

## The Morphological Expression of Endometrial Pinopodes During Implantation in Mice After Ovarian Stimulation and Progesterone Injection

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### Abstract

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**Introduction:** The aim of this study was to evaluate the expression of pinopodes as an implantation marker after ovarian hyperstimulation and progesterone injection using scanning electron microscopic studies.

**Material and Methods:** Three groups of NMRI adult female mice were used in the experiment. The control group (Group A) were untreated pseudopregnant mice. Group B mice were made pseudopregnant after superovulation treatment with hMG and hCG. Group C mice were treated the same as Group B and then received progesterone daily from day 1 of pseudo pregnancy. Animals were sacrificed by cervical dislocation 3.5 and 4.5 days after hCG injection. Tissues were obtained from the middle 1/3 part of uterine horns and processed for scanning electron microscopic studies.

**Results:** In the control group there were some pinopodes at 3.5 days of pseudopregnancy and the apical surface of all cells expressed these projections on day four. In the hyperstimulated group without progesterone injection no pinopodes were seen 3.5 days after hCG injection and some appeared on day 4. In the hyperstimulated and progesterone-injected group well developed pinopodes were expressed 3.5 days after stimulation and they became much smaller on day 4 after hCG injection.

**Conclusion:** The results showed that the life span of pinopodes is short and changeable during hyperstimulation and that progesterone causes premature expression of the pinopodes, suggesting that the implantation after ovarian stimulation might depend upon the timing of the pinopode expression.

**Key words:** Ovarian stimulation, Pinopode, Progesterone, Scanning Electron Microscopy



## Introduction

The epithelium of endometrium and the trophoctoderm of the blastocyst are closely apposed at the time of implantation and the interaction between these two types of epithelium is essential for successful implantation. Synchrony between embryonic development and endometrial receptivity is essential to attachment and implantation of embryos and is particularly relevant in the context of embryo transfer (1, 2). The endometrium is receptive for embryo implantation for a limited time described as the implantation window (3, 4). Several markers of the implantation window have been proposed including endometrial morphology, expression and secretion of some cytokine such as leukemia inhibitory factor and appearance of cytoplasmic microprojections known as pinopodes (1, 5).

Pinopodes have not the same function in all mammals (6). These endometrial cell surface projections are involved in the pinocytosis of uterine secretion and macromolecules in the mouse (7) and rat (8) but have no pinocytotic role in the cow (9), human (6) and rabbit (10). The surface of these processes may have receptors or act as an attachment site for adhesion molecules, which are essential for embryo-endometrium interaction and adhesion during implantation (11).

The pinopodes are present for only 24-48 hours during implantation, and their appearance is considered an indicator of the implantation window in mammals (12). The presence and development of pinopodes is dependent on the ovarian hormones, especially progesterone (13). High concentration of estrogen have been reported to interfere with the formation of pinopodes (14, 15). Exposure of the endometrium to supraphysiological level of estrogen after mouse ovarian hyperstimulation may have a detrimental effect on pinopodes formation or disappearance.

This study was done to evaluate alteration of pinopodes expression at the pre-implantation and implantation period after mouse ovarian hyperstimulation using human menopausal gonadotropic hormone (hMG) and human chorionic

gonadotropic hormones (hCG) and daily injections of progesterone.

## Material and Methods

### \* *Animals*

Animals were cared for and used according to the Guide for the Care and Use of Laboratory Animals at Tarbiat Modarres University. Thirty females NMRI mice aged 6-10 weeks were housed under conditions of 12h light: 12h dark and randomly divided into the following three groups:

Group A: The mice of the control group were rendered pseudopregnant.

Group B: Hyperstimulated group: The mice in this group were superovulated using an intraperitoneal injection of 10 i.u. hMG, followed 46h later by injection of 10 i.u. hCG. on the evening of the second injection the mice were rendered pseudopregnant as in the control group.

Group C: Hyperstimulated and progesterone injected group: After superovulation of the mice as in Group B and induction of pseudopregnancy, daily subcutaneous injections of progesterone (1 mg/mouse) were started on day 1 of pseudo pregnancy (16).

### \* *Tissue preparation*

The mice of each group were sacrificed by cervical dislocation on days 3.5 and 4.5 after hCG injection or artificial mating. The samples were obtained from the middle 1/3 part of the uterine horns immediately.

### \* *Scanning electron microscopy*

The lumen of the uterine horns were washed several times by flushing with buffer phosphate to remove mucous secretions and then washed with 0.2 M sodium cacodylate buffer (pH= 7.2). The samples were fixed first with 2.5% glutaraldehyde in cacodylate buffer (pH= 7.2), and then 1% osmium tetroxide. The specimens were dehydrated first in increasing concentrations of ethanol (50%, 70%, 80%, 90% and 100%) and then in acetone. They were dried, mounted and coated with gold particles and examined using a Philips scanning electron microscope.

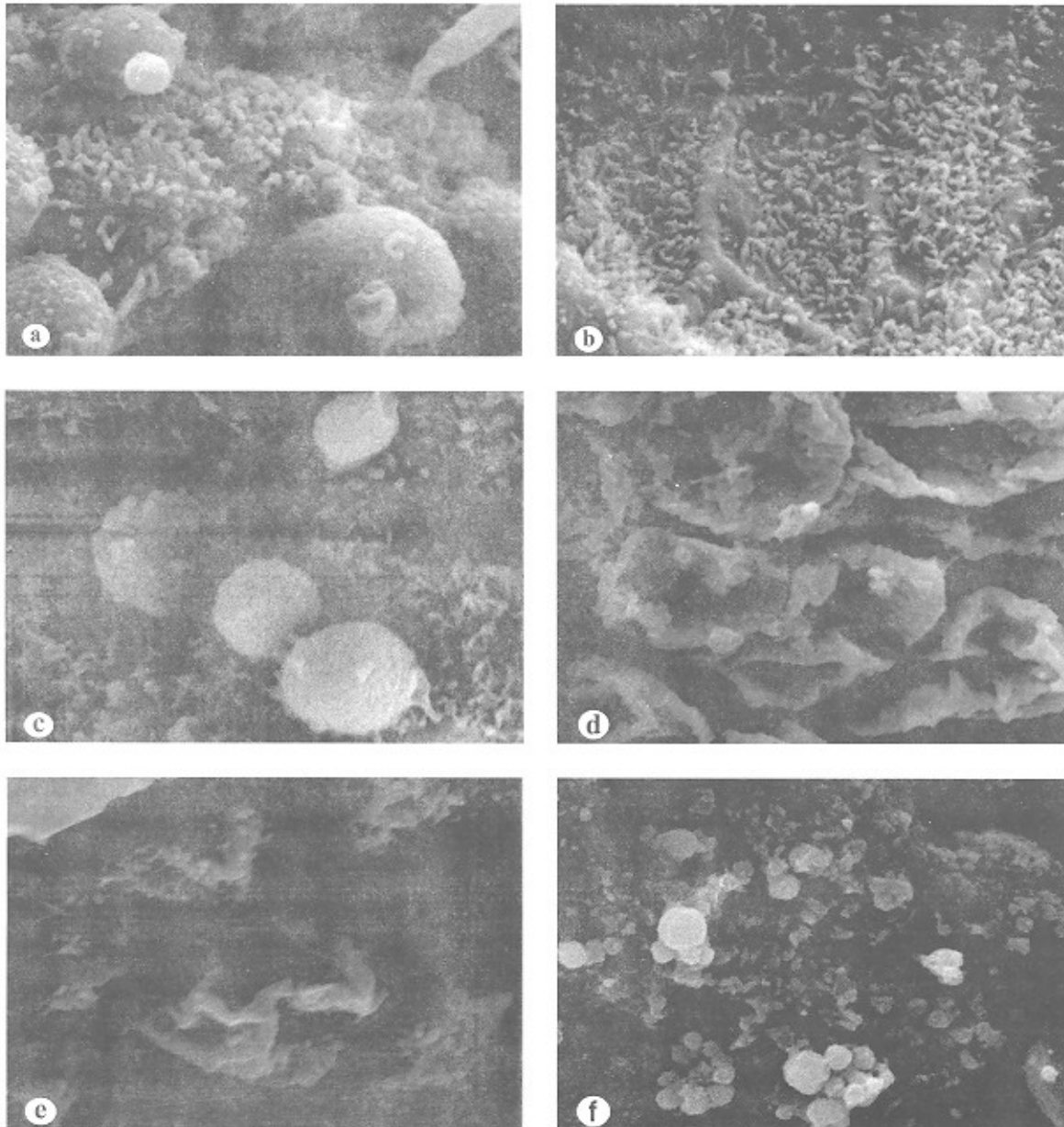


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The sections were scored according to morphology and stage of development (developing, fully developed and collapsed) of the pinopodes and on the percentage of the endometrial surface occupied by pinoposed (abundant= > 50%, moderate= 20-50%; few= <20%) (3, 12).

## Results

SEM studies demonstrated that the major cell population was secretory cells with a number of microvilli or pinopodes on their apical surface (Table 1). The morphology of surface of these cells in the hyperstimulated and control groups had distinctive differences as follow:



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Figure 1: Scanning electron microscopy of the mouse endometrial surface in (a) non-stimulated control group 3.5 days after pseudopregnancy. Two types of cells are visible, in one group of the cells some microvilli have disappeared and pinopodes have developed. b: Hyperstimulated group on the day 3 after hCG injection. Note the absence of pinopodes. All of the cells have microvilli and their apices are flat. c: Hyperstimulated and progesterone injected group 3.5 days after hCG injection. Well developed pinopodes are seen. d: Non-stimulated control group 4.5 days after pseudopregnancy. e: Hyperstimulated group in the fourth day of hCG injection; many pinopodes are present and some are collapsed. f: Hyperstimulated and progesterone-treated mice on 4.5 days after hCG injection: Note large pinopodes transformed to small-sized projection and the number of microvilli decreased. (Magnification of all figures were\*10000)

Table 1: Pinopodes and microvilli on the endometrial cell surface in three groups of mice

Group	Days after hCG or artificial mating	Pinopodes	Microvilli
A: Control	3.5	moderate	abundant
	4.5	abundant and collapsed	not visible
B: Hyperstimulated	3.5	not visible	abundant
	4.5	abundant	moderate
C: Hyperstimulated +Prog	3.5	abundant	moderate
	4.5	small and scattered	minimum

Treatments:

Group A: Pseudopregnant controls

Group B: Pseudopregnant after ovarian stimulation with 10 i.u.hMG followed 48 h later by 10 i.u.hCG

Group C: As for Group B then 1mg progesterone daily from day 1 of Pseudopregnancy

### \* Non-stimulated control groups

On day 3 after artificial mating the apical cell surface was dome shape and the border of the cells well defined. The microvilli of these cells were short and the tips of these microvilli were dilated. Some cells had no microvilli and were transformed to fungal shape projections (pinopodes) which were located at the corner of the cells while the remaining surface was smooth. The relative ratio between secretory cells with microvilli and those with pinopodes was 3:1 (Figure 1a). However, on day 4 all the apical cell surfaces expressed these projections most of which were collapsed (Figure 1d)

### \* Hyperstimulated groups

In the hyperstimulated group (Group B) 3.5 days after hCG injection the surface of the luminal epithelium was covered with hexagonal and flat apical border cells. No signs of pinopodes formation were seen. The microvilli of secretory cells were abundant and slender shaped but narrower than in the control group (Figure 1b). On the day of implantation (4.5 days after hCG injection) the ratio of the cell surface covered with microvilli and pinopodes appeared to be equal. Some pinopodes were well developed with small projection on them (Figure 1e). The microvilli of supporting cells were short and these cells had dome shape apical surfaces.

Hyperstimulated- progesterone injected groups  
In the hyperstimulated - progesterone groups (Group C) 3.5 days after hCG the cell borders of the luminal epithelium were indistinct and microvilli were present in

only half the cells. Pinopodes were formed and well developed as large spongy projections (Figure 1c). In this group distinctive morphological changes were observed at the implantation time (4.5 days after hCG). There was a great reduction in microvilli and large pinopodes had transformed to small projections (Figure 1f). The border of neighbouring cells was not distinguishable.

## Discussion

The ultrastructural studies described here show that the mouse endometrium is composed mainly of secretory cells bearing microvilli or pinopodes. These cells undergo a series of changes during the estrous cycle and implantation. Pinopodes have been considered a useful marker for endometrium receptivity in many species. These results showed that on day 3.5 of pregnancy the pinopodes are present but the number of these projections is low in comparison with that at implantation. In the hyperstimulated mice without progesterone injection (Group B) three days after hCG injection pinopodes were not present and all of the epithelial cells had microvilli on the apical cell surface, but on the 4th day well-developed pinopodes were present and some were collapsed.

These results show that after ovarian hyperstimulation without progesterone injection the duration of pinopodes expression is limited to a short time and their onset is delayed. It is suggested that the absence of pinopodes at pre-implantation may be due to the high levels of estrogen in hyperstimulated mice.

Estrogen has been shown to be essential for





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implantation in mice but there is no report of its effect in supraphysiological concentration on the expression of mouse endometrial pinopodes. During ovarian stimulation a large number of follicles develop and result in high levels of estrogen. It has been shown that small doses of estradiol potentiate the progesterone effect, whereas higher concentrations of this steroid almost completely block expression of progestational response (17). It follows that supraphysiological levels of estrogen may inhibit expression of pinopodes in mice because pinopodes expression is progesterone dependent (13). A similar effect has been reported in humans (13, 14). In contrast to our results there are some reports of premature appearance of pinopodes after hyperstimulation of animals and humans (17-19). On the hand Nikas et al. (20) showed that human ovarian stimulation did not affect the formation or duration of expression of endometrial pinopodes.

After administration of progesterone in a protocol similar to that used in hormone replacement therapy well organized pinopodes were expressed over the surface of the endometrium pre implantation but these projections were collapsed and transformed to small projections on the fourth day after hCG and start of progesterone injection. Examination by light and transmission electron microscopy showed that the cells of the projections were not apoptotic but had normal morphology and ultrastructure (21, 22).

These observations show that progesterone injection after hyperstimulation could cause premature expression of pinopodes before implantation time. Our

hypothesis is that the presence of pinopodes dependson the balance between concentration of progesterone and estrogen. Starveus-Evers et al. also showed thatpinopode formation in human endometrium isassociated with the concentration of progesterone and progesterone receptors (23).

In contrast to the results here it has been shown that with hormone replacement therapy in women the timing of the nidation window seems to be postponed for some two days (24), and that administration of progesterone antagonist on day 1 of rat pregnancy displaces the time of appearance of fully developed pinopodes from day 5 to day 6 or 7 (4).

Experience from oocyte donation programs has shown that women treated with oestradiol and progesterone in hormone-controlled cycles have a higher chance of conception that women undergoing ovarian stimulation (25). This may be due to a better priming of the endometrium and avoidance of high estradiol concentrations (23).

Although there are many differences between the mouse and human in implantation and pinopodes function, results reported here and elsewhere show that the life span of pinopodes is limited to a short time which is altered by hyperstimulation regimes. This alteration in expression of pinopodes results in an alteration in the implantation window which may be responsible for failure of implantation in animals (26) and women (27) undergoing ovarian hyperstimulation. Progesterone injection may be effective in controlling the premature development of pinopodes.

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