大韓不妊學會雜誌: 第25卷 第1號 1998
Kor. J. Fertil. Steril., Vol. 25, No. 1, 1998, 4

Pinopode Development 2-days after Oocyte Retrieval in the Human IVF Patients

Kyung-Ah Lee, Sei Yul Han, Dong Hee Choi, Woo Sik Lee, Tae-Ki Yoon and Kwang-Yul Cha

College of Medicine, Pochon CHA University, Infertility Medical Center, CHA General Hospital, Seoul 135-081

체외수정 환자에서 난자회수 2일째의 자궁 내막의 Pinopode의 발달

포천 중문의과대학교, 차병원 여성의학연구소

이경아 · 한세열 · 최동희 · 이우식 · 윤태기 · 차광열

= 국문초록 =

본 연구는 체외수정 프로그램에 참여하는 환자에 있어서 난자회수 이틀째의 자궁내막의 발달상태를 알아보기 위하여 pinopode의 발달상태, 에스트로겐 및 프로제스테론 수용체의 발현을 관찰하였다. 생검한 자궁내막 조직을 양분하여, 절반은 전사전자 현미경 (scanning electron microscope)으로 pinopode를 관찰하기 위하여 2.5% glutaraldehyde와 2% paraformaldehyde로 고정하였고, 나머지 절반은 dating 및 스테로이드 수용체의 면역조직화학적 측정 (immunocytochemistry)을 위하여 10% formalin으로 고정하였다. 모두 12명의 환자중 8명에서 pinopode가 관찰되었으며, pinopode 발달이 관찰되지 않은 환자들은 hCG 주사를 맞는 날의 estradiol (E2)의 혈중농도가 600 pg/mL 이하로 낮았다. 본 연구의 결과로부터 자궁내막의 발달상태를 알아 보기 위해서는 지금까지 일반적으로 사용되어 오던 dating이나 스테로이드 수용체의 면역조직화학적 측정법 이외에도 pinopode를 관찰함으로써 조금 더 정확한 진단을 할 수 있으리라고 사료되며, pinopode의 발달은 E2의 혈중농도와 관계가 있을 것으로 추정된다.

Key Words: Human, IVF, Endometrium, Pinopode development

INTRODUCTION

There are three factors for successful implantation. These are embryo quality, uterine receptivity, and synchronization between embryonic and endometrial development. Despite remarkable progress in investigating embryos in human IVF, there has been slow progress in exploring the implantation process. It may be due to two reasons as follow. First, it is difficult to directly investigate the mechanism of implantation in the human, because of ethical considerations. Second, there is no sensitive and widely accepted marker for assessing endometrial development. Since the finding of a novel standard for dating endometrial biopsy by Noyes *et al.* in 1950, there have been many attempts to identify suitable markers for uterine receptivity. Those include ultrasonographic changes (Ueno *et al.*, 1991; Grunfeld *et al.*, 1991), three dimensional morphological changes

이경아: 차병원 여성의학연구소 기초의학연구실, 서울시 강남구 역삼1동 606-5, Tel: 02-3468-3440, Fax: 02-501-8704, E-mail: kal1227@hitel.net

of the endometrium such as pinopode formation (Martel *et al.*, 1987; Martel *et al.*, 1991; Nikas *et al.*, 1995; Psychoyos & Nikas, 1994), integrin expression (Ilesanmi *et al.*, 1993; Lessey *et al.*, 1992; Lessey, 1994), and measurement of endometrial proteins (Bell, 1986; Fay & Grudzinskas, 1991).

Investigations in the rat (Martel *et al.*, 1991) and human (Martel *et al.*, 1987; Nikas *et al.*, 1995; Psychoyos & Nikas, 1994) suggested the presence of pinopodes as a marker for the receptive phase. A chronological barrier in uterine receptivity could be one of the major factors limiting IVF pregnancy rates. If we were able to manage the 'implantation window' we may be able to improve implantation and pregnancy rates in the human IVF program. In 1987, Martel *et al.* found early appearance of pinopodes in stimulated cycles for IVF compared to natural cycles in humans (Martel *et al.*, 1987). This effect was found in patients stimulated with clomephene citrate/hMG/hCG. The purpose of the present study was to evaluate the endometrial development in IVF patients stimulated with either by FSH/hMG/hCG or with GnRH agonist down regulation.

MATERIALS AND METHODS

Patients

Patients were stimulated with FSH/hMG or GnRHa/FSH/hMG and administered 10,000 IU hCG when there were at least two leading follicles at the size of >18 mm in diameter and/or serum E2 concentrations >600 pg/mL. For poor responders, who had <600 pg/mL serum E2, the decision to administer hCG was based on follicular and endometrial development rather than only on E2 levels. Oocytes were retrieved transvaginally with ultrasound guides 34~36 hours after the hCG administration. All patients were administered doxycycline monohydrate (Vibramycin, Pfizer, Korea) in daily doses of 200 mg, beginning one day before oocyte retrieval for four days, and 25 mg progesterone i.m. (Progest, Samil Pharm. Co., Ltd, Korea) from the day of oocyte retrieval. Endometrial biopsies were taken from 12 patients who had no embryos available for transfer on the scheduled day, 2-days after oocyte retrieval. Biopsies were taken by Pipelle or Novak curette. All patients were infertile due to tubal factor, male factor, or unexplained infertility. Women included in this study had similar hormonal profiles and length of follicular phase compared with those of patients who had successful fertilization.

Scanning Electron Microscopy

Half of the biopsied endometrium was rinsed thoroughly with saline and immediately immersed in 2.5% (w/v) glutaraldehyde containing 2% paraformaldehyde solution in PBS. The specimen was fixed in 1% (w/v) osmium tetroxide, dehydrated in ethanol, and dried in a critical-point drier (E3000, Polaron, Watford, England). Specimen was mounted, coated with gold palladium by ion sputter (JFC 1100, Jeol, Japan), and examined under a scanning electron microscope (Jeol, Japan).

Dating and Immunocytochemistry

The remaining half of the biopsied endometrium was rinsed in saline, then fixed in 10% formalin solution in PBS for further preparation for paraffin section. Histological assessment was performed by dating the endometrium according to standard criteria after hematoxylin and eosin staining. Immunocytochemical analysis of ER and PR was performed with commercially available antibodies. Technicians and pathologists analyzed samples in a blind fashion relative to patients' demographic characteristics.

Hormonal Evaluation

Serum E2 was measured by radioimmunoassay. Radioimmunoassay was conducted according to procedures described in the instruction of Spectria kit (Orion Diagnostica, Espoo,

Finland). The inter-assay and intra-assay coefficients of variations were 5.2% and 5.8%, respectively.

RESULTS

Patients

Demographic characteristics of the included patients were as follows. Mean E2 level was 395±66 pg/mL in the poor responders and 1328.03±215.18 pg/mL in the good responders, and mean age was 34.5±2.2 and 33.6± 0.9, respectively. Length of folliculogenesis in the two groups was statistically not different. The mean number of leading follicle size over 18 mm in diameter was statistically different as 2.5 in the poor responders versus 5 in the good responders ($p<0.05$).

Pinopode Formation

We classified endometrium with long, erect microvilli and no pinopodes as grade 0, with the starting point of pinopodes as +, and with apparent round swollen microvillous cells with short microvilli as +++. Table 1 summarizes results of the SEM observation of pinopode development. We analyzed our data in relation to ovarian stimulation protocols first, and found no consistent results (left side of Table 1). We observed various stages of pinopode formation from 0 to +++ in patients stimulated with either FSH/hMG or GnRH/FSH/hMG. Five out of 7 patients stimulated with FSH/hMG and 3 out of 5 patients stimulated with GnRH/FSH/hMG had developed pinopodes 2-days after oocyte retrieval.

When we reanalyzed the endometrial biopsies in relation to serum E2 concentrations on the day of hCG administration (right side of Table 1), we found significant results. Poor responders with lower serum E2 levels had delayed pinopode formation with long, erect microvilli compared to the good responders. Scanning electron microscopic pictures of the poor responders are shown in Figure 1. Four out of 6 patients showed grade 0. Whereas, well-developing pinopodes were found in patients with higher E2 values. We observed

Table 1. Pinopode formation in patients stimulated by FSH/hMG/hCG (CB) or with GnRH agonist down regulation (LA). Left column of the table was sorted by induction methods and listed according to age, while right column was sorted by serum E2 level on the day of hCG administration

Sorted by induction method					Sorted serum E2 level				
Patient's					Patient's				
IND*	ID	Age	E2	Pinopode	E2	ID	IND*	Age	Pinopode
CB	1	30	965.7	+++	131.2	5	CB	38	0
CB	1	30	965.7	+++	131.2	5	CB	38	0
CB	2	32	2308.4	+++	316.6	7	CB	36	+
CB	3	34	874.5	++	392.9	6	CB	41	0
CB	4	37	776.9	+++	407.6	9	LA	30	+~++
CB	5	38	131.2	0	544.0	12	LA	34	0
CB	6	41	392.9	0	578.9	8	LA	28	0
CB	7	36	316.6	+	776.9	4	CB	37	+++
LA	8	28	578.9	0	874.5	3	CB	34	++
LA	9	30	407.6	+~++	965.7	1	CB	30	+++
LA	10	32	1531.0	+~++	1531.0	10	LA	32	+~++
LA	11	34	1797.2	+++	1797.2	11	LA	34	+++
LA	12	34	544.0	0	2308.4	2	CB	32	+++

IND*: Induction methods

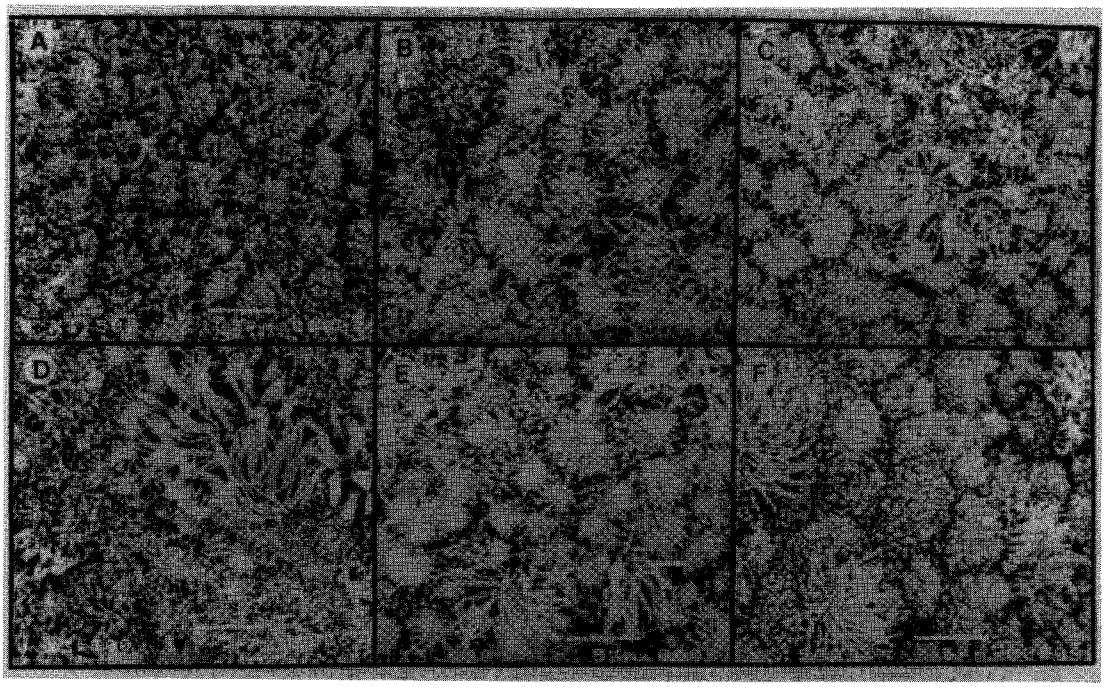

Figure 1. Scanning electron microscopy of the biopsied samples obtained 2-days after oocyte retrieval from the six different poor responders (x3,500). Patients A to D showed endometrium with long, erect microvilli and no pinopodes formation, while patients E and F showed early developing stage of pinopodes.

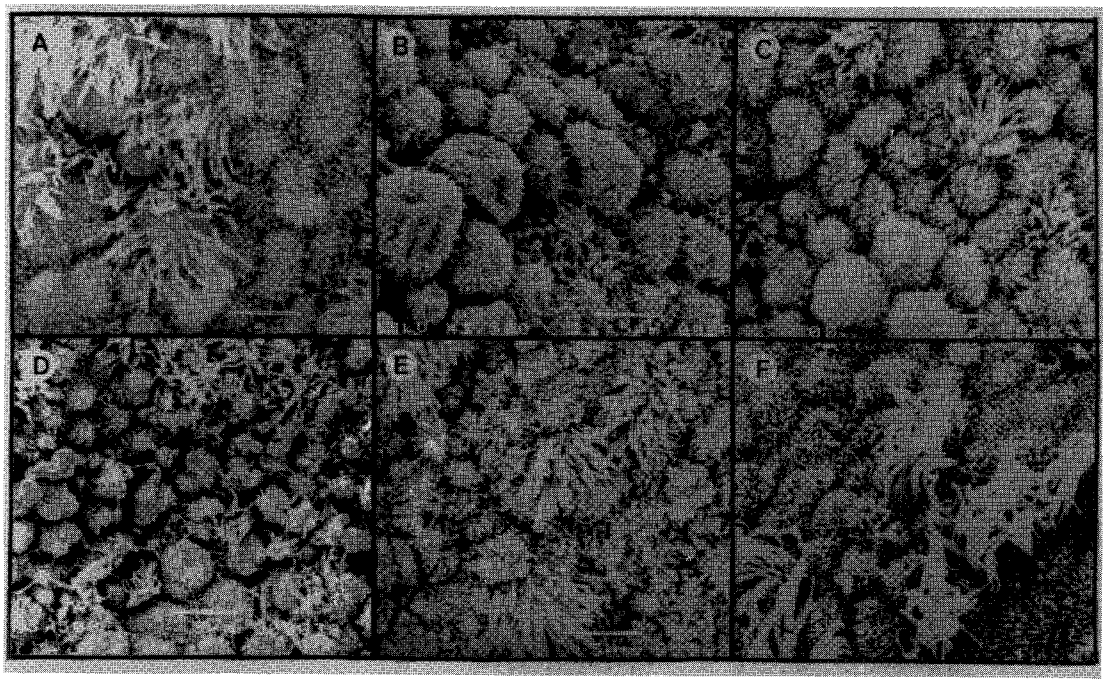

Figure 2. Scanning electron microscopy of the biopsied samples obtained 2-days after oocyte retrieval from the six different good responders (x3,500). Most of patients (A to D) showed swollen microvillous cells with short microvilli. Patients E and F showed delayed formation of pinopodes compared to patients A to D.

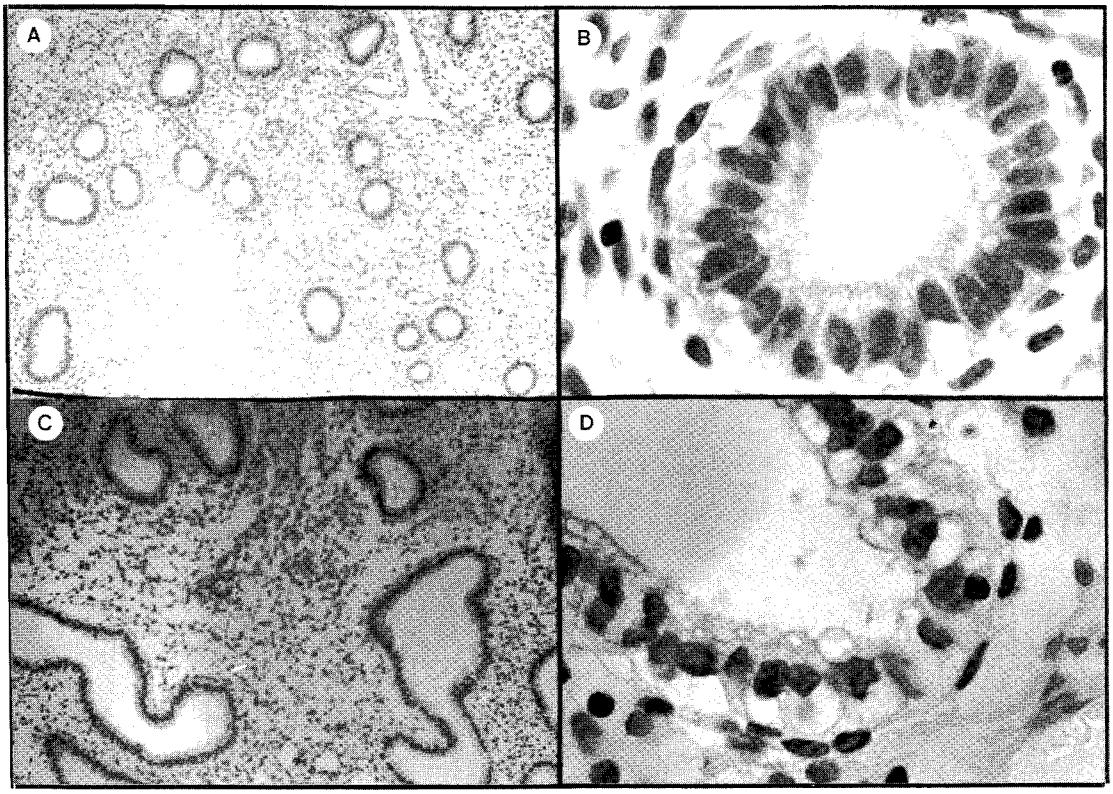

Figure 3. Typical microphotographs of the endometrium from poor responders (A, B) and good responders (C, D). A, C: x100; B, D: x1,000.

round lumpy pinopodes with short microvilli in 4 out of another 6 patients with higher E2 levels (Figure 2).

Dating

We observed simple tube shape of glands and less edema in stroma in poor responders (Figure 3A, B); while more advanced form of glands with prominent subnuclear vacuoles in glandular epithelium in the good responders (Figure 3C, D). Although we were able to find some consistency in the glandular development according to E2 levels, it was difficult to judge the status of the endometrium by dating alone due to the dyssynchrony between the glandular and stromal development.

Immunocytochemistry for Steroid Receptors

Glandular and endometrial epithelial cells stained more strongly with ER antibody than stromal cells. Staining in stroma cells was weak for ER. However, in the case of PR staining, stroma cells were stained equally as strong as the epithelial cells. Staining patterns were similar for ER and PR in all patients (data not shown).

DISCUSSION

The major findings of the present study were: 1) most of the patients stimulated with FSH/hMG/hCG or with GnRH down regulation (8/12) had developing pinopode formation 2-days after oocyte retrieval, 2) good responders in terms of follicle growth and estradiol secretion had more advanced pinopodes when compared to the poor responders, 3) it was possible to predict the development of pinopodes from the estradiol value on the day of

hCG administration, and 4) neither dating nor expression of steroid receptor was a sensitive marker for assessing status of the endometrium biopsied at the early luteal phase. However, results of dating and immunocytochemical staining of the steroid receptors were informative when analyzed together with the endometrial morphological changes observed by scanning electron microscopy.

It is important to know the endometrial development in the human IVF program. In 1987, Martel and co-workers found an advanced development of the pinopodes in the 4 out of 9 endometrial samples taken from the patients stimulated with clomiphene citrate/ hMG/hCG. They suggested that the hormonal treatment applied to induce ovulation can modify the normal development of the prenidatory endometrium, and may have a negative effect on the rate of egg implantation (Martel *et al.*, 1987). We also observed the advanced development of the pinopodes in the endometrium of patients stimulated with the different hormonal treatments, FSH/hMG/hCG or with GnRH agonist long protocol. It may be concluded that the endometrial development in the stimulated patient is more advanced than the normal cycles.

We found more advanced pinopode formation in the good responders with higher serum E2 levels on the day of hCG administration. When we grouped patients by their serum concentrations of estradiol, the cut off value was 600 pg/mL. Our rationale for using this value was that poor responders have fewer than two leading follicles 18 mm in diameter, and one leading follicle secretes around 300 pg/mL estradiol. In 1991, Martel and co-workers found that in the rat the appearance of pinopodes was strictly progesterone-dependent and also relative to the timing of estradiol administration and the dose administered (Martel *et al.*, 1991). Induction of endometrial receptivity requires a minimum of 3 days of priming of the endometrium with progesterone, and with minute amounts of estrogen at the end of this period (Martel *et al.*, 1991; Psychoyos, 1993). However, it may be difficult to apply these results to the human since those previous results were obtained in rats by administering exogenous progesterone and estradiol to ovariectomized animals. Unfortunately, it was not possible to evaluate biopsy results in relation to the serum progesterone levels in the present study. We also consider the steroid concentrations and steroid receptor expression at the tissue level would be more important than the circulating levels. Based upon our results, we only can suggest that the higher concentration of estradiol priming before progesterone supplement enhances endometrial development as well as pinopode formation in stimulated cycles. The regulation of the pinopode formation in the human endometrium by estradiol and progesterone requires further investigation.

Endometrial estrogen and progesterone receptors are regulated by estradiol and progesterone, thus the measurement of its receptors could be useful in evaluating the hormonal milieu in the endometrium. The pattern of steroid hormone receptor distribution has previously been defined by many investigators in normal menstrual cycles by using immunocytochemistry (Bergeron *et al.*, 1988; Garcia *et al.*, 1988; Lessey *et al.*, 1988; Snijders *et al.*, 1992). Our results were comparable to those of previous studies in that the glandular and endometrial epithelial cells stained more strongly for the estrogen receptor than stroma cells, whereas the stroma cells stained equally as strong as the epithelial cells for the progesterone receptor. We could not discriminate between good and poor responders by immunocytochemical staining only.

The most important finding in the present study was that we were able to predict the developmental status of the endometrium, histology as well as pinopode formation, from the estradiol value on the day of hCG administration. Rogers and co-workers suggested

that pinopode formation, previously thought to indicate uterine receptivity, may not always do so (Roger *et al.*, 1989). They found no correlation between pinopode formation and the circulating plasma estradiol and progesterone levels on the day of biopsy. However, the big differences between that report and the present study are, 1) biopsies were taken during the second half of the cycle in that study, and 2) plasma steroids were measured on the day of biopsy taken. Thus, their conclusion is not suitable for all cases.

Based on our results, we concluded that pinopode development shows a good correlation with results of dating and serum estradiol concentrations on the day of hCG administration. Time difference in endometrial development between the poor and good responders may have important implications for the successful implantation of the transferred embryos in the human IVF program.

Acknowledgment

The authors are grateful to Mr. In-Sik Lee at the Catholic Medical Center for his technical assistance in using the scanning electron microscope; Mr. Jin-Sub Jung and Hee Jung Ahn, M.D., at the Department of Anatomy and Pathology for their technical help in making paraffin sections and histological assessment. The authors are grateful to Prof. Michael Shapiro, M.A., at Dongyang University, Young-Ju, Korea for his critical review of the manuscript.

REFERENCES

Bell SC: Secretory endometrial/decidual proteins and their function in early pregnancy. *J Reprod Fertil (Suppl)* 1986, 36, 109-125.

Bergeron C, Ferenczy A, Toft DO, Schneider W, Shyamala G: Immunocytochemical study of progesterone receptors in the human endometrium during the menstrual cycle. *Lab Invest* 1988, 59, 862-878.

Fay TN, Grudzinskas JG: Human endometrial peptides: A review of their potential role in implantation and placentation. *Human Reprod* 1991, 6, 1311-1326.

Garcia E, Bouchard P, De Brux J, Berdah J, Frydman R, Schaison G, Milgrom E, Perrot-Applanat M: Use of immunocytochemistry of progesterone and estrogen receptors for endometrial dating. *J Clin Endocrinol Metab* 1988, 67, 80-87.

Grunfeld L, Walker B, Bergh PA, Sandler B, Hofmann G, Navot D: High-resolution endovaginal ultrasonography of the endometrium: A non-invasive test for endometrial adequacy. *Obstet Gynecol* 1991, 78, 200-204.

Ilesanmi AO, Hawkins DA, Lessey BA: Immunohistochemical markers of uterine receptivity in the human endometrium. *Microsopy Res Tech* 1993, 25, 208-222.

Lessey BA, Damjanovich L, Coutifaris C, Castelbaum A, Albelda SM, Buck CA: Integrin adhesion molecules in the human endometrium: Correlation with the normal and abnormal menstrual cycle. *J Clin Invest* 1992, 90, 188-195.

Lessey BA, Killam AP, Metzger DA, Haney AF, Greene GL, McCarty KS, Jr: Immunohistochemical analysis of human uterine estrogen and progesterone receptors throughout the menstrual cycle. *J Clin Endocrinol Metab* 1988, 67, 334-340.

Lessey BA: The use of integrins for the assessment of uterine receptivity. *Fertil Steril* 1994, 61, 812-814.

Martel D, Frydman R, Glissant M, Maggioni C, Roche D, Psychoyos A: Scanning electron microscopy of postovulatory human endometrium in spontaneous cycles and cycles stimulated by hormone treatment. *J Endocrinol* 1987, 114, 319-324.

Martel D, Monier MN, Roche D, Psychoyos A: Hormonal dependence of pinopode formation at the uterine luminal surface. *Human Reprod* 1991, 6, 597-603.

Nikas G, Drakakis P, Loutradis D, Mara-Skoufari C, Koumantakis E, Michalas S, Psychoyos A: Uterine pinopodes as markers of the nidation window in cycling women receiving exogenous oestradiol and progesterone. *Human Reprod* 1995,

10, 1208-1213.

Noyes RW, Hertig AI, Rock J: Dating the endometrial biopsy. *Fertil Steril* 1950, 1, 3-25.

Psychoyos A, Nikas G. Uterine pinopodes as markers of uterine receptivity. *Assisted Reprod Reviews* 1994, 4, 26-32.

Psychoyos A: The implantation window: Basic and clinical aspects. In: Mori T, Aono T, Tominaga T, Hiroi M, editors. Perspectives in Assisted Reproduction, Rome, Italy. *Ares Serono Symposia* 1993, 4, 57-62.

Rogers P, Murphy C, Cameron I, Leeton J, Hosie M, Beaton L, Macpherson A: Uterine receptivity in women receiving steroid replacement therapy for premature ovarian failure: ultrastuctural and endocrinological parameters. *Human Reprod* 1989, 4, 349-354.

Snijders MPML, de Goeij AFPM, Debets-Te Baerts MJC, Rousch MJM, Koudstaal J, Bosman FT: Immunocytochemical analysis of oestrogen receptors and progesterone receptors in the human uterus throughout the menstrual cycle and after the menopause. *J Reprod Fertil* 1992, 94, 363-371.

Ueno J, Oehninger S, Bryzshi RG, Acosta A, Philput CB, Muasher SJ: Ultrasonographic appearance of the endometrium in natural and stimulated in-vitro fertilization cycles and its correlation with outcome. *Human Reprod* 1991, 6, 901-904.