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RESEARCH ARTICLE

Immunohistochemical Expression of Beclin1 and Lc3 in the Ovary and Oviduct during Early Pregnancy in Rats

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Abstract

Proteins associated with autophagy are called (Autophagy related proteins) Atg proteins. Atg6 (Beclin 1) complex regulates autophagy at the molecular level and lyses cytosolic proteins, organelles and cytoplasmic components in the autophagosome during autophagy. LC3 is an autophagosomal marker. Monitoring autophagy-related processes is very important in understanding pregnancy metabolism. In this study, Beclin 1 and LC3 were immunohistochemically demonstrated in the ovary and oviduct on day 5 in the first pregnancy period. Accordingly, on the 5th day of pregnancy, it was determined that autophagy continued to increase in pregnancy with the immunohistochemical (+) of Beclin 1, LC3 in interstitial cells, germinative epithelium, follicle granulosa cells and theca interna/externa in the ovary and epithelial cells in the oviduct.

Key Words: Autophagy, beclin 1, lc3, pregnancy, wistar albino rat

Ratlarda Gebeliğin Erken Döneminde Ovaryum ve Oviduktta Beclin1 ile LC3'ün İmmunohistokimyasal İfadesi

Öz

Otofaji ile bağlantılı proteinler (Autophagy Related Proteins), Atg proteinleri olarak isimlendirilir. Otofajinin moleküler düzeyde düzenlenmesinde Atg6 (Beclin 1) kompleksi ve otofaji sırasında otofagozomda sitozolik proteinleri, organelleri, sitoplazmik bileşenleri lize eden LC3 ise otofagozomal işaretleyicilerdendir. Otofaji ile ilgili süreçlerin izlenmesi gebelik metabolizmasının anlaşılmasında oldukça önemlidir. Yapılan çalışmada gebeliğin birinci periyodu içinde 5. günde ovaryum ve oviduktta Beclin 1, LC3 immunohistokimyasal olarak gösterilmiştir. Buna göre gebeliğin 5. gününde ovaryumda intertisyel hücreler, germinatif epitelde, folikül granüloza hücrelerinde ve teka interna/eksternada, oviduktta ise epitel hücrelerinde, Beclin 1, LC3'ün immunohistokimyasal olarak (+) liği ile otofajinin gebelikte artarak devam ettiği belirlenmiştir.

Anahtar Kelimeler: Beclin 1, gebelik, lc3, otofaji, wistar albino rat

INTRODUCTION

The process by which lysosomes catalyze the breakdown of damaged components, leftover molecules, and metabolic cellular contents is known as autophagy (1). It contributes to the maintenance of homeostasis (2). For cell physiology to continue, basal autophagy and cytoplasmic contents must remain constant throughout growth and development (3,4). Autophagy, together with the ubiquitin-proteasome system, eliminates spermatozoon mitochondria after fertilization and thus contributes to heteroplasmy. It is an essential biological function that acts as an auxiliary pathway to the degradation of the ubiquitin-proteasome system (5). After the late two-cell stage, autophagy shows high activity, targeting maternal mRNAs and proteins that may be required in the zygotic process (6). Autophagy is also vital for pre-implantation embryo development, cell differentiation and organogenesis (7).

Autophagy is realized by 3 different mechanisms. The first is macroautophagy, the second is microautophagy and the third is chaperone-mediated autophagy. Over thirty autophagy-related proteins (Atg proteins) are known to exist (8). Autophagosomes arise in structures called anterior autophagosomal structures (PAS). It has been reported that autophagy is shaped by four steps at the molecular level. In the first step, the mTor complex: Atg1-Atg13-Atg17 kinase complex, and the PIP3 complex in the second step: Atg6 (Beclin 1) complex that regulates the activity of Vps34, the emergence of the Ubiquitin-like system in the third step, and Atg9 and the loop system in the fourth step After these processes, autophagy progresses in the cell as nucleation, membrane elongation, interaction with lysosome and destruction (9).

Beclin 1 initiates the formation of Vps34 autophagosomes in mammalian cells. Among the most basic processes for autophagic activity is the separation of Beclin 1 and Beclin 2. The microtubule-associated protein light chain 3 (LC3A/B-LC3I/II) protein complexes and the Atg12/Atg5/Atg16 complex regulate the elongation of autophagosomes (10). Cellular mTor mechanism control involves Beclin 1, Beclin 2, LKB1-AMPK-mTor, P53, and PI3K-Akt-mTor pathways (11). The endoplasmic reticulum, mitochondria, and perinuclear space are the locations of Beclin 1, which is expressed in human tissues. Beclin 1 is also involved in the formation of the double-membrane autophagosome, which is required for autophagy. Loss of Beclin 1 in humans leads to prostate, ovarian and breast cancers (12). The effect of Beclin 1 on luteinization and steroidogenesis in pregnancy has been investigated and it was found to be effective in progesterone synthesis. Beclin 1 is effective in the production of lipid droplets in steroidogenesis (13).

LC3 is a 1A/1B lightchain protein associated with microtubule formation. It is soluble in mammalian tissues and cultured cells with a molecular mass of approximately 17kDa. It lyses cytosolic proteins, organelles and cytoplasmic components in the autophagosome during autophagy. A cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine to form the LC3- phosphatidylethanolamine conjugate (LC3-II), which is taken up into autophagosomal membranes. Autophagosomes fuse with lysosomes to form autolysosomes and intra-autophagosome components are degraded by lysosomal hydrolases, degrading LC3-II. LC3-II is an autophagosomal marker. An effective technique for tracking autophagy-related activities, such as autophagy and autophagic cell death, is the detection of LC3 by immunoblotting or immunofluorescence (10).

This study aimed to investigate the intensity of autophagy in the ovary and oviduct in the first period of pregnancy in rats and to reveal the distribution of Beclin 1 and LC3, which are responsible for different steps of autophagy, immunohistochemically.

MATERIAL AND METHODS

Establishment of Experimental Protocols and Groups

Twelve 60-day-old female Wistar Albino rats obtained from Sivas Cumhuriyet University Experimental Animals Center were used. The rats were split into two groups, one for pregnancy induction and one for control, each with six animals. The animals in each cage were kept in an environment with a temperature of 22±2, 12 hours of light/12 hours of darkness, ad libitum water and food.

One male animal per female was housed in a separate cage for one night in the pregnancy group. The vaginal cytology procedure was then used to evaluate the swabs obtained from the female animals. This approach involved transferring the swab samples on a slide and fixing them in methyl alcohol for three minutes. After ten minutes of toluidine blue staining on the air-dried slides, the preparations were closed and assessed.

The animals considered to be on Day 0 of pregnancy were those in which spermatozoa were seen in the preparations under examination. The ovaries and the middle part of oviduct of the animals in the control group and those on the 5th day of pregnancy were removed.

Immunohistochemistry

Following a 24-hour fixation in 10% neutral buffered formaldehyde, the ovaries and oviducts were embedded in paraffin blocks after undergoing routine tissue processing steps. Serial slices 50µ apart and 5µ thick were obtained from the prepared paraffin blocks using a microtome (Leica RM 2125). The slices were put on adhesive slides and the streptavidin-biotin complex (sABC) staining technique was employed to assess the distribution and density of LC3 and Beclin-1. The antigen retrieval procedure was initiated following the sections' de-paraffinization in xylenes and dehydration in alcohol series. Slides dipped in a 10-fold diluted citrate buffer solution were cooked for 20 minutes at 600 watts in a microwave oven to retrieve antigen. Following a 20-30 minute cooling period, the sections were rinsed for 15 minutes in PBS solution and then incubated for 20 minutes in the dark in a 3% hydrogen peroxide–methyl alcohol solution to inhibit endogenous peroxidase activity. After incubation, the sections were washed again with PBS solution for 15 minutes. After this stage, Ultra V Block solution was dropped on the tissues to prevent nonspecific antibody binding, waited for 10 minutes, and then incubated with diluted primary antibody at $+4^0$C overnight without washing. As primary antibodies, Beclin-1 (Afbiotech-AF5128) and LC3 (Proteintech-14600-1-AP) were adminis-

tered at 1/100 dilution. Following primary antibody incubation, slices were submitted to standard immunohistochemistry techniques, followed by a 15 minute PBS wash, and the response was shown using AEC chromogen. In the negative control, PBS was used in place of the primary antibody following protein blocking, and the tissues were incubated with this solution for an entire night. Sections counterstained with Gill's hematoxylin were covered with a water-based covering medium and viewed under a microscope. For this, staining intensity was scored between 0-3 (0; no staining, 1; slight staining, 2; moderate staining, 3; strong staining) The immunohistochemical staining results were assessed semiquantitatively and displayed in a table 1.

Table 1. Semiquantitative representation of the density and distribution in Beclin 1, LC3 immunohistochemical staining in the ovary and oviduct in the control group and in the first period of pregnancy.

			Control	Day 5
OVARY	Germinal Epithelium	Beclin 1	+	++
		LC3	+	++
	Interstitial Cell	Beclin 1	+	+
		LC3	+	+
	Granulosa Cell	Beclin 1	+	+
		LC3	+	+
	Corpus Luteum	Beclin 1	++	++
		LC3	++	++
OVIDUCT	Oviduct Epithelium	Beclin 1	+	+
		LC3	+	+
	Lamina Propria	Beclin 1	--	--
		LC3	--	--

RESULTS

On the fifth day of pregnancy and in the control group, the ovary and oviduct were assessed. The staining intensity and staining distribution of LC3 and Beclin-1 staining were assessed. The germinative epithelial cortex [wall of follicles (primary, secondary), interstitial cells, luteal cells in corpus luteum] and medulla were evaluated in the ovary. Epithelium in the mucosa and muscular and serosal layers in the oviduct were examined and evaluated, semiquantitatively shown in the Table 1. Since no positive reaction could be detected in the muscularis and serosa layers of the oviduct, it was not added to the table (Table 1).

It was observed that Beclin 1 and LC3 immune reactions increased in the germinative epithelium of the ovary. It was determined that the reaction intensity was similar in the interstitial cells, corpus luteum and luteal cells on the 5th day of pregnancy (Figure 1A, B, C, D) (Figure 2A, B, C).

In the oviduct, on the 5th day of pregnancy and in the control group, Beclin 1 and LC3 (+) reactions were observed to be of similar location and intensity in the epithelium, and -/+ in places in the propria, muscles and serosa (Figure 3A, B, C, D).

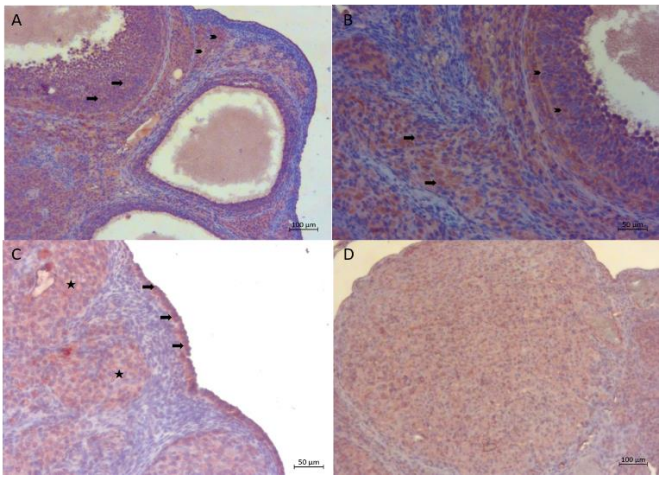

Figure 1. A. Ovary (Control) - Beclin 1. Immunopositive interstitial cells (arrowheads) and granulosa cells (arrows). (Scale bar: 100 µm). **B.** Ovary (Pregnancy, Day 5) - Beclin 1. Immunopositive interstitial cells (arrows) and granulosa cells (arrowheads). (Scale bar: 50 µm). **C.** Ovary (Control) – Beclin 1. Immunopositive interstitial cells (stars) and germinative epithelium (arrows). (Scale bar: 50 µm). **D.** Immunopositivity in the corpus luteum on Day 5 of pregnancy – Beclin 1. (Scale bar: 100 µm).

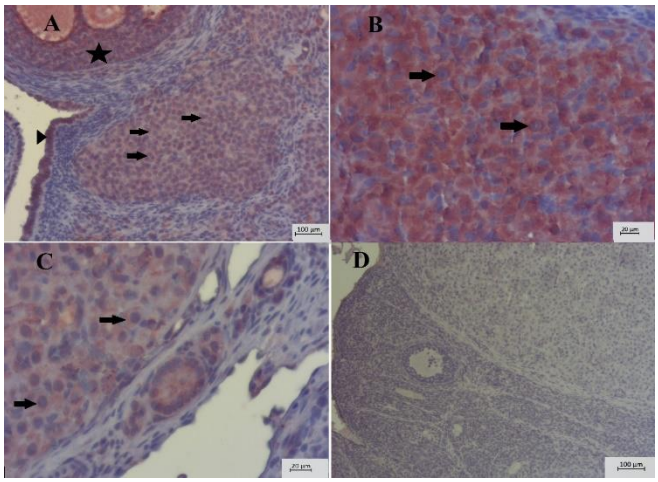

Figure 2. A. Ovary (Control) – LC3. Immunopositive interstitial cells (arrows), germinative epithelium (arrowhead) and granulosa cells (star). (Scale bar: 100 µm). **B.** Corpus luteum (Pregnancy, Day 5) – LC3. Immunopositive corpus luteum cells (arrows). (Scale bar: 20 µm). **C.** Ovary (Pregnancy, Day 5) – LC3. Immunopositive interstitial cells (arrows). (Scale bar: 20 µm). **D.** Ovary negative control (Scale bar: 100 µm).

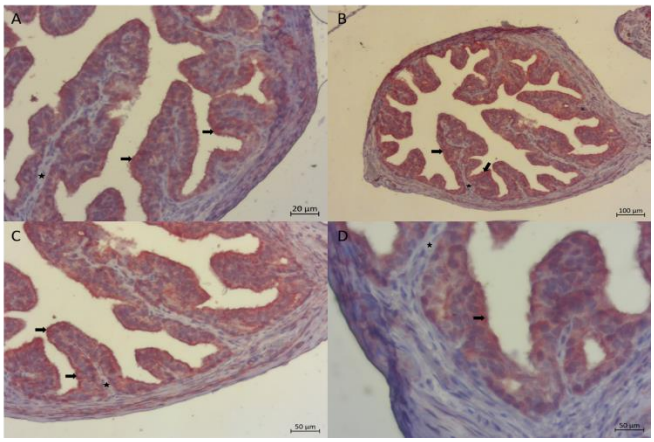

Figure 3. A. Oviduct (Control) – LC3. Immunopositive oviduct epithelium (arrows) and immunonegative lamina propria (star). (Scale bar: 20 µm). **B.** Oviduct (Pregnancy, Day 5) – Beclin 1. Immunopositive oviduct epithelium (arrows) and immunonegative lamina propria (star). (Scale bar: 100 µm). **C.** Oviduct (Control) – Beclin 1. Immunopositive oviduct epithelium (arrows) and immunonegative lamina propria (star). (Scale bar: 50 µm). **D.** Oviduct (Pregnancy, Day 5) – LC3. Immunopositive oviduct epithelium (arrow) and immunonegative lamina propria (star). (Scale bar: 50 µm).

DISCUSSION AND CONCLUSION

Autophagy demonstrates that damaged cell organelles are re-applied to provide new structural remnants to maintain homeostasis (15). Autophagy is essential for follicular growth and differentiation in the ovary, oocyte development, follicular atresia and follicular maintenance of reproduction (16,17). Proper functional autophagy is important in the process from oogonia to follicle development and degeneration (18). has confirmed the effect of autophagy on female reproductive functions starting from oocyte development to postnatal remodelling, including implantation, pregnancy maintenance, and preservation of placental physiology (19). It has been reported that the possibility of oocyte maturation defect and germ cell deformity is associated with the deficiency of basic autophagic molecules. Therefore, the role of autophagy in the female reproductive system needs to be clearly understood (20).

Beclin 1, an important protein related to autophagy, also plays a role in cell differentiation, anti-apoptosis and cancer suppression (21). Another protein widely used as a monitoring tool for autophagic activation is LC3 (22). In the research by Zhao et al. (21), the immunohistochemistry expression of Beclin 1 was statistically found to be 16.7% in the control group. It has been stated that Beclin 1 is localized in the cell cytoplasm in the ovary, but no detailed information is given about the positive regions. In this study conducted in the ovary, it was observed that the distribution and localization sites of Beclin 1 and LC3 in pregnant and non-pregnant animals were similar and that the (+) level was in the cytoplasm and the nuclear (+) level was not, as in the study conducted by Zhao et al. in 2014 (21).

In mammals, the corpus luteum (CL) is a unique and transient endocrine gland that develops from follicle remnants following ovulation, which is essential for hormone homeostasis and pregnancy (23-25). When the CL ceases to function in the absence of pregnancy or at the end of pregnancy, it ceases to produce progesterone and then undergoes a period of regression. Therefore, the luteolysis process is considered as an important step for the maintenance of ovarian homeostasis and the resumption of the next estrous cycle (24, 25). Previous investigations have indicated that apoptosis is the key process leading to the death of luteal cells and the regression of CL (24, 25), but the function of autophagy in pregnancy luteolysis remains unclear (26). Few studies have investigated the mechanism of autophagy in the initial stage of pregnancy luteolysis (26).

The LC3 protein levels in the corpus luteum of the control group grew considerably throughout the first trimester of pregnancy, according to the Western-Blot analysis of LC3 protein levels in the research by Tang et al. (26) with pregnant rats. Therefore, they stated that autophagy may play a role in the luteolysis of pregnant rats in the initial period of pregnancy. However, immunohistochemical staining was not performed for LC3 in the study. The immunohistochemistry distribution of LC3 and Beclin 1 was found to be comparable in this investigation; however, staining density and intensity differed from location to location. It was thought that this variability could be due to the reasons reached in the study results of Tang et al. (26). It was concluded that the ovarian tissue should be examined again in the later stages of pregnancy and the evaluation should be made.

While our investigation found Beclin and LC3 positivity in certain parts of the ovary and oviduct autophagy also plays a significant role in the regression of the corpus luteum during false pregnancy, and its significance in pregnancy luteolysis is yet unknown (26). As a result, the purpose of this study was to look at autophagy in rats' luteal processes during early pregnancy. The findings of autophagy-related protein LC-3 and Beclin-1 suggest that autophagy is relatively high during the first trimester of pregnancy. Growth retardation during pregnancy has been linked to autophagosome production and LC3 and Beclin 1 expression, and aberrant placentation has been linked to an imbalance in cell homeostasis (27-29). Autophagy has been reported to be elevated higher in placentas with fetal growth retardation than in normal placentas due to the expression of LC3, Beclin 1, and the Atg family (30). The function of autophagy in the ovaries and oviduct during pregnancy has not been completely clarified, even though the processes underlying autophagy during cellular proliferation and development are well understood. The fact that the distribution and density of autophagy in the ovary and oviduct are different during pregnancy has shown that autophagy is effective not only fatally but also maternally.

Gawriluk et al. (13) demonstrated that Beclin1 is required for female reproduction for the corpus luteum to function properly during pregnancy, and they stated that the success of mammalian reproduction is dependent on the production of hormones that not only promote germ cell development in the female but also ensure the establishment and maintenance of pregnancy. They found that a lack of the important autophagy gene Beclin1 in progesterone-producing cells in the ovary reduces circulating progesterone in pregnant mice and that exogenous progesterone administration causes a preterm labour phenotype, indicating that progesterone is an essential hormone in mammalian pregnancy. They added that these findings show that autophagy may have a role in steroidogenesis, and hence in successful reproduction, and that future research should focus on the autophagy-dependent and autophagy-independent activities of Beclin1 in the ovarian process. The findings of this study support the link between hormonal changes and autophagy.

Choi et al. (31) showed very weak LC3 immunoreactivity in the corpus luteum on day 2 of pseudopregnancy in pseudopregnancy rats. After day 7, immunoreactivity for LC3 increased, and very intense immunoreactivity for LC3 was seen in luteal cells on days 14 and 20. In addition, it was stated that intermediate fibroblasts and stromal cells in the corpus luteum showed very weak LC3 immunoreactivity throughout their study.

In their studies conducted on the ovary in the prepubertal period in rats (17), they reported that LC3 immunolocalization was very intensely positive in primordial follicles, primary and preantral follicles granulosa cells while in late antral follicles, the positivity was high in granulosa cells and weak in theca follicles. The same study also found that LC3 could not be detected in the cytoplasm of oocytes at any developmental stage. In the study, the (+) status was found to be similar in granulosa cells (17) while the (+) status was occasionally encountered in oocytes.

In conclusion, the study determined that the immunohistochemistry distribution of Beclin 1 and LC3 in the ovary

and oviduct is connected to the pregnancy period and hormonal process and that it fluctuates. It is thought that this study will contribute to the literature on this subject by forming the basis for studies to be conducted on autophagic molecules in the later stages of pregnancy.

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No support was received from any organization in the conduct of this research.

CONFLICT OF INTEREST

There is no conflict of interest to be declared by the authors.

AUTHOR CONTRIBUTIONS

SU participated in the planning, design, and writing of the study. ENTK contributed to data collection, processing, and editing of the manuscript. The analysis and interpretation of the data were performed by SU and FEB. All authors contributed to the critical review of the study.

ETHICAL STATEMENT

Approval was acquired from the Sivas Cumhuriyet University Animal Experiments Local Ethics Committee with the decision dated 22.09.2023 and numbered 65202830-050.04.04-776 and the study was carried out in compliance with the ethics committee instruction.

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