SECRETOMICS OF ENDOMETRIAL FLUID FOR NON-INVASIVE ASSESSMENT OF ENDOMETRIAL RECEPTIVITY

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The secretions produced by the endometrium provide an accessible and potentially non-invasive window on the intra-uterine environment. While much work has been done over the past 30 years to elucidate the nature, constituents and function of endometrial secretions, recent development of techniques capable of quantifying multiple factors at very low concentrations considerable progress in our understanding of the role and significance of endometrial secretions. This material is now to contain a number of mediators which modulate endometrial receptivity, and which may be involved in maintenance and nurturing of ascending spermatozoa and the preimplantation embryo. However, may questions remain as to the functions of this fluid, which represents the first uterine component which the preimplantation embryo interacts.

Composition of Endometrial secretions

The fluid in the uterine cavity is produced by glandular secretion and transudation from stromal blood vessels in the endometrium. The primary compenents are proteins, amino acids,e lectorlytes, glucose, urea, cytokines, growth factors, metalloproteinases and their inhibitor, and cells. The composition varies during the menstrual cycle in response to changes in ovarian steroid production. Early electrophoresis studies demonstrated the proliferative phase to be associated with an increased expression of proteins in the 34-45 kDa region, with post-ovulatory upregulation of proteins in the 12-18 kDA regions (Beier et al 1998) The most abundant proteins identified using two-dimensional electrophoresis are of serum origin and include immunoglobulins, alpha-1 antitrypsin precursor , haptoglobin and transferrin (Parmar et al 2008).

Individual assays have revealed the presence of a number of cytokines in uterine fluid. LIF, activin A, glycodelin and IL-18 have all been thus identified (Dimitriadis E, 2010, Van der Gaast et al 2009). Recently Luminex technology has enabled the analysis of multiple mediators in a small sample of endometrial fluid. The group of Macklon has developed this technique (van der Gaast, Boomsma), in which a 2 ml syringe connected to an embryo transfer catheter is introduced transcervically into the uterine cavity. and suction gradually applied. In order to prevent contamination by cervical mucus during catheter removal, the outer sheath of the embryo transfer catheter isadvanced to a depth of 4 cm from the external cervical os, following the application of suction. The inner catheter is then withdrawn through the outer sheath, which prevented contact with the cervix. The outside of the catheter is cleaned to remove any potential cervical mucus. The tip of the catheter was cut off and snap frozen in liquid nitrogen in an Eppendorf tube and stored at -80°C. If appropraiate an embryo transfer procedure can be carried out thereafter.

Aspirate volumes cannot be reliably measured, since the aspirates are highly viscous, and between 1 and 4 microliter. Therefore, total protein content of the aspirate is usually used for normalization purposes. However, the mean and range of concentrations of individual mediators in endometrial secretions shows considerable differences. In one study in which key mediators were identified to be included in a luminex array, a number were excluded from the panel, either because appropriate antibodies were not available (Glycodelin), or because of problems arising from cross-interference (IL-11, Leukemia Inhibitory Factor [LIF] and Macrophage Colony Stimulating factor [M-CSF]). The final panel therefore included IL-1ß, IL-5, IL-6, IL-10, IL-12, IL-15, IL-17, IL-18, TNF-α, IFN-γ, MIF, Eotaxin, IP-10, MCP-1, Dkk-1, HbEGF and VEGF IFN-γ was not detectable in any of the samples in spite of a comparable detection limit of the assay)Boomsma et al 2009a).

Although the analysis of aspirated endometrial secretions constitutes a potentially valuable means of assessing endometrial receptivity, certain limitations need to be addressed. In one study, one quarter of aspirates showed visual signs of blood contamination, which was shown to affect the results of a of measurements of IL-1β, IL-12, IL-17, IL-18, MCP-1 and Eotaxin. Since no mediators were detectable in blank samples with severe blood contamination, the altered cytokine concentrations measured in contaminated samples are most likely due to interference of laser readings of the microspheres in the cytokine assay by haemoglobin, rather than cytokines present in blood. Therefore, blood contamination should be included as a confounder when analysing the results of cytokine measurements. A second problem was that concentrations of certain mediators were frequently below the reliable detection limit.

Another non-invasive approach to assess endometrial receptivity, which can in principle be applied during the window of implantation, is by flushing of the uterine cavity (Berkkanoglu *et al.* 2006; Li *et al.* 2006; Olivennes *et al.* 2003). However, this approach results in a higher and variable degree of dilution of endometrial secretions, since 1-10 mL saline water is instilled and the amount of fluid recovered differs. Therefore, this may account for the inconsistent findings reported (Laird *et al.* 1997; Lédée et al. 2002; Olivennes *et al.* 2003). A further non-invasive approach proposed is the analysis of endometrial markers of receptivity in peripheral serum samples. However, since the

expression of cytokines differs in different compartments of the body due to the strong influence of the local micro-environment, it is unlikely that this approach would generate data representative of the intra-uterine milieu (Hoozemans *et al.* 2004).

In recent years our group has developed a novel non-disruptive technique for aspirating endometrial secretions immediately prior to embryo transfer which promises to 'open the blackbox' of human peri-implantation events. Endometrial secretions contain a range of soluble and cellular constituents which offer a window on the molecular activity of the endometrium. Moreover endometrial secretions represent the first maternal interface with which the embryo must interact as a chain of events which result in implantation or rejection of the embryo are set into play. In addition, there is evidence that glandular secretions play a role in support the nutrition of the peri-implantation embryo. Our group has shown in two matched controlled studies that endometrial fluid aspiration can be performed without negatively affecting implantation rates (Boomsma *et al*, 2009a;van der Gaast *et al*, 2003), and that the material can be obtained without contamination by cervical mucus(Boomsma *et al*, 2009a). We have demonstrated that many putative factors such as glycodelin and LIF can be quantified in these secretions, and that levels of glycodelin correlate closely with the degree of maturation as assessed by Noyes criteria (van der Gaast *et al*, 2009). We have also shown that endometrial secretion profiling enables the impact of interventions such as ovarian stimulation at the endometrial-embryo interface to be studied (Boomsma *et al*, 2009b), and in a large prospective study using this technique we identified a profile of cytokine concentrations in endometrial secretions which significantly correlated with the chance of successful implantation of the embryo transferred immediately after aspiration of the secretions (Boomsma *et al*, 2009c). The Salamonsen group have similarly analysed uterine lavages samples for cytokines and chemokines: the combined data provide strong information on this class of molecules. However, for this approach to be further developed a number of additional studies are now required.

Firstly, before analysis of endometrial secretions in a given cycle can be deemed to be representative, and hence 'diagnostic' of the level of endometrial receptivity in an individual patient, the degree of normal inter-cycle variation in the molecular profiles identified in secretions needs to be assessed and quantified. Secondly, it remains unclear how the contents of secretions vary normally between the follicular phase and the period of endometrial receptivity in the luteal phase. Thirdly, whilst data from each of our groups have identified a number of markers within a restricted profile using Luminex™ techniques, high throughput proteomic studies are now required to identify other, more sensitive and specific soluble markers of endometrial receptivity.

Recently, studies have begun to appear applying mass throughput techniques to the analysis of endometrial secretions (Ametzazurra *et al*, 2009;Casado-Vela *et al*, 2009;Scotchie *et al*, 2009). These small pilot studies have demonstrated the feasibility of applying a combination of 2D gel electrophoresis and mass-spectrometry to characterise the secretome of the endometrium. However, none have addressed questions relating to cycle to cycle variation, and all have been complicated by the presence of large quantities of proteins derived from serum transudation masking active glandular secretions, thus reducing the sensitivity of the technique for discerning the endometrial component to the material analysed.

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