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Changes in the Development of Uterine Pinopodes in Steroid Hormone Supplemented Cycles

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Summary

The endometrium acquires the ability to implant a hatched blastocyst only within a specific time termed the receptive phase. Ovarian steroid hormones are essential for structural and functional changes that prepare the endometrium to be receptive. Pinopodes have been suggested to be markers of uterine receptivity. The aim of this study was to compare the pinopode expression and serum levels of ovarian steroid hormones in the mid-luteal phase of the natural cycle and in a "mock" cycle in the same subject. Sequentional endometrial biopsies within 48 hours were obtained from women in the mid-luteal phase (ovulation +5, ovulation +7) of the natural cycle and in the "mock" cycle (progesterone supplementation +5 and +7). Biopsies were examined under a scanning electron microscope for pinopode detection. The expression of pinopodes was similar in both cycles, where pinopodes covered about 5 % of the endometrial surface. The developmental stages were also similar with a slight increase of fully developed pinopodes in both samples in the "mock" cycles. Our findings suggest that hormonal preparation of the endometrium do not change the timing of pinopode expression.

Key words

Endometrial receptivity • Pinopodes • Scanning electron microscopy • Steroid hormones

Introduction

Implantation is one of the most interesting biological events. Implantation failure is considered to be the major factor limiting the success of infertility treatment including *in vitro* fertilization (IVF). Endometrial receptivity is one of crucial factors for successful implantation. Dynamic changes in the endometrium during the natural menstrual cycle proceed under control of ovarian steroids – estrogens and progesterone. Ovarian stimulation as well as hormone substitution can modify normal development of the endometrium and may have a negative effect on embryo implantation (Nicas *et al.* 1995).

The endometrial epithelium consists of two types of cells – the ciliated ones and cells with microvilli. During the menstrual cycle, different hormone-dependent changes also occur in the endometrial surface especially in cells with microvilli. The morphology of cilliated cells does not change so much. These cells vary in size and

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shape in the proliferative phase and there is a gradual development of microvilli. After ovulation, cell size increases in the early luteal phase and microvilli are long, thick and upright. In the mid-luteal phase microvilli decrease in number and size in some cells and fuse or disappear (Ferenczy *et al.* 1972). A smooth membrane projection, a developing pinopode, is formed from the entire cell apex. The fully developed pinopodes look like mushrooms.

Pinopodes are transient apical protrusions of the endometrial epithelial surface that occur only during the receptive phase. The appearance of pinopodes is strictly progesterone-dependent (Stavreus-Evers *et al.* 2001). Their function in humans is still not fully understood. Because of their temporal and spatial expression, pinopodes have been suggested as suitable morphological markers of endometrial receptivity. The occurrence and lifespan of pinopodes is precisely regulated. The time correlation of pinopode expression and the period of blastocyst hatching as well as the preference of human blastocyst to attach to pinopodes suggest that pinopodes should be good indicators of the receptive phase (Bentin-Ley *et al.* 1999).

In this study the effect of oral hormone substitution on endometrial preparation for frozen-thawed embryo-transfer in infertile women with normally functioning ovaries was evaluated by means of comparison of pinopode expression and steroid hormone levels in a mock substituted cycle with a previous natural cycle in the same patient.

Methods

Twenty-one infertile women that had cryopreserved embryos from previous IVF cycle were recruited in a single infertility center over a three-year interval 2001-2003. All women gave their written consent to the study, which was approved by the Institutional Review Board of the Faculty of Medicine, Palacký University.

Women had a history of infertility of more than 12 months, were less than 40 years old, had a regular menstrual cycle, normal basal serum levels of gonadotrophins (FSH <10 IU/l) and prolactin. Infertility evaluation revealed endometriosis (1), tubal (8), idiopathic (5) and male (9) factors of infertility. Patients were examined in the course of two menstrual cycles.

1) *Spontaneous cycle*: All subjects monitored their own urinary LH excretion daily from cycle day 10 using commercial urinary kit (Simtech Biores Inc, USA). From cycle day 11, repeated vaginal ultrasound examination (Hewlett Packard, probe 7.5 MHz) and serum LH, estradiol (E_2) and progesterone (P) determination were performed within a 2-day interval till ovulation occurred (day 0). Endometrial biopsies and further blood sampling for E_2 and P determination were performed on luteal days +5 and +7.

2) *"Mock" (substituted) cycle:* Hormonal substitution was started on the first day of another cycle using progressively increasing doses of estradiol-valerate (2 mg/day from day 1 to 6, 4 mg/day from day 7 to 10 and 6 mg/day from day 11 to 15). From the 11th day of substitution serum levels of E_2 and P were measured as in the spontaneous cycle. If on day 15 the endometrial thickness reached 8 mm or more micronized progesterone was added in a dose 600 mg/day and the dose of estradiol-valerate was decreased to 4 mg/day. Endometrial biopsies as well as blood samples were taken on days 5 and 7 of P addition.

Hormonal analysis of E_2 and P was performed using commercial RIA kits Ref.1188 and 1663 supplied by Immunotech.

Uterine endometrial sequential biopsies were taken within the interval of 48 hours from the anterior wall and fundus using a Novak curette while patient was under sedation. The tissue was immediately placed into a solution of 2 % glutaraldehyde and 1 % formaldehyde in 0.1 M phosphate buffer, pH 7.2, for two hours.

The specimens for scanning electron microscopy were prepared as previously described (Novotný *et al.* 1999). Samples were examined under the microscope Tesla BS 340, magnification 3000x, by a single operator. To increase the likelihood of representative observation, since the endometrium shows variable morphology from one area to another, pinopode number was counted in 30 randomly selected areas of each tissue specimen. The average number of apical parts in 1000 areas of different specimens was 253 (at 3000x magnification). The mean count of pinopodes of each specimen was calculated. The total pinopode expression was then arbitrarily expressed as percentage of 250.

The developmental stages of pinopodes were morphologically classified according to Bentin-Ley *et al.* (1999) as developing, fully developed and regressing. Developing pinopodes are small, smooth, naked, mushroom-like protrusions sometimes still with short microvilli. Fully developed pinopodes are larger, completely smooth bulges without or with only some

wrinkles (Fig. 1). Regressing pinopodes have a wrinkled surface reminding a deflated ball (Fig. 2).

Statistical analysis of obtained data was carried out by t-test for unpaired observation, Wilcoxon matched-pairs test, Scheffe test and Kruskal-Wallis analysis.

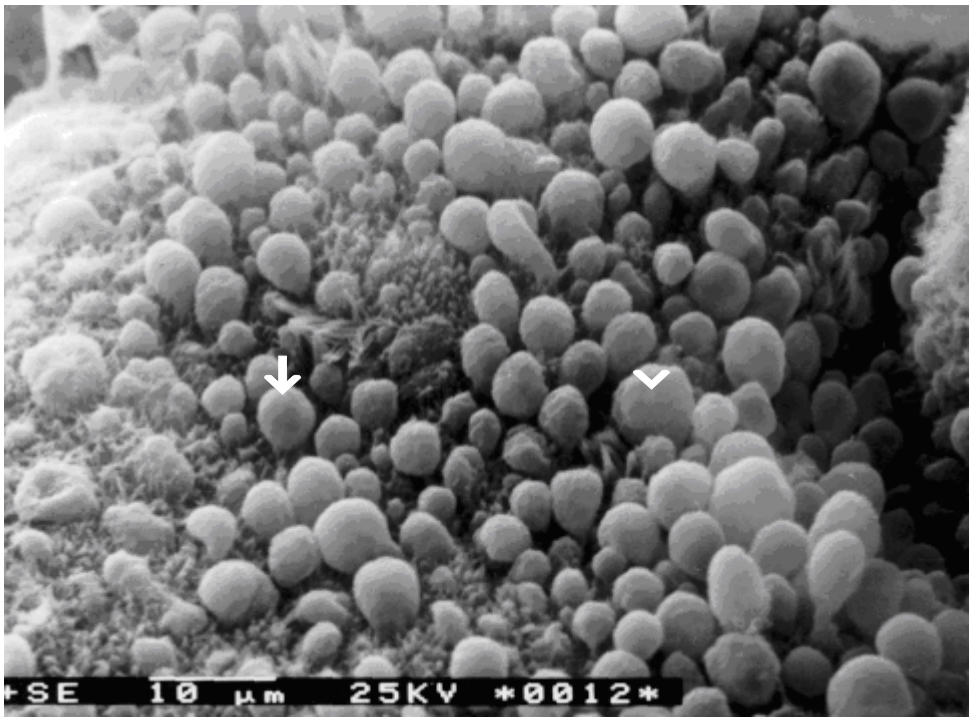

Fig. 1. Developing (white arrow) and fully developed (white arrow head) pinopodes in scanning electron microscopy on the luteal day 5 of the spontaneous cycle. Magnification 3000x.

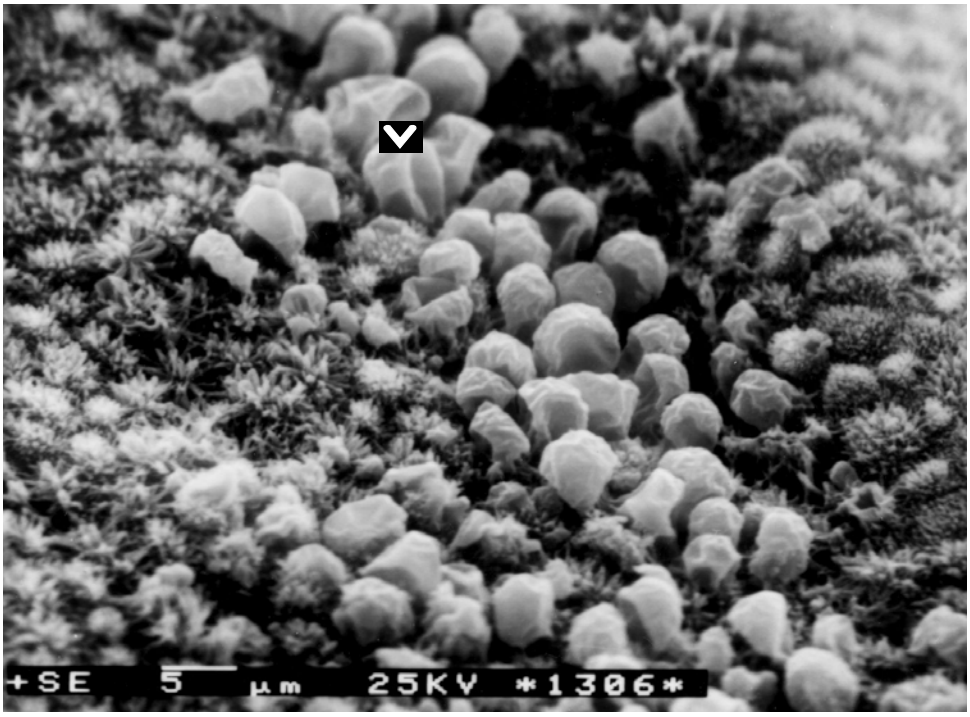

Fig. 2. Regressing pinopodes (white arrow head) in scanning electron microscopy on the luteal day 7 of the spontaneous cycle. Magnification 3000x.

Results

Hormone levels

The serum levels of E_2 in the late proliferative phase were similar in both cycles, whereas P levels were significantly lower in the "mock" cycles (P<0.001). In the mid-luteal phase of "mock" cycles, the levels of both E_2 (P<0.005) and P (P<0.001) were significantly higher, while the E_2 levels were only slightly increased. The P levels in the "mock" cycles mostly reached supraphysiological levels. Figure 3 shows the mid-luteal P levels in non-parametric Box-Whisker graphical representation.

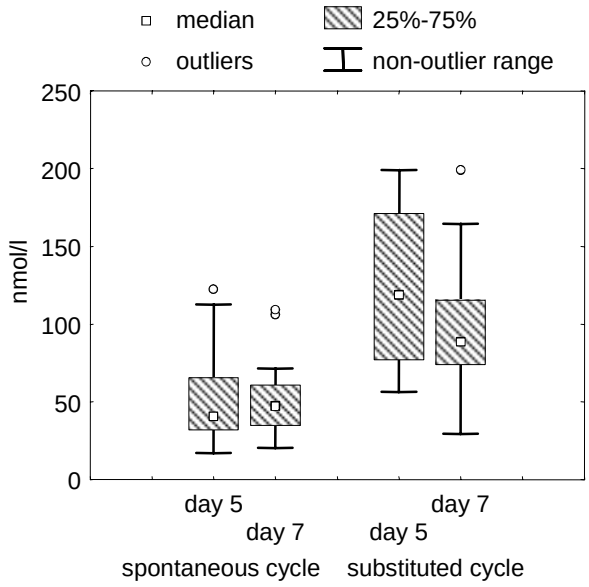

Fig. 3. Comparison of serum progesterone levels (nmol/l) in the mid-secretory phase (on luteal day 5 and 7) of the spontaneous and „mock" (substituted) cycles. Graphical representation in the non-parametric Box-Whisker diagram.

Pinopode expression

The endometrial epithelial surface was not uniform and showed variable morphology from one area to another. The endometrial surface was covered mostly by cells with microvilli and to a less extent by ciliated cells. There were marked differences in the pinopode expression between 30 separate randomly selected areas in each sample. Areas with no pinopodes as well as clusters containing more than 50 % of pinopodes could be found. The mean coverage of the surface by pinopodes in different developmental stages was between 3 to 6 % (Fig. 4, Box-Whisker graph). Their occurrence was slightly higher in the mock cycles but without statistical significance. In 6 patients, no pinopodes were found in 8 cycles, four times in a spontaneous and four times in a "mock" cycle, but always only in one biopsy sample. The absence of pinopodes was more frequent on day +7 in both cycles. On the other hand, in four patients we found exceptionally high pinopode expression in individual samples ranging from about 10 % up to 24 %.

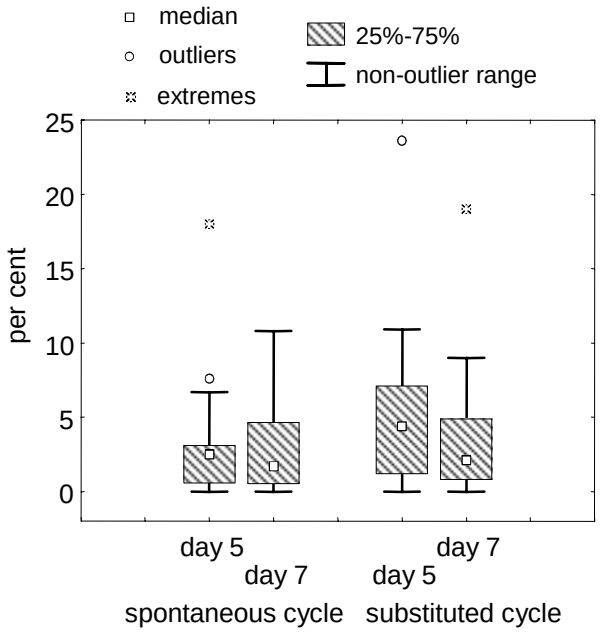

Fig. 4. Comparison of mean overall pinopodes expression (percentage of endometrial surface covered) in the mid-secretory phase (on luteal day 5 and 7) of the spontaneous and the „mock" (substituted) cycles. Graphical representation in the non-parametric Box-Whisker diagram.

Three developmental stages of pinopodes were evaluated as developing, fully developed and regressing (Fig. 5). Their presence was quantified by dividing into 6 categories (0 %, 20 %, 40 %, 60 %, 80 %, 100 %). In spontaneous as well as in the „mock" cycles the prevalence of fully developed pinopodes was found in both on days +5 and +7. Slightly higher occurrence of fully developed pinopodes in the „mock" cycles was found.

It is known that pinopode expression is progesterone-dependent. In an animal model a negative influence of higher level of E_2 was observed. Therefore we have compared the pinopode expression and serum levels of P as well as E_2/P ratio in both cycles. The data were treated by the Scheffe test and Kruskal-Wallis analysis and no correlation was found. Also supraphysiological levels of P often found in the "mock" cycles did not positively influence the occurrence of pinopodes. We are aware that our findings cannot unequivocally exclude a relationship between ovarian steroid levels and pinopodes expression because of high

inter-individual variability and hitherto a small number of investigated samples.

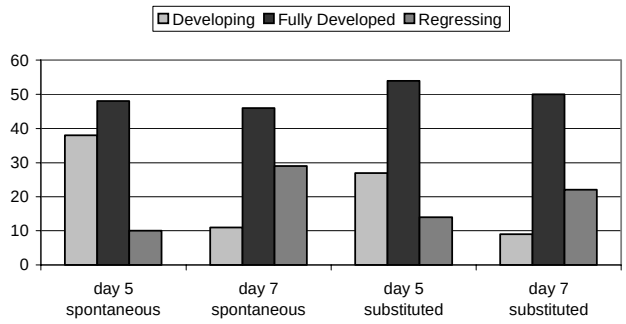

Fig. 5. Comparison of the mean proportion (in %) of developmental stages of pinopodes (e.g. developing, fully developed and regressing) in the mid-secretory phase (on luteal day 5 and 7) of the spontaneous and the „mock" (substituted) cycles.

Discussion

An ideal marker of the receptive uterus must fulfill a number of important criteria. It should be present in the endometrium, it should appear within the window of implantation or precede it by a short period of time and disappear with the termination of the receptive phase. In many species, the receptive phase is strongly associated with the generation of pinopodes (Carson *et al.* 2000). The functionality of pinopodes is not clear. Their formation is thought to be progesterone-dependent (Nicas *et al.* 1995) and associated with a decrease of progesterone receptors A and B in the glandular and luminal epithelium (Stavreus-Evers *et al.* 2001). It was also found that heparin-binding epidermal growth factor-like growth factor (HB-EGF) is present both inside epithelial cells and on the surface of pinopodes (Stavreus-Evers *et al.* 2002). HB-EGF stimulates epithelial expression of the key endometrial proteins including leukemia inhibitory factor, $ß_3$ integrin subunit and homeobox gene, which are biomarkers expressed during the implantation window (Lessey *et al.* 2002). The increase of HB-EGF expression was found in the mid-luteal phase.

Apposition is the initial contact between blastocyst and endometrial epithelium. It was demonstrated that adhesive forces between smooth epithelial surface (pinopode) and trophoblast are stronger than between rough epithelial surface structures (microvilli, cilia). Adhesion of the trophoblast to endometrial epithelial surface is a relatively slow process, but adhesive forces increase progressively (Thie *et al.* 1998).

It is well known that the endometrial epithelial surface is not uniform in morphology and undergoes cyclical changes under the control of ovarian steroids. Hysterectomy specimens were used for the evaluation of these cyclical changes on the uterine fundus, Fallopian tube and endocervix. The morphological changes of the tubal epithelium except fimbriated end exhibit the same changes as the uterine fundus, including formation of pinopodes. This fact may further promote the implantation of ectopic pregnancy in Fallopian tubes. The lower uterine segment exhibits only weak changes and no pinopode were found (Nicas 1999a,b).

In our study, all 30 randomly selected areas were included in the calculation of the mean occurrence of pinopodes to get representative data. Perhaps this is the reason why our findings are markedly lower than those found by others. Usadi *et al.* (2003) always selected only the most representative photograph out of 12 images for the counting of pinopodes. Nicas (1999a,b) did not specify the number of evaluated areas, but the number of pinopodes was roughly estimated by subjective estimation as abundant (>50 %), moderate (20-50 %), and few (<20 %). Similar classification without specifying the number of examined areas was used by Garcia-Velasco *et al.* (2001) and Adams *et al.* (2001) in artificial cycles and by Develioglu *et al.* (1999) in stimulated cycles. Our results are in agreement with findings of Bentin-Ley (2000) who described that pinopodes covered 5-10 % of the surface.

It is assumed that the lifespan of fully developed pinopodes does not exceed 24 hours because in the subsequent biopsies from oocyte donors fully developed pinopodes were seen only in a single biopsy of each patient (Nicas 1999a,b). In our group, two consequent biopsies were performed within the interval of 48 hours. In spontaneous as well as in "mock" cycles the prevalence of fully developed pinopodes was found on both the days +5 and +7. Nevertheless, a certain shift from the incidence of developing to regressing pinopodes in the „mock" cycles may suggest a tendency to an accelerated pinopodes development.

We therefore suggest that the fully developed pinopodes are present in human endometrium for a longer interval than it is generally believed, perhaps by more than 48 hours. This fenomenon was found both in natural as well as in the "mock" cycles.

Changes that occur within the receptive phase

and immediately following implantation can be divided into three distinct phases. The first phase, regulated by ovarian steroids, is characterized by changes in the endometrial epithelial cells in preparation for blastocyst apposition and attachment (Fazleabas and Strakova 2002). It is known that human blastocysts preferentially attach to pinopode formation. The embryonic signals, such as hCG, lead to a further modulation of the endometrium in the second phase (Alexander *et al.* 1998). According to ultrastructural studies pinopode apical plasma membrane does not directly participate in the embryo-endometrial interaction, but trophoblast cells contact with pinopodes at their lateral plasma membranes by sharing junctional complexes (Bentin-Ley 2000, Lopata *et al.* 2002). The final, third phase is associated with trophoblast invasion, where trophoectoderm ectoplasmic extensions intrude between uterine epithelial cells and embryo-maternal junctions replace the junctional complexes between endometrial cells (Fazleabas and Strakova 2002). Thus during the receptive phase dramatic changes proceed in the uterine endometrium. In agreement with others we propose that pinopode expression coincides with the initiation of implantation but their expression probably covers a longer period of time than was previously reported. Further studies addressed on the function of pinopodes and particularly *in vitro* studies of the interaction between embryo and endometrium are needed.

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