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Original Article

The effect of sperm activation on pinopod formation in endometrial epithelium

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ABSTRACT

Introduction: Endometrial receptivity is crucial in implantation of the developing embryo in the endometrium and formation of the pregnancy. In this study, possible effect of sperm and uterine endometrial contact on formation of pinopod, an important element in morphological differentiation necessary for implantation, was investigated.

Materials and methods: In this experimental study, 42 female Spraque–Dawley albino rats and 14 male Spraque–Dawley albino rats (total 56 rats) were used. Vasectomy was performed in half of the male rats. For each group, two distinct branches were formed with 21 females and 7 males: Group 1 (non-vasectomized) and Group 2 (vasectomized). Cases were sacrificed and evaluated every day from Day 1 to Day 3. Scanning electron microscopic (SEM) images were analyzed according to different stages of pinopod development on different days. Pinopods were classified as developing, developed and regressing pinopod. The average number of pinopods were calculated by counting the pinopods at four endometrial regions examined for each rat and total number was divided by 4. Same procedure was done for all rats in every group. Results were compared among the groups. For statistical analysis among the groups, *Independent Samples Test (Mean ± Std) and **Mann Whitney *U* Test (Median (25–75%)) were used.

Results: The most important finding in SEM examination of uterus removed on the first day following mating from female rats that were copulated with non-vasectomy male rats which comprised the first group of the study was that heads of the sperms in the uterus were embedded in endometrial epithelium. Similarly, examination of the endometrium of uterus that were removed on postcoital second day revealed small number of developed pinopods (average 0.39) ($P = 0.902$). Examination was done by taking the developing pinopods within image area into account and number of developing pinopods in endometrium epithelium of the rats in first group (average 20.61) was higher than that of second group (average 12.86) ($P < 0.001$). Examination of endometrium of the uterus that were removed on third postcoital day revealed less number of developing pinopods in both groups. Examination of first group revealed an average of 1.21 developing pinopod, whereas the average number of developing pinopod in second group was 2.25 ($P = 0.011$). Examination based on the count of developed pinopods revealed that number of pinopods in first group (average 13.79) was higher than second group (average 8.96) ($P < 0.001$). Regressing pinopod images were observed in only endometrium that were taken on postcoital third day in the second group.

Discussion: In this study, it was clearly shown that sperms were entered into endometrial epithelium with their heads. It can be suggested that they might have a facilitating effect for pinopod formation by reacting with endometrial epithelium as a result of this invasion. It would be beneficial to demolish the other factors triggering pinopod formation to investigate whether presence of sperm alone in the uterus has an effect on pinopod formation.

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1. Introduction

Endometrium receptivity and implantation have been an important topic of investigation for scientists for a long time. Several hormones and cytokines that play a role in endometrium receptivity have been established by studies. LIF (Leukemia Inhibitory Factor), Prostaglandin E2 and Platelet Activating factor dominate among these substances.[1–4] Besides it has been shown in certain studies that secretion of heparin binding epidermal growth factor (HB-EGF) occurs prior to attaching of blastocycts to endometrium epithelium.[5–8] However, although many studies were conducted and results of scientific evaluation were described, there is no consensus about the factors that endometrium receptivity depends on.[9]

Aside from the hormones and cytokines that are thought to be effective on endometrium receptivity, there are several studies claiming that pinopods play an important role on endometrium receptivity.[10,11] However, studies suggesting that this topic is controversial are also present.[9,12] On the other hand, it has been reported previously that there is no correlation between LIF and MMP2 (Matrix Metallo Proteinase) secretion during the implantation window and pinopod formation.[13] In addition to this, Oborna and colleagues did not find any difference in the duration of pinopode formation between cycles where steroid hormone was added and not added.[14] Furthermore, a study conducted with oopherectomized mice, did not show any correlation between pinopod and integrin expression.[15]

While menstruation is seen in humans, it is not observed in several mammalian species. When Emera et al. investigated the reason of the case, they proposed various hypotheses.[16] One of these hypotheses suggests that spontaneous decidualization takes place, while another one claims that clearance of sperm-originated pathogens from the environment occurs. According to this theory, menstruation occurs in order to protect uterus from colonization of pathogens transported by sperms.[16–19]

Electron microscopic studies established the presence of pinopod formation in both rodents and humans. However, there are significant differences in pinopod formation and developmental periods between both species due to the marked difference in cycle days.[9,20–23] Therefore, caution should be taken when studies on rodents are designed and evaluated.

Many theories were suggested regarding pinopod formation mechanisms. There has also been a controversy about whether pinopods have an effect on endometrium receptivity or not. Here in this study, we have investigated if sperms in the uterus following coitus have any effect on pinopod formation.

2. Materials and methods

In this experimental study, 42 female Spraque–Dawley albino rats and 14 male Spraque–Dawley albino rats (total 56 rats) were used. Female and male rats were divided into two groups. Vasectomy was performed in half of the male rats. During vasectomy procedure, ketolar-rompun anesthesia was given. Following vasectomy, one month was allowed for wound recovery.

1. Twenty-one female rats were divided into three groups with seven rats in each and they were placed in same place with the non-vasectomized male rats. First group of female rats were sacrificed on Day 1 after copulation, while the second group was terminated on Day 2. Finally the last group was sacrificed on Day 3 after copulation. Uterus were harvested and procedures were carried on for examination under SEM.
2. Twenty-one female rats were divided into three groups with seven rats in each and they were placed in same place with the vasectomized male rats. Seven female rats were sacrificed on Day 1 after copulation, 7 were sacrificed on Day 2 and remaining 7 female rats were terminated on Day 3 after copulation. Uterus were removed and procedures were carried on for examination under SEM.

Tissues were treated with phosphate-buffered-glutaraldehyde solution for 5 h and fixated in 1% osmium tetroxide solution for 2 h. After the dehydration of tissues with alcohol, tissues were treated with amyl acetate and critical point dryings were done. Dried tissues were covered with gold–palladium and examined with JEOL SEM 5600.

Analysis of electron microscope images was done according to observation of distinct phases of pinopod formation on different days. Pinopods were classified as developing, developed and regressing pinopods according to Bentin-Ley et al.[24] and Develioglu et al.[25] All four regions showing pinopod formation were detected in each uterus since total pinopod area on endometrial epithelium could comprise approximately between 5.5% and 20% of the uterus.[25–27] Uterus were examined with SEM under 3000 magnification and pinopods within image area were counted one by one in a manner appropriate with classification. Number of pinopods in four areas was added up in each rat and average number of pinopod was found by dividing the total number by four. Same procedures were carried out for the rats in all groups and average number of pinopods was found. For statistical analysis among the groups, *Independent Samples Test (Mean ± Std) and **Mann Whitney *U* Test (Median (25–75%)) were used.

3. Results

Statistical analyses were summarized in Table 1. Although in previous studies, the count of pinopods, classified according to developmental stages, was evaluated as little if pinopod formation was less than average of 20% in each area, as moderate if it was 20–50% and numerous if it was above 50%,[14,24,28] we herein did not use the percentage method for evaluation as we could count pinopods one by one in each image area.

Pinopod formation and development process were tried to be shown with studies conducted with mice and it was found that formation of fully developed pinopods peaked on Days 4 and 5.[29,30] Although formation of fully developed pinopods is involved in classifications of other studies, here in this study it is not included because of the termination of our experimental model before Day 5. The reason for not waiting until fifth day is that a regression in pinopods might be seen after Day 3 since there is no embryo development in female rats which are copulated with vasecto-

Table 1
Pinopod formation stages according to groups and statistical assessments.

	Group	Mean ± Std	Median (25–75%)	*P*
Day 1 – Developing[a]	1	0,5000 ± 0,204	0,50 (0,25–0,75)	*P* = 0,258
	2	0,3571 ± 0,244	0,25 (0,25–0,50)	
Day 2 – Developing[a]	1	20,61 ± 1,65	20,75 (19,75–22,00)	*P* < 0,001
	2	12,86 ± 1,35	13,00 (11,75–14,25)	
Day 2 – Developed[b]	1	0,39 ± 0,197	0,25 (0,25–0,50)	*P* = 0,902
	2	0,39 ± 0,13	0,50 (0,25–0,50)	
Day 3 – Developing[b]	1	1,21 ± 0,78	1,00 (0,50–2,00)	*P* = 0,011
	2	2,25 ± 0,289	2,25 (2,00–2,50)	
Day 3 – Developed[b]	1	13,79 ± 0,57	13,75 (13,50–13,75)	*P* < 0,001
	2	8,96 ± 1,07	9,75 (7,75–9,75)	
Day 3 – Regressing[b]	1	0 ± 0	0 (0–0)	*P* < 0,001
	2	2,32 ± 0,73	2 (1,75–3,00)	

[a] Independent Samples Test (Mean ± Std).
[b] Mann Whitney *U* Test (Median (25–75%)).

mized male rats, as a result no appropriate comparison could be done with the group that was copulated with non-vasectomized males. Other than this, statistical evaluations were not performed on Day 1 for developed and regressing pinopods and on Day 2 for regressing pinopods since such formations were not observed in any group.

The most important finding in SEM examination of uterus removed from the female rats which were copulated with non-vasectomized males (first group) on postcoital day one is that the heads of sperms in the uterus were buried in endometrial epithelium. Tail parts of the sperms however were observed clearly outside of the endometrial epithelium (Fig. 2A). Small number of pinopods (average 0.5) observed in uterus removed on postcoital first day (Fig. 1A and Table 1) were suitable to be classified as developing pinopods (Fig. 2B and Table 1). However, no developed or regressing pinopods were seen. Therefore, these values are not shown in Table 1. Similarly, in examination of endometrium of female rats that were copulated with vasectomized males (second group) and whose uterus were removed on Day 1 following copulation, moderate number of (average 0.3571) developing pinopods were detected (Fig. 1A and Table 1). There was not any statistically significant differences when these two values were compared ($P = 0.258$).

In examination of endometrium of uterus removed on postcoital second day, small number of developed pinopod (average 0.39) was seen in each group (females copulated with non-vasectomized and vasectomized males) (Fig. 1C and Table 1) and the difference was not significant ($P = 0.902$) when these two groups were compared. In examination based on developing pinopods within the image area, number of developing pinopods in endometrial epithelium of the rats in first group (average 20.61) (Fig. 2C and D) was higher than those in second group (average 12.86) (Fig. 1B and Table 1) with a statistically significant difference ($P < 0.001$). No regressing pinopods were seen in both groups examined on postcoital second day. It was also observed on postcoital second day that sperms buried in the endometrial epithelium on postcoital day one were still seen as their tails outside of the epithelium.

Examination of endometrium of uterus removed on postcoital third day revealed small number of developing pinopods in both groups. Examination of first group revealed an average of

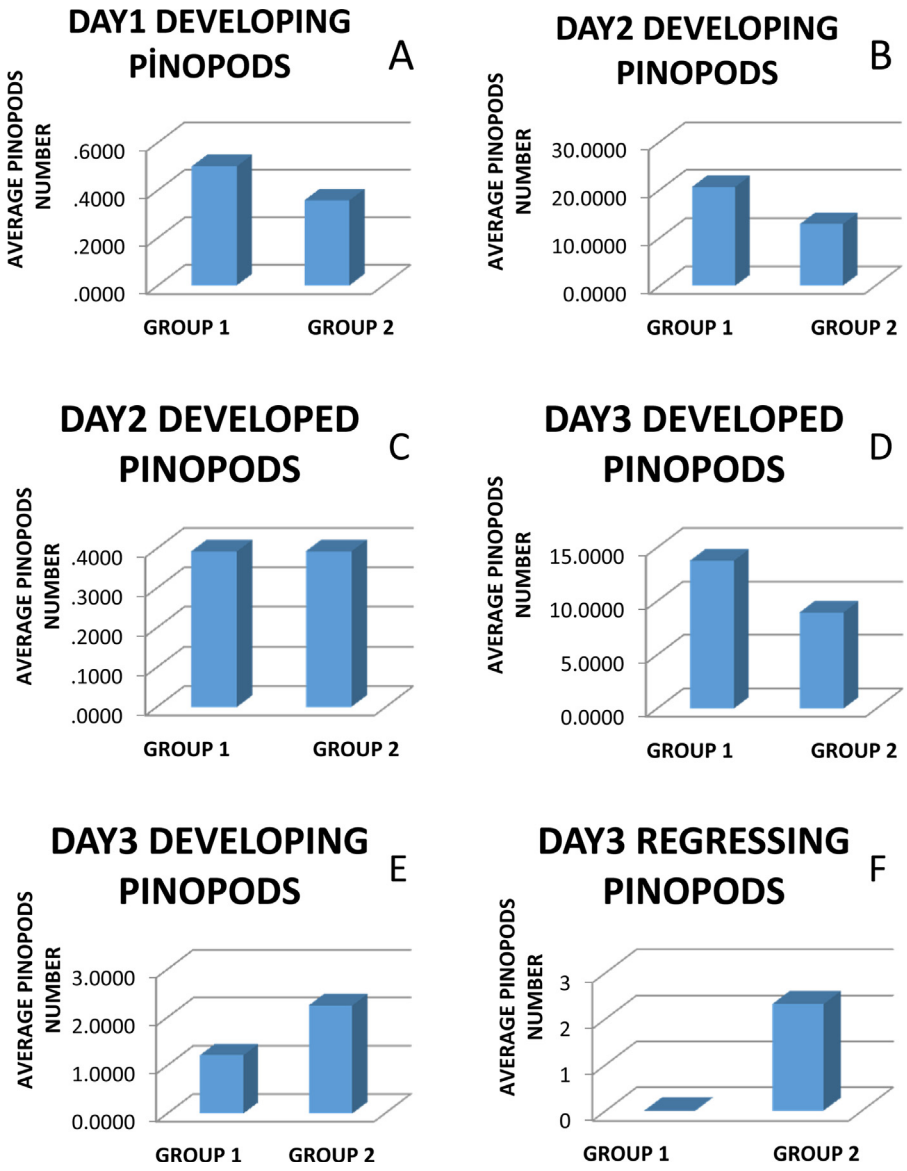

Fig. 1. Graphs showing the average numbers of pinopods of experimental groups. Group 1 indicates females copulated with non-vasectomized male rats while Group 2 indicates same with vasectomized male rats.

1.21 developing pinopod, whereas the average number of developing pinopod in second group was 2.25 (Figs. 1D and 2E and F and Table 1). There was a statistically significant difference ($P = 0.011$) when these two values were compared. Examination based on the count of pinopods revealed that the number of developed pinopods in first group (average 13.79) was significantly higher than second group (average 8.96) ($P < 0.001$) (Fig. 1E and Table 1). Regressing pinopod images were observed only in endometrium taken on postcoital third day in the second group (Fig. 1F and Table 1).

4. Discussion

The first requirement for the occurrence of pregnancy in humans and other mammalians is fertilization of oocyte by sperm, to achieve this, the sperm must complete the capacitation in the female genital tract. While capacitation starts with the interaction of sperm with cervical mucous, it continues with the female-sperm epithelium interaction. Reeve and Ledger established that Arg-Gly-Asp (RGD) series has a role in the interaction of human uterine tubes epithelium with sperm.[31] It is important to show that to accomplish the interaction, RGD sequence acts as a mediator of sperm to identify and bind to integrin receptors. In the same study, it has also been indicated that RGD is not present in ampullary region of the tubes, it is resided in isthmic region which implies that fallopian tubes are not just a simple passage way for sperms, but they also constitute a regulatory environment for sperm functions.[31] Baillie and colleagues have also demonstrated the difference of isthmic region and ampulla part.[32] The interaction between sperm and female genital tract is important for sperm to bind to oolemma since sperm gains capacitation during this interaction and should complete capacitation and acrosomal

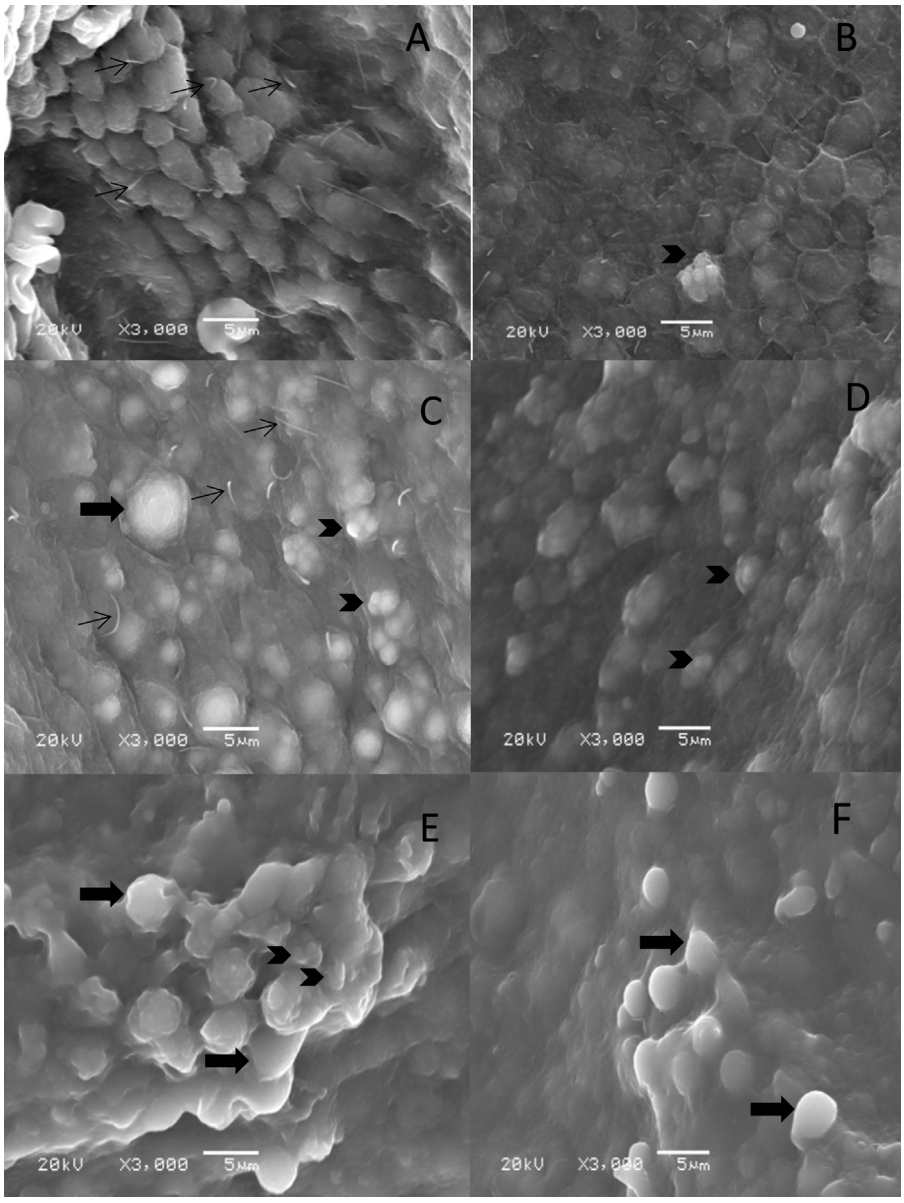

Fig. 2. Sperms (⟶), developing (➤) and developed (➡) pinopod formations are shown in endometriums of uteruses removed on different days by SEM. Sperms and developing pinopods on the surface of endometrium are shown in uterus samples removed postcoital first day from female rats copulated with nonvasectomized male rats in A and B. Sperms, developing and developed pinopods on the surface of endometrium are shown in uterus samples removed postcoital second day from female rats copulated with non-vasectomized male rats in C. Developing pinopods on the surface of endometrium are seen in uterus samples removed postcoital second day from female rats copulated with vasectomized male rats in D. Developed and developing pinopods in endometriums of uteruses removed from female rats copulated with vasectomized male rats on postcoital third day are seen in E. Developed pinopods in endometriums of uteruses removed from female rats copulated with vasectomized male rats on postcoital third day are seen in F.

reaction in order to express integrins such as fibronectin and vitronectin necessary for binding to oolemma.[31] In a study, showing that co-incubation of human oviductal epithelial cells and human sperm induces sperm capacitation, it was found that sperms that have not completed capacitation remained in fallopian tube.[33] Probably, the goal of female epithelium from this interaction is obtaining crucial factors for implantation. However, studies are showing that this interaction between sperm and epithelium takes place with uterus epithelium as well, focusing on the findings suggests that the sperm capacitation is the ultimate goal. When the role of isthmic part of fallopian tube on sperm capacitation is taken into account, it can be suggested that this effect is continued in epithelium of tubal neighboring area because of the importance of uterine epithelium on capacitation.

The second crucial factor for conception is the acceptance of the developing embryo into the endometrium after fertilization in other words endometrial receptivity. Consequently, we herein plan to establish the possible effects of sperm and uterine epithelium interaction on pinopod formation as one important morphologic differentiation necessary for implantation. Endometrial receptivity, that is required for implantation, is a condition in which epithelial morphology differentiates into pinopod. The formation of membrane projections resembling a single flower from the uterinal epithelium by the fusion of ciliary cell microvilli referred as ''pinopod''.[21]

It was interesting to be shown in a comparative study conducted in fertile and menopausal women that cells with cilia and microvilli were shown in only fertile women.[34] It is also interesting to note that pinopod formation displays individual diversity. In addition to this, although pinopods are present in normal fertile women on 6–8 days post-ovulation, they are seen 1–2 days earlier in patients who receive controlled ovarian hyperstimulation.[35]

In a study established using SEM, it was found that sperm heads look like brushes which are opposite to grass look ciliary epithelium. Small extensions or microvilli were covered with secretions of secretory cells that provide a moistened and nutritious environment for sperm and embryo.[36] One of the important factors in endometrial receptivity is the expression of e-cadherin. The expression of e-cadherin is calcium (Ca) dependent. Alteration in intercellular Ca concentration triggers the reformation of cell adhesion molecules which in turn affects the cohesion and polarization of epithelial cells.[37] On the other hand, ATP secreted from endometrium epithelium plays an important role on the regulation of endometrial functions, coordination of sperm migration and sperm capacitation. Another function of ATP is steroid dependent activation of estrodiol receptors.[38] First step to start sperm capacitation is the exit of cholesterol out of the cell membrane and subsequent entry of Ca and bi-carbonite through sperm membrane.[39] It might be possible that exiting cholesterol out of the cell has a role on cell organization in endometrial epithelium. For this reason, as a result of sperm and endometrium epithelium reaction, Ca secretion can occur and be inducted through some signals. However, further studies should be established to verify our hypothesis. Researchers believed that their SEM findings, suggesting ciliary epithelial cells are in close relation with sperm, and are related to the sperm capacitation process. However, the nonexistence of studies on possible gains of epithelium from the interaction of sperm and endometrial microvilli epithelium, necessitates examination of this issue in regard to this aspect.

It has been controversial for a long time whether there is a relation between pinopods localized on endometrial epithelial surface and endometrial receptivity. To address this, several investigators continued on Nilsson's electron microscopy studies conducted in 1958.[40] Nikas and Makrigiannakis evaluated pinopods as a marker of endometrial receptivity.[12] The period when pinopods reside in endometrial epithelium is within a time called implantation window. Studies conducted with rats showed that fully developed pinopod structure formed between 4th–5th days of conception.[29,30] It has been suggested by several studies conducted so far, that many hormones, cytokines etc. are important mediators of pinopod formation.[14,15,39] However, some researchers indicated that there is no correlation between LIF and MMP2 expression and pinopod formation.[13] In our previous study published in 2009, we suggested that pinopod formation occurs through the autocrine paracrine interactions between fallopian tube epithelium and oocyte and embryo.[4] Of course as these studies point out, there are many factors facilitating the formation of pinopod and hence endometrial receptivity.

In this study, all stages of pinopod formation were monitored from the first day through the third day. Findings related to pregnant rats that were copulated with non-vasectomized male rats – constituting the first group of our study – were consistent with the results of previous studies.[10,12,14] However, the results of a second group in which we experimented on female rats copulated with vasectomized male rats differ from the first group in that the number of formed pinopods were less. Even though it has been established as a fact that there are numerous factors triggering pinopod formation, no studies has been published about sperms being a factor involved in pinopod formation. Emera et al. have suggested several theories on the occurrence of menstruation.[16] One of these theories suggests that endometrium reacts in order to protect itself from pathogens carried by sperms and menstruation ensues. However, we previously suggested that presence of oocyte and embryo in fallopian tube acts on pinopod formation.[4] In this study, no embryo formed in the second group in which vasectomized rats were studied, therefore this could be a reason for the decrease in the number of pinopods. If we re-evaluate the results of our previous study where we performed tubal ligation on a group of female rats, in the light of present study, pinopod formation rate of 25% achieved by copulating the tubal ligated rat-group might be the consequence of invasion of sperms into endometrial epithelium.

In our third day assessment, we observed regressing pinopod formation in the second group whereas we did not see any regressing pinopod in the first group. We interpreted this finding as an indicator for initiation of regression of pinopods beginning from the third day in case no conception occurs.

In this study it was clearly shown that sperms were entered into endometrial epithelium with their heads. It can be suggested that they might have a facilitating effect for pinopod formation by reacting with endometrial epithelium as a result of this invasion. It would be beneficial to demolish the other factors triggering pinopod formation to investigate whether only the presence of sperm in the uterus has an effect on pinopod formation. The improvements made in cryopreservation techniques have led few or no detrimental effects to the embryo and have not provided any advantages to the offspring when compared to fresh embryos; this has allowed reproductive practitioners to create the freeze-allpolicy. On the other hand, there are increasing concerns about the adverse effects associated with COS (controlled ovarian stimulation) over the endometrial and uterine environments. It has been suggested that obstetric and perinatal outcomes in pregnancies resulting from fresh ET (essential thrombocythemia) are poorer when compared with those that occur after FET (frozen embryo transfer). There is a growing evidence in the literature suggesting better IVF outcomes when adopting the freeze-all policy instead of fresh ET.[40] There have been reports of greater implantation and pregnancy rates with FET than with fresh autologous embryo transfer, suggesting superior endometrial receptivity in the absence of ovarian stimulation.

Particularly for the elimination of undesired situations due to induction – such as hyperstimulation or changes in steroid levels, frozen cycle applications may be predicted to be more preferable applications. In such cycles, applications increasing the pinopod formation may have the potential of greater success rate through increasing the endometrial receptivity, resulting in increased rate of endometrial implantation. In these cases, it is thought that pregnancy rates may be increased through sperm-endometrial stimulation. Particularly for some infertile cases with tubal factor and for selected indications, receptivity-pinopod increase can be ensured by endometrial sperm application. However, future supportive studies are needed on this issue.[41]

Author's roles

Study design: Orhan Özatik, Tamer Mungan.

Experiments carried out by Orhan Özatik.

Tissue preparation for electron microscope and analysis: Orhan Özatik, İlknur Dağ.

Article, discussion script and analysis: Orhan Özatik, Tamer Mungan.

Statistical analysis: Ahmet Musmul.

Conflicts of interest

The authors have none to declare.

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