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# Investigation of the Presence of Stem Cells in Rat Uterus Tissue in Postnatal Development Periods*

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**Abstract:** Uterine tissue is an organ with a high proliferation capacity where regeneration, differentiation and shedding are seen. Although this tissue is regenerated every month, the source of it hasn't been determined yet. Studies are carried out on stem cells being a source that can provide regeneration of the endometrium and the markers expressed by these cells. The purpose of this study was to examine the immunreactivity of CD9 and CD13 expressed by essential population of uterus and CD34, marker of hematopoietic stem cells, at various stages of the postnatal developing process. In this study, was used 42 female Wistar-albino rats split into six groups; Group I; newborn (2-days), Group II; pubertal (38-days), and Group III: fertile group (12 weeks), by identifying the stages of the estrous cycle in rats a) proestrus, b) estrus, c) metestrus, d) diestrus. The expression of CD9, CD13, and CD34 in uterine tissues excised from rats was investigated. In the groups of 38-days and 12-weeks, CD34-expressing cells were present in the stroma of the endometrium next to the myometrium, but such cells were absent in the 2-days group. All groups' uterine epithelium displayed CD9 expression, except for group 2-days. Endometrial stromal cells that expressed CD13 showed only little immureactivity in groups 2 and 38-days, while CD13 expression is noticeable in group 12-weeks. It was thought that the uterine endometrium could be regenerated in puberty and adulthood with the contribution of bone marrow-derived stem cells and uterine-derived epithelial and stromal cells.
**Keywords:** Endometrium, immunohistochemistry, smear

**Doğum Sonrası Gelişim Dönemlerindeki Sıçan Uterus Dokusunda Kök Hücre Varlığının Araştırılması**

**Öz:** Uterus dokusu yenilenme, farklılaşma ve dökülmenin görüldüğü yüksek proliferasyon kapasitesine sahip olan bir organdır. Bu dokuda yenilenme her ay olmasına rağmen, yenilenmenin kaynağı halen belirlenememiştir. Kök hücrelerin endometriyumun rejenerasyonunu sağlayabilen bir kaynak olduğu ve bu hücrelerin eksprese ettiği belirteçler ile ilgili çalışmalar yapılmaktadır. Bu çalışmanın amacı, uterusun temel popülasyonu tarafından eksprese edilen CD9 ve CD13'ün ve hematopoetik kök hücrelerin belirteci olan CD34'ün doğum sonrası gelişim sürecinin çeşitli aşamalarında immünreaktivitesini incelemekti. Bu çalışmada 42 adet dişi Wistar albino rat altı gruba ayrıldı; Grup I yenidoğan (2 günlük), Grup II pubertal (38 günlük) ve geri kalan gruplar, Grup III: fertil grup (12 hafta), sıçanlarda östrus döngüsünün evrelerini belirleyerek a) proöstrus, b) östrus, c) metestrus, d) diestrus. Deney sonunda, sıçanlardan alınan uterus dokularında CD9, CD13 ve CD34 ekspresyonları araştırıldı. 38 gün ve 12 haftalık postnatal gruplarda, miyometriyumun yanındaki endometrium stromasında CD34 eksprese eden hücreler mevcutken, postnatal 2 günlük grupta böyle hücreler bulunmamaktaydı. CD9 ekspresyonun da uterus epitelinde 2 günlük grup dışındaki tüm gruplarda gözlendi. Endometriyum stromasında eksprese edilen CD13 ise 2 günlük ve 38 günlük sıçanlarda zayıf olmasına rağmen, 12 haftalık sıçanlarda belirgin CD13 ekspresyonu olduğu belirlendi. Uterus endometriyumunun puberte ve erişkin dönemlerde, kemik iliği kaynaklı kök hücrelerin ve uterus kaynaklı epitelyal ve stromal hücrelerin katkısı ile yenilenebileceği düşünülmüştür.
**Anahtar kelimeler:** Endometriyum, immünohistokimya, smear

## Introduction

Throughout a woman's reproductive cycle, the human endometrium experiences over 400 times of regeneration, differentiation, and shedding (Jabbour et al., 2006). This tissue is composed of two sublayers, the pars functionalis and the pars basalis. The endometrium's damaged layer is known as the pars functional. The general opinion related to the regeneration of the pars functionalis is that a particular cell group in the basal region of the endometrium grows and expands rapidly to reconstitutethe endometrium (Padykula, 1991). The ability of the endometrium to

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regenerate itself is thought to be a result of stem cells, according to a widely held belief today. Research on endometrial stem cells has benefited from the most recent technical advancements employed to identify adult stem cells in many tissues (Kyo et al., 2011). Endometrial stem cells, which are the cells that make up the uterus in each menstrual/estrous phase, are thought to originate from the endometrial glands and stromal cells. In addition, there are new views on the existence of an extrauterine source and the fact that these cells are responsible for the repair of the uterus following menstruation and delivery. It has been suggested that one of these cell sources may be bone marrow-derived mesenchymal stem cells (Bruscia et al., 2006). Hematopoietic stem cells (HSC) are found in hematopoietic tissues such as bone marrow, cord blood, and mobilized peripheral blood. CD34, which is expressed by 0.5–5% of human bone marrow cells, is one of the most significant markers of HSCs. Early progenitor cells contain CD34, while more mature cells do not express it (Civin et al., 1990). CD9 is a 24-27 kD glycoprotein that the glandular epithelium's cell surface actively expresses during the menstrual phase (Park et al., 2000). CD13, known as N aminopeptidase, is expressed in the endometrial stroma during the menstrual phase, and its expression is more pronounced in the secretion phase compared to the proliferative phase (Seli et al., 2001). These findings demonstrate the use of CD9 and CD13 as endometrial glandular and stromal cell surface markers (Kato et al., 2007).

The endometrium is a tissue that can reproduce physiologically, shed, and then renew itself. Studies show that stem cells may be involved in this regeneration. It is also believed that endometrial stem cells play a role not only in regeneration, but also in the development of gynecological diseases such as endometriosis and endometrial cancers (Kato, 2012). The regeneration of the endometrium depends on cell proliferation in the proliferative phase, and with this regeneration, the endometrium reaches the required thickness. An endometrium that is not fully developed as a result of insufficient proliferation and that is thinner than it can be considered as one of the causes of infertility. Bone marrow stem cells' role in the proliferative stage is poorly understood. Although CD9/CD13 has been used as differentiation markers in the uterus in studies, there has not been enough study on the expression of these proteins in the uterus. This study used immunohistochemistry to assess how CD9/CD13 and CD34 immunoreactivities affected uterine alterations at various postnatal developmental stages.

**Materials and Methods**

The Ethics Council of Experimental Animals at Erciyes University in Turkey granted ethical approval for the use of Wistar albino rats in this work. We utilized 42 female Wistar albino rats. The Experimental and Clinical Research Center at Erciyes University supplied the rats. According to the guidelines for animal experimentation, all procedures for the experiment were administered at Erciyes University, Faculty of Medicine (Kayseri, Türkiye) (date: November 14, 2012, decision no:11/116). Throughout the experiment, rats were housed in plastic cages with standard conditions of 60% humidity, temperature (22.5°C) and appropriate illumination (light cycle:12h/dark cycle:12h). The study consisted of a total of 42 rats, with six groups and seven rats in each group. 1- newborn group (two days), 2- pubertal group (38 days), 3 - Group III: fertile group (12 weeks), by identifying the stages of the estrous cycle in a) proestrus, b- estrus, c- metestrus, d- diestrus. The phases of the estrous cycle were determined by taking vaginal smears from 12-weeks rats. For the smear, the rat vagina was washed with a plastic pipette with 10µl saline (NaCl 0.9%) drawn, and the vaginal secretion was collected and the vaginal fluid was spread on the slide. A different slide was used for each rat. After the smears were stained with hematoxylin-eosin (H-E), the stages of the estrous cycle were determined by examining them under the microscope.

Uterine tissue samples were taken from rats belonging to all experimental groups using ketamine-xylasine (50 mg/kg-15 mg/kg) under general anesthesia. Excised tissues were fixed with 10% formaldehyde solution for histological examinations and were blocked by embedding in paraffin after routine tissue follow-up. 5-6 µm sections from paraffin blocks were taken to polylysine-coated slides. Using standard histological techniques, prepared slides were deparaffinized with xylene and diluted by passing through a graded alcohol series. The sections were H-E stained, and the Olympus BX51 microscope was utilized to look at them in order to view the general histological structure (Baran et al., 2022).

Avidin-biotin-peroxidase method and immunohistochemical techniques were applied to determine CD34, CD9 and CD13 molecules in the uterine tissues of the rats in the experiment. In order to rehydrate sections that had been held at 60°C over night, they were first put through a succession of xylene and then graded alcohol. Following a thorough cleaning with distilled water, for 5 minutes, the sections were heated in 10% citrate buffer at 600 W in a microwave for antigen recovery, and then allowed to cool for 10 minutes at room temperature in the same buffer solution. After sections were cleaned with phosphate buffer (PBS), endogenous peroxidase activity was inhibited for 12 minutes using 3% hydrogen peroxide ($H_2O_2$). The following stages involved using a staining kit for the Large Volume Detection System (Thermo Scientific, TP-125-HL). To guarantee that the regions outside the antigenic zones were covered, Ultra V block was added to the sections

rinsed with PBS. Immediately afterwards, CD34 (Abnova: PAB18289), CD9 (NBP1-40752) and CD13 (NBP1-19793) antibodies were applied to the sections and incubated for 30 minutes at room temperature after 1 night at +4°C. Following washing, the sections were incubated for 10 minutes with a biotin-secondary antibody before going through the washing procedure once more. Then, the sections were exposed to streptavidin peroxidase for 10 minutes. To make the immunoreactivities visible, they were treated for 1 to 5 minutes with the kit's peroxidase substrate that had diaminobenzidine (DAB) characteristics. Distilled water was used to rinse the sections for five minutes. Sections that had been Gill hematoxylin counterstained were rinsed with distilled water. After eliminating the water from the sections with an increasing alcohol series and passing them through xylene, they were sealed with closure solution (Entellan®, Merck) and inspected under an Olympus BX51 microscope (Baran et al., 2022).

Immunohistochemical results were evaluated by counting the cells expressing CD34 in the part of the endometrium adjacent to the myometrium, and the immunoreactivity intensities of CD9 expressed in the glandular structures of the endometrium and CD13 expressed in the endometrial stroma using the Image J program. Ten different areas from random sections taken from tissues were included in the measurement. The mean immunoreactivity intensity was determined for all groups (Onder et al., 2021).

### *Statistical analyses*

All statistical analyses were performed using the SPSS 20.0 program. The Shapiro-Wilk test was used to verify whether the data conformed to the normal distribution, and the Levene homogeneity test was used to determine variance homogeneity. The Mann Whitney U test was used to conduct and assess the difference tests in cases of difference, and the Kruskal-Wallis Analysis was used to compare groups in variables that did not exhibit a normal distribution. Statistics were regarded as significant at $P<0.05$.

## Results

When the H&E-stained smear samples belonging to different periods of the estrous cycle were examined in light microscopy, nucleated epithelial cells as well as non-nucleated epithelial cells were observed in the proestrus phase. The estrus is the phase of aucleated cornified cells with acidophilic staining. In the metestrus phase, which shows basophilic staining, leukocytes were observed among the cornified cells. In the diestrus phase, there were many leukocytes and small nucleated epithelial cells (Figure 1).

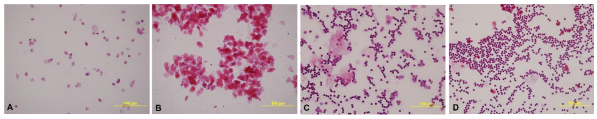

**Figure 1.** Cytological evaluation of hematoxylin-eosin (H&E) stained vaginal smears X40. (A) Proestrus (B) Estrus (C) Metestrus (D) Diestrus.

In the uterus of a newborn (2-days) rat, the epithelium located on a prominent basement membrane was observed to be a single layered columnar epithelium. Diffuse blood vessels and fibroblast-like cells were observed in the lamina propria layer located under the epithelial layer. It has been determined that there are several rows of muscle layers in the myometrium (Figure 2. A1, A2, A3). In the pubertal (38-days) rat uterus, as in the 2-days rat, the uterine lumen was surrounded by a single-layered columnar epithelium, and the nuclei of the cells forming the epithelium were observed to be oval shaped and located basally. It was observed that the lamina propria, which is loose connective tissue, is rich in blood vessels. It was also noted that the endometrial glands in the lamina propria were less in number than those in the 12-weeks adult group. The circular and longitudinal muscle bundles in the myometrium layer were regularly located (Figure 2. B1, B2, B3).

In the sections of the uterine tissues belonging to the proestrus stage in adult rats, it was observed that the single-layered columnar epithelial cells surrounding the lumen had a centrally located oval nucleus and prominent nucleoli. At this stage, a small amount of leukocyte infiltration was observed both in the epithelium surrounding the endometrial lumen and in the epithelium of the endometrial gland. The histological structures of the circular and longitudinal muscle bundles in the myometrium layer were found to be normal (Figure 2. C1, C2, C3). During the oestrus stage, the lumen was surrounded by a tall columnar epithelium in the adult rat uterus. Leukocyte infiltration was observed both in the endometrial gland epithelium and among the epithelial cells lining the degenerating uterine lumen (Figure 2. D1, D2, D3). In the metestrus stage, the endometrial lumen epithelium in the uterus of adult rats was shown to have shorter epithelial lengths than those in the proestrus and estrus groups. Leukocyte infiltration of the endometrial epithelium as well as degradation were both seen. The stromal cells next to the myometrium were less numerous, whereas the cells next to the basement membrane of the lamina propria layer were more abundant. The myometrium layer, which makes up a significant portion of the uterine wall, was found to have circular and longitudinal muscle bundles that were regularly positioned (Figure 2. E1, E2, E3). In diestrus adult rats, the endometrial epithelium was seen as low prismatic or cuboidal. However, it was noteworthy that the nuclei of the epithelial cells had

also taken a round shape in accordance with the shape of these cells. The height of the uterine glands epithelium with a prominent lumen in the lamina propria of the uterus was higher than the epithelium surrounding the uterine lumen. In addition, the cells in the stroma were distinguished by volume. The myometrium layer of 12-week-old adult rats had normal histological structure (Figure 2. F1, F2, F3).

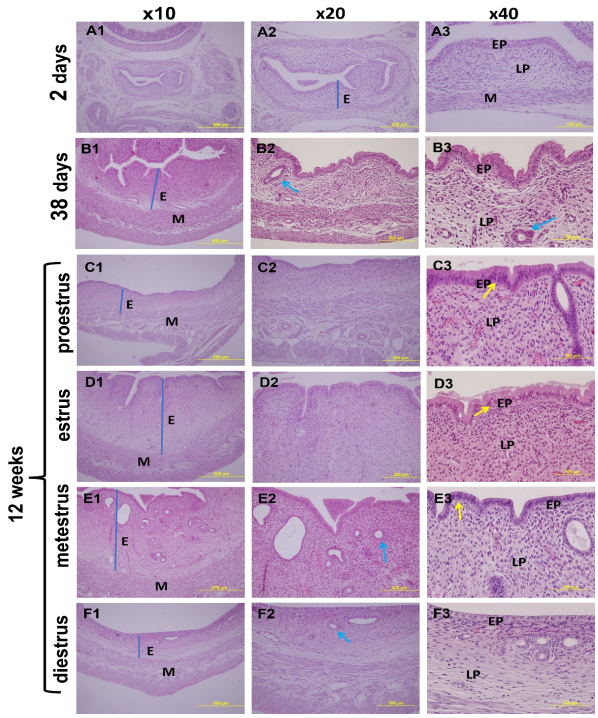


**Figure 2**. Uterine tissues from the groups at different stages of postnatal development. A1, A2, A3) 2 days group; B1, B2, B3) 38 days group; 12 weeks groups, C1, C2, C3) Proestrus group; D1, D2, D3) Estrus group; E1, E2, E3) Metestrus group; F1, F2, F3) Diestrus group. Endometrium (E), Myometrium (M), epithelium (EP), lamina propria (LP), uterine gland (blue arrow), leukocyte infiltration (yellow arrow). H&E, 1, 2, 3: x10, x20, x40.

### *Immunohistochemistry findings*

CD9 and CD13 immunoreactivities expressed by epithelial and glandular cells in the uterus population and CD34 positive cells expressed by hematopoietic stem cells in this population were immunohistochemically evaluated. The mean CD9 and CD13 immunoreactivity of the uterine tissues of the experimental groups and the mean CD34 positive cell numbers are shown in Table 1.

When CD34 expressions were examined immunohistochemically, in the 2-days experimental group, expression was observed in the endothelial cells vessel walls in the lamina propria. In the 38-days and 12-weeks experimental groups, CD34 expression was observed in the cytoplasm of the cells in the endometrium adjacent to the myometrium (Figure 3). According to the number of cells expressing CD34, there was a statistically significant difference between the 2 -day group and all other groups, while a statistically significant difference was only found between the 38-day group and the estrus ($P<0.05$) and metestrus ($P<0.05$) groups. In the 12-weeks groups, there was a statistically significant difference only between the proestrus group and the estrus group ($P<0.05$).

When CD9 expression was examined immunohistochemically, CD9 expression was not found in the uterine tissue sections of the 2-days experimental group, while CD9 was expressed in the uterine lumen epithelium and glandular epithelium in the lamina propria in the 38-days and 12-weeks experimental groups (Figure 3). According to CD9 epithelial immunoreactivity measurement results, an increase in CD9 expression was observed in the period from estrus to proestrus. However, no statistically significant difference was found between the groups ($P>0.05$).

When CD13 expression was examined immunohistochemically, it was observed that CD13 was expressed in the cytoplasm of cells in the lamina propria of the 12-weeks experimental groups, while no significant CD13 expression was found in uterine tissue sections in the 2-days and 38-days experimental groups (Figure 3). According to the CD13 immunoreactivity in the endometrial stroma, a statistically significant difference was found between the 2-days group and the 38-days ($P<0.05$) and oestrus ($P<0.05$) groups when the 2-days group was compared with the other groups. A statistically significant difference was found between the 38-days group and all other groups ($P<0.05$).

**Table 1.** The immunreactivity of CD34, CD9 and CD13 in all experimental groups

| | Groups | CD34 | CD9 | CD13 |
|---|---|---|---|---|
| | **2 days** | 0±0 | 12.08±10.08 | 83.30±7.10 |
| | **38 days** | 5.90±3.03 | 113.53±10.82 | 85.10±4.95 |
| **12 weeks** | **proestrus** | 5.41±2.84 | 117.77±9.91 | 89.84±8.29 |
| | **estrus** | 4.67±4.09 | 115.83±12.69 | 90.42±5.52 |
| | **metestrus** | 5.127±4.25 | 115.33±12.16 | 90.06±5.08 |
| | **diestrus** | 5.35±4.14 | 116.34±17.17 | 88.85±7.72 |

***Note:*** *Immunohistochemical data are expressed as mean ± standard deviation only.*

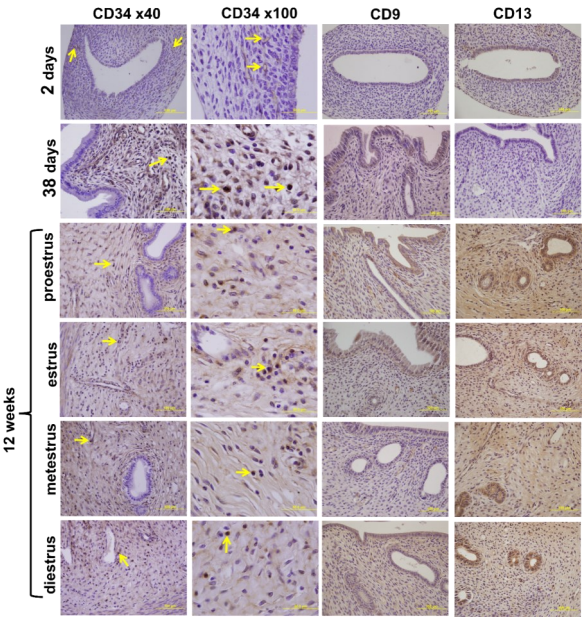


**Figure 3.** Light micrographs of uterine tissues. Immunohistochemical localization of CD34 immune positive cells; x40, x100 in experimental groups. The levels of CD9 and CD13 in uterine tissues of rats (CD9 and CD13 immunoreactivity, x40).

## Discussion and Conclusion

Morphogenesis of the uterus begins in fetal life, but the tissue-specific structure is completed in the postnatal period, including the formation of endometrial glands (Justyna, 2013). Although the development of endometrial glands occurs in the fetus in humans and continues postnatally (PND) and is completed at puberty, it begins postnatally in rodents (Gray et al., 2001). Simple epithelium and undifferentiated mesenchyme support the simple epithelium in the newborn mouse and rat uterus, while endometrial glands are absent. On the 5$^{th}$ day of PND, epithelial invaginations representing the formation of glandular epithelial branches are seen (Brody and Cunha, 1989). Only PND 7 in mice and PND 9 in rats did endometrial gland development become apparent. In rats, continued from day PND 9 to PND 15 days and formed simple tubular glands (Branham et al., 1985). It has been stated that the perinatal mouse uterus consists of an endometrial layer, a stromal layer without endometrial glands, a single-layered columnar epithelium surrounding the lumen supported by this stromal layer, and the myometrium consists of prominent circular and longitudinal smooth muscle layers (Teixeira, 2008). In this study, it was observed that the 2-days rat uterine lumen was surrounded by a single layer of epithelium and there were no endometrial glands in the cell-rich lamina propria. In addition, the muscle layer of the uterus was not evident.

Significant histological changes are observed during the estrous cycle in the uterus, which is the target of ovarian hormones, in rodents without menstruation. The height of the uterine lumen epithelial cells varies during the estrous cycle, with the longest estrus and the shortest diestrus. In contrast to metestrus and diestrus, proestrus and estrus have significant levels of mitotic activity in the uterine epithelium. In uterine glandular cells, the mitotic rate is at its lowest in the estrus phase, and the mitotic rate gradually increases and reaches its highest level in the proestrus phase (Sato et al., 1997).

The human endometrium has features such as cyclic regeneration and tissue destruction under the influence of estrogen and progesterone (Masuda et al., 2010). In these continuously regenerating tissues, adult stem cells are in charge of cell formation. Adult stem cells or progenitor cells thought to dwell in the basalis layer of the endometrium are thought to be responsible for the monthly cyclical regeneration of the functionalis layer of the endometrium. In the studies carried out to define these putative cells, it was determined whether adult stem cells have functional properties such as self-renewal, differentiation into one or more cell lines, and high proliferation potential (Gargett et al., 2007). Chan et al. (2004) supported the view that these cells are hormone-independent cells because the colony forming capacity of epithelial and stromal cells in active and inactive endometrium does not change. At the same time, since these cells are dense in the basalis of the endometrial layer and not in the functionalis, it confirms that the basalis contains a niche for endometrial stem cells. In this study, we aimed to determine the contribution of the endometrium's own population and the contribution of non-endometrial bone marrow-derived stem cells to

the regeneration of the endometrium at different stages of development.

Chan and Gargett (2006) described that 3% of epithelial cells (primarily luminal) and 6% of stromal cells next to the luminal epithelium exhibit mitotic activity in the endometrial-myometrial junction. Cervello et al. (2007) determined that there were stromal cells in a similar location, but no cells showing LRC characteristics were found in the epithelial compartment. In another study, LRC in the stromal area was not evaluated, but the presence of epithelial LRCs in the glandular epithelium was determined intensively (Kaitu'u-Lino et al., 2010). Thus, a consensus on the location of cells with long-term mitotic activity could not be obtained.

One of the methods used to identify stem cells in the endometrium is the isolation of cells with the side population (SP) phenotype (Masuda et al., 2010). They showed that SP cells were found in uterine regions that showed immunoreactivity as $CD9^-CD13^-$. It has been stated that $CD9^-CD13^-$ cells form gland or stroma-like cells. $CD9^+CD13^-$ cells formed gland-like cells, and $CD9^-CD13^+$ cells formed stroma-like cells. CD9 and CD13 expression levels were determined by immunohistochemistry at the secretion stage of cultured endometrial cells. CD9 showed only glandular expression, while both glandular and stromal expressions of CD13 were observed. As negative markers for immature endometrial cells, CD9 and CD13 have been reported to be useful (progenitor cells) (Kato et al., 2007). In another study, Kato et al. (2012) stated that SP cells differ from non-SP cells with their features such as decreased expression of differentiation markers (CD9 and CD13), long-term proliferation capacity in cell culture, and in vitro self-renewal. CD9 and CD13, whose presence in the uterus was determined by different methods in previous studies, were evaluated immunohistochemically in our study. While CD9 expression was not observed in the 2-days group, which was examined at different developmental stages, CD9 expression was observed in the cytoplasm of the uterine luminal epithelium and glandular epithelium cells in the 38-day and 12-weeks groups. CD13 was expressed in cells in the endometrial stroma. CD13 expression was evident in the 12-weeks group, and statistically, the difference between all groups was significant. It was thought that the reason why both CD9 and CD13 expression did not show expression in the 2-days group may be related to the absence of cyclical changes in the endometrium yet. Although the immunoreactivity values of CD9 in the 38-days and 12-weeks groups varied between the groups, these changes were not statistically significant, suggesting that CD9 is not sensitive to hormonal changes. It was suggested that CD13 might be sensitive to the changes in the oestrous phases and possibly to the hormonal changes in these phases.

In humans and rodents, bone marrow is considered a potential source for generating endometrial epithelial and stromal stem/progenitor cells (Taylor, 2004). Du and Taylor (2007) described Y chromosome positive endometrial epithelial and stromal cells in women who underwent bone marrow transplant from male donors. Mint et al. (2008) performed mouse bone marrow transplantation using male donors and identified donor-derived endothelial cells in recipient endometriums. The idea that the bone marrow is a significant source of endometrial stem cells with the ability to develop into parenchymal and endothelial cell types has been supported by studies.

According to a study, endometrial cells can be produced by donor-derived bone marrow cells and that non-uterine stem cells, such as bone marrow stem cells, can also aid in the regeneration of endometrial tissue (Taylor, 2004). In a certain microenvironment, according to Zhang et al. (2012), bone marrow mesenchymal stem cells can differentiate into endometrial epithelial cells, and the right concentration of 17-estradiol promotes this differentiation.

Cho et al. (2004) identified stem cells in the uterus from the fetal to the postmenopausal stage using CD117, CD34, Bcl-2, and Ki-67 primary antibodies. They claimed that, aside from during the embryonic and gestational periods, the only place CD34, a bone marrow stem cell marker, is found in the stroma right next to the glands at the base of the endometrium. They demonstrated using immunohistochemistry that CD34 was solely expressed in the stromal cells of the basalis layer. In order to prevent cyclic shedding, it was also mentioned that these markers are typically found in the stroma of the basalis. In our study, CD34 -expressing cells were present in 38-days and 12-weeks adult animals, and this study was similar to the location of these cells in the uterus.

As a result, according to the data obtained from CD9, CD13 and CD34 immunohistochemical staining, it was thought that the uterine endometrium could be regenerated with the contribution of bone marrow stem cells and uterine epithelial and stromal stem cells in 38-days and 12-weeks groups.

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