVF in the future.

Prospect

The continuing high rate of implantation failure observed in assisted reproduction technologies has driven the search for clinical markers of endometrial receptivity. Many candidate markers have been proposed, including integrins, glycodelin and LIF [26, 56, 57]. However, none have as yet been shown to be valuable clinically.

Endometrial secretion aspiration immediately prior to embryo transfer is well tolerated, safe to perform, and the aspiration provides sufficient material for analysis. This technique may therefore offer a clinically useful approach to study the endometrial factor in embryo implantation. Several studies have been undertaken to explore that protein profile expression in endometrial secretions. Although further work will be required to confirm the findings of above preliminary study, this approach opens the possibility for studying the complex intrauterine regulatory networks before implantation and the identification of further important regulators of endometrial maturation and receptivity. In contrast to more invasive techniques, endometrial secretion aspiration may make it possible to correlate such markers directly with successful implantation. Clinical applications could include the assessment of endometrial maturation during an IVF treatment cycle, allowing embryos to be frozen rather than transferred to a suboptimal intrauterine milieu, and the evaluation of interventions designed to improve endometrial receptivity. However, as yet, the clinical value of endometrial secretion analysis in terms of predicting endometrial receptivity and consequently fertility are limited. Although it is a promising technique, endometrial secretion analysis has a number of limitations amenable to improvement. First, certain proposed markers of the window of implantation were excluded in the multiplex immunoassay. In order to refine the array, novel potential markers for endometrial receptivity should be included in future studies. In addition, one-quarter of endometrial secretions showed visual signs of blood contamination, which was shown to affect the results of a number of mediators. The other problem was that concentrations of certain mediators were frequently below the reliable detection limit, which may complicate statistical analyses. Furthermore, as yet, proteomic analysis of endometrial secretions using proteomic techniques has not been able to constitute a potentially valuable means of assessing endometrial receptivity. Therefore, further development and application of such techniques are required to elucidation of what

constitutes the optimal periconceptional uterine environment for implantation, and the impact of ovarian stimulation on endometrial receptivity. In particular, future studies should be performed to ascertain more specific and sensitive predictors of implantation. Such markers would be of significant clinical value, especially for women undergoing IVF procedures.

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Disclosure of conflict of interest

None.

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