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Immunohistochemical Investigation of Autophagy in the Uterus during the First Trimester of Pregnancy in Rats

Dilara USLU[1] Sema USLU[2,*]

[1]*Sivas Cumhuriyet University, Faculty of Veterinary Medicine, 58140, Sivas, Türkiye*
[2]*Sivas Cumhuriyet University, Faculty of Veterinary Medicine, Department of Histology, 58140, Sivas, Türkiye*

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ABSTRACT During placental development, autophagy has an important role at the molecular level, especially in cases such as trophoblast cell proliferation and cell death. Abnormal placental development due to trophoblast dysfunction causes serious gynaecological diseases and various fetal malformations. In the study conducted to investigate autophagy on the 5th day of pregnancy, in the pregnant and non pregnant group uterus tissues, uterine glands LC3 and Beclin 1 (+), in the evaluation of myometrium and perimetrium, weak (+) was observed in myometrium cells in pregnancy, while (+) reaction could not be distinguished in perimetrium. In this study, it was concluded that the immunohistochemical increase in LC3 and Beclin 1 intensity in the uterus, especially in the endometrial areas in the first trimester of pregnancy compared to the control group tissues is related to the physiology of pregnancy, homeostasis in pregnancy and hormonal mechanism.

Keywords: *Autophagy, Beclin1, LC3, Pregnancy, Rat, Uterus.*

ÖZ

Ratlarda Gebelikte Birinci Trimesterde Uterusta Otofajinin İmmunohistokimyasal Olarak Araştırılması

Plasental gelişim sırasında otofaji, özellikle trofoblast hücre proliferasyonu ve hücre ölümü gibi durumlarda moleküler düzeyde önemli bir role sahiptir. Trofoblast disfonksiyonuna bağlı anormal plasental gelişim, ciddi jinekolojik hastalıklara ve çeşitli fetal malformasyonlara neden olmaktadır. Gebeliğin 5. gününde otofajiyi araştırmak için yapılan çalışmada gebe ve gebe olmayan grupta uterus dokularında, uterus bezlerinde LC3 ve Beclin 1 (+), myometrium ve perimetriumun değerlendirilmesinde gebelikte myometrium hücrelerinde zayıf (+) gözlenirken, perimetriumda (+) reaksiyon ayırt edilememiştir. Bu çalışmada, kontrol grubu dokularına kıyasla gebeliğin ilk trimesterinde uterusta, özellikle endometrial alanlarda LC3 ve Beclin 1 yoğunluğundaki immünohistokimyasal artışın gebelik fizyolojisi, gebelikteki homeostaz ve hormonal mekanizma ile ilişkili olduğu sonucuna varılmıştır.

Anahtar Kelimeler: *Beclin1, Gebelik, LC3, Otofaji, Rat, Uterus.*

INTRODUCTION

Autophagy means self-digestion, derived from the roots of auto (self) and phagy (eat). Autophagy is the process of catalyzing degraded parts, waste molecules and metabolic parts within the cell by lysosomes (Ozpolat and Benbrook 2015). Dysfunction of autophagy is an underlying mechanism for neurodegenerative transmission, muscle diseases, cancer and heart diseases. During autophagy, double-membrane autophagosomes are formed to catabolize intracellular components, including organelles such as mitochondria. Autophagosomes fuse with lysosomes to form autolysosomes and are degraded by lysosomal hydrolases (Rubinsztein et al. 2005; Deretic et al. 2006; Munz 2006; Wileman 2006).

Autophagy is activated by other physiological stresses such as hypoxia, energy depletion, endoplasmic reticulum stress, high temperature, hormonal stimulation, various pharmacological agents and innate immune signals, as well as in diseases such as bacterial, viral and parasitic infections, acute pancreatitis, heart disease and protein aggregopathies. In contrast, autophagy suppression is frequently associated with some diseases, such as a subset of cancers, neurodegenerative diseases, infectious diseases, and intestinal inflammation, and decline in autophagy function is the most frequently observed feature of aging (Eskelinen 2005).

It is emphasized that autophagy is especially stimulated by starvation, electromagnetic radiation, stress, endoplasmic reticulum (ER) stress and chemotherapeutic agents (Yoshida 2017). The continuity of basal autophagy and cytoplasmic contents during the growth and development

✉ *Corresponding author: semauslu@cumhuriyet.edu.tr

process is very important for the continuity of cell physiology. It appears that autophagy is very important during cell development (Al Rawi et al. 2011; Sato and Sato 2011). After fertilization, autophagy destroys spermatozoon mitochondria together with the ubiquitin-proteasome system, which leads to heteroplasmy (Song et al. 2016). By focusing on maternal mRNA and proteins that might be necessary for the zygotic process, autophagy exhibits considerable activity after the two-cell late stage (Stitzel and Seydoux 2007). According to Di Bartolomeo et al. (2010), autophagy is also essential for the development of pre-implantation embryos, cell differentiation, and organogenesis. Autophagy helps mature erythrocytes get rid of organelles like ribosomes and mitochondria that aren't needed for cell activity (Mortensen et al. 2010).

Autophagy-related proteins (Atg proteins) were first detected in studies conducted in yeast, and more than 30 Atg genes were found (Xie and Klionsky 2007). A portion of these proteins aid in the development of autophagosomes. Pre-autophagosomal structures (PAS) are the structures in which autophagosomes develop. There are four phases that form the autophagy mechanism. 1. The mTor complex, which is the Atg1-Atg13-Atg17 kinase complex 2. Complex PIP3: Atg6 (Beclin 1) complex, which regulates the activity of Vps34 3. Ubiquitin-like system 4. Atg9 and the cycle system After these steps, autophagy in the cell; It progresses as nucleation, membrane elongation, fusion with lysosome and destruction (Arslan et al. 2011).

The mTor pathway is responsible for initiating autophagy in response to starvation conditions in the cell. Accumulation of intracellular free radicals, lack of amino acids and low amount of ATP inhibit the mTor pathway. Following these events, Unc51-like kinase (ULK1) is activated. Beclin 1 serves as a substrate of ULK1; On the other hand, by attaching to Beclin 1, the anti-apoptotic protein B-cell leukemia/lymphoma (Bcl-2) prevents autophagy (Kim et al. 2002). In mammalian cells, Vps34 initiates autophagosome formation, and especially the dissociation of Beclin 1 and Beclin 2 is the most fundamental event for autophagic activity. The Atg12/Atg5/Atg16 complex and microtubule-associated protein light chain 3 (LC3A/B-LC3I/II) protein complexes control the elongation of autophagosomes (Mooren and Krüger 2015). The mTor mechanism in cells is regulated by the Beclin 1, Beclin 2, LKB1-AMPK-mTor, P53, and PI3K-Akt-mTor pathways (Chen et al. 2010). Each gene plays a role in different steps of autophagy. LC3 is one of these genes. LC3-1 is located in the cytoplasm and is 18 kDa, while LC3-II is located on the outer surface of the autophagy membrane and is 16 kDa (Yang et al. 2005). Beclin 1 is expressed in human tissues and located in the endoplasmic reticulum, mitochondria and perinuclear space. Moreover, double-membrane autophagosomes, which are essential for autophagy, are formed by Beclin 1. Human prostate, ovarian, and breast cancers are caused by loss of Beclin 1 (Aita et al. 1999).

Autophagy has an important role at the molecular level during placental development, especially in situations such as proliferation of trophoblast cells and cell death (Gong and Kim 2014). Abnormal placental developments due to trophoblast dysfunction cause serious gynecological diseases and various fetal malformations (Fisher 2015).

The aim of this study was to determine the distribution of autophagy markers LC3 and Beclin 1 in the uterus on day 5 of gestation and to examine their development during pregnancy.

MATERIAL AND METHODS

Ethical Approval

Approval was obtained from Sivas Cumhuriyet University Animal Experiments Local Ethics Committee with the decision numbered 65202830-050.04.04-662 dated 16.06.2022; The study was conducted in accordance with the ethics committee guidelines.

Establishment of Experimental Groups and Experimental Protocol

The animal material used in the study was 60-day-old twelve female Wistar Albino Rats obtained from Sivas Cumhuriyet University Experimental Animals and Experimental Research Center. The rats were divided into the nonpregnant (control) group and the pregnant group, with 6 animals for each group. Animals in each cage were housed under 12-hour light/12-hour dark cycle at a temperature of 22±2 °C. Rats were given ad libitum access to food and water.

The female animals in the group to be pregnant were kept in separate cages for one night, with one male animal each female (to increase the possibility of mating). Afterwards, swabs taken from female animals were examined by vaginal cytology method. Swab samples taken according to this method were transferred to the slide and fixed in methyl alcohol for 3 minutes. Air-dried slides were stained with toluidine blue for 10 minutes. Animals with spermatozoa in the examined preparations were accepted on day 0 of pregnancy. The animals that reached the 5th day of pregnancy and the control group animals were decapitated and their uteruses were removed.

After being fixed in 10% neutral buffered formaldehyde (Formaldehyde Merck: 103999; Distilled Water; NaH2PO4. H2O; Na2HPO4 Sigma-Aldrrich) for 24 hours, the uteruses went through routine tissue tracing stages and were embedded in paraffin blocks (Paraplast Plus, Sigma: P3683). Serial sections of 5µ thickness were taken from the prepared paraffin blocks with the help of a microtome (Leica RM 2125).

Immunohistochemistry

The prepared sections were placed on adhesive slides and the strept avidin-biotin complex (sABC) staining method was applied to determine the distribution and density of LC3 and Beclin-1 in the sections. After the sections were deparaffinized in xylene (Xylene-Merck Millipore: 108661) and dehydrated in alcohol series, the antigen retrieval process was started. For antigen retrieval, the slides were immersed in a 10-fold diluted citrate buffer (Citrate Buffer Heat-Induced Epitope Retrieval pH: 6 Thermo Scientific: AP- 9003-999) solution and boiled in a microwave oven at 800 watts for 20 minutes. After this process, the sections were cooled for 20-30 minutes, washed in Phosphate buffered saline (PBS Sigma: P4417) solution for 15 minutes, and then incubated in 3% hydrogen peroxide (Hydrogen peroxide 30% Merck: 108597) solution in the dark for 20 minutes to block endogenous peroxidase activity. When the incubation was completed, the sections were washed again with PBS solution for 15 minutes and then treated with 0.2% Triton X 100-PBS solution for 15 minutes to help the primary antibody pass through the cell membrane. After this stage, to prevent nonspecific antibody binding, Ultra Vision Block (UltraVision™ - Ultra V Block, Large Volume Detection System anti-Polyvalent, HRP (Ready-To-Use) Thermo Scientific: TP-060-HL) solution was dropped on the tissues and waited for 10 minutes, then diluted without washing. (Large Volume UltrAb Diluent Plus Thermo Scientific: TA-125-UDX) was

incubated with the primary antibody at 4 °C overnight. As primary antibody, LC3 (Proteintech-14600-1-AP) was applied at 1/100 dilution and beclin-1 (Afbiotech-AF5128) was applied at 1/100 dilution. After the primary antibody incubation, the sections were washed with PBS for 15 minutes and subjected to routine immunohistochemistry and AEC chromogen (AEC Substrate System Thermo Scientific: TA-060-HA) was used to demonstrate the reaction. Sections counterstained with Gill's hematoxylin were covered with water-based covering medium (Lab Vision™ Vision Mount Thermo Scientific: TA-125-UG) and examined under a microscope. In the negative control, after protein blocking, PBS was used instead of primary antibody and the tissues were incubated with this solution overnight. Immunohistochemical staining results were evaluated semiquantitatively and presented in tabular form. The staining intensity was graded from 0 to 3 (0 being no staining, 1 being faint staining, 2 being moderate staining, and 3 being high staining (Tatar et al. 2023).

RESULTS

The uteruses of the control group were taken and evaluated on the 5th pregnancy day. LC3 and Beclin-1 stainings were evaluated in terms of staining intensity and staining distribution. A semiquantitative evaluation was made in tabular form by examining the endometrium, myometrium and perimetrium sections of the uterus. The lamina epithelialis and lamina propria in the endometrium, smooth muscles in the myometrium and perimetrium were evaluated without stratification. Lamina epithelialis and subepithelial area, stratum functionalis and stratum basalis in lamina propria and stratum vascularis in myometrium were examined. Endometrial epithelium was clearly distinguished in LC3 and Beclin 1 staining in the control group and tissues taken from the 5th day of pregnancy. In the 5th day of pregnancy staining, more intense staining was observed in the preparations examined than in the control groups. Cells with LC3 and Beclin1 (+) reactions were identified in the decidual cells found in the lamina propria (Figure 1). Uterine glands distinguished in the control group and pregnant uterine tissues were detected as having LC3 and Beclin 1 (+) reactions in all groups. In the evaluation of the myometrium and perimetrium, weak staining was observed in the myometrium cells during pregnancy, while a (+) reaction could not be distinguished in the perimetrium.

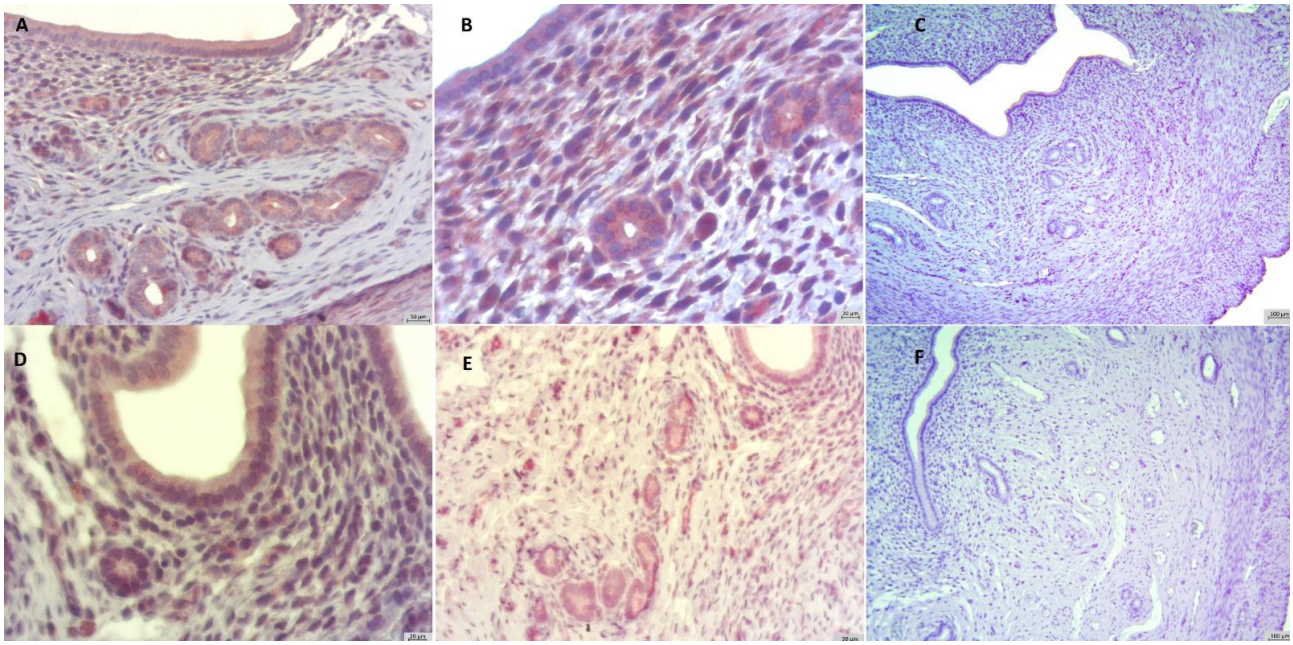

Figure 1: A: Uterus pregnancy day 5 Beclin 1 (Bar: 50 µm). **B:** Uterus pregnancy day 5 LC3 (Bar: 20 µm). **C:** Uterus negative control (Bar: 100 µm). **D:** Uterus control Beclin 1 (Bar: 20 µm). **E:** Uterus control LC3 (Bar: 50 µm). **F:** Uterus negative control (Bar: 100 µm).

Table 1: Semiquantitative score table.

			LC3 Control	LC3 Day 5.	Beclin1 Control	Beclin1 Day 5.
Endometrium	L. Epithelialis		++	+++	++	+++
	L. Propria	Gl. Uterina	++	++	++	+++
Myometrium	Musculus		+/-	+/-	+/-	+/-
	Vascularisation in St. Vasculare		+/-	+/-	+/-	+/-
Perimetrium	-		-	-	-	-

DISCUSSION AND CONCLUSION

Autophagy is the process by which autophagosomes and lysosomes break down a portion of the cytoplasm to carry out homeostatic tasks. Autophagy includes processes whose stages have been recently investigated and the molecular mechanisms in the stages have been tried to be revealed (Chifenti et al. 2013). Autophagy involves a series of interrelated products. LC3 is one of the proteins that has two forms, conjugated and cytosolic, which are necessary for the final autophagosome formation. Beclin-1, on the other hand, regulates the autophagic mechanism with its interactions and, unlike LC3, which is the final autophagosome formation marker, is associated with proteins that participate in the nucleation of the autophagic vesicle in the early stages of autophagy, and plays a role in many biological events such as stress adaptation, endocytosis, development, tumor formation, aging and cell death is another protein (Wirawan et al. 2012). In this study, it is thought that the selection of these two proteins, which have very important roles in the autophagic mechanism, will support the revealing of the biological mechanism that occurs in the placenta, a temporary organ structure that provides immune support and is vital for the fetus in many mammalian species, including humans. In humans, LC3 and Beclin 1 activities were investigated in cells identified as syncytiotrophoblasts, cytotrophoblasts, and stromal cells in the first trimester of pregnancy, and the connection between preeclampsia, intrauterine period, and autophagy was revealed (Oh et al. 2008; Curtis et al. 2013). In this study, uterine samples taken on the 5th day of pregnancy in rats were examined, and LC3 and Beclin 1 activities were investigated immunohistochemically and semiquantitative evaluations were made. Chifenti et al.'s (Chifenti et al. 2013) study revealed that autophagy increased compared to the control group, in parallel with the results of Western Blot studies in humans. The increase in autophagic activation and the similarity of LC3 and Beclin 1 immune localization reveal that autophagy may play a control role in intrauterine development in the first trimester. Factors such as trimesters of pregnancy and the age of the mother may change autophagic needs during pregnancy. Distribution variability between cytotrophoblasts and syncytiotrophoblasts may also differ depending on the gestational period of autophagy. It is thought that the distribution of LC3 and Beclin 1 will vary according to the gestational period, but may be higher in the first trimester, early placentation, and the distribution of immune reactions in the endometrial glands due to the more effective microenvironmental factors.

In the third trimester of a typical pregnancy, Yang et al. (2020) looked into the impact of nutritional support on the levels of LC3 and Beclin 1. They found that although Beclin 1 did not change statistically, the amounts of LC3 increased with nutritional support.

LC3 (autophagy-related gene 8 (Atg-8)) and Beclin 1 expression and autophagosome formation have been observed in Intrauterine Growth Retardation (IUGR) patients, and it has been reported that the IUGR-causing abnormal placentation is associated with cell homeostasis imbalance (Hung et al. 2012; Gong and Kim 2014).

In placentas with fetal growth restriction, Beclin 1, Atg family and LC3 expression are all determinant and autophagy is increased in these individuals compared to normal placentas (Wirawan et al. 2012). Autophagy was found to be increased in placentas with IUGR compared to normal placentas. The presence of placental dysfunction and abnormal fetal development in the same study suggests that autophagy might be crucial in gynecological diseases. Although the mechanisms of autophagy during cellular proliferation and development are known, the role of autophagy in the placenta has not been fully elucidated. A study suggested that urotensin II (UT II) and LC3 release from the placenta increases in hypoxic conditions and that this situation stimulates placental autophagy (Gong and Kim 2014; Pan et al. 2018). In another study, UT II was found to be positively correlated with autophagy in the placenta (Pan et al. 2018).

Chifenti et al. (2013) reported in their study that autophagy is effective in the regulation of placental function, continuity of pregnancy, and utero-placental circulation. In this study, the distribution of LC3 and Beclin 1 autophagic markers supports the importance and existence of autophagy in pregnancy starting from the first trimester (Chifenti et al. 2013). Oh et al. (2008), according to their study on pathologies such as preeclampsia, the amount of LC3 and Beclin-1 in the first trimester of preeclamptic pregnancy was determined to be higher than in the first trimester of normal pregnancy.

Alleire et al. (2019) stated in a study that apoptosis and autophagy work with synergistic metabolism, that these two molecular systems are effective in homeostasis in the placenta, that ULK1, Beclin-1, LC3, Parkin and PINK1 are included in these mechanisms, and that their amounts may change during pregnancy supports the results.

In conclusion, our study revealed an immunohistochemical increase in LC3 and Beclin 1 density in the uterus, particularly in the endometrial areas.

CONFLICTS OF INTEREST

The authors report no conflicts of interest.

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AUTHOR CONTRIBUTIONS

Idea / Concept: DU, SU
Supervision / Consultancy: SU
Data Collection and / or Processing: DU, SU
Analysis and / or Interpretation: DU, SU
Writing the Article: DU, SU
Critical Review: SU

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