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Investigating Autophagy Genes Expression and their Possible Relations with Apoptosis in PBMCs of Patients with Thin Endometrium

Lida-Aslanian Kalkhoran[1,2,3], Mohammad Sadegh Soltani-Zangbar[1], Ali Aghebati-Maleki[4], Mahya AhmadpourYoushanlui[1], Mehdi Yousefi[1], Leili Aghebati-Maleki[1]*

[1]Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran; [2]Department of Immunology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran; [3]Student Research Committee, Tabriz University of Medical Sciences, Tabriz, Iran; [4]Department of Molecular Medicine, Faculty of Advanced Medical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran

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ABSTRACT

Background: Autophagy genes are essential for proper uterine function, reproductive physiology, and the maintenance of endometrial atrophy (EA).

Objective: This study aims to clarify how these genes impact endometrial thickness and their significance in the apoptosis of peripheral blood mononuclear cells (PBMCs) from patients with thin endometria.

Methods: Blood samples were collected from 40 patients with thin endometrium and 40 healthy controls, both groups being in the non-pregnancy stage. Real-Time PCR was used to measure the expression levels of autophagy genes ATG5, ATG7, LC3B, Beclin1, FOXO1, FOXO3a, FOXO4 and FOXO6 in 40 women with thin endometrium and 40 healthy women. Also, apoptosis was assessed by flow cytometry. To further elucidate the biological pathways and processes associated with the differentially expressed autophagy genes, we conducted a KEGG pathway enrichment analysis using the EnrichR tool.

Results: Evaluation of the expression levels of autophagy genes showed a significant difference between the studied groups, with the expression levels of ATG5, ATG7, LC3B, Beclin1, FOXO1, FOXO3a, FOXO4, and FOXO6 higher in the patient group. Moreover, there was a positive correlation between LC3B expression and the frequency of apoptotic cells in the studied patients. The EnrichR tool identified the enriched pathways: “Shigellosis,” “FOXO signaling,” “Fruptosis,” and to a lesser extent, “Longevity regulating pathway,” “Autophagy,” and “Mitophagy.”

Conclusion: Our results showed that autophagy genes associated with apoptosis in PBMCs may be involved in endometrial thinning in EA patients.

Keywords: Autophagy; Endometrium; Apoptosis; ATG; FOXO

Corresponding author:
Leili Aghebati-Maleki,
Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
Email: leili_aghebati_maleki@yahoo.com
Aghebatil@tbzmed.ac.ir

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INTRODUCTION

Embryo implantation into the maternal endometrium is a critical step in human conception (1, 2). The endometrium provides the structural and functional support required for placental development during pregnancy (3-5). A thin endometrium is a well-recognized factor associated with reduced implantation rates and implantation failure. Therefore, assessment of the endometrial characteristics, particularly endometrial thickness, is an essential component of assisted reproductive procedures (6). In most studies, an endometrial thickness of less than 7-8 mm is classified a thin endometrium. Pregnancy rates are generally lower when the endometrium is thin, however, several reports have documented successful pregnancies with endometrial thickness measurements as low as 4- 4.6 mm (7, 8). One of the challanges in assisted reproduction is the concern caused by a thin endometrium, which is frequently associated with lower pregnancy success rates after treatment. The etiology of thin endometrium is multifactorial, and are not yet fully understood, making effective management difficult. Endometrial thinning can result from various causes, with inflammatory and iatrogenic factors being the most common. However, some women naturally have a thin endometrium (9). Pathological causes of thin endometrium include Asherman's syndrome, uterine fibroids, previous intrauterine surgery, certain medications, hormone disorders, and premature ovarian failure (10-12).

Recently, the key role of autophagy-related genes in both physiological and pathophysiological states of the human endometrium has been increasingly recognized (13, 14). Autophagy is a cellular catabolic process, literally meaning "self-eating," in which cells degrade and recycle cytoplasmic components (15). Autophagy-related genes are induced under various cellular stress conditions and participate in regulating multiple cellular processes. Stimuli such as hypoxia and starvation induce autophagy primarily through on activation of AMP-activated protein kinase (AMPK) and inhibition of the mammalian target of rapamycin complex 1 (mTORC1) (16). The conserved ATG proteins orchestrate the initiation and progression of autophagy (17). Initially, in response to cellular stress signals, the phagophore is formed, typically originating from regions near the endoplasmic reticulum (ER). Two ubiquitin-like conjugation systems are essential for autophagosome membrane: the conjugation of ATG12 with ATG5, which associates with ATG16 to localize at the phagophore, and downstream, the conjugation of ATG8 (LC3 in mammals) tophosphoethanolamine (PE), which is incorporated into both the growing phagophore and the autophagosomal membrane (18). The interaction of ATG12-ATG5 conjugate and ATG16L1 leads to the formation of the multimeric ATG5-ATG12-ATG16 complex. Together with members of the ATG8 protein family -including ATG3, ATG7, microtubule-associated protein light chain 3 (LC3) and the GABARAP subfamilies- the autophagosomal membrane undergoes expansion and maturation to form a mature autophagosome. The E1-like enzyme ATG7 plays a key role in the autophagy conjugation systems, including the ATG12–ATG5 pathway and the lipidation of LC3. LC3 exists in two forms: the cytosolic soluble form (LC3I) and the lipidated, membrane-bound form (LC3II). In mammalian tissues, LC3 is expressed as three isoforms -LC3A, LC3B, LC3C- with LC3B being the most commonly used marker of autophagy (19). Members of the FOXO family proteins (FOXO1, FOXO3a, FOXO4, and FOXO6) play important regulatory roles in the autophagy pathway (20). One of the critical genes involved in the initiation of autophagy is Beclin-1, which is essential for autophagosomes nucleation and the activation of other autophagy-inducing factors (21). Several studies have highlighted the role of autophagy in maintaining endometrial homeostasis and supporting successful pregnancy. Low estrogen levels can lead to thinning of the endometrium, a condition

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known as endometrial atrophy, and molecular mechanisms involving autophagy-related genes have been implicated in this process (22). In atrophic endometrial cells, autophagic activity is associated with a higher number of autophagosomes and an enhanced apoptotic cell death response. A key early event in this process is the ER stress–mediated inactivation of the AKT (AKT serine/threonine kinase)-MTOR (mechanistic target of rapamycin kinase) signaling pathway. The aim of this study is to investigate the expression levels of genes involved in autophagy in women with thin endometrium compared with healthy controls, and to assess their correlations with apoptosis levels in PBMCs from these patients.

MATERIALS AND METHODS

Study Population

This study was approved by the Research Ethics Committee of Tabriz University of Medical Sciences (IR.TBZMED.REC.1401.733). Forty non-pregnant women with thin endometrium (mean age: 27.19 ± 4.13) who presented to Valiasr Hospital between September 2021 and April 2022 were enrolled. Forty healthy non-pregnant women (mean age: 26.34±3.74) with a history of pregnancy and at least one successful delivery served as the control group (women without thin endometrium). Exclusion criteria included genetic and uterine abnormalities, hematological disorders, infectious and immunological conditions, and hormonal disorders. Inclusion criteria for the thin-endometrium group were:: an endometrial thickness (EMT) less than 7 mm on days 11-13 of the menstrual cycle and absence of significant intrauterine adhesions. All participants provided written informed consent prior to enrollment.

Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

After collecting 10 ml of peripheral blood from patients and healthy controls under sterile conditions and using EDTA-K2 as an anticoagulant, PBMCs were isolated density-gradient centrifugation using Ficoll (1.077 g/mL; Biosera, UK) at 450 g for 25 min. Following separation of the layer (23), the cells were divided into two portions: one for assessing the frequency of apoptotic cells by flow cytometry, and the other for RNA extraction and subsequent gene expression analysis.

Apoptosis Assay

Apoptosis was assessed using flow cytometry (BD FACSLyric™). In this method, apoptotic cells are detected by staining with annexin V and propidium iodide (PI), followed by flow cytometric analysis (24). Under normal conditions, phosphatidylserine is located on the inner leaflet of the plasma membrane (cytoplasmic side). During early apoptosis, phosphatidylserine becomes exposed on the outer membrane surface, where it can be detected by Annexin V. PI, which is impermeable to intact cell membranes, enters only necrotic or late-apoptotic cells with compromisedmembrane integrity, allowing differentiation between apoptotic and necrotic populations. After 48 hours of cell culture, 50 µL of annexin-binding buffer was added to 3×10^5 cells. Cells were then stained with 5µL of annexin V-FITC and incubated for 15 minutes at room temperature in the dark. Following incubation, 200 µl of binding buffer was added and PI was introduced at a final concentration of 50 µg/ml. The Annexin V-FITC apoptosis detection kit was purchased from BD Biosciences (CA, USA).

Real-time PCR

The expression levels of ATG5, ATG7, Beclin1, LC3B, FOXO1, FOXO3a, FOXO4, and FOXO6 were evaluated by real-time PCR in patients with thin endometrium and healthy control women. After isolation of PBMCs, total RNA was extracted using RNX-PLUS solution (Sina Clone, Tehran, Iran). Complementary DNA (cDNA) was synthesized using the Revert Aid Reverse

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Transcriptase kit (Thermo Fisher, Waltham, MA, USA), according to the manufacturer's instructions. Gene expression was quantified on a Roche LightCycler® 96 system (Roche Diagnostics, Mannheim, Germany) using gene-specific primers and SYBR Green. The sequence of primers designed with OLIGO v. 7.56 are presented in Table 1. Relative expression levels were calculated using the 2-ΔΔCT.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software; San Diego, CA, USA). An unpaired t-test was used to compare the frequency of apoptotic cells and the expression of autophagy-related genes between the studied groups. Pearson's correlation coefficient was applied to evaluate the relationship between the expression of autophagy-related genes and apoptosis levels. A $P<0.05$ was considered statistically significant.

Bioinformatics Analysis

To further investigate the biological pathways associated with the autophagy-related genes examined in this study, we conducted a bioinformatics analysis using the EnrichR tool (25). The list of differentially expressed autophagy genes, including ATG5, ATG7, LC3B, Beclin1, FOXO1, FOXO3a, FOXO4, and FOXO6, was submitted to the EnrichR web-based platform (https://maayanlab.cloud/Enrichr/). We conducted a pathway enrichment analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) 2021 database (26) to identify significantly enriched biological pathways associated with the input genes. EnrichR reports a combined score that integrates the p-value from Fisher's exact test with the z-score measuring deviation from the expected rank. In this study, a combined score greater than 2 was considered indicative of significant pathway enrichment (25).

RESULTS

Laboratory Findings

The general characteristics of the study groups are summarized in Table 2. The mean ages of the patient and control groups was 27.19±4.13 and 26.34±3.74 years, respectively.

Table 1. Primer sequences of evaluated genes.

Gene	Primer	Sequence (5′→3′)
ATG5	Forward	GCAGATGGACAGTTGCACACAC
	Reverse	GAGGTGTTTCCAACATTGGCTCA
ATG7	Forward	CGTTGCCCACAGCATCATCTTC
	Reverse	CACTGAGGTTCACCATCCTTGG
Beclin1	Forward	CTGGACACTCAGCTCAACGTCA
	Reverse	CTCTAGTGCCAGCTCCTTTAGC
LC3B	Forward	GAGAAGCAGCTTCCTGTTCTGG
	Reverse	GTGTCCGTTCACCAACAGGAAG
FOXO1	Forward	CTACGAGTGGATGGTCAAGAGC
	Reverse	CCAGTTCCTTCATTCTGCACACG
FOXO3a	Forward	TCTACGAGTGGATGGTGCGTTG
	Reverse	CTCTTGCCAGTTCCCTCATTCTG
FOXO4	Forward	ACGAGTGGATGGTCCGTACTGT
	Reverse	CCTTGATGAACTTGCTGTGCAGG
FOXO6	Forward	CCTGCGCATCAAGGGCAAG
	Reverse	GCACTCGGGGAGCTGTCGTC
β-actin	Forward	CACCATTGGCAATGAGCGGTTC
	Reverse	AGGTCTTTGCGGATGTCCACGT

ATG: Autophagy-related; Beclin1: A mammalian ortholog of yeast ATG6; LC3B: Microtubule-associated proteins 1A/1B light chain 3B; FOXO: Forkhead box transcription factors

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Table 2. Clinical characteristics of the studied population

Characteristics	Control (Mean±SD) N=40	Thin endometrium (Mean±SD) N=40	*p* value
Maternal age (years)	26.34±3.74	27.19±4.13	NS
BMI (kg/m2)	25.82±3.31	26.94±3.69	NS
Systolic blood pressure (mmHg)	113.5±11.87	117.8±14.52	NS
Diastolic blood pressure (mmHg)	73.55±7.38	75.14±6.94	NS
Fasting Blood Sugar (mg/dl)	101.4±14.88	105.2±11.35	NS
Triglyceride (mg/dl)	132.8±28.5	133.4±25.32	NS
Cholesterol (mg/dl)	157.8±21.36	161.4±24.17	NS
HDL- Cholesterol (mg/dl)	53.24±6.16	52.18±6.72	NS
LDL-Cholesterol (mg/dl)	105.7±17.81	108.3±16.46	NS

No significant differences were observed in routine laboratory parameters between the thin-endometrium group and the healthy controls.

Flow Cytometry and Apoptosis Assay

Apoptosis in isolated PBMCs was assessed using flow cytometry and Annexin V-PI staining. Flow cytometry analysis revealed a significant increase in apoptosis in the patients compared with the controls. The percentage of apoptotic cells in women with thin endometrium was significantly higher than in healthy control women (p=0.0002, Fig. 1A). Fig. 1B illustrates the proportions of apoptotic cells in unstained, control and patient samples, demonstrating a higher apoptotic rate in the patient group.

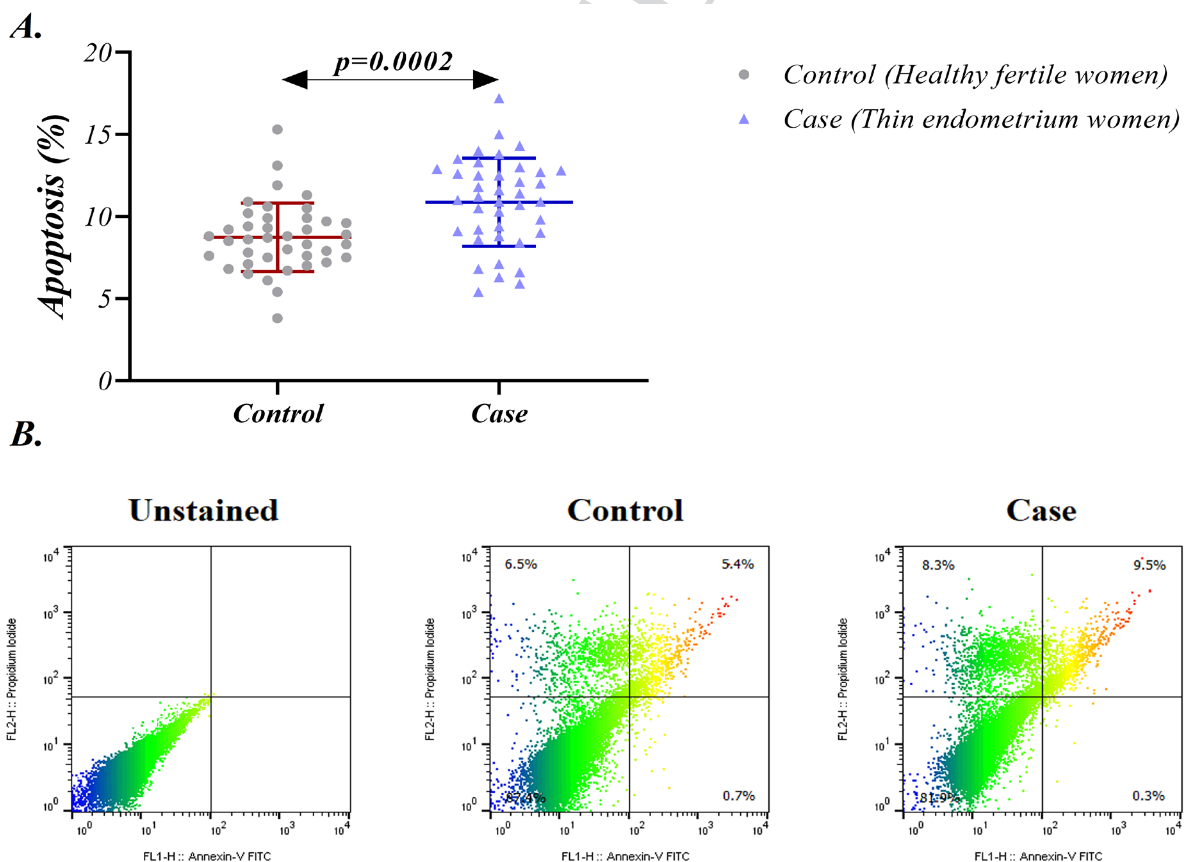

Fig. 1. Cell apoptosis assessed by flow cytometry technique in patients with thin endometrium and healthy controls. A) Comparison of apoptosis levels between patients with thin endometrium and healthy controls. B) Representative dot plots showing the frequency of apoptotic cells in the unstained control, healthy contols and thin endometrium group, respectively.

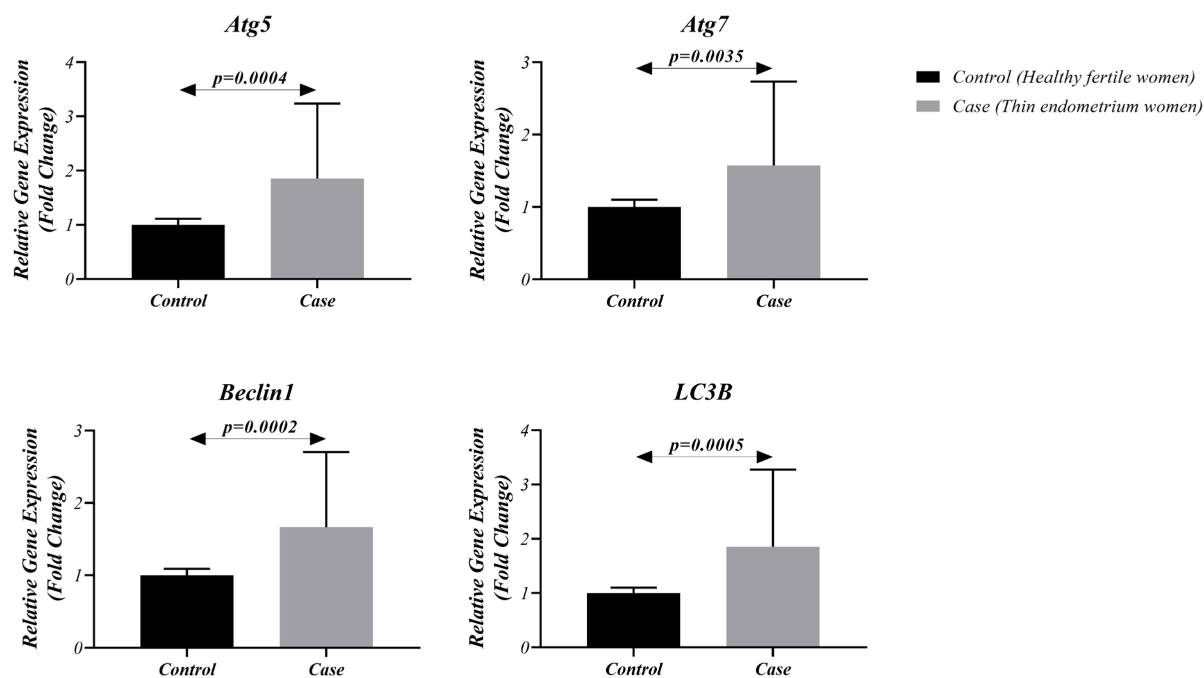

Fig. 2. Expression levels of ATG family genes in patients with thin endometrium compared with healthy controls. $p<0.05$ was considered statistically significant

Table 3. Molecular changes in women with thin endometrium compared with healthy fertile women

Real-Time PCR (Fold Change)			
Target	**Control (Mean±SD) N=40**	**Thin endometrium (Mean±SD) N=40**	***p* value**
ATG5	1.000±0.1129	1.855±1.383	0.0004
ATG7	1.000±0.1004	1.573±1.161	0.0035
Beclin1	1.000±0.09249	1.666±1.037	0.0002
LC3B	1.000±0.1015	1.856±1.420	0.0005
FOXO1	1.000±0.1038	2.043±1.275	<0.0001
FOXO3a	1.000±0.1199	2.275±1.671	<0.0001
FOXO4	1.000±0.08839	2.767±1.809	<0.0001
FOXO6	1.000±0.1172	1.981±1.556	0.0003
Flow cytometry			
Apoptosis (%)	8.735±2.084	10.87±2.689	0.0002

Data are presented as mean±SD. $p<0.05$ considered statistically significant. ATG: Autophagy-related; Beclin1: A mammalian ortholog of yeast ATG6; LC3B: Microtubule-associated proteins 1A/1B light chain 3B; FOXO: Forkhead box transcription factors.

Gene Expression

The expression levels of autophagy-related genes in women with thin endometrium and healthy controls were measured using Real-time PCR (Table 2). A significant difference in the expression of these genes was observed between the patient and control groups.

Increased Expression Levels of ATG Genes in Women with Thin Endometrium

Our results demonstrated significantly elevated expression levels of ATG5 (0.0004), ATG7 (0.0035), Beclin1 (0.0002), and LC3B (0.0005) in women with thin endometrium compared to healthy pregnant controls (Fig. 2; Table 3). Overall, the expression levels of these genes were markedly higher in the thin-endometrium group than in healthy non-pregnant women.

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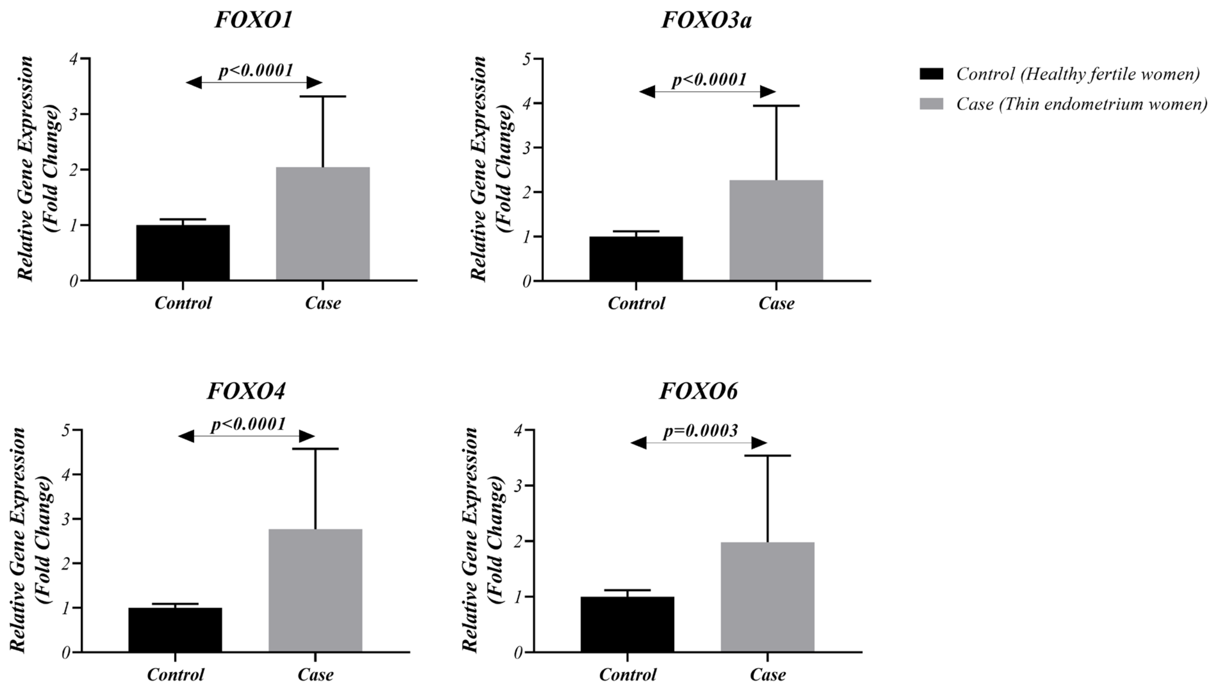

Fig. 3. Expression levels of FOXO family genes in patients with thin endometrium compared with healthy non-pregnant women. Statistical significance was considered at $p<0.05$

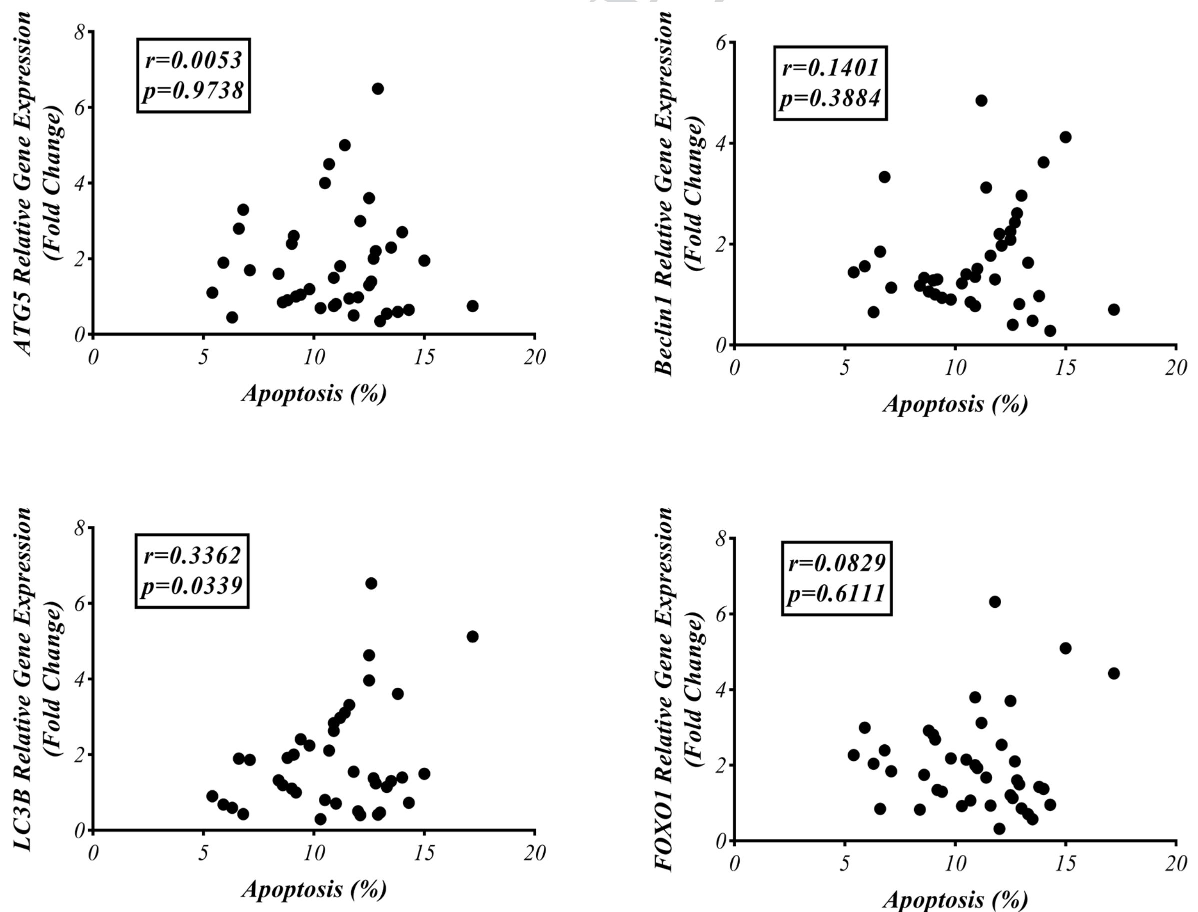

Fig. 4. Correlation between expression levels of autophagy-related genes and apoptosis frequency in PBMC from patients with thin endometrium. Statistical significance was considered at $p<0.05$

Our findings showed significantly elevated expression levels of FOXO1 ($p<0.0001$), FOXO3a ($p<0.0001$), FOXO4 ($p<0.0001$), and FOXO6 ($p=0.0003$) in women with thin endometrium compared with healthy controls (Fig. 3; Table 3).

Correlation Analysis

The correlation between the percentage of apoptotic PBMCs and the expression levels of ATG5, LC3B, Beclin1, and FOXO1 genes was evaluated in patients with thin endometrium. Among the analyzed genes, a significant positive correlation was observed between L3CB expression and the percentage of apoptotic cells ($p=0.0339$, r=0.3362, Fig. 4). The correlation between the percentage of apoptotic PBMCs and the expression levels

Table 4. Results of Bioinformatics Analysis.

Term	*p* value	Adjusted *p* value	Odds Ratio	Combined Score	Genes
Shigellosis (host cell stress / autophagy-related signaling)	1.50E-06	3.16E-05	81.61157	1094.192	FOXO6;FOXO4; FOXO1;ATG5
FOXO signaling pathway (apoptosis & autophagy regulation)	1.50E-05	1.58E-04	93.1125	1034.156	FOXO6;FOXO4; FOXO1
Ferroptosis (regulated cell death)	1.14E-04	7.97E-04	170.5385	1548.508	ATG7;ATG5
Longevity regulating pathway (FOXO-associated survival)	7.07E-04	0.003711	66.30667	481.0329	FOXO1;ATG5
Autophagy (macroautophagy pathway)	0.001269	0.005332	49.02963	326.9852	ATG7;ATG5
Mitophagy (selective mitochondrial autophagy)	0.026883	0.076562	42.48401	153.6333	ATG5
RIG-I-like receptor signaling (immune stress response)	0.027664	0.076562	41.24845	147.9839	ATG5
Prostate cancer (FOXO-linked transcriptional pathways)	0.038154	0.076562	29.60714	96.70056	FOXO1
AGE-RAGE signaling in diabetic complications (oxidative stress)	0.039314	0.076562	28.70563	92.89679	FOXO1
Glucagon signaling pathway (metabolic stress response)	0.042014	0.076562	26.80054	84.95102	FOXO1
Insulin resistance (FOXO metabolic regulation)	0.042399	0.076562	26.54873	83.91052	FOXO1
AMPK signaling pathway (autophagy modulation)	0.047012	0.076562	23.85714	72.93978	FOXO1
Thyroid hormone signaling pathway (metabolic regulation / FOXO link)	0.047395	0.076562	23.65714	72.13611	FOXO1
Insulin signaling pathway (FOXO regulatory axis)	0.053513	0.080269	20.85714	61.06622	FOXO1
Cellular senescence (FOXO-regulated stress response)	0.060733	0.085026	18.28295	51.21548	FOXO1
NOD-like receptor signaling pathway (inflammatory stress response)	0.070159	0.086661	15.72381	41.77794	ATG5
Neutrophil extracellular trap formation (innate immune activation)	0.073158	0.086661	15.04863	39.35412	ATG7
Transcriptional misregulation in cancer (FOXO target pathways)	0.074281	0.086661	14.81002	38.50463	FOXO1
Ras signaling pathway (cell survival / apoptosis)	0.089133	0.098516	12.22078	29.54525	FOXO4
Human papillomavirus infection (cell cycle & apoptosis)	0.125	0.13125	8.511688	17.69958	FOXO1
Pathways in cancer (FOXO-associated oncogenic signaling)	0.193707	0.193707	5.245822	8.610534	FOXO1

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of ATG5, LC3B, Beclin1, and FOXO1 was evaluated in patients with thin endometrium. Among the analyzed genes, a significant positive correlation was observed between LC3B expression and the percentage of apoptotic cells (r=0.3362, *p*=0.0339; Fig. 4). No statistically significant correlations were found for the other genes.

Bioinformatics Analysis

To further elucidate the biological pathways and processes associated with the differentially expressed autophagy-related genes, we conducted a KEGG pathway enrichment analysis using the Enrichr tool (Table 4). The analysis identified several significantly enriched pathways (combined score > 2) in the thin endometrium group. The top-ranked pathways included Ferroptosis (combined score=1548.508), *Shigellosis* (combined score=1094.192), and the FOXO signaling pathway (combined score=1034.156). Other significantly enriched pathways comprised Longevity regulating pathway (combined score=481.0329), *Autophagy* (combined score=326.9852), and Mitophagy (combined score=153.6333). A graphical representation of the gene interactions and the observed

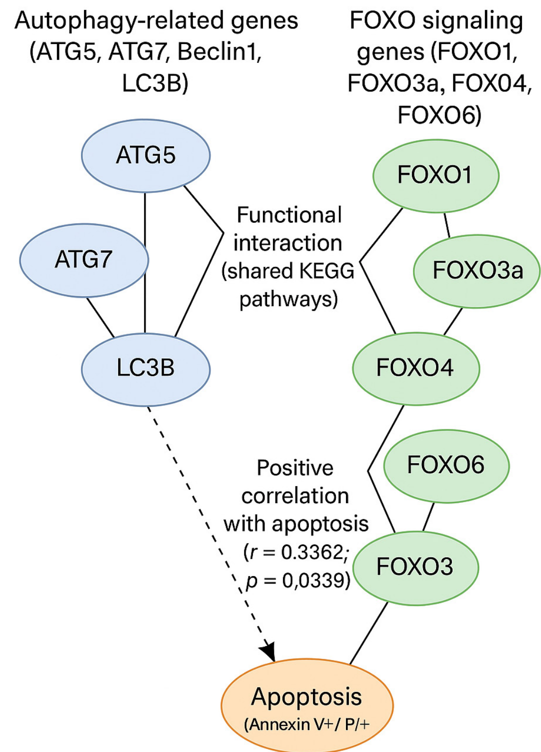

Fig. 5. Gene interaction and correlation diagram. Schematic representation of functional clustering of autophagy-related genes (ATG5, ATG7, Beclin1, LC3B) and FOXO family genes (FOXO1, FOXO3a, FOXO4, FOXO6) based on shared KEGG pathway enrichment analysis. The dashed line indicates the significant positive correlation between LC3B expression and apoptotic cell frequency.

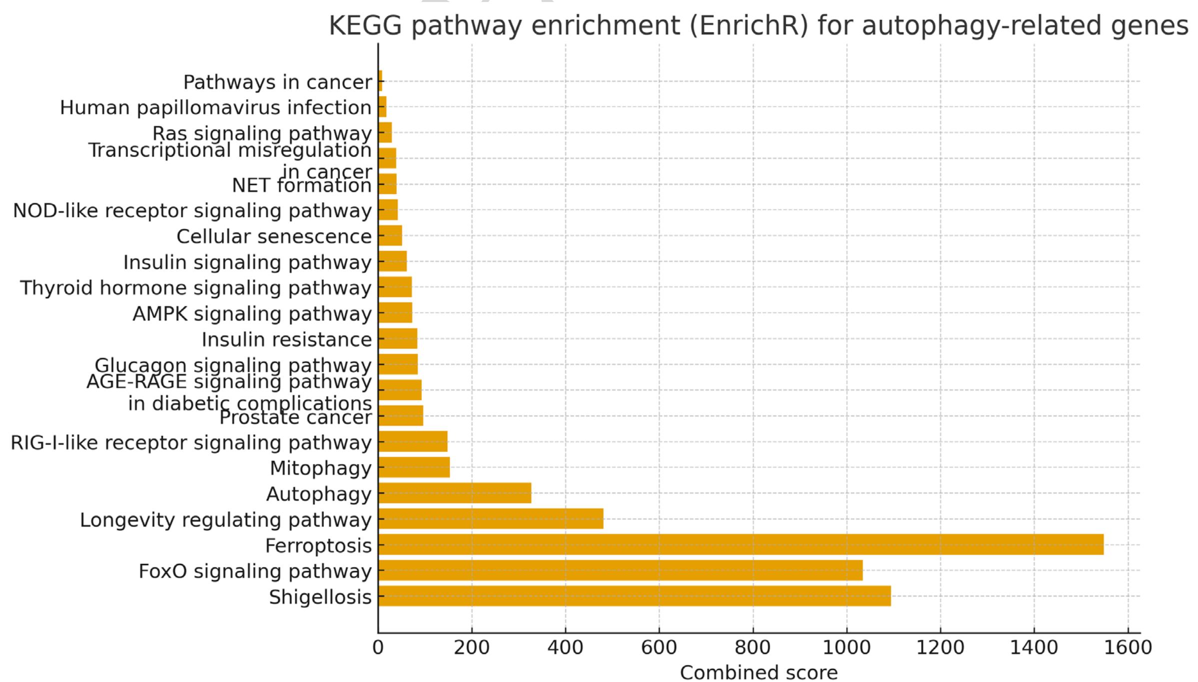

Fig. 6. KEGG enrichment bar plot. Bar plot illustrating the Combined Scores of significantly enriched KEGG pathways associated with the differentially expressed autophagy-related genes.

correlation between LC3B expression and apoptotic cell frequency is presented in Fig. 5. a bar plot depicting the Combined Scores of the enriched pathways is shown in Fig. 6.

DISCUSSION

Endometrial thickness is a critical factor in successful pregnancy. Higher likelihood of achieving and maintaining a full-term pregnancy is associated with an adequately thick endometrium. Optimal endometrial thickness facilitates proper embryo implantation and supports the supply of nutrients required for early embryonic development. Consequently, endometrial thickness is routinely evaluated during in vitro fertilization (IVF) as an important indicator of endometrial receptivity (27). Several studies have reported reduced pregnancy rates in women with a thin endometrium (28, 29). A persistent clinical debate concerns the minimum endometrial thickness required to support successful conception. In general, an endometrial thickness of less than 7 mm on ultrasound is considered suboptimal for in vitro fertilization and has been associated with lower pregnancy success rates.

Autophagy plays a critical and multifaceted role in the endometrium. Emerging evidence supports its central importance in maintaining normal endometrial physiology-including cyclic regeneration, menstruation, implantation, and decidualization during pregnancy–as well as in the development of endometrial pathologies such as hyperplasia, endometriosis, endometrial cancer, and atrophic endometrium (30-33). Autophagy can contribute to cell death through the degradation of essential cellular components, and it may also be activated by apoptosis-inducing stimuli. In studies conducted by Choi et al (33) autophagy was shown to induce apoptosis-mediated cell death in granulosa and luteal cells (34, 35). Based on these previous reports, we first quantified the frequency of apoptotic cells in the patient and control groups. Our findings demonstrated a significant increase in the proportion of apoptotic cell in women with a thin endometrium compared with the controls. Consistent with our observation, Choi et al (33) reported that autophagy plays an essential role in regulating the endometrial cell cycle through apoptosis. In the second stage of our analysis, we assessed the expression levels of key autophagy-related genes, including ATG5, ATG7, Beclin1 (ATG6), LC3B (ATG8), FOXO1, FOXO3a, FOXO4, and FOXCO6. Our results showed a significant difference in gene expression between patients with thin endometrium and healthy non-pregnant women. Specifically, the expression levels of the investigated autophagy-related genes were significantly higher in the patient group compared with controls. The KEGG pathway enrichment analysis revealed that several significantly enriched pathways were associated with the differentially expressed autophagy genes in the thin endometrium group, including "Shigellosis," "FOXO signaling pathway," and "Ferroptosis". The "Shigellosis" pathway, which includes FOXO1, FOXO4, FOXO6, and ATG5, suggests a possible connection between bacterial infection, inflammation, and dysregulated autophagy, all of which may contribute to chronic inflammation and endometrial atrophy (36, 37). Shigellosis, caused by the Shigella species, is known to trigger host cell autophagy as a protective response (38). However, Shigella can also subvert the autophagy machinery to enhance its own intracellular survival and replication (39). Dysregulation of this pathway may disrupt the balance of the endometrial immune response, potentially leading to chronic inflammation and impaired tissue homeostasis, which are key features of endometrial atrophy (40). Dysregulation of this pathway may disrupt the balance of the endometrial immune response, potentially leading to chronic inflammation and impaired tissue homeostasis—both key features of endometrial atrophy (40). The

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"FOXO signaling pathway" was also among the top enriched pathways, underscoring the important role of FOXO transcription factors (FOXO1, FOXO4, FOXO6) in the regulation of autophagy and other cellular processes. FOXO proteins are key regulators of autophagy, cell cycle progression, and cell survival (41). Dysregulation of the FOXO signaling pathway may result in altered endometrial cell proliferation, differentiation, and apoptosis (42), processes that are essential for maintaining normal endometrial thickness and function. Impaired FOXO signaling can disrupt the delicate balance between cell renewal and cell death (43), potentially contributing to the development of endometrial atrophy. In addition, the "Ferroptosis" pathway links dysregulated autophagy to increased cell death in the endometrium (44), implicating autophagy-related genes such as ATG7 and ATG5. Ferroptosis is a form of regulated cell death characterized by the accumulation of lipid peroxides and iron-dependent oxidative stress.The involvement of the autophagy-related genes such as ATG7 and ATG5 in this pathway suggests a potential connection between dysregulated autophagy and increased ferroptosis in the endometrium. Elevated ferroptosis, together with impaired autophagy, may lead to excessive cellular damage and loss of endometrial cells, ultimately contributing to the development of endometrial atrophy. Other enriched pathways, including the "Longevity regulating pathway," "Autophagy," and "Mitophagy," further underscore the central role of autophagy-related genes in maintaining endometrial homeostasis. Dysregulation of these pathways may impair essential cellular processes thereby contributing to endometrial atrophy. Collectively, these findings provide a foundation for exploring targeted therapeutic strategies aimed at restoring normal endometrial function. In a study, Liu and colleagues (45) demonstrated that the embryo survival following transfer is influenced by endometrial thickness, with lower survival rates observed in cases of reduced endometrial thickness. The role of autophagy in endometrial atrophy remains poorly understood. However, several studies have investigated the role of autophagy-related genes in endometrium- associated diseases. For example, Feng et al reported increased expression of the autophagy adapter SQSTM1 in a mouse model of endometrial hyperplasia (32). Many studies have highlighted dysregulated autophagy in endometrial cancer (EC) and the potential of anticancer agents to therapeutically modulate this process. For example, in endometrial adenocarcinoma, increased expression of Beclin1 has been reported and associated with poor prognostic features, including higher tumor grade and myometrial invasion (46). In contrast, another study found reduced Beclin1 expression during neoplastic transformation (47). In the final stage of the study, we assessed the correlation between autophagy-related gene expression and the rate of cell death in patients. Contrary to our expectations, only one gene demonstrated a significant positive correlation with PBMC apoptosis. Specifically,LC3B expression was positively and significantly correlated with the percentage of apoptotic cells. These findings suggest a close association between apoptosis in patients with thin endometrium. However, additional functional and mechanistic studies are required to better interpret the strength and biological significance of the observed correlation coefficient. Accordingly, targeting the interplay between autophagic and apoptotic pathways may represent a promising therapeutic strategy for for the management of thin endometrium.

Limitations of the Study

The main limitation of this study was the relatively small number of participants who met the inclusion and exclusion criteria, particularly among women with thin endometrium. Additionally, the present study analyzed only PBMC samples. Future studies that include analysis of endometrial tissue alongside PBMC samples would provide

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a more comprehensive understanding of the uterine biological environment and the underlying mechanisms.

ABBREVIATION LIST

EA: Endometrial atrophy
ATG: *Autophagy-related*
LC3B: Microtubule-Associated Protein 1 Light Chain 3 beta
FOXO: Forkhead Box O
AMPK: AMP-activated protein kinase
mTORC: Rapamycin complex 1
ER: Endoplasmic reticulum
PE: Phosphoethanolamine
LC3: lipidation of microtubule-associated light chain 3
PBMCs: Peripheral blood mononuclear cells
KEGG: Kyoto Encyclopedia of Genes and Genomes
IVF: In vitro fertilization
EC: Endometrial cancer

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CONFLICT OF INTEREST

The authors declare no relevant financial or non-financial conflicts of interests.

DATA AVAILABILITY

Data will be made available upon reasonable request.

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