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Original Article / Özgün Araştırma

Comparison of Exosome Presence and Morphologic Changes Between Implantation and Inter-Implantation Areas in Rat's Utero by Confocal Microscope and SEM

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Abstract

Aim: In this study, it was aimed to compare the differences between the implantation and interimplantation sites in terms of the presence and release density of exosomes.

Method: Wistar albino strains were used in this study. The rats were considered pregnant with the presence of vaginal plugs after mating. Then the rats were sacrificed on the 6th day when the embryos first attach to the uterus and implantation started. Implantation and inter-implantation sites were easily identified by staining the implantation sites with intravenous Chicago Blue dye given just before sacrification. After tissue preparation, sections were placed on slides. Exosomes detected with anti-CD63 fluoresence staining and imaged by confocal microscope. Further these sites were evaluated by Scaning electron microscopy (SEM).

Result: When implantation and inter implantation sites compared, it was observed that amont of exosomes was higher than inter-implantation sites in confocal images. Additionally, SEM images confirmed the confocal results of these sites.

Conclusion: Our study is the first in the literature to compare implantation and inter-implantation areas in terms of the presence of exosomes. These results probably suggested that the exosome plays an important role in implantation for the embryo to find the correct implantation site. Probably these exosomes must carry the necessary signals to find the right implantation site. However, further studies are needed to reveal the function of exosomes in implantation sites.

Keywords: Anti-CD63; Embryo implantation;Exosome;Rat; Uterus.

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Sıçanlarda İmplantasyon ve İnter-İmplantasyon Alanlarının Morfolojik Değişiklikleri ve Eksozom Varlığı Açısından Konfokal ve Elektron Mikroskobu ile Değerlendirilmesi

Öz

Amaç: Bu çalışmada sıçanlarda implantasyon ve inter-implantasyon alanlarının eksosom varlığı, yoğunluğu ve bu alanların morfolojisi bakımından karşılaştırmayı amaçladık.

Yöntemler: Çalışmada wistar albino susu sıçanlar kullanıldı. Katımın ertesi günü vajinal plak izlenen sıçanlar gebe kabul edildi.Embriyonun uterusa ulaştığı ve implantasyonun başladığı zamana karşılık gelen gebeliğin 6.günü sakrifikasyon planlandı. Sakrifikasyondan hemen önce intravenöz uygulanan Chicago Blue boya sayesinde implantasyon ve inter-implantasyon alanları kolaylıkla ayırt edildi. Doku takibi sonrasında lizinli lamlara kesit alındı. Eksozomlar, anti-CD63 işaretlemesi ile Konfokal mikroskopta görüntülenerek bu alanlar eksosom varlığı açısından görüntülendikten sonra aynı alanlar SEM ile daha yüksek çözünürlükte görüntülendi.

Bulgular: İmplantasyon ve inter-implantasyon alanları arasındaki farklılıklar, eksozom varlığı ve morfoloji açısından karşılaştırıldı. İmplantasyon alanlarındaki eksozom sayısı, inter-implantasyon alanlarındakinden daha fazla olduğu görüldü. Konfokal ve SEM görüntülemeleri birbirini doğruladı.

Sonuç: Çalışmamız eksozom varlığı açısından implantasyon ve inter-implantasyon alanları karşılaştıran ilk çalışmadır. Implantasyon alanlarında eksosom miktarının daha fazla görülmesinin nedeni muhtelemen embriyonun uterusta doğru implastayon alanını bulmasıyla ilgili olmalıdır. Bunu da eksosomlar taşığı sinyal molekülleri ile yönlendiriyor olmalılar. Fakat bu soruların cevaplarını aydınlatmak için daha fazla çalışmaya ihtiyaç vardır. Bizim çalışmamız, eksosom varlığının implantasyon ve interimplantasyon alanları arasındaki farkını ortaya koyarak bundan sonraki çalışmalara ışık tutmaktadır.

Anahtar Kelimeler: Anti-CD63; Embriyo implantasyonu;Eksozom; Rat; Uterus.

INTRODUCTION

Extracellular vesicles (EVs) are formed directly or indirectly through the plasma membrane. Exosomes make up the smallest group of extracellular vesicles known to be secreted by cells. Vesicles are 40-100 nm in diameter and surrounded by a double phospholipid layer. Surface markers are used to distinguish exosomes from other vesicles. The best known surface marker of the exosome is considered CD63. Exosomes are released by almost all cells in the body. They are found in all body fluids. İnitial researches suggested that exosomes only play a role in removing waste from the cells. However, in recent studies, it has been revealed that the cargo contents of exosomes consist not only of waste molecules but rather of nucleic acids, proteins, miRNA, mRNA, nucleoproteins, and various enzymes used in essential roles like intercellular communication, immune regulation, cell differentiation, cell migration, and signal transduction[1]. They are released by many cells, such as placental and endometrium cells. Although exosomes are thought to play a role in ovarian follicular biology and implantation, their role has not been fully elucidated. Recent studies have shown that the exosomes secreted by blastocysts stimulate the development of endometrial receptivity[2].

Implantation is a dynamic process in which the embryo and endometrium are actively involved. This process is only possible in a short period called the implantation window. The implantation window was defined as between the 20th and 24th days of the 28-day menstrual cycle or 6-10 days after ovulation in humans[3].Morphological and functional characteristics of both the embryo and endometrium changed due to signal transduction between the embryo and endometrium[4]. The implantation window in rats is between the 5-6th day of gestation and

the implantation and inter-implantation sites are separated. Embryo implantation in rodents occurred within a special crypt (implantation site) formed by the progression of the endometrium luminal epithelium towards the anti-mesometrial (AM) pole[5]. The mechanism of formation of this event is not fully known. Pinopods are apical membranes of the epithelial cells, lining the uterine space during implantation, losing their microvilli, and developing membrane extensions[6]. They are markers of embryo implantation. Wnt5a- ROR signaling has been shown to play a role in epithelial differentiation, crypt formation, and regulation of embryo insertion space at implantation[7]. Inter-implantation areas separated implantation areas and these two regions were different in the molecular structure[8].

Successful implantation depends on the information exchange between the embryo and the endometrium, a phenomenon called cross-talk. Exosomes were thought to take part in this process[9].Extracellular vesicles positive for exosome, CD63 and HSP70 were isolated in uterine lumen fluid of cyclic and pregnant sheep[10]. In human studies, extracellular vesicles were isolated from the uterine fluid of women at different stages of the menstrual cycle. In the endometrium, the apical surfaces of both luminal and glandular epithelial cells have been shown to express exosomal markers CD9 and CD63. It has been established that exosomes transmit information directly between the endometrium and the blastocyst and thus participate in implantation[11].

The embryo implantation site is ready and evident even before its uterine adhesion. Implantation and inter-implantation areas can be easily distinguished from each other macroscopically in rats. So, what differences between these two areas cause the embryo to go and adhere to that area? In our study, we hypothesized that exosomes play a role in determining the implantation site. We aimed to compare exosome presence and morphological changes between embryo implantation and inter-implantation sites in rats.In the literature, exosomes have been shown at the implantation sites, but the implantation and inter-implantation sites have not been previously compared for the exosome presence. Endometrial luminal epithelial surface features, pinopod structures, and exosomes were visualized morphologically by scanning electron microscope and confocal microscope. In a confocal microscope, the CD63 exosome marker was immuno-labeled to detect exosomes.

METHODS

Animals

Experimental procedures were approved by the Dokuz Eylul University Local Ethical Committee of Animal Experiments (Protocol no. 10/2019). This study was done in March 2019 at xxxxxxx Animal Laboratory.

In this study, 7 Wistar albino female rats obtained from Dokuz Eylul University Medical Faculty Research Unit were used. Wistar albino was chosen due to its availability in Dokuz Eylul University Faculty of Medicine Experimental Animal Laboratory and suitability with the literature. All subjects were housed in a standard animal room and cages at 20–22 0C room temperature with 50-60% relative humidity in 12/12 hours of dark/bright periods in theexperimental animal laboratories and fed with rested tap water and standard pellet feed (ad libitum).

Experimental Design

Our study was a prospective experimental study. Virgin females were mated with males overnight, and vaginal plug formation in the morning (08:00) was determined as the first day of gestation. Seven pregnant rats were fallowed for six days after vaginal plug

determination. The rats were sacrificed on the 6th day of gestation (09:00 AM). Rats were euthanized 10 minutes after tail-vein injection of 0.1 ml of 1% Chicago blue dye (Sigma-Aldrich, USA) in saline to detect implantation sites. Each animal's uterus was flushed with sterile saline individually[12,13].

On the 6th day of pregnancy, the implantation sites (IS) and inter-implantation sites (IIS) of the uterus were collected for immunofluorescence staining and electron microscopy. IS was separated from IIS and fixed with paraformaldehyde (Sigma, 158127). While these areas were directly scanning under scanning electron microscope (SEM), 0.5 µm sections fixed and embedded in paraffin were obtained for the confocal microscope.

Immunofluorescence (IF) Staining

Paraffinized tissues were deparaffinized with ethanol and xylene. The tissues were then boiled with citrate buffer (pH 6.0) (Cat No. AP-9003-125 Labvision). After cooling, they were blocked with 1% bovine serum albumin (BSA; BioVision) and incubated for 1 hour with the anti-CD63 antibody (ab59479, abcam) at room temperature. They were then washed with phosphate-buffered saline (PBS; Sigma aldrich) with three timesand incubated with a secondary antibody (Alexa fluor 488, ab201540) for 1 hour in the dark and washed with 3 times. After washing, the samples were covered with a mounting medium and imaged with aconfocal microscope (Zeiss LSM 880 confocal microscope-Germany)[14].

Scanning Electron Microscope (SEM)

The uterus fixed with 4% PFA was washed with PBS and dehydrated in graded ethanol series, then samples were dried and covered with goldparticles by Quorum Q1150R coater. Samples were imaged with the Zeiss Sigma 500 Electron Microscope (Germany)[15,16].

Comparison of Implantation and Inter-implantation Areas

Implantation and inter-implantation areas were compared morphologically for epithelial features and the presence and intensity of exosomes from Confocal and SEM images.

RESULTS

The presence of exosomes in the implantation and interimplantation sites was compared in rat uterus on the 6th day of gestation, when embryos first started implantation by adhering to the uterus. The average number of implantation sites in rats on day 6th was 12 (Figure 1A).

The lumen of the inter-implantation site was wide lumen, and the luminal epithelium had low prismatic cells as compared with epithelium of implantation site (Figure-1B). It was observed that the amount of exosomes especially in the luminal region was lower than in the implantation region. (Figure-1B), SEM images also showed that the amount of exosome was very low in the luminal epithelium in inter-implantation sites. SEM images were consistent with confocal images.(Figure-1C).

The implantation site's lumen was narrow, and the luminal epithelium had high prismatic cells (Figure-1D). In confocal images, it was observed that the amount of exosomes in the implantation areas was considerably higher than in the interimplantation areas, and these exosomes were especially concentrated in the luminal area (Figure-1D). Likewise, in SEM images, it was observed that exosomes are concentrated in the luminal area, which is a proof of exosome secretion from the endometrium of implantation site. In addition, the SEM results confirm the confocal microscopy results (Figure-1E).

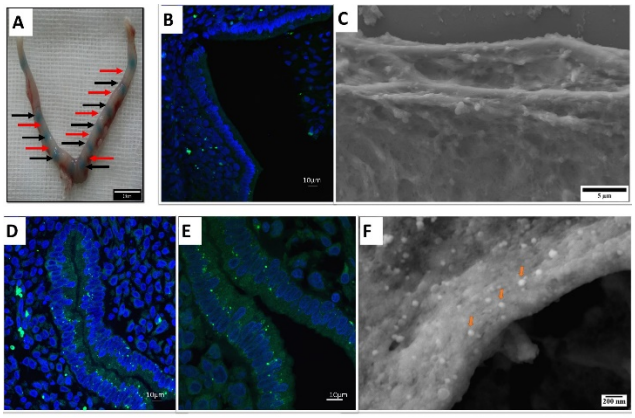

Figure 1.A: Morphology of implantation and inter-implantation site on the 6th day (D6) of gestation in the rat uteri. Black arrows show implantation site and red arrows show inter-implantation site (scale bar:0,5cm).

Figure 1.B-C: The inter-implantation site on the 6th day (D6) of gestation in the rat uteri. B. It was immunostained using anti- CD63 antibody, and fluorescence intensity was determined using confocal microscopy (25X, scale bar:10µm), C. Scanning electron microscopy showed exosomes at inter-implantation site on D6 (scale bar:1µm). **Figure 1.D-F:** The implantation site on the 6th day (D6) of gestation in the rat uteri. D. It was immunostained using anti- CD63 antibody, and fluorescence intensity was determined using confocal microscopy, Confocal microscopy (40X, scale bar:10µm) E. Confocal microscopy (63X, scale bar:10µm) Fluorescence intensity in perinuclear region was compared between groups F. Scanning electron microscopy showed exosomes (yellow arrows) at implantation site on D6 (scale bar:200nm)

As a result, a significantly higher amount of exosomes was observed at the implantation site compared to the interimplantation sites.

DISCUSSION

Implantation is the interaction of an active embryo with a receptive endometrium.In this process, mutually released signal molecules regulate the embryo-maternal communication called 'cross-talk'. In this way, embryo development and endometrial functions are regulated. Exosomes are involved in cell-cell interaction.If we can understand the function of exosomes in implantation, they can be used as biomarkers in IVF-ET for the detection and prognosis of embryo quality and endometrial receptivity.

In our study, we hypothesized that exosomes play an important role in determining the implantation site.

Therefore, we compared implantation and interimplantation sites for the presence of exosomes.There were few studies investigating the correlation between exosome and embryo implantation. Machtinger et al. searched three different databases on the roles of extracellular vesicles in gamete maturation, fertilization, and embryo implantation.They concluded that functions of EVs in reproduction were discussed in 2016 but not yet confirmed by specific empirical evidence[9]. We focused only on the implantation window period in our study. We evaluated the presence and density of exosomes by preparing micrometer sections from the implantation sites that we observed macroscopically. After fluorescence imaging, it was observed that the presence of exosomes in the implantation sites was more intense than in the interimplantation sites.

There must be a harmony between the embryo and the endometrium for successful implantation. EVs can contribute to this communication process called cross-talk. EVs are released from the endometrial epithelium into the uterine cavity may affect the blastocyst or the adjacent endometrium. It can stimulate endometrial receptivity and ensure successful implantation. Another study reported that exosome markers CD63 and HSP70 was positive in the uterine fluid of pregnant sheeps[10].When these vesicles were examined, miRNAs and proteins found in the trophectoderm and endometrial epithelium were observed. Another gruop revealed that exosomal markers such as CD9 and CD63 were detected in endometrial epithelial cells, and it was argued that endometrial epithelium was a possible source of exosome[11]. Presence of EVs in endometrial fluid suggested that EVs play a

crucial role in embryo-endometrial communication in the endometrium. In our project we evaluated the uterus on the 6th day of pregnancy in rats with CD63 immunofluorescence staining for the presence of exosomes. The high exosome density observed during early pregnancy, as a result of confocal imaging, supported studies highlighting the role of exosomes in the implantation process.

Desrochers et al. demonstrated that exosome-treated blastocysts developed faster and had higher implantation success. They proved the role of the exosome in embryo-endometrium communication[17]. Burns et al. reported that ex vivo studied extracellular vesicles in sheep isolating them from uterine fluid and labeled them with PKH67 immunofluorescent staining. They then introduced these exosomes to pregnant or non-pregnant sheep and observed where they would go[18]. Our study compared the presence of exosomes at implantation and interimplantation sites from the rat uterus without interfering with in vivo conditions, and observed significant differences between the two sites.

Su et al. examined that the expression of autophagy markers ATG5 and LC3 on days 4, 5, and 6 of pregnancy (D4, D5, and D6) to determine the role of autophagy in the uterus of pregnant mice during the preimplantation and implantation stage. Down-regulation of autophagy-dependent markers were observed at D5 and D6 compared to preimplantation day D4[19]. In our study, we recorded higher exosome density at day 6th. This result indicated that the exosomes can play specific role in implantation process.

Our study focused on the 6th day of pregnancy. The implantation day and post-implantation days were not examined. We aimed to compare the 5th, 6th and 7th days of pregnancy in terms of the presence and amount of exosomes.

Previous studies on implantation and exosome have not evaluated implantation and interimplantation sites for exosomes. In conclusion, our study is the first to compare the implantation and inter-implantation sites for the presence of exosomes.

Our findings showed that during the implantation the release of exosomes increases in implantation sites. It may be related to the determination of the implantation site by the embryo. Further studies are needed to understand the role of exosomes in the implantation site.

Ethics Committee Approval: Experimental procedures were approved by the Dokuz Eylul University Local Ethical Committee of Animal Experiments (Protocol no. 10/2019). This study was done in March 2019 at Dokuz Eylul University Animal Laboratory.

Conflict of Interest: The authors declared noconflicts of interest.

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