European Society of Human Reproduction and Embryology

COURSE 4

Implantation

Special Interest Group Early Pregnancy

Sepcial Interest Group Endometriosis and Endometrium

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Course 4 - A joint pre-congress course organised by the

Special Interest Groups Early Pregnancy and the

Special Interest Group Endometriosis and Endometrium

"Implantation"

PROGRAM

Course Coordinators: SIG Early Pregnancy: E. Jauniaux (UK) N. Exalto (NL), SIG Endometrium and Endometriosis: T. D'Hooghe (B), J. Horcajadas (E)

Course description: An update on basic and clinical aspects of implantation.

09.00 - 09.45	Mediators of implantation - **P. Bischof (CH)**
09.45 - 10.30	Endometrial cell-surface barrier - **TBA**
10.30 - 11.00	*Coffee break*
11.00 - 11.30	Molecular mechanisms of decidualization - **J. Brosens (UK)**
11.30 - 12.00	Genomics of human endometrial receptivity - **J. Horcajadas (E)**
12.00 - 12.30	The role of the endometrium in early pregnancy nutrition – **G. Burton (UK)**
12.30 - 13.30	*Lunch*
13.30 - 14.15	Time of implantation - **D. Baird (USA)**
14.15 - 15.00	Role of ultrasound in endometrial evaluation - **D. Timmerman (B)**
15.00 - 15.30	*Coffee break*
15.30 - 16.15	Implantation and recurrent miscarriage, clinical aspects – **S. Quenby (UK)**
16.15 - 17.00	Myometrial contractility and implantation - **D. De Ziegler (CH) (UK)**

Mediators of implantation

Paul Bischof and Marie Cohen
Department of Obstetrics and Gynaecology
University of Geneva
Laboratoire d'Hormonologie
Maternité
1211 Geneva 14
Switzerland

Learning Objectives

After this presentation, the participants should be able to:

- Appreciate the biological importance of trophoblast invasion as a model for embryo implantation.
- Realise the instrumental role of matrix metalloproteinases and their inhibitors in the process of implantation.
- Identify the major mediators of trophoblast invasion.
- Understand the similarity between embryo implantation and tumour metastatisation.

The biological importance of trophoblast invasion as a model for embryo implantation.

During embryo development, mitotic divisions of the blastomeres form the morula and later the blastocyst. Until the 4- or 8-cell stage, the blastomeres are distinct and can easily be counted; the embryo has no polarity. After the 8-cell-stage, each blastomere interacts with its neighbours through homotypic cell-surface adhesion molecules. This interaction is known as compaction. The most significant event occurring at compaction is the emergence of two distinct blastomere populations: those remaining in contact with the outside (zona pellucida) destined to form the trophectodermal lineage (TE, the future placenta and its membranes), and those inside the embryo, destined to form the embryo proper.

Cytotrophoblastic cells (CTB) are derived from the TE and represent a heterogeneous population during early pregnancy. After initial attachment of the blastocyst to the uterine lining, mononuclear CTBs that surround the embryonic disc fuse to form a syncytium. These multinucleated, terminally differentiated giant cells invade the pseudo-decidualised endometrium. Once the placental villi are formed, some CTBs of anchoring villi (that contact the uterine wall) acquire a transiently invasive phenotype and invade the decidualised endometrium. Meanwhile, the CTBs of floating villi (in the extra-villous space bathed by maternal blood) remain attached to the villous basement membrane. Therefore, CTBs follow one of two existing differentiation pathways (Bischof & Irminger-Finger 2005).

- Villous CTBs, considered as stem cells, form a monolayer of polarised epithelial cells that proliferate and eventually differentiate by fusion to form the syncytiotrophoblast (STB).
- Villous CTBs can also form multilayered columns of proliferating mononuclear cells that differentiate into non-polarised and invasive CTB. These motile and highly invasive CTBs are found in the maternal decidua, the intima of the endometrial spiral arteries and the proximal third of the myometrium.

Villous CTBs are proliferating cells, in contact to one another and to STB through the adhesion molecule E-cadherin. They express epidermal growth factor (EGF) receptors and actively secrete EGF, transforming growth factor-beta (TGF) and interleukin-1 beta (IL-1).

The laminin receptor (integrin 6 4) anchors these CTBs to the villous basement membrane. Being in a rather hypoxic environment, these cells express hypoxia induced factor (HIF-1) and the von Hippel-Lindau (VHL) proteins.

Invasive CTBs do not proliferate and express HLA antigens, HIF-1 and VHL and integrins: the distal CTBs express 1 1, 5 1, v 1 and v 3, whereas the proximal CTBs do not express these integrins and still retain the 6 4 laminin receptor. These invasive cells when cultured in vitro maintain their invasiveness and are thus used as a model for the invasive stage of the implantation process.

The instrumental role of matrix metalloproteinases and their inhibitors in the process of implantation.

The motile and highly invasive CTB, as well as tumour cells, are invasive because they secrete proteases capable of digesting the surrounding extra cellular matrix (ECM). Serine proteases, cathepsins and metalloproteinases have been implicated in the invasive process of tumours as well as CTBs (Cohen M, Meisser A, Bischof P., 2006).

Metalloproteinases (MMP) also termed matrixins; represent a family of at least 26 human zinc-dependent endopeptidases, collectively capable of degrading essentially all components of ECM. According to their substrate specificity and structure, members of the MMP gene family can be classified into 4 subgroups:

1. Gelatinases (MMP-2 and MMP-9) digest collagen type IV (the major constituent of basement membranes) and denatured collagen (gelatine).
2. Collagenases, (MMP-1, 8, 13) digest collagen type I, II, III, VII and X. They are thus appropriately designed for digesting the collagen of the interstitium ECM.
3. Stromelysins (MMP-3, 7, 10, 11 and 12) have a relatively broad substrate specificity and digest collagen type IV, V, VII, as well as laminin, fibronectin, elastin, proteoglycans and gelatin.
4. The substrate of the membrane MMPs (MMP-14, 15, 16) is essentially proMMP-2 and these enzymes allow activation of MMP-2 at the cell surface of the invasive front.

Most MMPs are secreted as inactive pro-enzymes (zymogens) that become activated in the extra-cellular compartments with the exception of MMP-11 and MT-MMPs. Activation occurs by dissociation of a Zn^{2+}-Cys interaction that leads to the loss of a pro-peptide. Several enzymes are capable of activating the pro-matrixins, the most prominent of them being plasmin. The activity of MMPs in the extra-cellular space is specifically inhibited by tissue inhibitors of MMP (TIMP), which bind to the highly conserved zinc-binding site of active MMPs at molar equivalence. The TIMP gene family consists of four structurally related members, TIMP-1, -2, -3 and –4. TIMP-3 is produced by CTBs and plays an important role in regulating CTB invasion.

In vitro, CTBs invade a reconstituted basement membrane (Matrigel); behaving thus, like metastatic cells. This invasive behaviour is due to the ability of CTBs to secrete MMPs, since TIMP inhibits their invasiveness. Furthermore, cultured CTBs secrete MMPs, but CTBs from early pregnancy are more invasive and secrete more MMPs than CTBs isolated from the term placenta. All MMPs are not equally important for trophoblast invasion. Gelatinase B (MMP-9) has been shown to mediate CTB invasion into Matrigel; however, it is unknown if this is also true in vivo, particularly since MMP-9 knock-out mice are partially fertile although they do not respond to experimental metastasis.

The major mediators of trophoblast invasion.

Although CTB (and TE of the blastocysts) behave like metastatic cells, in contrast to tumour cells, CTB are only transiently invasive (first trimester) and their invasion is normally limited only to the endometrium and to the proximal third of the myometrium. This temporal and spatial regulation of trophoblast invasion is believed to be mediated in an autocrine way by trophoblastic factors and in a paracrine way by uterine factors. Several types of regulators have been investigated such as hormones, cytokines, growth factors and ECM glycoproteins (Bischof and Campana 2000).

Hormones, such as progesterone and hCG are secreted by trophoblast and are present in large amounts at the foeto-maternal interface. Progesterone down regulates the production of MMP-9 in 1st trimester CTB. This effect must be mediated intracellularly by other factors since there is no progesterone response element in the regulatory region of the MMP-9 gene hCG could play a previously unsuspected autocrine role in the regulation of trophoblast invasion. Indeed we observed that in CTB, hCG specifically increased MMP-9 secretion (more than 10 times). These results are in line with studies that evaluated the degree of trophoblast invasion in ectopic pregnancy in correlation with circulating hCG.

Cytokines and growth factors are known to affect the invasive behaviour of cells and CTB are no exceptions. This abundant literature has been reviewed recently. Factors such as Il-1, -6, -10, -15 as well as tumour necrosis factor**,** EGF, leukaemia inhibitory factor**,** TGF**,** insulin-like growth factor binding protein-1 and insulin-like growth factor II have all been shown to modulate MMP secretion and/or invasion in human trophoblastic cells.

Components of the ECM. are known to influence adhesion, spreading, migration and differentiation of cells through specific cell surface receptors called integrins. Villous CTB predominantly express the α6ß4 integrin whereas invasive CTB modulate their integrin repertoire from being α6ß4 positive and α5ß1 negative to becoming α6ß4 negative and α5ß1 positive. These changes in integrin expression are linked to the acquisition of an invasive phenotype.

The similarity between embryo implantation and tumour metastatisation.

The establishment of an invasive phenotype in trophoblast as well as in tumours involves a host of cellular processes and an array of expression and/or repression of several genes such as those involved in cell adhesion, composition of the ECM, matrix digestion, angiogenesis, apoptosis or cell cycle arrest. It is now commonly admitted that trophoblast invasion and tumour progression depend on the same biochemical and molecular mediators.

The transcription factor and tumour suppressor p53 plays a key role in malignant transformation. Almost all invasive human tumours have either an inactivated p53 gene or a defect in the p53 pathway. P53 has been called the “Gatekeeper of the genome” (Levine 1997). Indeed, genotoxic stresses that induce DNA breaks, induce p53 phosphorylations. This phosphorylated and thus stabilised protein acts as an onco-supressor by inducing either cell cycle arrest (allowing the DNA to be repaired) or apoptosis. This important mechanism thus

avoids the DNA damage to be carried over to daughter cells. If the p53 gene is mutated or if the p53 pathway is defective, then p53 is no longer protective and becomes oncogenic.

It has been shown that p53 mediates activation of MMP-2 and potently inhibits expression of MMP-1 and MMP-13 gene transcription. Given that human MMP-9 promoter has several features in common with the promoters of these MMPs and since it was shown that MMP-9 over-expression was strongly associated with the functional loss of p53 in different carcinoma cells, we hypothesized that MMP-9 expression could also be regulated by wild type p53 (wt-p53) in the human trophoblast. To test this hypothesis, transcription assays were performed with chloramphenicol acetyl transferase reporter driven by serial deletion of hMMP-9 promoter, co-transfected with expression vector of p53 in HT1080 cells and CTBs. The unpublished results have demonstrated that wt-p53 down-regulates the –670, but neither the –531 nor the -90 promoters activity of hMMP-9. On the other hand, the tested p53 mutants had partially lost this repressive activity. Experiments with mutated hMMP-9 promoters revealed that NF-kB at position -600 and Ets at position –541 are involved in p53 trans-repression of MMP-9. Moreover, results from band shift assays demonstrated that MMP-9 promoter was inhibited by p53, through a NF-kB dependent but a NF-kB binding independent mechanism. Taken together, these observations indicate that the hMMP-9 gene is a target gene for p53 repression.

In experiments designed to test the effects of endogenous trophoblastic p53, we observed that inhibition of endogenous p53 with pifithrin alpha or p53 siRNA did not increase the activity or expression of MMP-9 in CTBs. This indicates that in CTBs p53 was indeed expressed and decreased by p53siRNA, but that it was functionally incompetent (as has been described for tumour cells) since it could not modify MMP-9 expression. Our surprise was even greater when we performed immunohistochemistry on first trimester trophoblast with antibodies raised to p53. With the antibody Ab 5, recognising a wild type conformation of p53, no staining could be observed in trophohoblast whereas it was positive in decidua. In contrast, antibody Ab 3, recognising a mutated conformation of p53, showed an abundant cytoplasmic localisation of p53 in villous and extra-villous trophoblast. We speculate that the functional incompetence and the cytoplasmic localisation of p53 are linked and that this particular status of trophoblastic p53 could be instrumental to the invasive behaviour of CTB and of blastocysts.

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Molecular mechanisms of decidualization

***Jan J Brosens*[1]**
***Birgit Gellersen*[2]**
[1]Institute of Reproductive and Developmental Biology
Imperial College London
UK
[2]Endokrinologikum Hamburg
Hamburg
Germany

Introduction

Pregnancy depends on the protection, controlled invasion and growth of the semi-allogenic placenta within the maternal uterine environment. In the endometrium, the maternal response to pregnancy is characterised by influx of specialized uterine natural killer (uNK) cells, remodelling of the spiral arteries, and differentiation of the endometrial stromal cells (ESCs) into decidual cells. In most species, this maternal uterine response is triggered by signals derived from the implanting blastocyst. In humans, however, the decidual process occurs independently of pregnancy during the mid-secretory phase of each cycle. This apparent switch from embryonic to maternal control of the decidual process has profound consequences for human reproduction. For instance, 'spontaneous' or conceptus-independent decidualization of the endometrium also occur in the few other species that menstruate, such as Old World monkeys, the elephant shrew and certain bats, suggesting that these processes are causally linked. Cyclic decidualization and menstrual shedding occurs on average 400 times during the reproductive years of women in developed countries. Consequently, abnormal uterine bleeding is one of the most common disorders in women and a major indication for surgical intervention. Furthermore, emerging evidence suggests that reproductive disorders that affect the preconceptual endometrial milieu predispose to a spectrum of pregnancy complications associated with impaired trophoblast invasion, including miscarriage, pre-eclampsia, fetal growth restriction and preterm labour. A detailed understanding of the decidual process is therefore important from a biological and a clinical perspective.

Morphological and biochemical aspects of decidualization

The first morphological signs of decidualization become apparent in stromal cells surrounding the terminal spiral arteries of the superficial endometrial layer approximately 9 days after ovulation and coincide with the end of the limited period of uterine receptivity. Upon differentiation ESCs progressively enlarge and multiple projections appear on the surface of the decidual cells that extend freely into the extracellular matrix or indent the cytoplasm of adjacent cells. This distinct morphological appearance is underpinned by biochemical changes. Major secretory products of decidualized stromal cells, such as prolactin (PRL) and insulin-like growth factor binding protein-1 (IGFBP-1), have traditionally been used as markers of the differentiated state. Recently, microarray-based genome-wide expression profiling has been used to interrogate gene expression in purified ESC cultures exposed to a decidualizing stimulus *in vitro*. These studies confirmed that the decidualization process involves sequential reprogramming of functionally related

families of genes involved in extracellular organization, cell adhesion, cytoskeletal organization, signal transduction, metabolism, differentiation and apoptosis.

Upon biochemical reprogramming, decidualizing endometrial stromal cells acquire many new functions necessary for successful trophoblast invasion and placenta formation. For instance, decidualizing stromal cells surrounding the spiral arteries highly express tissue factor, the initiator of the extrinsic coagulation pathway, and plasminogen activator inhibitor-1 indicating that they play a primary role in maintaining vascular stability prior to menstruation and during endovascular trophoblast invasion. Differentiating ESCs secrete a variety of factors, such as macrophage inflammatory protein-1β, interleukin (IL)-11, IL-15 and PRL, which are thought to provide chemotactic, proliferative and differentiating signals for uNK cells. On the other hand, the decidua also expresses the tryptophan-catabolising enzyme indoleamine 2,3-dioxygenase and Fas ligand, which are implicated in the suppression of T cell-dependent immune responses to fetal alloantigens. Remodelling of the extracelluar matrix and secretion of growth factors and binding proteins such as IGFBP-1 are thought to critically regulate coordinated trophoblast invasion and differentiation. Upon differentiation, stromal cells become resistant to inflammatory signals and express enzymes such as manganese superoxide dismutase that are involved in oxidative stress defences. These observations suggest that the decidua serves as a buffer that protects the developing conceptus from environmental stress.

Intiation of the decidual process

Many protocols for *in vitro* decidualization have been developed over the last 25 years. Three major points can be deduced from these studies. First, treatment with progesterone for 8 or more days, alone or in combination with oestradiol, can induce expression of decidual markers such as PRL or IGFBP-1 in primary cultures, albeit at modest levels. Second, ligands that trigger a persistent increase in intracellular cyclic AMP (cAMP) levels much more rapidly induce expression of decidual marker genes, although the levels are not maintained in long-term cultures. Finally, full decidualization and sustained expression of the differentiated phenotype requires both elevated cAMP levels and progesterone.

Convergence of cAMP and progesterone signalling is also required for the timed expression of the decidual phenotype during the mid-secretory phase of the cycle. The endometrial stroma in the secretory phase of the cycle is not only exposed to progesterone but also to a number of factors which bind to G-protein coupled receptors (GPCRs) present in stromal cells. Binding of these receptors results in activation of adenylate cyclase, formation of the second messenger cAMP and activation of protein kinase A (PKA). Such ligands include prostaglandin E_2, corticotrophin releasing hormone, relaxin and perhaps the circulating pituitary gonadotrophins. In pregnancy, the endometrium is under the continued support of steroid hormones as well as blastocyst-derived signals such as human chorionic gonadotrophin (hCG), which provides the hormonal milieu for full decidualization of the entire endometrium.

Autocrine/paracrine signals of decidualization

Once the decidual process is initiated, differentiating cells secrete a number of cytokines and growth factors involved in propagating this process. In cAMP-treated primary ESC cultures, induction of IL-11 expression parallels that of PRL and IGFBP-1, and inhibition of IL-11 signalling attenuates the expression of these differentiation markers. IL-11 and its receptor subunit IL-11Rα are localized in the decidualized stromal cells of the mid-late secretory endometrium. Moreover, female IL-11R-deficient mice are infertile due to a defective post-implantation decidual response. Cyclic AMP signalling also induces expression of heparin-binding epidermal growth factor (HB-EGF), both the soluble form and the transmembrane precursor, as well as its two cognate receptors, EGFR and ErbB4/HER4, in human ESCs. Inhibition of HB-EGF signalling not only attenuates PRL and IGFBP-1 production in differentiating ESCs but also sensitises these cells to apoptosis induced by pro-inflammatory signals. Notably, HB-EGF induces IL-11 secretion by cultured ESCs. Members of the TGF- superfamily, including inhibins, activins and follistatin, have also been implicated in the paracrine/autocrine regulation of the decidual process. Production of activin A, a dimer of βA subunits, is induced in ESCs upon cAMP-induced differentiation. It rises in parallel with PRL secretion between days 2 and 6 of treatment. It has been shown that activin A itself promotes the expression of decidual markers *in vitro*, an effect inhibited by co-treatment with the activin-binding protein follistatin. Another TGF- family member highly expressed upon cAMP stimulation of ESCs is Lefty-A, which was originally identified as an endometrial bleeding-associated factor (EBAF). Like other TGF- members, Lefty-A is expressed as a polypeptide that requires processing for its activity.

Progesterone signalling

Progesterone regulates endometrial differentiation predominantly by activating the nuclear progesterone receptors (PRs), members of the superfamily of ligand-activated transcription factors. Two isoforms exist, PR-A and PR-B, which arise from different promoter usage in a single gene. PR-B differs from PR-A in that it contains an additional 164 amino acids at the N-terminus (B-upstream sequence, BUS). Although the PR isoforms display indistinguishable hormone- and DNA-binding affinities, several studies have shown that, depending on the cell- and promoter context, PR-A and PR-B have remarkably different transcriptional activities. In general, the PR-A isoform is transcriptionally much less active and functions as a dominant inhibitor of transcription by PR-B and various other steroid receptors. PR-A shares with PR-B the activation functions AF-1 and AF-2 but lacks AF-3, which is situated in the BUS segment specific to PR-B. Binding of progesterone induces a conformational change in the receptor, resulting in phosphorylation, dissociation from heat shock proteins, dimerization, sumoylation of a subpopulation of receptor molecules, binding and activation of specific response elements in the promoter region of target genes, and recruitment of the basal transcriptional machinary. The latter requires further interaction of the AF-2 region with steroid receptor co-activators (SRCs) resulting in recruitment of other SRC-associated histone acetyltransferases (CREB binding protein and pCAF) and the methyltransferase CARM1 involved in modifying the chromatin template. Conversely, silencing of gene expression by steroid hormones or other transcription factors requires binding to co-repressors, such as SMRT and N-CoR, that mediate transcriptional repression. There is overwhelming evidence to

suggest that PR-A is the dominant isoform in ESCs. Moreover, gene ablation studies in mice have shown that PR-A, in contrast to PR-B, is indispensable for decidualization.

Convergence of progesterone and cAMP signalling

In recent years, the regulation of major decidual marker genes, such as PRL, IGFBP-1, and TF, has been widely used as a paradigm for the dissection of the cross-talk between cAMP and progesterone signalling in ESCs. Notably, none of the major decidual genes appears to be under direct transcriptional control of activated PR, in keeping with the inability of progesterone to induce ESC differentiation in short-term cultures. However, emerging evidence suggests a major role for the activated PR-A, as a scaffold for the recruitment of transcription factors activated, directly or indirectly, in response to cAMP signalling. Direct physical interaction has indeed been demonstrated between PR and STAT5, C/EBP , or FOXO1. By hijacking these transcription factors, the activated PR acquires control of the diverse gene families involved in decidualization. More recently, it has become apparent that the cAMP and progesterone signalling pathways also differentially control the expression and activity of a limited number of transcription factors involved in cell fate decisions.

Cross-talk between progesterone and cAMP signalling occurs at multiple as illustrated by the regulation of FOXO1 expression and activity. FOXO transcription factors (FOXO1, FOXO3a, and FOXO4) are critical mediators in cell fate decisions in response to growth factor, hormonal and environmental cues Of the three human FOXO proteins, FOXO1 is markedly induced upon decidualization both *in vivo* and *in vitro*, and is involved in regulating the expression of decidual marker genes, such as PRL and IGFBP-1. Furthermore, the expression and activity of FOXO1 itself is subject to intricate control mechanisms involving both the PKA pathway and the ligand-activated nuclear PR. Within 3 days of cAMP treatment, cultured ESC up-regulate FOXO1 mRNA and protein, and this response is markedly enhanced by progestin although treatment with progestin alone does not induce FOXO1 expression. A known FOXO target gene is *BIM* (*BCL2L11*), which encodes the proapoptotic Bcl-2 homology 3 domain-only protein Bim. Bim expression increases in the endometrium prior to menstruation and in culture upon activation of the cAMP pathway in a FOXO1-dependent manner. However, despite the fact that FOXO1 levels are even higher in cells treated with a combination of cAMP and progestin, Bim expression is inhibited under these conditions. The solution to this paradox lies in the fact that cAMP causes nuclear acccumulation of FOXO1 while added progestin induces nuclear exit and thus inactivation of a large fraction of the total FOXO1 protein pool. Withdrawal of progestin in turn results in rapid nuclear re-accumulation of FOXO1, increased expression of Bim and increased cell death. These findings suggest a critical role for FOXO1 in mediating the proaptototic pathway initiated by progesterone withdrawal at the end of the menstrual cycle. This is underpinned by the observation that silencing of FOXO1 expression in differentiating ESC completely abrogates apoptosis induced by progestin withdrawal, suggesting that progesterone serves as a survival factor in decidualized ESC through partial cytoplasmic retention and inactivation of FOXO1.

Summary

Decidualization involves extraordinary reprogramming of many cellular functions. In recent years, the number of signals, signal transduction cascades and transcription factors implicated in this process has grown exponentially. At first, the complexity of orchestrating the expression of so many diverse gene sets appears beyond comprehension. Yet, despite this complexity, a relatively simple and elegant model has emerged. In humans, differentiation of ESCs is initially triggered by local factors capable of elevating cellular cAMP levels and sustained activation of the PKA signalling pathway. This in turn elicits the expression of a number of factors that feed-back on the decidualizing cells, thereby activating subsidiary signalling pathways and downstream transcription factors which converge onto the activated PR. By recruiting decidua-specific transcription factors, the activated PR acquires control of the diverse gene sets necessary for cellular differentiation and survival. Consequently, homeostasis of differentiating endometrium, in the presence or absence of pregnancy, becomes entirely dependent upon progesterone.

Selected Reading

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- Giudice LC 2004 Microarray expression profiling reveals candidate genes for human uterine receptivity. Am J Pharmacogenomics 4:299-312. - *An in-depth overview of relevant microarray studies.*
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Genomics of Human Endometrial Receptivity

José A. Horcajadas, PhD
Fundación IVI, C/ Guadassuar, 1 Bajo, Valencia, Spain
Phone: 00 34 963 45 55 60 Fax: 00 34 963 45 55 12
e-mail: jhorcajadas@ivi.es

Learning objectives

(1) To define endometrial receptivity
(2) To integrate the available information of genomic analysis of human endometrial receptivity
(3) To understand application of the new technologies for diagnosis of infertility of endometrial origin.

Introduction

The human endometrium is a complex tissue and its cyclic regulation requires the successful interaction of an uncountable number of factors. This organ is hormonally regulated being non-adhesive to embryos throughout most of the menstrual cycle in humans and other mammals. In this environment, endometrial receptivity refers to a hormone-limited period in which the endometrial tissue acquires a functional and transient ovarian steroid-dependent status allowing blastocyst adhesion[1]. The scientific knowledge of the endometrial receptivity process is fundamental for the understanding of the mechanisms that govern embryonic implantation and human reproduction[2]. This important knowledge can potentially be used to improve fertility in infertile patients whereas the opposite can be applied as an interceptive approach to prevent embryo implantation[3].

Advances in gene expression profiling facilitated by the development of DNA microarrays[4] represent a major progress in global gene expression analysis. The availability of this technology has made possible to investigate the endometrial receptivity process from a global genomic perspective. These works will be reviewed here.

Endometrial receptivity

We know that the endometrium is a specialized, hormonally regulated tissue that is non-adhesive to embryos during most of the menstrual cycle in humans and other primates. Endometrial receptivity is a self-limiting period in which the endometrial epithelium acquires a functional and transient ovarian steroid-dependent status that favors blastocyst adhesion[5]. The endometrial epithelial cells (EEC) become receptive mainly due to the presence of progesterone (P) after appropriate 17β-estradiol (E2) priming. This period, termed the "window of implantation", begins 4-5 days after P production or administration and finalizes a further 5 days later[6]. In this way, the receptive window is limited to days 19-24 of the menstrual cycle in humans and 8-10 days post ovulation in other primates[6]. Indeed, the administration of P antagonist or E2 antiserum during the pre-implantation period disrupts endometrial receptivity in primates[7-10].

The embryo may be relevant in the induction of the receptive status[11], a role which has been demonstrated in the nonhuman primate (Papio anubis). Therefore, the regulation of these mutually interactive mechanisms is mediated by hormones (endocrine) and by the embryo (paracrine /yuxtacrine).

To acquire the receptive phenotype, the EEC suffers structural and functional changes. The morphological changes include modifications in the plasma membrane[12] and cytoskeleton[13,14]. The apical plasma membrane develops transitional adhesive properties when it undergoes structural changes; long thin, regular microvilli are gradually converted into irregular, flattened projections, a process named plasma membrane transformation[12]. It is possible that the remodeling of the epithelial organization from a polarized to a non-polarized phenotype prepares the apical pole for cell-to-cell adhesion[13].

The significant histological, biological and physiological changes that occur in the endometrium during the proliferative, secretory and menstrual phases of the cycle are ultimately the result of changes that occur at the level of gene transcription. In this sense, microarray technology studies have contributed enormously to the knowledge of this complex and significant process.

Wide genomic analysis of human endometrial receptivity

Initial studies were performed using macroarrays that contained 375 genes[14]. Soon, researchers working on human reproduction realized the powerful capacity of microarrays and four works using microarrays were published between 2002 and 2003[15-18]. Very recently it has been published the last one[19]. Although all of them have used the same technology, some differences in the experimental design and data analysis require our attention. Table 1 summarizes the differences among these studies. The menstrual day for sample collection, number of endometrial biopsies used, pooling or not the RNA isolated, are important differences between these papers. Furthermore, the data analysis and statistical method used; four of them established a minimal fold-increase of 2.0 to consider evident gene regulation. However, Riesewijk and co-workers used a 3.0 fold-increase. It is remarkable that in this study they performed the microarray analysis using samples coming from the same woman at two different stages of a single menstrual cycle. This approach minimizes biological variability between samples[18].

A partial comparison of microarray data from five works published is presented in the last of the five papers[19]. The differences in study designs are reflected in the lists of differentially expressed genes identified. Curiously, only one gene is present in all the studies, osteopontin, which was clearly up-regulated in the five comparisons. This structural protein is present in several organs. In the endometrium, it has been found in glands and secreted into the lumen[20] and although it has assigned adhesive functions its role in human embryonic implantation is still unknown[21].

Several molecules are present in four out of five papers. Some of them are proteins previously described in the endometrium with or without an assigned role in endometrial receptivity. If we examine the function of the consensus genes during the WOI we can find a wide range of functions: genes involved in lipid metabolism (apolipoprotein D), immune response (decay accelerating factor for complement, serine (or cysteine) proteinase, interleukin 15), regulation of cell cycle (growth arrest and

DNA-damage-inducible, alpha), ion binding (annexin IV) or enzymes with different functions in different tissues (monoamino oxidase A).

It seems that lipid metabolism is constantly controlled during endometrial development. Kao's work[16] underlines the presence of members of Wnt family in the list of up-regulated genes. The marked up-regulation of Dickkopf-1, present in all four studies is of particular interest. Dickkopf-1 inhibits Wnt signalling by binding LRP5/6[22]. Wnt7A (-/-) null mice are infertile and have complete absence of uterine glands and a reduction in mesenchymally derived uterine stroma[23]. The role of the Wnt family in human endometrium and implantation should be considered in future investigations. These data, offer the opportunity to develop an endometrial database of genes expressed during the WOI in the natural cycle. It is important to accept that these published data are complementary, not only due to different study designs, but also due to differences in the software and statistics used for analysis of the hybridization data. Most of the laboratories have their favourite protein or molecule and they are working in the dissection of its function in endometrial receptivity. But, at the moment, functional studies have not demonstrated the existence of a magic molecule in endometrial receptivity. Probably, we will never be able to understand this complex process with the regulation of one gene because it is the result of an equilibrated expression of tens of genes and this has to be into account in future studies.

New diagnosis of infertility of endometrial origin

Microarray technology has bursted into many research fields allowing scientists to analyze widely expression of many genes in quick and efficient experiments. In reproductive medicine, researchers have used microarrays to understand the molecular mechanisms involved in endometrial receptivity. The significant histological, biological and physiological changes that occur in the endometrium during the proliferative, secretory and menstrual phases of the cycle are ultimately the result of changes that occur at the level of gene transcription. Endometrial receptivity at the time of embryonic implantation is a crucial moment on the menstrual cycle with a high relevance. To know the gene expression profile of endometrial receptivity at the time of implantation has been one of the main goals for researchers working in human reproduction in the last years. Endometrial receptivity is a very complex process in which an uncountable number of genes are involved. However, the studies published have also demonstrated that a limited number of candidates are always presents in that studies and that endometrial receptivity could be perfectly explained only with their participation. This is the time for genomic era and a complete overhaul is necessary for the complete implementation of microarray analysis in research, drug testing, diagnostic and tissue evaluation. Future directions in endometrial receptivity studies will also require the implementation of better in vitro models and the continuous use of genomics and proteomics in these models. Although non-primate animal models have distinct advantages in monetary and temporal cost, vast amounts of genetic information, and the ability to be genetically modified, they remain inherently limited in their ability to elucidate the physiological mechanisms of endometrial receptivity. However, studies in non-human primates have shown high fidelity to human implantation, suggesting their potential as models for investigation endometrial receptivity, embryo implantation, early pregnancy and endometrium-related diseases such as endometriosis.

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Table 1. Summary of studies using microarray analysis performed in endometrium for analyzing endometrial receptivity during the WOI.

Study	Samples	RNA pooled	First sample (day of cycle)	Second sample (day of cycle)	Fold-change	UP	DOWN
Kao	11	NO	Prolif. phase (8-10)	LH+(8-10) (21-23)	>2.0	156	377
Carson	6	YES	LH+(2-4) (15-17)	LH+(7-9) (20-22)	>2.0	323	370
Borthwick	10	YES	Prolif. phase (9-11)	LH+(6-8) (19-21)	>2.0	90	46
Riesewijk	10	NO	LH+2 (15)	LH+7 (20)	>3.0	153	58
Mirkin	8	NO	Early-luteal (16)	Mid-luteal (21)	>2.0	49	58

The role of the endometrium in early pregnancy nutrition

Professor Graham J Burton
Department of Physiology, Development and Neuroscien,
Anatomy School
Downing Street
Cambridge CB2 3DY
UK

Learning Objectives:

- to appreciate that the maternal arterial circulation to the human placenta is highly restricted during the first trimester
- to realise that the uterine glands deliver secretions into the intervillous space of the placenta during the first trimester, and that maternal glycoproteins are phagocytosed by the trophoblast (histiotrophic nutrition)
- to set the human situation in the context of comparative placentation
- to understand the potential benefit of histiotrophic nutrition during the period of organogenesis in terms of protection against free radical mediated teratogenesis

Maternal placental circulation during the first trimester

The advent of high-resolution ultrasound imaging has led to a major change in our understanding of the status of the maternal circulation to the placenta during early pregnancy. In most textbook accounts it is stated that the invading syncytiotrophoblast breaks into the maternal vessels shortly after implantation, and that a circulation is established in the developing placenta during the second week post-fertilisation. These accounts have been based on the examination of a few histological samples alone, which of course provide only static images. They were first challenged over a decade ago when it was appreciated that moving echoes indicative of significant blood flow cannot be detected within the placenta until 10-12 weeks of pregnancy[1]. Further investigations involving the ex-vivo perfusion of pregnancy hysterectomy specimens confirmed that in first trimester samples little perfusate entered the placenta, whereas in second trimester cases inflow was rapid. The explanation for the difference was provided by morphological studies, which revealed that invading extravillous trophoblast cells occlude the tips of the spiral arteries during early pregnancy. Shortly after implantation is complete a sub-population of trophoblast cells, the extravillous trophoblast, migrate from the developing placenta into the endometrium and inner third of the myometrium. The presence of these cells is associated with the loss of smooth muscle from the walls of the spiral arteries, and their physiological conversion into dilated flaccid conduits. There are two lines of migration; the interstitial trophoblast cells migrate through the endometrial stroma and invade the vessels from the outside, whilst the endovascular trophoblast migrates down the lumens of the arteries. It transpires that the volume of migrating endovascular cells is sufficient to effectively plug the tips of the arteries, for hysteroscopy has revealed that the placental intervillous space is filled with a clear fluid prior to 10 weeks of pregnancy[1]. This fluid may represent a plasma filtrate that percolates through the network of intercellular spaces within the plug. The pink hue of maternal blood was only seen after 12 weeks when the plugs dissipate. Recent studies have shown that when the maternal circulation is established it begins in the periphery of the placenta where trophoblast invasion is least, and hence where plugging of the spiral arteries is likely to be less extensive. Locally high levels of oxidative stress induced in the placental tissues may lead to villous regression and formation of the chorion leave[2].

Uterine glands as an alternative source of nutrition during the first trimester

In view of these new findings we have investigated other sources of nutritional support for the conceptus during the first trimester by reviewing an archival collection of placenta-in-situ histological sections. We have observed that the endometrium is still well-developed during early pregnancy, being approximately 6 mm thick at 6 weeks gestational age. The uterine glands are still highly active, and their lumens are packed with heterogeneous carbohydrate-rich secretions containing numerous lipid droplets. The glandular cells have an irregular columnar appearance at this stage, and their apical portions are packed with glycogen as during the late secretory phase of the normal cycle[3]. The glands open through the developing basal plate into the intervillous space and the secretions can be observed dissipating between the villi. Hence, the secretions may also contribute to the clear fluid observed in the space during the first trimester[1]. We have followed the fate of these secretions by performing immunohistochemistry for maternal-specific proteins, such as MUC-1 and glycodelin (PP14), the mRNAs for which are not expressed in the placenta. These studies have shown that the maternal proteins are phagocytosed by the syncytiotrophoblast, where they enter the lysosomal pathway. Our findings suggest the maternal proteins are broken down, raising the possibility that the constituent amino acids are re-cycled in anabolic pathways in the placenta and fetus. Intact maternal proteins have also been traced into the amniotic and coelomic fluid, from where they appear to be taken up by the outer mesothelial covering of the yolk sac[3].
Communications between the uterine glands and the intervillous space can be observed until at least 13 weeks of gestation, but by then the endometrium has undergone considerable regression and now is only 1-2 mm thick. The glandular epithelial cells have also become cuboidal, and appear more quiescent with no intracellular accumulations of glycogen. The overall impression is therefore one of a gradual decline in activity of the glands during the first trimester.

Comparative placentation

In all species the initial nutritional support of the conceptus is provided by the glandular secretions of first the oviduct and then the uterus. The period for which this histiotrophic form of nutrition is important varies between species depending on the type of implantation and placentation that has evolved. In the non-invasive species, such as the sheep, pig and horse, where the placental membranes are simply apposed to the uterine epithelium, the period may last for several weeks. In the horse, for example, the conceptus does not attach to the uterine wall until around day 40 post-fertilisation. It is supported in the interim by copious secretions form the uterine glands, which on account of their high lipid content have been referred to over the years as uterine milk.

The importance of these secretions has been demonstrated experimentally in the sheep, for if development of the glands is suppressed then the conceptus fails to develop once it enters the uterus and the pregnancy fails[4]. An increasing number of growth factors and cytokines have been identified in the sheep secretions, and so it is likely that the secretions play major roles in regulating placental cell proliferation and differentiation, as well as providing simple nutrient support. Furthermore, it has been proposed that a hormonal dialogue between the conceptus and the glands influences production of the secretions in a servo-mechanistic manner.

The human is almost unique amongst mammals in displaying such precocious and invasive implantation. However, the new appreciation of the reliance on histiotrophic nutrition during the first trimester brings the human situation into closer agreement with the general mammalian condition than previously recognised. Growth factors have also been identified within the glandular epithelial cells during the first trimester, suggesting again a greater role for the glands in the regulation of placental development than nutrition alone[5].

Benefits of histiotrophic nutrition during organogenesis

The lack of significant maternal blood flow to the human placenta during the first trimester ensures that organogenesis takes place under a relatively low oxygen environment. The oxygen concentration measured in the intervillous space of the placenta is less than 20mm Hg prior to 10 weeks of gestation, and then rises to approximately 60mm Hg by 12 weeks[6]. The conceptus is not compromised energetically however, and appears to employ the phylogenetically old polyol pathways to maintain ATP levels within the normal range. Looking across the mammalian spectrum it is likely that the same is true in most species, although equivalent measurements of oxygen concentrations have not been performed. High activity of the polyol pathways has however been observed in the sheep during early pregnancy.

One of the principal effects of maintaining tissues under low oxygen conditions is that production of potentially damaging oxygen free radicals will be reduced. There is no doubt that free radicals can be teratogenic in conditions such as diabetes, and that antioxidants can protect against congenital malformations in experimental animal models[7]. We have therefore proposed that the human placenta protects the embryo during the main period of organogenesis by excluding maternal blood from the intervillous space, and utilising nutrients from the uterine glands instead[8]. Once organogenesis is complete and the fetus is ready to grow then an effective maternal circulation to the placenta is established at the start of the second trimester. The transition represents a dangerous period, and it is essential that onset of the circulation is gradual. If onset is precocious and disorganised then overwhelming oxidative stress of the placental tissues may contribute to pregnancy failure[6].

Conclusion

The endometrium is essential for the normal development of the conceptus during the first trimester, providing a rich source of nutrients, growth factors and cytokines that may play important roles in supporting and regulating feto-placental development.

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Time of Implantation

Donna Day Baird
Epidemiology Branch
National Institute of Environmental Health Sciences
National Institutes of Health
DHHS, Research Triangle Park, NC 27709, USA
E-mail: baird@niehs.nih.gov
Tel: (919) 541-2786

Learning Objectives

1. Identify the biological evidence for estimating the timing of implantation.

Direct Observation -- Hertig and Rock's hysterectomy study
In vitro development of embryos
Endometrial window of implantation
Detection of hCG in maternal blood or urine

2. Identify 3 necessary characteristics for a study of implantation based on hCG measures.

3. Describe the observed distribution of implantation days based on hCG data from the North Carolina Early Pregnancy Study.

4. Identify pattern of pregnancy survival in relation to timing of implantation.

5. How does the pattern of hCG rise vary in relation to timing of implantation?

6. How do patterns of corpus luteum rescue differ in relation to timing of implantation?

7. Identify three research questions regarding timing of implantation that are important for fertility treatment programs.
   a) Do fertility drugs result in a less defined endometrial window of receptivity, thus allowing lower quality embryos to implant?
   b) Does later implantation indicate a slower growing conceptus?
   c) What are the biological effects of a progesterone surge at time of implantation?

Lecture Summary

Biological Evidence for Estimating the Timing of Implantation
Implantation involves apposition, attachment, and invasion of the blastocyst stage embryo to the uterine endometrium. There are several ways of estimating time of implantation.

Direct observation of implantation

The initial study of timing of implantation is also the most direct. Hertig, a pathologist, and Rock, a gynecologist, spent 15 years on a study designed to yield direct evidence of early pregnancy. They enrolled women planning to have hysterectomies. Entry criteria were: 1) married and living with husbands, 2) prior history of at least 3 full-term pregnancies (demonstrated fertility), 3) willing to participate. Participants were told about the study goals: to learn about early human embryo development and implantation of the conceptus in the uterine lining. The date for surgery was set, and participants were asked to send a postcard to the clinic whenever they had unprotected intercourse. Participants also recorded menses if it came before the surgical date. Surgical dates were not changed in order to allow women additional cycles to become pregnant, but since women knew the purpose of the study and were interested in helping, many presumably attempted to conceive. Hertig examined excised uteri, used the endometrial tissue to estimate day of the cycle, and performed a detailed search for early embryos whenever an early pregnancy seemed possible given the timing of intercourse.

Summaries of Hertig et al.'s data were published in 1956-19599.

Post-ovulation Day & Stage		**Nos. of Embryos** Observed/Expected/Possible
2-3	(early preimplantation)	1/5/9
4-5	(preimplantation blastocyst)	7/9/15
6-	(implantation begins?)	0/3/5
7-8	(early implant, open to lumen)	2/15/25
9-10	(early implant, invasive)	3/10/17
11-14	(visible implant, buried)	18/18/31

In their day-9 embryos they find evidence of early utero-placental circulation. In their 11-14 day embryos the circulation is further developed with lacunar spaces filled with maternal blood.

In vitro development of embryos

In vitro fertilization data on embryo development show blastocyst formation on days 4-5 post insemination, hatching and attachment day 6, and attachment with extensive outgrowth day 8.

Endometrial window of implantation

Endometrial window for implantation is thought to be open for an estimated 2-6 days beginning perhaps as early as day 5 post-ovulation

Detection of human chorionic gonadotropin (hCG) in maternal blood or urine

The pre-implantation conceptus shows gene expression for hCG as early as the 8-cell stage. Production increases rapidly during early pregnancy, and hCG is used as the standard test for pregnancy. Theoretically, a very sensitive assay could identify the first day that it appears in mom's circulation. That would be expected to occur when the invading trophoblastic tissue is in proximity to maternal circulation (1-3 days after attachment). Concentrations in serum and urine are similar. Urinary measurement is the only noninvasive method of studying timing of implantation in large community-based populations. In addition to a very sensitive assay for hCG, there needs to be prospective collection of urine with short intervals between collection times (at least daily). Ideally, time of fertilization would mark the start of the pregnancy, but day of ovulation serves as a surrogate.

The North Carolina Early Pregnancy Study – hCG detection

Objective

The primary objective of the study was to estimate the number of pregnancies lost early, before women even know they are pregnant.

Study methods

Participants (n = 221) -Volunteer women from the communities around Research Triangle Park, NC enrolled when they discontinued their method of birth control in order to become pregnant. Couples with known fertility problems or with major chronic diseases like diabetes were excluded.

Protocol - Women collected daily first morning urine specimens from enrollment and continuing through the eighth week of pregnancy or for six months if no recognized pregnancy occurred. Specimens were stored in home freezers for up to two weeks before study pick up. They were then stored at -20^{o} C in study freezers. The women were very conscientious about collecting urine specimens (only 2% of collection days were missing). Daily record cards were used to record menstrual bleeding.

Day of ovulation – Estrogen and progesterone metabolites were measured in urine. The ratio of these metabolites rises as estrogen rises, then drops precipitously when estrogen falls and progesterone begins to rise at time of ovulation. An algorithm was developed and validated to identify a day of ovulation based on this drop in the metabolite ratio.

Definition of a pregnancy (n = 199) – A very sensitive assay for hCG (detection limit of .01ng/ml =~ .13 mIU/ml) was measured in daily urine specimens. Three consecutive days with levels >0.025 was defined as a pregnancy. Clinical pregnancies were those that lasted at least 6 weeks post LMP. Early losses were those ending within 6 weeks of LMP

Day of implantation – First day of pregnancy when hCG exceeded 0.015 ng/ml. Data were complete for detection of day of implantation for 189 (95%) of the pregnancies (141 clinical pregnancies and 48 early losses).

The data across menstrual cycles for a single woman can be pictured on separate panels for steroid hormone metabolites, the ratio of the estrogen and progesterone metabolites, intercourse and hCG, with menses and ovulation shown by vertical shading and dotted line, respectively (Fig. 1).

Study results - implantation times

Day Post-ovulation	**No.**	**%**	
6	3	2	
7	8	4	
8	29	15	For clinical pregnancies, 84%
9	62	33	implanted on days 8-10.
10	52	28	
11	23	12	
12	9	5	Early losses tended to implant later.
13	1	<1	
14	0	0	
15	0	0	
` 16	1	<1	
17	0	0	
18	1	<1	

Study results – correlates of early or late implantation

Late implanting pregnancies are more likely to be lost early (Fig. 2).

Among clinical pregnancies, the rate of hCG rise is slower for late-implantatng pregnancies. The hCG rise for pregnancies implanting on luteal-day 10 or earlier vary little by day of implantation, but clinical pregnancies implanting later have a slower hCG rise. The doubling time of the most linear portion for the first week after implantation is ~22 hours for clinical pregnancies implanting by day 10. Surviving pregnancies that implanted later than day 10 have a doubling time of ~30 hours for this same time interval.

Though the signal from the conceptus, as reflected by the rise of hCG, varies little by day of implantation for those implanting by day 10, the corpus luteum rescue appears weaker for early implantations (Fig. 3). By examining the changes in daily concentration of the

urinary progesterone metabolite during the week after implantation (7 days of data beginning on day of implantation), we could identify three different corpus luteum responses among clinical pregnancies. Some showed a surge of progesterone almost immediately after implantation (within 2 days), the Early PdG Rise group. Others showed a delayed surge (rise on days 3-6 post implantation), the Late PdG Rise group. The remaining clinical pregnancies maintained midluteal levels of PdG, but did not show a PdG surge within the first week. Pregnancies that implanted early were more likely to show the No Rise pattern of CL response.

Other Studies on Timing of Implantation using hCG Detection

IVF studies are limited in determining day of implantation because hCG is injected prior to egg retrieval. Clearance of this exogenous hCG takes a week or more, so detection of embryo-produced hCG is not possible until exogenous hCG is cleared to below levels produced by the conceptus. Therefore, studies that estimate time of implantation in IVF pregnancies generally are unable to identify the hCG rise associated with the conceptus until later than in natural conception cycles. However, the data suggest that there may be more late implantations than is seen in naturally-conceived pregnancies. If this is the case, it suggests that the endometrial window of implantation may be wider in embryo transfer cycles and/or that the IVF embryos proceed through preimplantation growth and the process of implantation (apposition, attachment, and invasion) at a slower rate than in natural conceptions.

There are no large studies of implantation other than the North Carolina Early Pregnancy Study with large numbers of participants who have no fertility problems. Some of the small studies that have been published show the relationship between late implantation and early pregnancy loss that was so strong in the NC Early Pregnancy Study, but others have not seen this pattern. One reason other studies do not see this relationship is because the early losses that die very early (with only a few days of faltering hCG production) are missed; the hCG assays commonly used are less sensitive that that used in the NC study. If we used an hCG value for our detection cutoff for detection that was the minimum hCG value seen on day of detection in a California study, only 65% of our 48 losses would have been detected (78% of those that implanted by day 10, but only 48% of those that implanted after day 10). The late implantations are selectively lost when the hCG assay lacks sensitivity.

In the majority of clinical pregnancies, implantation was followed with a week by a surge in progesterone. A surge in progesterone in response to implantation has also been seen in other primate species. The function of this hormonal surge is not well understood.

Research Questions

What is the cause of late implantations? Is the conceptus slow growing and/or aneuploid? Is the endometrium at fault with delayed receptivity or a poorly defined window of receptivity?

How does in vitro fertilization and early development affect the development of the conceptus regarding its ability to implant in a timely fashion? What are optimal growth rates? How do fertility drugs impact on endometrial development and control of the implantation process?

What is the biological importance of a progesterone surge at time of implantation?

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This paper describes the rescue of the corpus luteum (reflected by change in concentration of the progesterone metabolite) in a sample of the pregnancies identified in the North Carolina Early Pregnancy Study.

Figure legends

Figure 1. Longitudinal data from a single participant showing cyclical hormonal changes during her participation. Vertical shading shows days of menses. The top panel shows changes in estrogen and progesterone metabolites. The second panel shows the ratio of the two metabolites with the vertical line designating the estimated day of ovulation based on the algorithm developed for this study. The lower panel shows hCG. There is a rise and fall associated with an early pregnancy loss, and then there is the hCG rise associated with a clinical pregnancy.

Figure

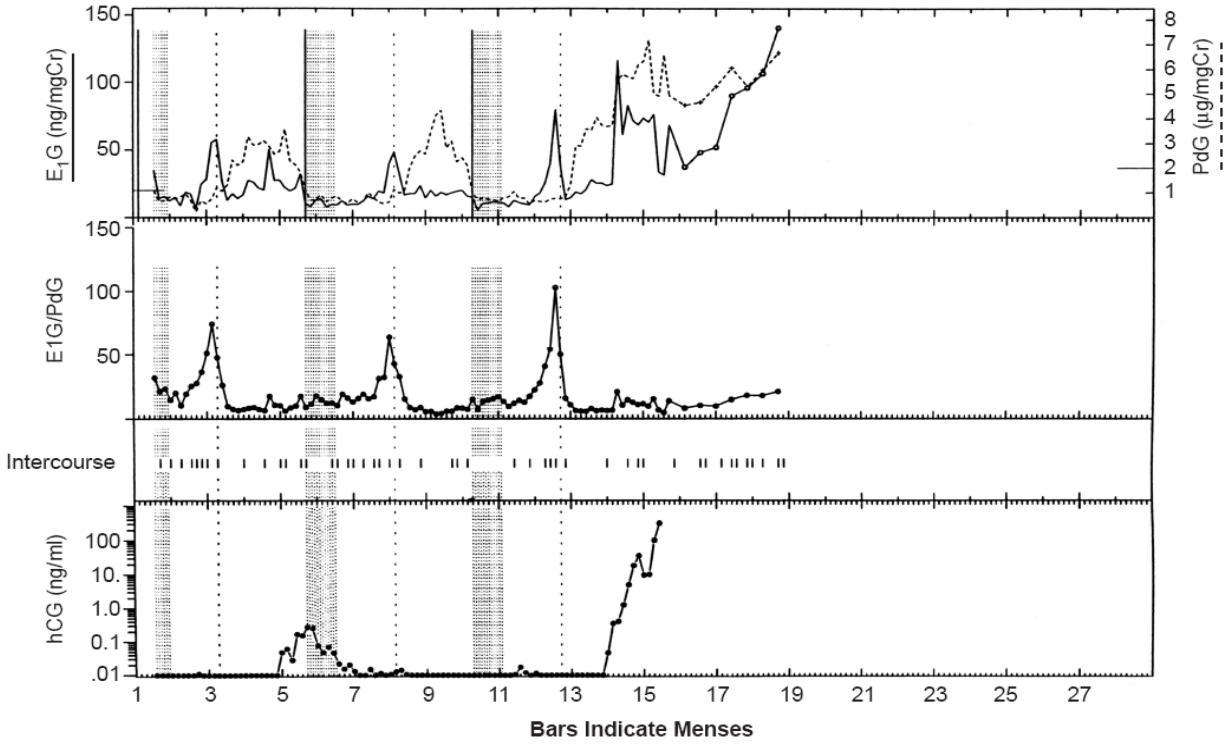


Figure 2. Timing of implantations in 189 naturally-conceived pregnancies and the risk of early loss. Overall, 141 pregnancies lasted at least six weeks after the last menses to become clinically recognized (top panel). Fifteen of these clinical pregnancies ended in spontaneous abortion (shaded area, top panel). The other 48 pregnancies ended in early loss (loss within six weeks after the last menses) (middle panel). The bottom panel shows the increasing proportion of early loss with later implantation (P for trend, <0.001). The day of ovulation was designated as day 0.

Figure 2

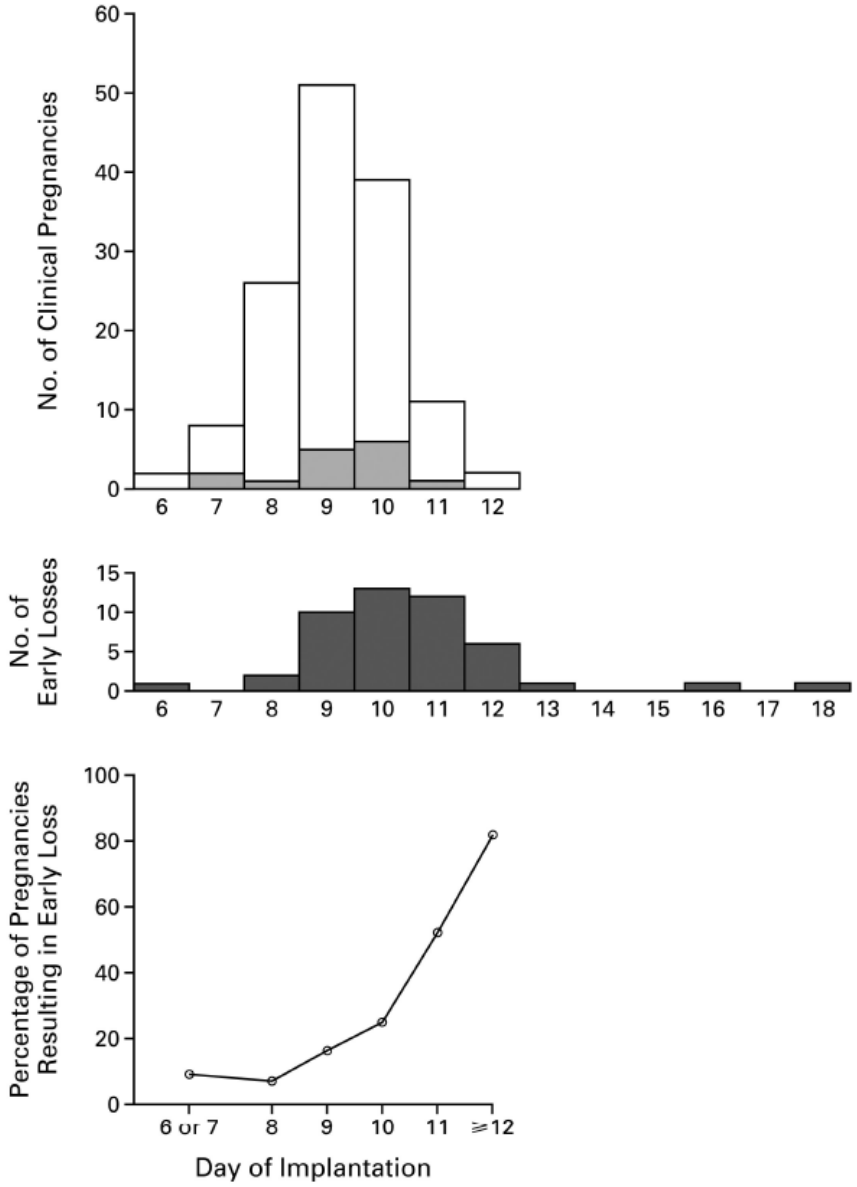
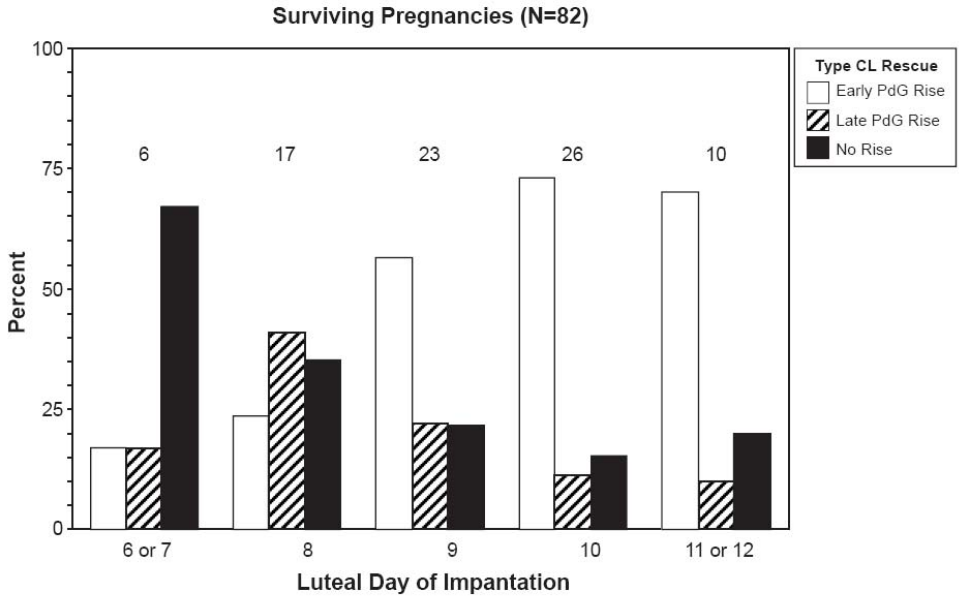


Figure 3. The type of corpus luteum rescue in relation to time of implantation in the North Carolina Early Pregnancy Study (N = 82, the sample of the study pregnancies for which the progesterone metabolite was measured during pregnancy). The Early PdG Rise group showed a progesterone surge within two days of implantation. The Late PdG Rise group showed a progesterone surge occurring 3 - 6 days after implantation. The No Rise group showed no progesterone rise during the week after implantation. Numbers above histogram show the number of pregnancies at each day of implantation.

Figure 3



Implantation and recurrent miscarriage, clinical aspects

Siobhan Quenby
School of Reproductive and Developmental Medicine
University of Liverpool
First Floor
Liverpool Women's Hospital
Crown Street
Liverpool
L8 7SS
UK
Email squenby@liv.ac.uk

Learning Objectives

Consider clinical problems of miscarriage in terms of feto-maternal interactions.

Use currently available diagnostic tests to understand the patho-physiology of early pregnancy loss.

Use this knowledge for patient counselling and treatment.

Why do miscarriages occur?

In order for pregnancy to be successful fetal trophoblast must invade the maternal decidual tissues. The principal cell type in these processes is extravillous trophoblast. Trophoblast invasion lays the foundations for placentation, as the extent of invasion determines the quality of anchorage and depth of the placenta, ensuring that it will not detach.

There are three reported primary pathologies in early pregnancy failure:

- Failure of the fetal extravillous trophoblast (EVT) to invade the maternal decidua (Jauniaux and Burton 2005)
- Failure of extravillous trophoblast to plug spiral arteries and protect the trophoblast from hyperoxia (Jauniaux and Burton 2005)
- Failure of villous vascularisation (Hakvoort et al.,2006)

These failures can occur because of poor fetal trophoblast function or a maternal environment hostile to the invading fetal tissue.

Poor fetal tissue

Karyotypic abnormality
We know that the most common reason for miscarriage is karyotypical abnormality. Thus chromosomally abnormal trophoblast appears to be poor at invading the mother and plugging her spiral arteries. New data from couples presenting with recurrent miscarriage (RM) illustrates this point very well. The subsequent pregnancy of women with diagnosed balanced translocations and RM was investigated for unbalanced translocations, which were present in 38% of miscarriages but 0% of ongoing pregnancies (Stephenson and Sierra 2006). Thus, trophoblast with abnormal karyotype does not appear to be able to implant well enough to maintain pregnancy in the first

trimester. However, the exact pathophysiology of pregnancy failure associated with abnormal fetal karyotype is yet to be elucidated.

V*illous Vascularization*
Poor or absent villous vascularization has consistently been identified in miscarried gestation sacs and fetal deaths (Hakvoort et al., 2006) again suggesting a fetal problem in the pathogenesis of miscarriage.

Hostile maternal environment

Antiphospholipid syndrome (APS) is the most investigated hostile maternal environment associated with miscarriage. There is an increasing body of evidence that factors in the serum of women with APS directly prevent trophoblast invasion (Sebire et al., 2002) and differentiation (Quenby et al., 2005) and increase trophoblast cell death by apoptosis (Bose et al., 2004). Thus, APS causes miscarriages by directly inhibiting trophoblast function.

Thrombophilia
Other thrombophilic disorders have been related to single and recurrent early pregnancy loss (Rey et al., 2003) however, the patho-physiology of these thrombophilia -related miscarriage is unknown. It is assumed that maternal thrombosis plays and important role in these pregnancy failures although the role of the fetal thrombophilic genome is still under investigation.

uNK cells
The uterine decidua is not necessary to trigger EVT invasion, but is likely to limit its extent and to accelerate the onset of EVT migration. In the maternal decidua immunocompetent maternal cells and allogenically different EVT co-exist. The most abundant immunocompetent cell in the maternal decidua is the uterine NK cell (uNK). These cells are also known as Large Granular Cells (LGL) or CD56 positive cells. The origin and function of these cells is unclear (Quenby and Farquharson 2006). Increased numbers of uNK cells were found in the pre-implantation endometrium of RM patients compared to controls (Quenby et al., 1999). There were more activated leucocytes in the decidua from the miscarriages of women with unexplained RM and a normal fetal karyotype compared to that from women with RM and abnormal fetal karyotype. This suggests different cellular immunity associated with the miscarriage of karyotypically normal and abnormal pregnancies (Quenby et al., 2002).

Endometrium

There are two paradigms in which to envisage the role of the endometrium in recurrent miscarriage. One is that the endometrium is hostile or inadequately receptive to the implanting embryo. Thus trophoblast invasion is poor and miscarriage is the result of the failure of the pregnancy to establish. This paradigm is supported by low endometrial integrin expression. This first paradigm is supported by the work of Jauniaux and Burton (2005) who describe deficient trophoblast invasion, failure of trophoblast to plug the lumen of spiral arteries leading to the miscarriage. We have proposed an alternative hypothesis that RM is the result of an intrauterine environment that allows the implantation of karyotypically abnormal pregnancy, destined to fail. The implantation of recurrently abnormal pregnancies then present clinically as miscarriage (Quenby et al 2002). This second paradigm is supported by the lack of barrier molecule MUC 1 in the endometrium of women with RM. It is very possible that different endometrial pathology is associated with the loss of a normal compared to an abnormal pregnancy.

Ultrasound detection of poor trophoblast invasion

Ultrasound is the gold standard tool for the detection of miscarriage. It has also been used to investigate the pathophysiology of miscarriage. Excessive entry of maternal blood at a very early stage into the developing placenta has been shown to result in oxidative stress and subsequent degeneration of villous tissue. Colour doppler is thought to be useful in detecting blood flow in the intervillous space in early pregnancy. Blood flow prior to 10 weeks gestation indicating failed trophoblast plugging of maternal arteries (Jauniaux et al., 2005).

Prevention of miscarriage

Improving trophoblast invasion remains a large clinical challenge. The following treatments are being investigated:

1. Heparin has been shown to attenuate the effects of APS - associated poor trophoblast invasion in vitro (Bose et al, 2004) and has been shown to be of benefit in preventing miscarriage in clinical trials.
2. However, heparin in the absence of APS may have a deleterious effect on trophoblast function (Quenby et al., 2004).
3. Antioxidants may prevent damage to trophoblast due to oxidative stress.
4. Prednisolone may suppress uNK cell numbers and improve early pregnancy placentation.
5. Progesterone may improve endometrial decidualisation and thereby make the maternal environment more favourable for early pregnancy.

These potential therapies (2-5) need to be tested in good quality randomised controlled trials before being introduced into clinical practise.

Counselling

Women may benefit from an appreciation that their fetal trophoblast failed to invade rather than feeling guilty that their body somehow rejected a wanted pregnancy.

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Myometral contractility and implantation

Dominique de Ziegler MD,
Marina Bellavia, MD
Patricia Ventura MD,
Victoria Ibechole MD
Nicolas Vulliemoz MD
Gabriel de Candolle MD
Division of Reproductive Endocrinology and Infertility
University Hospitals Geneva and Lausanne
Switzerland

Address for correspondence:
Pr. D. de Ziegler
Endocrinologie Gynécologique et Médecine de la Reproduction
Dépt. de Gynécologie-Obstétrque
Hôpital Universitaire de Genève
30 Bd de la cluse
1211, Genève
Tel.: +41-22 382 4302
Fax: +41-22 382 4313
E-mail: ddeziegler@bluewin.ch

Introduction.

There is currently ample evidence that the non-pregnant uterus is the site of contractions that take place throughout the menstrual cycle. The interest for studying uterine contractility was recently revived with the availability of high-resolution vaginal ultrasound (US) probes that allowed direct visualization of the contractile process. Typically, three distinct patterns are recognized during the menstrual cycle:

- During the *luteo-follicular transition* or inter-cycle interval (i.e., during menses), uterine contractility (UC) is characterized by elevated resting tone pressure and contractions of increased amplitude that involve the whole 3 myometrial layers. Typically, this pattern of contractility is involved in the proper external expulsion of endometrial debris and blood shed as a result of progesterone withdrawal upon demise of the corpus luteum (CL).
- In *the late follicular phase* of the menstrual cycle, UC frequency increases to approximately 5 UC/min under the influence of rising E2 levels.
- Finely, *following ovulation*, i.e., during *the luteal phase*, UCs abruptly abate with a pattern characterized by a state of uterine quiescence under the influence of the smooth muscle relaxing properties of progesterone (P4).

The recent renewed interest for uterine contractility stems from the possibility of directly visualizing UC on images generated by high-resolution ultrasound probes. The present review will summarize our understanding of the physiological role of uterine contractility on reproductive function with particular interest for its impact on endometrial receptivity. The review will primarily focus on the patho-physiology of UC at the time of ET and the means existing for limiting these effects.

The luteo-follicular transition: UC during menses

Physiology
During the luteofollicular transition and early follicular phase (at the time of menses), UCs are predominantly constituted of antegrade contractions that propagate from the fundus to the cervical end of the uterus (1, 2). This pattern of contractility is instrumental in the forward emptying of uterine contents (menstrual blood). At this stage in the menstrual cycle, UC is also a primary factor in hemostasis. Characteristically, the prevailing pattern of contractility encountered during menses represents a miniature replica of the expulsive contractions of labor. During this phase of the menstrual cycle, UCs are most often perceived by patients. On occasion, UCs can even become frankly painful (a condition known as dysmenorrhea) to the point of requiring specific medication and/or time off from work. Studies based on intrauterine pressure (IUP) recordings and US findings typically showed a rather low (by reference to the late follicular phase) UC frequency of approximately 2-3/min (range, 0.5-6), but with a characteristically high mean UC amplitude of up to 60mm Hg. The mean resting tone is also elevated during menses, when it commonly averages 40mm Hg, the highest value observed during the menstrual cycle. The elevated resting tone and high UC amplitude during menses reflect the active involvement of all layers of the myometrium during the contractile process. By contrast, solely the subendometrial layers of the myometrium participate in UCs at other times in the menstrual cycle (2). Recently, US-based studies have abounded. By and large, these all concurred to confirm the early IUP data with reports of similar UC frequencies at the time of menses (3, 4).

While the US based approach has been validated for the measurement of UC frequency, its value for studying UC displacement toward the fundus (retrograde) or cervical end (antegrade) of the uterus remains controversial. US also fail to assess uterine tone (pressure) and UC amplitude, 2 parameters that characterize the UC pattern encountered during menses. This methodological limitation explains that US have not yet been able to distinguish the physiologic (normal) pattern(s) of uterine contractility during menses from the dyskinetic ones (abnormal) that are associated with dysmenorrhea.

Studies relying on intra uterine pressure (IUP) recording using multiple tip pressure detectors have identified that UCs mainly feature antegrade displacement (from fundus to cervix) during the luteo-follicular transition (1). Most ultrasound-based studies looking at the apparent displacement of the contractile event on accelerated image sequences have also concurred to describe the predominance of antegrade contractions over all other forms of UC during the early follicular phase (menses).

Alternative methods have currently been proposed for assessing UC direction. Among them, interesting data have been generated from studying the displacement of uterine contents identified by various contrast media and/or makers, notably, Tc-99-labeled macroalbumin aggregates (MAA) (5) or X-ray contrast medium (6).

The specific characteristics of UCs experienced during the early follicular phase (menses) and their association with the subjective perception of painful cramps (potentially leading to frank dysmenorrhea) have not yet been fully clarified. By inference from studies of uterine contractility during other phases of the menstrual cycle (when UCs are never perceived, let alone painful), we can formulate the following hypothesis: During the luteofollicular transition (menses), the *transmural* involvement of UCs (i.e., the implication of all the myometrial layers rather than just the subendometrial ones) is likely the primary factor linked to their painful perception. Probably, this latter characteristic is also crucial to another

function of uterine contractility at the time of menses, hemostasis. We are still in need of investigative tools capable of providing precise, reliable, and clinically usable markers that reflect the painful characteristics of UC during the early follicular phase. We must recognize however, that to this day, the results of US studies have not provided the awaited help originally hoped.

Patho-physiology
Primary dysmenorrhea is a clinical condition characterized by painful, possibly invalidating uterine cramps that occur just prior to or at the time of menses. A common disorder that mainly affects young women, dysmenorrhea can become invalidating for many women, tracking from one to several days each month. In women with dysmenorrhea, early follicular phase uterine contractility is characterized by an increase in resting IUP (resting tone) and UC amplitude, duration, and frequency (7). These characteristics of dysmenorrhea can all be assessed by IUP recording; which unfortunately it is an invasive method. Yet to this day, despite high expectation placed on ultrasound evaluations, only one of the characteristics of dysmenorrhea, UC frequency, can reliably be assessed. Unfortunately, this parameter of dysmenorrhea, the increase in UC frequency measurable by US, is the weakest of all UC characteristics for reflecting the extent of menstrual cramps and dysmenorrheal (8). Hence, despite the advent of ultrasound, we still need a reliable marker(s) of UC alterations responsible for dysmenorrhea that could easily and non-invasively serve in large-scale clinical trials.

Furthermore, because the endometrium is inherently thin at the time of menses, US imaging can never reach the quality that we are accustomed to during the end-follicular phase and early to midluteal phase studies. US is therefore of little help in studying dysmenorrhea and comparing the efficacy of different treatments. The inherent characteristics of early follicular phase uterine contractility and its disruption in dysmenorrhea still evade all forms of noninvasive scrutiny.

End follicular phase contractility.

Physiology
Irrespective of the method used, investigators in the field nearly unanimously described an increase in UC frequency in the late follicular phase that peaks at or near the time of ovulation (1, 3, 9-11). A consensus also exists for identifying E_2 as the primary utero-stimulant in the menstrual cycle (9-11). Hence, logically, it is the increase in E_2 levels occurring in the late follicular phase that has been seen as the primary triggering stimulus for the preovulatory increase in UC frequency. Finely, just prior to ovulation, mean UC frequency peaks at approximately 4-5 UC/min (9-11).

Characteristically, however, despite the relatively high UC frequency (up to 5 UC/min), these are notoriously painless during the late follicular phase. By and large, US-based data have all tended to confirm older electrophysiologic studies suggesting that the subendometrial layers of the myometrium are solely involved in late follicular phase contractility (9). Hence, late follicular UCs have sometimes been dubbed *subendometrial contractions* by investigators using US-based approaches. The impression of intense displacement given by viewing US scans has also led other investigators to describe late follicular phase UCs as *wavelike contractions*.

As stated earlier, most investigators using either IUP or US-based approaches have concurred in describing the predominance of retrograde contractions during the late follicular phase.

Because sperm has been found in the pelvic cavity within minutes of intercourse (well before it could have traveled there on its *own steam*), retrograde uterine contractility that predominates in the late follicular phase has been seen as instrumental in the rapid transport of sperm (12). Hence, contractility of the female tract (uterus and tubes) stands out as the primary motor that assures the rapid transport of sperm from the cervical area to the distal end of the fallopian tubes where fertilization normally takes place.

IUP-based studies have attempted to document UC direction by computing time lags between the high points of contractions recorded in different sections of the uterus. Using triple-ended catheters, Martinez-Gaudio et al. (1) showed that retrograde displacement of IUP waves predominated during the late follicular phase. Yet, these investigators observed that the opposite (antegrade displacement towards the cervical end) also occurred occasionally. Sometimes, complete reversal of UC displacement with a switch from retrograde to antegrade propagation of the contractile wave was described in the same patient less than 10 minutes after a typical retrograde pattern had been observed (1) (Fig. 1).

During the late follicular phase, US-based studies have also concurred in describing the predominance of retrograde displacement based on the subjective assessment of the direction of contractility on fast plays of US sequences (3, 9, 11). But, as stated before, interpreting UC direction based on US visualization has never been validated and is possibly misleading. To more precisely study the actual displacement of uterine contents under the influence of uterine contractility, Leyendecker's team adapted an existing Tc-99-based hysterosalpingo-scintigraphy (HSS) technique (13, 14). Until then, HSS had been developed to replace hysterosalpingograms (HSG). This had failed because HSS images are less reliable than conventional HSGs in anatomical studies of the fallopian tubes. Leyendecker's team should be credited, however, for expending HSSs' indications for studying the functionality of the uterotubal contractile unit rather than solely assessing its anatomical characteristics, as done with HSGs. Hence, while we think that HSSs will not replace HSGs for studying tubal patency, they definitely can play a role in studying retrograde contractility and its disorders. In their studies, Leyendecker's team showed that Tc-99-labeled MAA placed in the vaginal fornix rapidly traveled towards the uterus and the fallopian tubes (13). According to these investors, retrograde transport towards the fallopian tubes only occurred if a developing follicle >16 mm was present in the ovary. And then, retrograde transport exclusively occurred towards the tube on the side of the developing follicle (13, 14).

Deviating from the HSS approach, we used an x-ray-based procedure (6) for studying retrograde transport of uterine contents in the late follicular phase in cycling women and oligoanovulators primed with exogenous E_2. For this, we duplicated the clinical conditions that commonly prevail in intrauterine insemination (IUI) protocols, the basis for most treatment offered to patients diagnosed with *unexplained* infertility in whom uterine dyskinesia is most likely to occur. For this, we used an embryo transfer catheter (Frydman's catheter) and gently deposited approximately 0.5 ml of an iodine-based contrast medium (Isteropack) in the middle portion of the uterine cavity and followed its displacement either retrogradely into the tubes or antegradely back in the vagina with successive spot x-rays (1-3 images). Studying a population of infertile women whose tubes were proved patent, we observed prompt emptying of uterine contents towards the fallopian tube and the pelvic cavity in approximately 60% of women. By contrast, all the x-ray medium was rapidly expelled from the uterus back into the vagina in the remaining 40% of women. In 100% of women showing positive retrograde transport, this took place towards both tubes (the one facing the developing follicle and the opposite one). Hence, in sharp distinction from data reported by Leyendecker's team (13, 14), our x-ray-based studies did not show the characteristic

lateralization of retrograde transport (6) reported by these investigators when using a Tc-99 HSS approach. We postulate that the observed discrepancy with Leyendecker's data reflects differences in sensitivity between of the two investigative methods used.

Patho-physiology
Using HSS, Leyendecker's group reported a characteristic disruption of the retrograde transport of Tc-99 MAA normally seen in the late follicular phase, in women with endometriosis. Globally, dyskentic alterations of the normal patterns of UC at various moments in the cycle are discussed in a dedicated section of this syllabus.

Utero quiescent effects of P4

Physiology
The utero relaxing properties of P4 have long been recognized in all mammalian species where they constitute an indispensable step toward embryo implantation and pregnancy. The utero-relaxing properties of P4 are in part dependant upon non-genomic effects that this hormone directly exerts on cell membranes. These are mediated either directly by P4 or by its metabolite, allopregnanolone, which binds to the hormonal site of the GABA-$_A$ cell membrane receptor complex (15, 16). Allosteric activation of the GABA-$_A$ receptor complex increases the resting potential by activating the Cl^- pump system. This ultimately leads to a decrease in cell excitability. Using an ex-vivo uterine perfusion model, Bulletti et al. demonstrated that these effects are detected electromechanically within hours of exposure to P4 (17). In an in vivo model, we demonstrated that these utero-relaxing properties of P4 were also exerted by vaginal administration of micronized P4 (18).

While utero-quiescence is the predominant feature of P4's effects on the uterus, certain have questioned the presence of finer more subtle UC possibly bidirectional in origin that might partake in the proper positioning of the embryo. This property of P4 might ultimately improve IR and PR. In rodents, a role for P4 has been advocated in the proper placement of embryo implantation sites along both uterine horns. Conversely, hormonal imbalances and notably an excess of E2 has been reported to interfere with the proper migration of embryos, which tend to accumulate toward the uterine end of the oviduct.

In humans the anti conceptive properties of large doses of E2 (DES) used in the past as emergency contraception has been linked to inhibition of the conceptus' transport toward the uterine cavity. As DES has been replaced by other approaches for emergency contraception, this hypothesis has never been confirmed and probably will never be in humans. It remains however that hormonal imbalances encountered in COH (particularly when clomiphene citrate was used) have been blamed for the increase in ectopic pregnancy observed even when the tubes are anatomically normal.

Patho-physiology
As discussed in a later section of this syllabus, COH alters the profile of UC encountered in the luteal phase which appears to hamper IR and PRs.

UC alterations encountered in COH

In an original work, Fanchin et al. observed that an increase in UC frequency at the time of ET bore an ominous prognosis on IVF outcome in a population of women whose ovarian response was good and all had 3 nice looking embryos available for transfer (19). In this study, UC were measured by US recording just prior to ET, yet the effects of ET on

contractility were not assessed. Also of note is the fact that in this program, luteal support was not initiated at the time of this study prior ET. This observation sparked interest for assessing UC at the time of EC and for possibly minimizing them in the intent of optimizing IVF outcome.

The report that embryos were found on the external portio of the cervix following ET speaks for the possibility that embryos might be expelled for the uterine cavity following classical ET (20). The mechanism explaining the increase in UC in IVF still eludes our comprehensive understanding, yet clues are pointing at certain phenomenon as likely cause for the increase in contractiliy.

In a prospective trial, we followed UC frequency beginning on the first day of the luteal phase in the menstrual cycle that just preceded IVF and in the IVF cycle itself, each patient being her own control (21). Results are depicted in Fig. 2 A and B. UC measurement were initiated on the day of LH surge and hCG administration in IVF and repeated every 2 days thereafter for 6 days.

Interestingly, UC frequency was identical (~5UC/min) on the day of LH surge (menstrual cycle) and hCG administration (IVF) when measurements were conducted in the same patients. This indicates that the markedly elevated levels of E2 (Fig. 2A) that characterize IVF do not further stimulate UC frequency over what is encountered in the menstrual cycle. In the menstrual cycles, UC frequency remained elevated 2 days after LH surge but droped significantly on the 4th day (Fig. 2B) to <1UC/min (21). On the contrary, UC frequency remained elevated on the 4th day after hCG when the same women underwent IVF. In our eyes, this indicates that the high levels of E2 encountered in IVF do not further enhance UC frequency at the end of the follicular phase (UC frequency maximum?) but induces some degree of resistance to the utero relaxing properties of P4. On the 6th day post hCG, UC frequency also decreased when women underwent IVF (as seen 2 days earlier in the natural cycle). Of possible importance however is the fact that on the 6th post hCG administration, women had received luteal support with exogenous P4 (600mg/day) for 2 days, starting immediately after ET. Confirming these results, Fanchin et al reported that UC frequency was significantly lower at the time of blastocyst transfers (day 5 post oocyte retrieval) than encountered 2 days after oocyte retrieval (22).

In a different trial, Fanchin et al. indicated that an earlier initiation of luteal support with vaginal P4 (on the day of oocyte retrieval) was capable of decreasing UC frequency at the time of ET. Globally the earlier onset of luteal support was also associated with a trend toward higher IR and PR (23). A different group of investigator could however not document that an earlier onset of luteal support translated in better IVF outcome (24). Logically these authors concluded that vaginal P4 at the dose of 400 mg started on the day of oocyte retrieval did not increase implantation or pregnancy rates when compared to the same dose started on the day of embryo transfer (24). Further fueling the controversy on the role of UC on embryo implantation, Woolcott and Stanger reported that higher UC frequency on the day of ET did not correlate with lower PR (25).

UC alterations and endometriosis.

Foll phase:
Based on their findings, Leyendecker and Wildt's groups claimed that endometriosis is associated with a state of hyperkinetic dyskinesia in women identified to suffer from endometriosis (5, 13). Furthermore, the disordered contractility that prevails in women

affected by endometriosis has been postulated to also alter the proper retrograde transport of sperm that normally takes place in the late follicular phase of the menstrual cycle. Interestingly, we made concordant observations in our x-ray-based study of late follicular phase contractility (6). Using our mock IUI paradigm, we also found that most women with proven endometriosis failed to show proper retrograde transport of the uterine contents towards the fallopian tubes on the day of the luteinizing hormone surge. The latter finding provides a plausible explanation for the poor success of IUI treatment in endometriosis. The proposition that endometriosis is associated with uterine dyskinesia led to the hypothesis that functional alterations of uterine contractility associated with this disease affect all the UC patterns seen during the menstrual cycle. Hence, *following this line of thought*, alterations affecting early follicular UCs will affect the proper emptying of uterine contents at the time of menses with the net result of increasing retrograde bleeding. Similarly, during the late follicular phase, alterations in normal retrograde contractions will affect the rapid transport of sperm and alter fertility. This hypothesis, therefore, offers new interesting views for explaining both the genesis of endometriosis (retrograde bleeding) and the infertility that accompanies even the most benign forms of this disease (mild to moderate endometriosis). To date, however, retrograde contractility has not been tested in conditions that normally accompany intercourse, that is, exposure of the uterus to the stimulating influences of prostaglandins present in semen and delivered to the vagina.

Endometriosis, a serious and potentially devastating disease of unknown origin, mainly affects gynecological and other lower pelvic organs. One of the two hypotheses put forth to explain the growth and development of endometrial implants in various areas of the pelvic cavity proposes a culprit role for retrograde bleeding. This view is commonly referred to as *Sampson's retrograde menstruation theory* (26). According to this classical hypothesis, retrograde bleeding recurring months after months will likely disseminate endometrial fragments into the pelvic cavity, where they will in turn implant and develop.[7] Sampson's theory of retrograde menstruation has long been opposed to the alternate hypothesis of "coelomic metaplasia." Until now, however, no compelling evidence has led to the overwhelming supremacy of either one of these two theories. Furthermore, breaking from a lingering opposition between these two concepts, the views that now prevail favor complementary rather than antagonizing roles for these two mechanisms. Hence, in this multimechanism view of endometriosis, retrograde bleeding is seen as one of the phenomena (rather than the sole mechanism) that fuels pelvic endometriosis. In an ultrasound-based study, unfortunately not confirmed by further work or even reproduced by others, Salamanca *et al.*[8] observed predominantly retrograde UC at the time of menses in women with documented endometriosis. On the contrary, in the experience of these investigators, antegrade contractions predominated during menses in unaffected controls. These findings were quick to gather adepts to the concept that endometriosis is intimately linked to some not yet identified form of dyskinetic alterations in uterine contractility during the early follicular phase. A limiting factor in this study, however, is the lack of validation of ultrasound studies for assessing the direction of contractility.

Inferences from studies of other forms of smooth muscle dyskinesia, notably, the irritable bowel syndrome (27), have led some to postulate that the alterations in uterine contractility found at the heart of endometriosis are probably of the hyperkinetic type. During the early follicular phase, the net result of these dyskinetic changes is likely to be variable degrees of impediment to proper antegrade emptying of menstrual blood. This, because of the high-pressure environment prevailing in the uterine cavity, will in turn lead to a disorganized evacuation of menstrual blood that will tend to exit the uterine cavity through all possible openings (including retrograde bleeding through the tubes). Hence, according to this

hypothesis, it is the loss of the proper antegrade pattern of UC at the time of menses (rather than its outright reversal) that ultimately leads through various patterns of chaotic uterine contractility to an increase in retrograde bleeding. Ultimately, retrograde bleeding will be one of the factors fueling the development of endometriotic implants through direct seeding of endometrial tissue in the pelvic cavity and activation of chronic inflammation.

Reports of women undergoing peritoneal dialysis at the time of menses provided the first hard-line evidence underscoring that most, if not all, women encounter some degree of retrograde bleeding, at least some of the time (29). Other reports assessed the amount of endometrial debris present in the pelvic cavity just after menses (30).

Measures for minimizing UC at the time of ET.

Bernarbeu et al. (31) reported a prospective trial looking at the effect of administrating indomethacin prior to ET with the intent of reducing Pg production and thereby UC in order to optimize UC efficacy. Their investigation was conducted prospectively in 173 consecutive IVF and IVF-ICSI cycles (31). Seventy two of these patients received 3 doses of 100mg of indomethacin rectally every 12h, starting on the evening prior to ET. The control group received no indomethacin. All other parameters were identical. The number of embryos transferred in both groups were identical as were all other common parameters used to assess IVF candidates (age, etc) and their response to COH. PR and IR at 59.7 and 27.8% and 59.4 and 26.4% in controls and patients receiving indomethacin respectively, were identical. Concluding on the results of their trial, the authors do not recommend using indomethacin prior to ET for the sake of reducing UC and optimizing PR (31). While prospective and randomized, the authors announced that their study was not blinded to patients and conceded that the doses used might not have been the optimal one for the intended effect. Of note also in this study, P4 supplementation was initiated on the afternoon of oocyte pickup, at the does of 200mg TID.

In a small trial published in the form of an abstract only, Schoolcraft et al. studied the effect of ritordrine administration prior to ET. In their hands too, the utero relaxing treatment was without effects on PR and IR. This group too initiates P4 supplementation on the day of oocyte retrieval.

Mansour et al (32) conducted a prospective trial looking at the effects of inducing a gentle pressure on the portiovaginalis of the cervix (Fig. 3) by releasing the blades of the speculum before ejecting the embryos and maintaining for 7 min afterwards. In the hands of these investigators, clinical PR was significantly higher in the study group as compared to controls (67% vs. 47.8%; odds ratio [95% CI] 1.39; [1.11-1.74]). IR was also significantly higher in the study group (33.3% vs. 21.5%, 1.54; [1.26-1.89]).

The actual use of a tenaculum for exerting traction on the cervix may actually trigger UC and in turn lower PR and IR (33), a phenomenon which may be mediated by OT secretion by the pituitary. In a prospective trial, Dorn et al. measured serum oxytocin (OT) at the time of ET (33). In their trial, serial blood samples were collected every 20 s during the ET procedure and serum OT concentration was measured. In the absence of tenaculum placement, none of the procedures associated with ET led to an increase in serum OT concentration. On the contrary, when a tenaculum was used, it was temporally (four out of five patients) associated with an elevation in OT level, which remained elevated until of the end of ET procedure. This reports confirms, if needed be, that the use of a tenaculum should be avoided if at all possible.

OT may actually be secreted at mid cycle independently of ET related procedures. Shukovski et al. (34) reported that serum OT increases during the follicular phase of the menstrual cycle, reaching a peak 1 day after the LH surge, and decreasing thereafter during the luteal phase. Serum OT was positively correlated with serum E2 values during the first part of the cycle ($P<0.01$). These authors concluded that that OT increases at mid cycle under an influence of E2. This led some investigators to use OT antagonists at the time of ET without clear with suggestion from their pilot trial for a benefice (abstract publication).

Along the line of minimizing endometrial trauma, which may increase UC (but not only), there are reports that soft end ET catheter provide better PR and IR results than their firm counterpart. In their meta-analysis, Abou-Setta et al. (35) report that soft the soft versus firm catheters ($P = 0.01$; OR[95%CI]: 1.39, [1.08–1.79]). When only the truly RCT were analyzed, the results favored soft ET catheters with even greater significance ($P < 0.00001$; OR[95%CI]: 1.49, [1.26–1.77]) (35).

Conclusion:

Characteristically, 3 patterns of uterine contractility are encountered in the non pregnant woman. The end follicular phase is characterized by abundant yet not perceived sub endometrial contractions, which is followed a period of utrine quiescence during the luteal phase.

In COH as commonly undertaken for the purpose of undertaking IVF, the proper pattern of UC encountered in the menstrual cycle appears disrupted with an increase in UC frequency at the time of ET. The latter appears to mainly reflect a state of relative resistance to the utero relaxing properties of P4 brought by the supra physiological levels of E2 encountered in COH.

The most effective measures for minimizing the possible deleterious effects of increased UC at the time of ET include: 1) Starting luteal support with vaginal P4 on the day of oocyte retrieval, 2) delaying transfers until the blastocyst stage. On the contrary, direct measures such using a utero-relaxant medication at the time of ET have so far been granted with no definitive benefit for IVF outcome.

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Figures

Fig. 1
Sperm transport through the uterus and tubes mainly depends upon peristaltic contractions that propel sperm forward to the distal end of the tube where fertilization takes place.

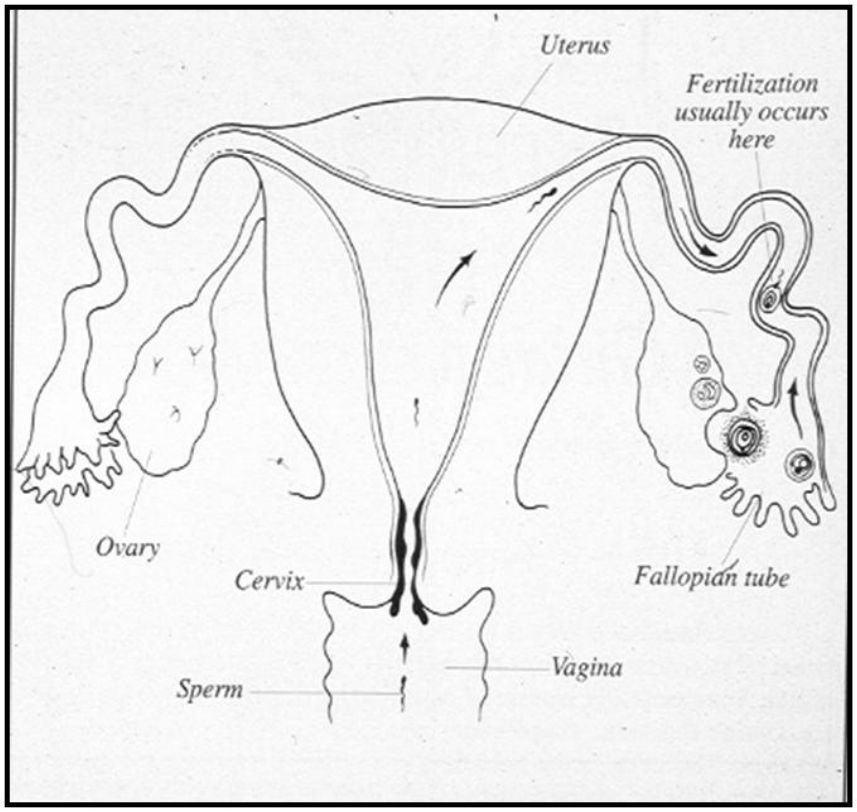

Fig. 2 Analysis of UCs in the preceding menstrual cycle and during IVF itself.
Fig. 2A Shows E2 levels, which are markedly higher **in IVF**

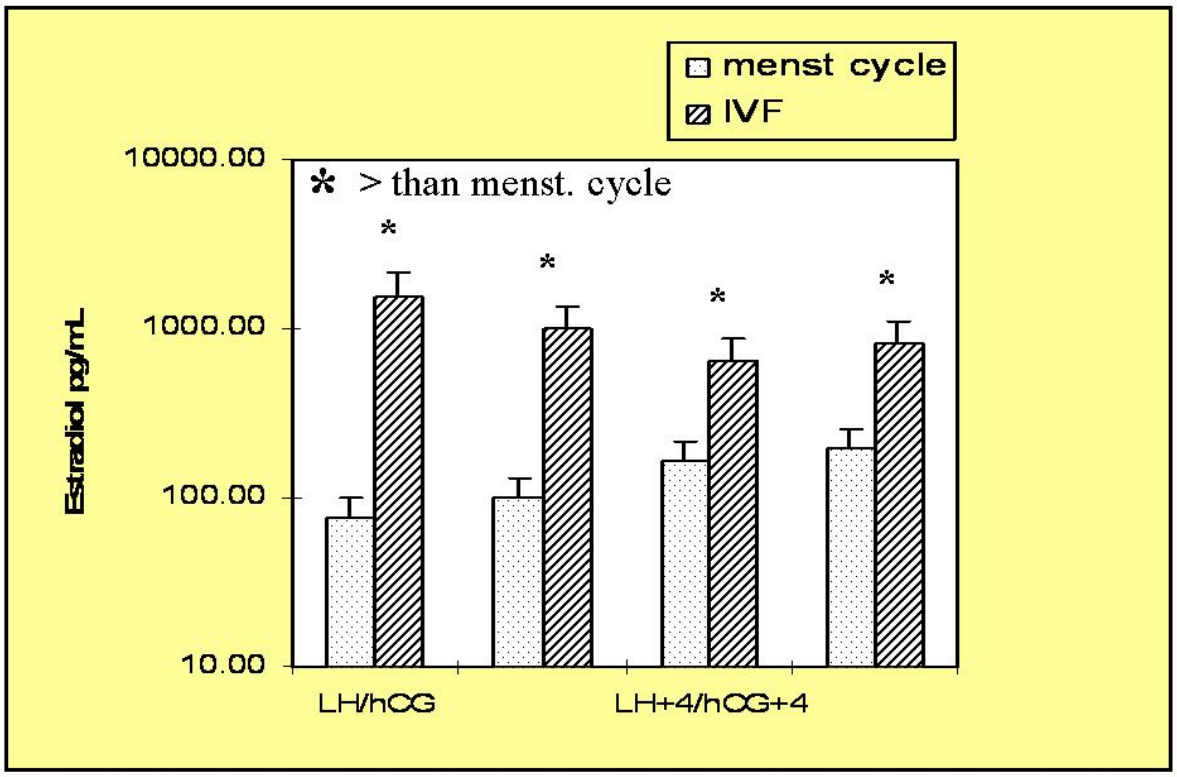

Fig. 2B Shows UC frequency following LH surge in the menstrual cycle and hCG administration in IVF (From Ayoubi et al. 21).

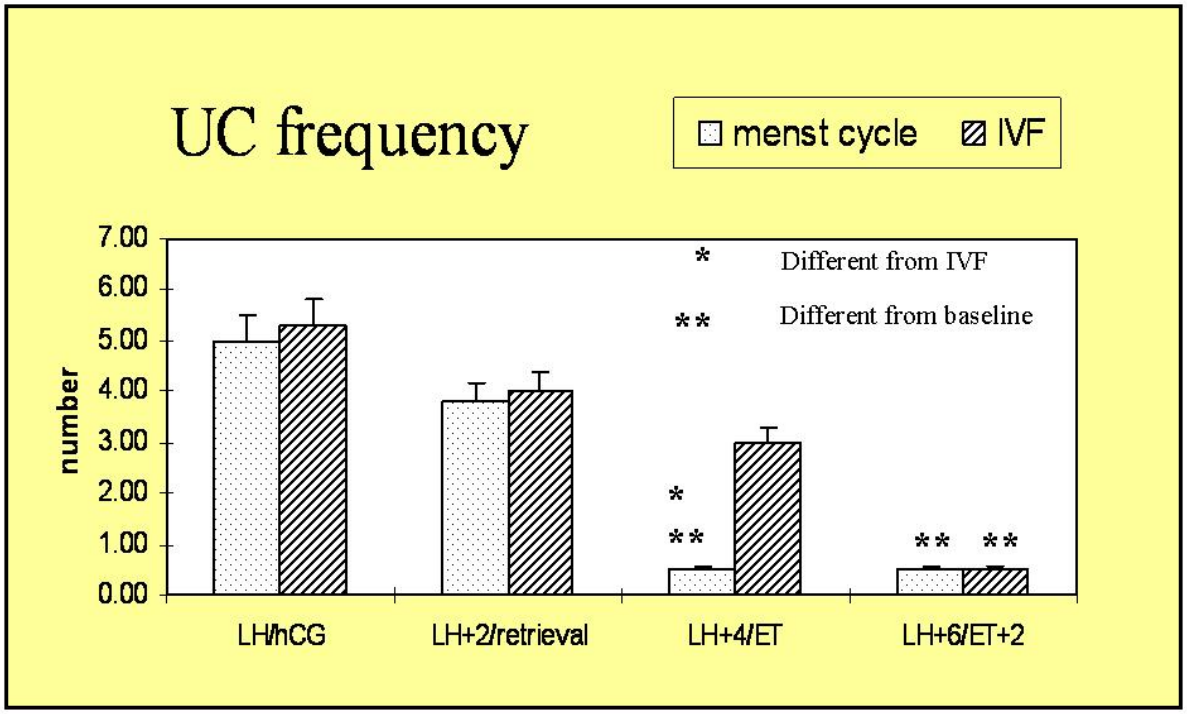

Fig. 3
Mansur et al. (32) recommend releasing the screw of the speculum prior to injecting embryos inside the cavity in order to exert some closing pressure on the cervix. Their prospective trial indicated better results.