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RESEARCH ARTICLE

The investigation of apelin and apelin receptor expressions in mouse endometrium during peri-implantation period

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ABSTRACT

Background: Fertilization, pre-implantation embryo development, implantation, and decidualization are critical for a healthy pregnancy. Successful implantation requires a competent blastocyst and a receptive uterus. Apelin was purified from the bovine stomach in 1998. Apelin receptor (APJ) is a member of G protein-coupled receptors. Apelin/APJ system's physiological role was shown in cardiovascular system, immune response, stress response, fluid regulation, nutrient uptake, angiogenesis, and adipoinsular axis; however, whether apelin/APJ system plays a role in implantation is unknown. In our study, we aimed to evaluate the localization and expressions of the apelin/APJ system in the peri-implantation period mouse uterus.

Methods: Uteri and implantation sites were collected from mice on the estrous phase and the 1st, 4th, 5th, 6th, and 8th days of pregnancy. Also, inter-implantation sites were collected from the 5th day of the pregnancy group. Localization and expressions of apelin and APJ were determined by immunohistochemistry and Western blot, respectively.

Results: Apelin and APJ were expressed in the luminal and gland epithelium, the stroma of all experimental groups. Two isoforms of apelin-8 and 16 kDa were detected by Western blot. While apelin expression increased from the estrous to the 8th day of pregnancy, APJ expression increased from the estrous to the 4th day of pregnancy, reached the highest expression level, then decreased.

Conclusions: Our findings suggest that the apelin/APJ system might be involved in implantation and decidualization. Our findings will guide further studies and may help elucidate the underlying causes of implantation failure and pregnancy loss.

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1. Introduction

Successful pregnancy in mammals depends on a series of crucial events, including fertilization, embryo development, embryo implantation, and decidualization. During the pre-implantation period, the embryo enters the uterine cavity and becomes a blastocyst with healthy development and differentiation for implantation to the uterus. In addition to the healthy development and differentiation of the blastocyst, the uterus must be in a receptive state. In mice, uterine receptivity is divided into 3 stages: pre-receptive (neutral), receptive, and non-receptive (Tu et al., 2014). In mice, the uterus becomes receptive only on the night of the 4th day of pregnancy (the day of implantation), on the 5th day, the uterus becomes non-receptive and does not respond to the presence of blastocyst. In mice, implantation occurs at the anti-mesometrial side of the luminal epithelium, which is the opposite side of the blood vessel-rich mesometrium (Ye, 2020). After the attachment, cells of the adluminal epithelium in mice and humans undergo apoptosis, so the embryo is embedded in the stroma (Zhang et al., 2013). Moreover, embryo implantation initiates the decidualization process, that includes the differentiation of stromal cells to decidual cells. This cellular differentiation process is a prerequisite for a successful pregnancy (Ramathal et al., 2010).

Abbreviations: AKT, Protein Kinase B; AMPKα, Adenosine Monophosphate-Activated Protein Kinase Alpha; APELA, Apelin Receptor Early Endogenous Ligand; APJ, Apelin Receptor; APLNR, Apelin Receptor-; BSA, Bovine Serum Albumin; DAB, 3,3'-diaminobenzidine; E2, Estrogen; ELA, Elabela; ERK, Extracellular Signal-Regulated Kinase; GPCRs, G Protein-Coupled Receptors; hESCs, Human Embryonic Stem Cells; HUVECs, Human Umbilical Vein Endothelial Cells; HRP, Horseradish Peroxidase; H-SCORE, Histological Scoring; IL, Interleukin; NMDA, N-methyl-D-aspartic Acid; NO, Nitric Oxide; NOS, Nitric Oxide Synthase; P4, Progesterone; PE, Pre-eclampsia; PI3K, Phosphoinositide 3-Kinase; PVDF, Polyvinylidene Difluoride; SDS, Sodium Dodecyl Sulfate; STAT3, Signal Transducer and Activator of Transcription 3; TBS, Tris-Buffered Saline; TGFß, Transforming Growth Factor Beta; VEGF, Vascular Endothelial Growth Factor

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Table 1
Establishment of experimental groups. Female mice with large, anucleated squamous epithelial cells in the smear were included in the estrous group. Female mice with a vaginal plug were admitted on the 1st day of pregnancy, thereby forming the experimental groups; D1: 1st day of pregnancy; D4: 4th day of pregnancy; D5: 5th day of pregnancy; D6: 6th day of pregnancy; D8: 8th day of pregnancy.

	Estrous (n = 10)	Large and anucleated squamous epithelial cells in the smear						
		Days of pregnancy						
		1	4	5		6		8
Experimental Groups	**D1 (n = 10)**	Vaginal plug checked; Recovering embryos from the oviducts						
	D4 (n = 10)	Vaginal plug checked	Recovering embryos from the uterus					
	D5 (n = 10)	Vaginal plug checked		Chicago blue injection; Implantation sites were demarcated by discrete blue bands				
	D6 (n = 10)	Vaginal plug checked				Chicago blue injection; Implantation sites were demarcated by discrete blue bands		
	D8 (n = 10)	Vaginal plug checked						Implantation sites were visible

The main focus of this study was to investigate the potential roles of apelin/apelin receptor during the peri-implantation period in mice. Apelin was first purified from bovine stomach extracts in 1998 (Tatemoto et al., 1998). Apelin is synthesized as 77 amino acids preproapelin (Reaux et al., 2001) and undergoes enzymatic degradation into fragments such as apelin-36, 31, 28, 19, 17, 16, 15, 13, and 12 which was summarized in a review (Antushevich and Wojcik, 2018). The apelin receptor (APJ) is a member of the G protein-coupled receptors (GPCRs) and was defined as the orphan receptor until apelin was discovered (Reaux et al., 2001). The physiological roles of the apelin/APJ system were shown in many events such as the cardiovascular system (Galanth et al., 2012), immune response (Horiuchi et al., 2003), stress response (Newson et al., 2013), fluid regulation (Takayama et al., 2008), food intake (Sunter et al., 2003), and angiogenesis (Kalin et al., 2007). Also, apelin is an essential angiogenic factor for normal blood vessel development and endothelial cell proliferation (Cox et al., 2006). The apelin-APJ signal is necessary for embryonic angiogenesis and is upregulated throughout tumor angiogenesis (Kalin et al., 2007). Apelin activates nitric oxide synthase (NOS), causing endothelium-dependent vasodilation and triggering nitric oxide release from endothelial cells (Zhou et al., 2016), Nitric oxide (NO) must be at the optimal level for embryo implantation (Ota et al., 1999). Apelin is known to be expressed in the human placenta (Cobellis et al., 2007; Telejko et al., 2010). The presence of apelin in the human placenta points out the importance of this peptide for pregnancy. However, the role of the apelin/APJ system in embryo implantation and decidualization processes is not known.

We hypothesized that the apelin/APJ system has a role in uteri during the peri-implantation period in mice. In this study, we determined the localization and expressions of apelin and APJ in peri-implantation period mouse uteri and implantation sites via immunohistochemistry and Western blot techniques, respectively.

2. Material and methods

2.1. Animals

6–8 weeks old Balb/C female mice and 6–8 weeks old Balb/C male mice were obtained from Akdeniz University Animal Research Unit, Antalya, Turkey, and the experimental protocol was approved by the Animal Care and Use Committee of Akdeniz University Faculty of Medicine. Animals were placed in a temperature (21 ± 2 °C) and humidity (60 ± 5 %) controlled room in which a 12–12 h light-dark cycle was maintained. Animals were fed with standard laboratory food and tap water in type 3H cages. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This study was performed in line with the principles of the Declaration of Helsinki. The experimental protocol was approved by the Animal Care and Use Committee of Akdeniz University Faculty of Medicine (approval no.: 722/2018.01.019).

2.2. Establishment of experimental groups

Non-pregnant female mice in the estrous phase were included in the estrous group (n = 10). For the estrous group, vaginal smears were taken from non-pregnant female mice and stained with crystal violet (0.1 %) (Cresyl violet (acetate), #105235, Merck, Darmstadt, Germany) to determine the estrous phase. Mice with large, anucleated squamous epithelial cells in the smear were included in the estrous group. Two or three female mice were left in the same cage with male mice overnight for mating, and the vaginal plug was checked the next morning. Female mice with a vaginal plug were admitted on the 1st day of pregnancy, thereby forming the experimental groups; D1: 1st day of pregnancy (n = 10), D4: 4th day of pregnancy (n = 10), D5: 5th day of pregnancy (n = 10); D6: 6th day of pregnancy (n = 10); D8: 8th day of pregnancy (n = 10). The 1st and

4th days of pregnancy were confirmed by recovering embryos from the oviducts and uterus, respectively. On the 5th and 6th days of pregnancy, 0.1 ml 1 % Chicago blue (#C8679, Sigma Aldrich, St. Louis, MO, USA) in saline was injected intravenously then implantation sites were demarcated by discrete blue bands. Implantation sites were visible for the 8th day of pregnancy without Chicago blue injection. The establishment of experimental groups is summarized in Table 1. Mice were sacrificed by cervical dislocation following anesthesia (Ketamine-Xylazine, 0.1 ml/10 g, #K113 Sigma Aldrich, St. Louis, MO, USA) on determined days, and uteri/implantation sites were collected. Also, inter-implantation sites between implantation sites on the 5th day of pregnancy were collected (D5-I). Uteri, implantation, and inter-implantation sites were fixed in 10 % formalin (#15512, Merck, Darmstadt, Germany) for tissue processing and subsequent immunohistochemistry, stored in liquid nitrogen for Western blotting.

2.3. Tissue processing

Uteri, implantation, and inter-implantation sites were fixed in 10 % formalin at room temperature for approximately 24 h. Formalin was removed by washing under tap water for 3 h. Then tissues were dehydrated in 70 %, 80 %, and 90 % ethanol (#100986, Merck, Darmstadt, Germany) for 24-h each and 100 % ethanol for 4 h. After dehydration, tissues were cleared with submersion in xylene (#16446, Sigma Aldrich, St. Louis, MO, USA) for approximately four minutes and embedded in paraffin wax (#107337, Merck, Darmstadt, Germany).

2.4. Immunohistochemistry

Formalin-fixed paraffin-embedded samples were cut into 5 µm sections, and sections were deparaffinized in xylene and rehydrated through a graded series of ethanol. To unmask antigens, an antigen retrieval procedure was performed by treating the samples in 10 mM citrate buffer (#100242, Merck, Darmstadt, Germany), pH6.0, in a microwave oven at 750 W for five minutes, three times. Endogenous peroxidase activity was blocked by incubation in methanol containing 3 % H_2O_2 (#108597, Merck, Darmstadt, Germany) for 15 min. Afterward, sections were incubated in a blocking solution (Ultra UVBlock, #TA-125UB, LabVision Corporation, Fremont, CA, USA) for 7 min. Then, sections were incubated with rabbit polyclonal anti-apelin antibody (#bs2425R, Bioss, Woburn, Massachusetts, USA) at 1:250 dilution and rabbit polyclonal anti-APJ antibody (#bs2430R, Bioss, Woburn, Massachusetts, USA) at 1:100 dilution at + 4 °C overnight. The primary antibody was substituted with the normal rabbit IgG (sc-2027; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in the same dilutions for isotype control. The next day after washing out the primary antibody, slides were incubated with biotinylated anti-rabbit secondary antibody (#BA-1000, Vector Laboratories, Burlingame, USA) at 1:500 dilution for 45 min at room temperature, followed by incubation with horseradish peroxidase (HRP)-conjugated streptavidin (#TS125HR, Thermo Scientific, Fremont, CA, USA) for 20 min at room temperature. Sections were treated with 3,3'-diaminobenzidine (DAB) chromogen (#D4168, Sigma Aldrich, St. Louis, MO, USA) for two minutes and then washed in tap water. Mayer's Hematoxylin (#109249 Merck, Darmstadt, Germany) was utilized to counterstain the sections, and they were dehydrated and mounted with Entellan mounting medium (#107961; Merck, Darmstadt, German). Sections were examined and photographed by a Nikon Eclipse E200 microscope.

2.5. Histological scoring (H-SCORE)

In sections of experimental groups, evaluation of the immunohistochemical labeling of apelin and APJ in the luminal epithelium, glandular epithelium, and stroma was performed using H-SCORE (Acar et al., 2014). Immunopositive cells were counted in five different areas under 200 × original magnification under the Nikon Eclipse E200 microscope. Three sections were randomly selected from every group. The evaluations were recorded as percentages of positively stained cells of all types in each of four intensity categories which were denoted as 0 (no staining), 1 + (weak but detectable above control), 2 + (distinct), 3 + (intense). The evaluation was conducted by two blinded observers. An H-SCORE value was derived by summing the percentages of cells stained at each intensity multiplied by the weighted intensity of the staining [H-SCORE = $\sum$.Pi (i + 1) for each tissue, where i is the intensity score. Pi is the corresponding percentage of the cells].

2.6. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and Western Blotting

Protein extraction and immunoblot analysis were performed as described previously (Acar et al., 2012). Samples were subjected to SDS polyacrylamide gel electrophoresis. At the end of electrophoresis, the proteins were transferred onto PVDF (polyvinylidene difluoride) membrane (#1620177, Bio-Rad, Portland, ME, USA) overnight. The next day, the membranes were blocked for 1 h with 5 % bovine serum albumin (BSA) (#A9647, Sigma Aldrich, St. Louis, MO, USA) prepared in Tris-buffered saline (TBS; pH 7.2–7.4). Then, membranes were incubated with 1:300 dilution of rabbit polyclonal anti-apelin (#bs2425R, Bioss, Woburn, Massachusetts, USA) and 1:500 dilution of rabbit polyclonal anti-APJ (#bs2430R, Bioss, Woburn, Massachusetts, USA) overnight at + 4 ºC. After washing, the membranes were incubated with horseradish peroxidase (HRP) conjugated secondary antibody (1:4000) (#PI-1000, Vector Laboratories, Burlingame, USA) for 1 h at room temperature. The membranes were incubated for five minutes within the Super Signal Chemiluminescence (CL)-HRP substrate system (#34080, Thermo Fisher Scientific, Rockford, USA), and the signals on membranes were then transferred on to hyper film (#28-9068-37 Amersham Hyperfilm ECL, GE Healthcare, Buckinghamshire, UK) in a dark room. The film was processed through a developer (#1757855, Ilford, England) and fixative (#1984565, Ilford, England), followed by washing with distilled water and drying. Thus, the quantities of apelin and APJ protein expressions were determined. Membranes were also labeled by an identical protocol for binding of a 1:5000 dilution of a rabbit monoclonal anti-beta actin antibody (#4970, Cell Signaling Technology, Danvers, MA, USA) as an internal control to confirm the equal loading of the samples. The bands were quantified using NIH image analysis software (Image J Version 1.36b; National Institutes of Health, MD, USA).

2.7. Statistical analysis

Statistical analyses were performed using one-way ANOVA followed by post hoc Tukey's HSD test. Probability values of less than 0.05 were considered significant; values are presented as mean ± SEM (standard error of the mean).

3. Results

3.1. Immunohistochemistry results

3.1.1. Apelin immunohistochemistry

Apelin showed cytoplasmic and nuclear localization, and its expression intensity showed differences between the luminal epithelium, glandular epithelium, and stroma of all experimental groups. In the luminal epithelium, apelin expression was the highest in the estrous group compared to other groups ($p \leq 0.0001$). During the peri-implantation period, apelin expression was lower in the uteri of

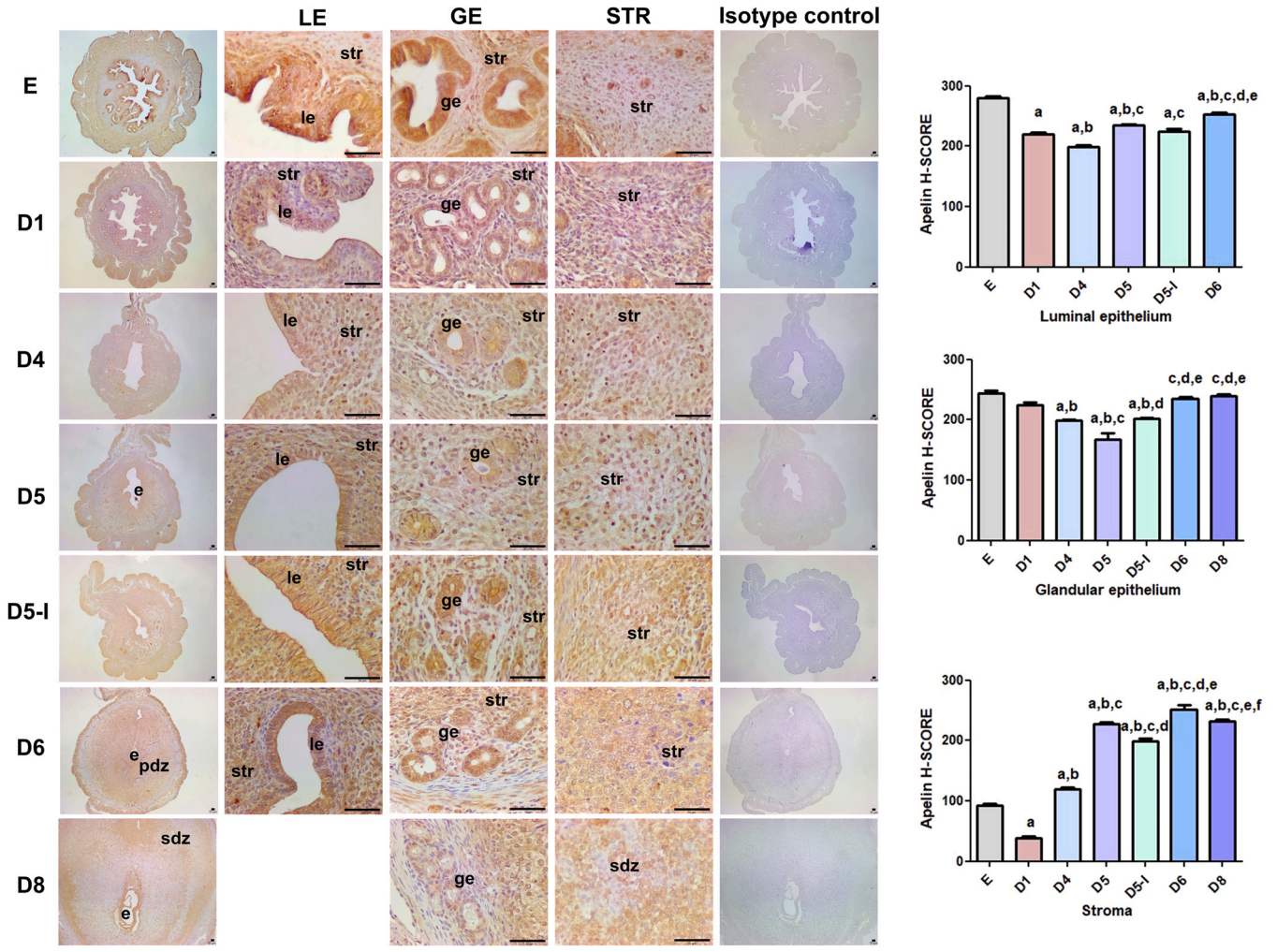


Fig. 1. Apelin immunohistochemistry and histological scoring (H-SCORE) in uterine luminal epithelium (le), glandular epithelium (ge), and stroma (str) of the experimental groups. Scale bar: 50 µm. A significant difference was accepted as $p < 0.05$. Significant difference compared to: [a]E (Estrous) group; [b]Day 1 group; [c]Day 4 group; [d]Day 5 group; [e]Day 5-I group; [f]Day 6 group. (e: embryo, pdz: primary decidual zone, sdz: secondary decidual zone).

the D4 group compared to the D1 group. However, apelin expression increased from the 4th to 6th days of pregnancy in the luminal epithelium. On the 6th day of pregnancy, apelin expression in the luminal epithelium was statistically significantly higher compared to the 1st, 4th, and 5th days of pregnancy ($p \leq 0.0001$). In the glandular epithelium, apelin expression decreased from the estrous group to the 5th day of pregnancy, and then it was increased through the 6th and 8th days of pregnancy. Apelin expression in the stroma of the estrous and D1 groups was statistically significantly lower compared to other groups ($p \leq 0.0001$). Apelin expression in the stroma was increased on the 4th day of pregnancy and through the 5th, 6th, and 8th days of pregnancy (Fig. 1).

3.1.2. Apelin receptor (APJ) immunohistochemistry

Apelin receptor showed membranous, cytoplasmic and nuclear localization, and its expression intensity showed differences between the luminal epithelium, glandular epithelium, and stroma of all experimental groups. In the luminal epithelium, APJ expression was the highest in D4 and D5 groups compared to other groups ($p \leq 0.0001$). APJ expression in the luminal epithelium was statistically significantly decreased in the D6 group compared to D4 and D5 groups ($p \leq 0.0001$).

APJ expression in the glandular epithelium showed a similar pattern to the luminal epithelium. APJ expression was the lowest in the estrous and D1 groups compared to other groups ($p \leq 0.0001$). APJ expression in the glandular epithelium was the highest in the D5 group, and it was downregulated in the D8 group. APJ expression in the uterine stromal cells showed a similar pattern to the luminal and glandular epithelium. It was the lowest in the estrous and D1 groups compared to other groups ($p \leq 0.0001$). On the 4th day of pregnancy, the stromal cells had the highest APJ expression ($p \leq 0.0001$). Then APJ expression was downregulated from the 4th to 8th days of pregnancy (Fig. 2).

3.2. Western blot results

3.2.1. Apelin western blot

Apelin protein has two isoforms, which are approximately 8 and 16 kDa. Both 8 kDa and 16 kDa apelin expressions gradually upregulated from the estrous phase to the 6th day of pregnancy. However, this gradual increase was not observed in the inter-implantation sites on the 5th day of pregnancy. This might be due to implantation does not occur at this site. On the 5th day of pregnancy, 8 kDa apelin expression was statistically significantly higher than the estrous and D1 groups; however, there was no significant difference between these groups for 16 kDa apelin expressions. Both 8 kDa and 16 kDa apelin expressions were statistically significantly higher in the D6 group compared to the estrous, D1, D4, and D5-inter groups ($p \leq 0.001$); nevertheless, only 16 kDa apelin expression was statistically significantly higher than the D5 group ($p \leq 0.001$). Both 8 kDa

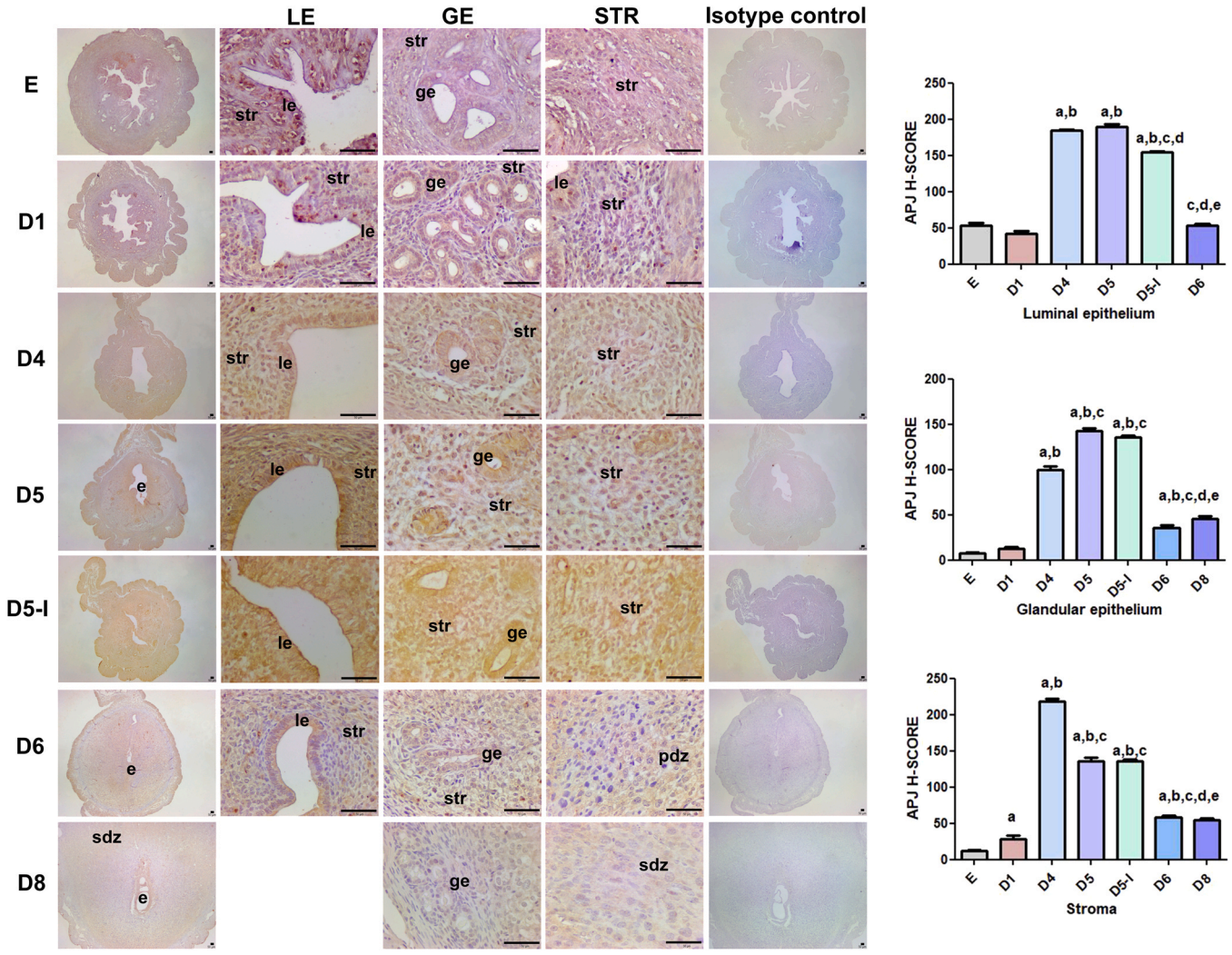


Fig. 2. Apelin receptor (APJ) immunohistochemistry and histological scoring (H-SCORE) in uterine luminal epithelium (le), glandular epithelium (ge), and stroma (str) of the experimental groups. Scale bar: 50 µm. A significant difference was accepted as $p < 0.05$. Significant difference compared to: [a]E (Estrous) group; [b]Day 1 group; [c]Day 4 group; [d]Day 5 group; [e]Day 5-I group. (e: embryo, pdz: primary decidual zone, sdz: secondary decidual zone).

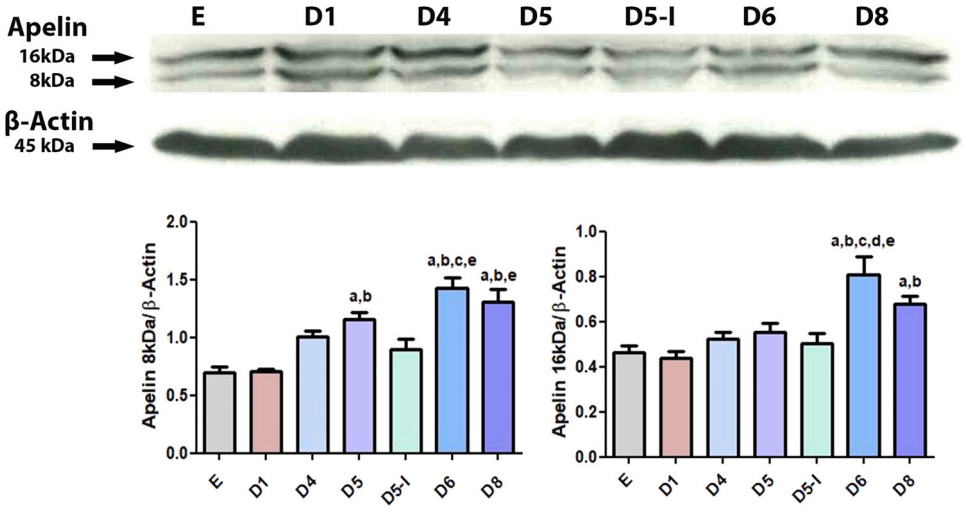


Fig. 3. 8 kDa and 16 kDa apelin and Beta actin Western blot bands of the experimental groups. A significant difference was accepted as $p < 0.05$. Significant difference compared to: [a]Estrous group; [b]Day 1 group; [c]Day 4 group; [d]Day 5 group; [e]Day 5-I group; [f]Day 6 group.

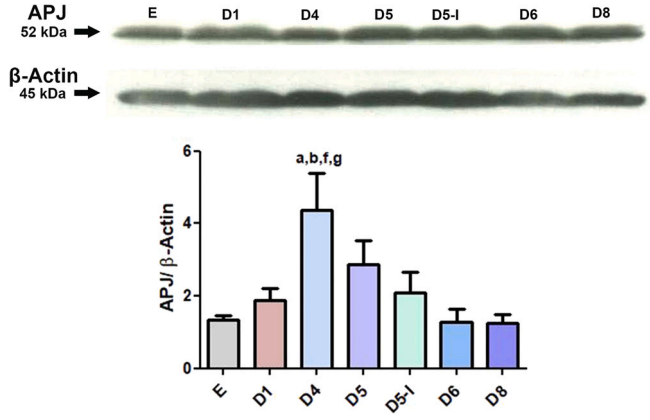


Fig. 4. APJ and Beta actin Western blot bands of the experimental groups. A significant difference was accepted as $p < 0.05$. Significant difference compared to: [a]Estrous group; [b]Day 1 group; [c]Day 4 group; [d]Day 5 group; [e]Day 5-I group; [f]Day 6 group; [g]Day 8 group.

and 16 kDa isoforms were the highest in the D6 group compared to other groups. On the 8th day of pregnancy, apelin expression was decreased, but there was no statistically significant difference compared to the D6 group (Fig. 3).

3.2.2. Apelin receptor (APJ) western blot

APJ expressions gradually upregulated from the estrous phase to the 4th day of pregnancy and reached the highest level on this day of pregnancy. APJ expressions gradually downregulated from day 4 to day 8 of pregnancy. APJ expression in the implantation sites on the 6th and 8th days of pregnancy showed a statistically significant decrease compared to the uteri on the 4th day of pregnancy ($p \leq 0.001$). On day 5 of pregnancy, APJ expression was downregulated compared to the 4th day of pregnancy; however, no significant difference was observed ($p > 0.05$) (Fig. 4).

4. Discussion

The apelin/APJ system plays important and diverse roles in the physiology and pathophysiology of many organs, including blood pressure regulation, cardiac contraction, angiogenesis, metabolic balance, cell proliferation, apoptosis, and inflammation (Lv et al., 2017). Recent studies showed that the uterus expresses components of the apelinergic system. Apelin expression was demonstrated in the uterus via purified apelin-36 isoform from rats' uterine tissue extracts (Kawamata et al., 2001). High expressions of APJ mRNA and apelin-13 binding sites were observed in the mouse uterine endometrium (Pope et al., 2012). Also, the mRNA expression of B78/apj -the rat homolog of the human APJ receptor- was observed in the stromal cells adjacent to glandular epithelial cells, as well as in myometrial smooth muscle cells in the rat uterus (O'Carroll et al., 2000).

Additionally, apelin has been shown to regulate uterine contraction. In an in vitro study conducted on human myometrial tissue, apelin was shown to inhibit contractility (Hehir and Morrison, 2012). On the other hand, Kacar et al. reported that apelin levels increased on the 21st day of pregnancy compared to the diestrous phase in rats. Furthermore, they observed that apelin induced contractions in myometrium contraction trials (Kacar et al., 2018). Even though the apelinergic system has been demonstrated to exist in the uteri of mice, rats, and humans, there are no studies investigating its kinetic expressions in mouse uteri during the peri-implantation period, which comprises embryo implantation and decidualization. In this study, expression of apelin and APJ were determined in the estrous phase and peri-implantation period uteri in a mouse model. Apelin and APJ were expressed at different intensities in the luminal epithelium, glandular epithelium, and stroma in all experimental groups.

In the luminal epithelium of the estrous group, apelin expression was the highest compared to other experimental groups. Apelin expression was gradually decreased in the glandular epithelium of the experimental groups from the estrous phase through the 5th day of pregnancy. Although the lowest apelin expression in the stroma was seen on the 1st day of pregnancy, it was also low in the estrous group compared to other experimental groups. APJ expression in the luminal epithelium, glandular epithelium, and stroma cells was low in the estrous phase compared to other experimental groups. 8 kDa apelin expression in the estrous group uterus was statistically significantly lower compared to the implantation sites of the 5th, 6th, and 8th days of pregnancy. Also, in the estrous group, 16 kDa apelin expression was statistically significantly lower than the implantation sites on the 6th and 8th days of pregnancy. We evaluated apelin localization and expression by H-SCORE in different compartments -luminal epithelium, glandular epithelium and stroma- of uterus and implantation sites. On the other hand we determined apelin expressions in total uterus and implantation sites via Western blot. Although apelin expression is high in luminal epithelium and glands, it is relatively less in stroma in estrous phase. Compared to luminal and glandular epithelium cells, stroma cells are more abundant in total uterine tissue. For this reason low apelin expression in the stroma is reflected in our Western blot results.

On the 1st day of pregnancy, apelin and APJ expressions showed a similar pattern with the estrous group. On this pregnancy day, although apelin expression was high in the luminal and glandular epithelium, it was at the lowest level in stroma cells compared to other experimental groups. On the 1st day of pregnancy, APJ expression was lower in the luminal epithelium cells than other groups. On this pregnancy day, in the glandular epithelium and stroma, APJ expression was lower than in other experimental groups, except for the estrous group. From the 1st to the 3rd day of pregnancy, the uterus is in the pre-receptive phase, and the proliferation of the uterine luminal epithelium begins via the effect of estrogen (Paria et al., 2001). Our results suggest that the apelin/APJ system may not be involved in the proliferation process of the luminal epithelium via estrogen in the first days of pregnancy.

Different immune responses and micro-environments are evolved during pregnancy. The pro-inflammatory immune response progresses, which allows for implantation and placentation, and then shifts to the anti-inflammatory environment for supporting fetal development and growth. At the end of the pregnancy, the anti-inflammatory environment shifts back to a pro-inflammatory micro-environment for labor (Förger and Villiger, 2020). In a study, apelin expressions were shown to be low in fetal membranes of term non-laboring women and women after spontaneous labor and delivery. Apelin knockdown in amnion cells significantly increased interleukin (IL)-1β-induced IL-6, IL-8 release, cyclooxygenase-2 mRNA expression, and resultant prostaglandin E_2 and $F_{2\alpha}$ release. This study supported the role of apelin in the regulation of pro-inflammatory and prolabor mediators in fetal membranes (Lim et al., 2013). Our results showing low apelin expressions in the uteri on the first day of pregnancy may suggest the formation of a pro-inflammatory environment required for embryo implantation.

Our Western blot results showed that 8 kDa and 16 kDa apelin expressions in the uterus on the 4th day of pregnancy increased compared to the estrous phase and the 1st day of pregnancy. Meanwhile, apelin expression in stromal cells was significantly increased on the 4th day of pregnancy compared to the estrous phase and 1st day of pregnancy. APJ expression was highest on the 4th day of pregnancy compared to all groups according to our Western blot analysis. Moreover, APJ expression was at the highest level in stroma cells of the uteri on the 4th day of pregnancy. Recently, it has been

shown that APJ and kappa opioid receptors form a heterodimer together, leading to the phosphorylation of extracellular signal-regulated kinase (ERK), resulting in increased cell proliferation (Li et al., 2012). APJ-mediated ERK phosphorylation may be involved in the proliferation of stromal cells on the 4th day of pregnancy.

The apelin receptor APJ binds to two distinct classes of endogenous ligands, apelin (Tatemoto et al., 1998) and Elabela (Chng et al., 2013). Elabela (ELA; also known as apelin receptor early endogenous ligand peptide /APELA) was discovered in 2013 as an isolated peptide from zebrafish, and it was shown that deletion of Elabela results in cardiac developmental malformations (Chng et al., 2013). It was reported that ELA treatment promoted cell viability and anti-apoptotic effects, and also induced phosphorylation of phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) and ERK1/2 in mesenchymal stem cells cultured in hypoxic and ischemic conditions (Fu et al., 2020). In another study, it was shown that PI3K/Akt signaling pathway is activated by ELA, which enhances the viability, migration, and tube-forming abilities of human umbilical vein endothelial cells (HUVECs) (Wang et al., 2020). These results showed that ELA may affect cell viability, migration, and regulate apoptosis through the ELA-APJ axis and PI3K/Akt signaling. According to our Western blot results, APJ expression was highest among the experimental groups, and higher than apelin expression on the 4th of pregnancy. Based on the literature and our findings, we think that APJ may also interact with ELA besides apelin and thus contribute to cell viability, proliferation, and angiogenesis on the 4th day of pregnancy.

On the 4th day of pregnancy, the uterus becomes receptive with the effect of pre-implantation ovarian estrogen (Wang and Dey, 2006). In mice, expression of E_2 promotes proliferation and differentiation of the luminal and glandular epithelial cells during the first two days of pregnancy. Progesterone (P_4) expression from the newly formed corpus luteum combines with the pre-implantation E_2. Stromal cell proliferation is activated by pre-implantation E_2, which is expressed on the 4th day of pregnancy, the day of implantation. The first sign of the attachment reaction in mice is seen at night on the 4th day of pregnancy. In addition, vascular permeability increases at the implantation site on the day 4th of pregnancy. Angiogenesis also occurs during the implantation and decidualization processes (Wang and Dey, 2006). The initiation of angiogenesis begins during early implantation and is supported by angiogenic factors and circulating hormones, which play a critical role in endometrial differentiation. Estrogen and progesterone have different effects in vivo: Estrogen increases uterine vascular permeability, inhibits angiogenesis, and progesterone has a little effect on vascular permeability and stimulates angiogenesis (Ma et al., 2001). In addition, it was reported that apelin is an angiogenic factor required for normal blood vessel growth and endothelial cell proliferation (Cox et al., 2006). According to our results, apelin and APJ expression were low while the estrogen level is high in the estrous phase and the first day of pregnancy, and the pre-implantation estrogen level, expression of apelin, and APJ increase on the 4th day of pregnancy. These results suggest that there may not be a relationship between estrogen, apelin, and APJ. When progesterone level is increased, expression of APJ also increases on the 4th day of pregnancy. However, in the later days of pregnancy, although progesterone levels remained high, apelin expression is increased, and APJ expression is decreased. This suggests that there may be a linear relationship between apelin and progesterone, but not for APJ. This made us think that the apelin/APJ system and progesterone may have a role in vascular permeability and angiogenesis on the 4th day of pregnancy.

We observed that apelin expression in the glandular epithelium was significantly higher on the 5th day of pregnancy compared to the 1st and 4th days of pregnancy. In a study, Mercati et al. showed high expressions of apelin and apelin receptor mRNA in the glandular epithelium of non-pregnant ewe uterus. As a result of their findings commented that apelin may play a role in providing nutrition to the pre-implantation period embryos by enhancing the secretion of various substances from the uterine endometrium, such as peptides, hormones, and cytokines (Mercati et al., 2019). High expression of apelin in the glandular epithelium during the early days of pregnancy may provide nutrition to the mouse embryo before implantation.

The 8 kDa and 16 kDa apelin expression was higher on the 5th day of pregnancy compared to the uteri of estrous phase and 1st and 4th days of pregnancy, and inter-implantation sites on the 5th day of pregnancy. On the other hand, the expression of apelin on the 5th day of pregnancy was lower than on days 6 and 8 of pregnancy. APJ expression on the 5th day of pregnancy was the second-highest level compared to the 4th day of pregnancy. Progesterone is secreted from the corpus luteum and continues to affect the uterus (Wang and Dey, 2006). After the embryo reaches the implantation site, the attachment of the blastocyst to the uterine epithelium initiates the decidualization process, that includes the differentiation of stromal cells to decidual cells. This progesterone-induced cellular differentiation process is a pre-requisite for a successful pregnancy (Ramathal et al., 2010). Based on our findings, we think that the apelin-APJ system may have a role in decidualization since apelin expression increases on the 5th day of pregnancy when stroma cells differentiate into decidua cells and form the primary decidual zone.

There are some studies showing that apelinergic system ligands may interact with receptors other than APJ. It was shown that apelin receptor early endogenous ligand peptide (APELA/ ELA) promotes cell migration and growth in ovarian surface epithelial cells. Interestingly, these cells were reported not to express apelin receptor (APLNR/APJ), suggesting APELA functions via independent of APLNR (Yi et al., 2017). In another study, ELA was shown to bind to an unknown cell surface receptor and activate PI3K/Akt phosphorylation and transforming growth factor beta (TGFß) signaling to promote self-renewal and cell growth of human embryonic stem cells (hESCs) that do not express APLNR (APJ)(Ho et al., 2015). Retinal ganglion cell death induced by N-methyl-D-aspartic acid (NMDA) was alleviated by intravitreal injection of apelin-36 in mice. So it was concluded that apelin-36 acts directly on NMDA receptors and antagonizes NMDA binding to NMDA receptors without utilizing the APJ (Sakamoto et al., 2016). In line with the literature and our results showing that APJ expression was low but apelin expression was high on the 5th day of pregnancy, we consider that apelin may have receptor/receptors other than APJ. Also, apelin and APJ expressions were lower at the inter-implantation sites where the embryo is not implanted than at the implantation sites on day 5 of pregnancy. This confirms the roles of both apelin and APJ at the implantation sites where the embryo is implanted. We think that angiogenesis during the implantation and decidualization processes on the 5th day of pregnancy may occur via the contribution of progesterone and apelin.

The highest apelin expression was observed on the 6th day of pregnancy among all experimental groups, while APJ expression was at the lowest level on this pregnancy day. At the implantation site on the 6th day of pregnancy, the stroma is divided into two zones; a primary decidual zone close to the embryo and a secondary decidual zone far from the embryo. On this day, while proliferation continues in the secondary decidual zone, proliferation is stopped in the primary decidual zone. According to our immunohistochemistry results, apelin and APJ were expressed in both the primary and secondary decidual zones. Since proliferation stops in the primary decidual zone, we think that the apelin-APJ system in this zone may play a role in the differentiation process. Since proliferation continues in the secondary decidual zone, the apelin-APJ system may have an ERK-mediated proliferation role in the secondary decidual zone.

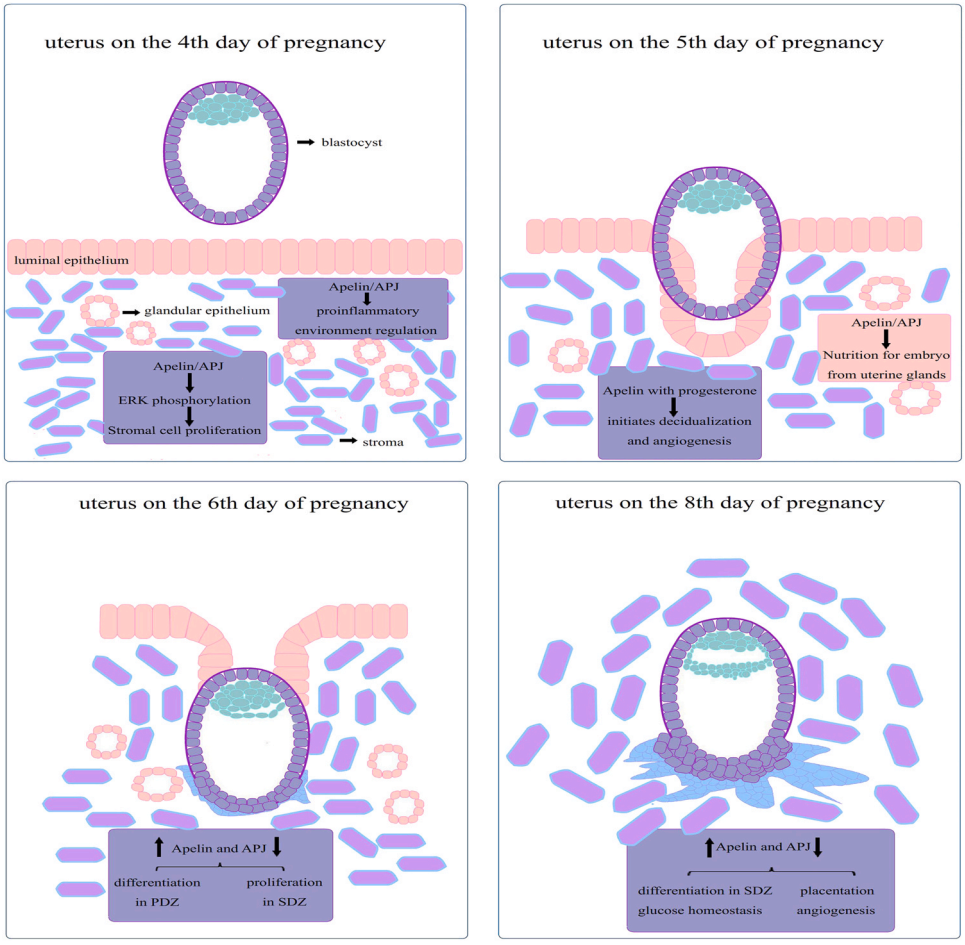


Fig. 5. Schematic illustration of the potential role of Apelin and APJ during peri-implantation period mouse uterus.

The 8 kDa and 16 kDa apelin expressions on the 8th day of pregnancy showed an increase compared to all experimental groups except on the 6th day of pregnancy. APJ expression on the 8th day of pregnancy was at the lowest level among the experimental groups. However, according to our immunohistochemistry and Western blot results, apelin expression is high, and APJ expression is low on the 6th and 8th days of pregnancy. It is possible that apelin binds to unknown receptor/receptors rather than APJ. On the 8th day of pregnancy, the primary decidual zone disappears, and only the secondary decidual zone is present. As the pregnancy progresses, the secondary decidual zone transforms into a thin layer of cells called the decidua capsularis. Mesometrial decidual cells later form the decidua basalis. Eventually, decidua cells join the placenta. The presence of apelin and APJ have been demonstrated in the human placenta, indicating the important role of the apelin/APJ system in placental development (Cobellis et al., 2007; Telejko et al., 2010). Cobellis et al. showed that apelin protein was high in the first trimester placentas of healthy women, but then it was decreased towards the third trimester, but APJ levels were increased in the cytotrophoblasts and endothelial cells. They also found that the apelin and APJ levels were statistically significantly increased in the placentas of women with pre-eclampsia (PE) (Cobellis et al., 2007). Mlyczynska et al. showed the expressions of apelin and APJ in syncytiotrophoblast (BeWo) and cytotrophoblast (JEG-3) cell lines. They also suggested that apelin may promote trophoblast cell proliferation by APJ and ERK1/2, signal transducer and activator of transcription 3 (Stat3), and adenosine monophosphate-activated protein kinase alpha (AMPKα) signaling and regulate early placentation (Mlyczynska et al., 2020). It is important to note that disruptions in placental formation and its functions may lead to pregnancy disorders such as preterm birth, pre-eclampsia, intrauterine growth restriction, and gestational diabetes mellitus resulting in health complications (Tsai et al., 2021). An analysis of the placentas obtained from pre-eclamptic women revealed that apelin and APJ expression levels increased dramatically in cytotrophoblasts, syncytiotrophoblasts, and stromal cells (Cobellis et al., 2007). On the other hand, it was reported that apelin-36 and apelin mRNA levels were lower in the placentas of women with hypertensive disorders complicating pregnancy compared to placentas of healthy women (Liao and Qiao, 2007). Some studies reported that serum apelin levels were significantly decreased in PE patients compared to normotensive pregnant women (Gurlek et al., 2020; Temur et al., 2020). Inuzuka et al. reported that apelin mRNA and protein were significantly decreased in pre-eclamptic placentas compared to normotensive pregnancies; however, apelin protein levels were higher in the maternal circulation in PE patients (Inuzuka et al., 2013). These results raise the possibility of the dysfunctional apelin-APJ system contributing to PE via reduced placentation. Additionally, studies have shown the positive effects of apelin on PE. Chronic apelin administration reduced maternal hypertension and proteinuria and improved fetal growth in a rat model of PE (Wang et al., 2017). In another study, apelin administration to rats with pre-eclamptic features reduced blood pressure and normalized heart rate variability and baroreflex sensitivity (Yamaleyeva et al., 2019) The deletion of the elabela gene -another ligand besides apelin- in pregnant mice led to defective placental angiogenesis and caused pre-eclampsia symptoms, including proteinuria and elevated blood pressure. Infusion of exogenous elabela to these knockout pregnant

mice showed that elabela administration could prevent hypertension, proteinuria, and birth weight (Ho et al., 2017). This new endogenous peptide ligand of APJ might be a therapeutic option for PE.

As it is known, trophoblasts are an interface between fetal and maternal tissues and are defined as a source of angiogenic growth factors. In mice, vascular endothelial growth factor (VEGF) expression begins in uterine stromal cells on the 3rd day of pregnancy and begins in trophoblast cells on the 8th day of pregnancy (Chakraborty et al., 1995; Halder et al., 2000). According to our results, the apelin-APJ system is likely to play a role in angiogenic activity in early pregnancy. The apelin and APJ during the peri-implantation period may indicate a functional role in implantation, decidualization, and placentation processes.

There is ample evidence that apelin is present in many tissues, including adipose tissue, and is involved in glucose homeostasis in obesity and diabetics (Recinella et al., 2020). As a result of insulin-induced apelin production in adipose tissue, plasma levels are elevated in obese hyperinsulinemic mice and humans (Boucher et al., 2005). Gestational diabetes mellitus, one of the metabolic disorders seen during pregnancy, is characterized by glucose intolerance and hyperglycemia (Landon and Gabbe, 2011). In their study, Hanssens et al. showed that in insulin-resistant obese pregnant mice at term, apelinemia, as well as placental apelin and APJ mRNA levels, were increased, whereas placental release of apelin was drastically reduced compared to controls (Hanssens et al., 2016). In another study, maternal serum apelin-36 levels were found to be significantly higher in women with gestational diabetes mellitus compared to healthy pregnant women (Aslan et al., 2012). Boucher et al. showed that the plasma apelin and insulin levels were significantly higher in obese patients (Boucher et al., 2005). Based on these studies, insulin may influence blood concentrations of apelin. Considering the elevated levels of apelin expression in our study, we think apelin may have a role in glucose homeostasis for embryo nutrition during the peri-implantation period.

5. Conclusion

Our results suggest that the apelin/APJ system may be involved in angiogenesis and proliferation during embryo development, implantation, decidualization, and placentation processes (Fig. 5). Our findings also suggest that apelin expression may be regulated by progesterone but not by estrogen, and APJ expression may be regulated by neither estrogen nor progesterone. Furthermore, our study suggests that apelin may contribute to embryo implantation and decidualization with another receptor besides APJ on specific days of pregnancy. Additionally, APJ may interact with ELA rather than apelin and contributes to cell viability, proliferation, and angiogenesis on the 4th day of pregnancy. More detailed studies should be conducted to reveal the role of the apelin-APJ system during implantation, decidualization, and placentation. It will be very precious to determine whether the apelin-APJ system also plays a role in human implantation and decidualization processes. We believe that our results will guide future studies and help elucidate the underlying causes of various pathologies, such as implantation failure and pregnancy losses'.

CRediT authorship contribution statement

Cemre Nur Balci and **Ezgi Golal**: Performed experiments, obtained and interpreted data, analyzed data, **Ismail Ustunel**: Assisted in analyzing data, **Cemre Nur Balci**: Drafted the manuscript, **Nuray Acar**: Supervised the study, assisted in interpreting and analyzing data, revised the manuscript, and interpreted data in the manuscript.

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Ethical Statement

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This study was performed in line with the principles of the Declaration of Helsinki. The experimental protocol was approved by the Animal Care and Use Committee of Akdeniz University Faculty of Medicine (approval no.: 722/2018.01.019).

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Code availability

Not applicable.

Data Availability

Available upon request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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