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Effects of metformin and ganirelix on subcutaneous endometriosis in a mouse model of autophagy-related cell death

Otofaji ile ilişkili hücrelerde subkütan endometriozis fare modelinde metformin ve genireliksin etkisi

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Abstract

Objective: This study aimed to investigate the efficacy of metformin and ganirelix on subcutaneous endometriotic tissues created in an experimental mouse model.

Materials and Methods: Five groups were formed with eight animals in each group. One of the groups was set as the control group. Endometriotic lesions were created by transplanting 40 mouse autologous endomyometrial tissues into the mouse subcutaneous tissue to a highly vascular surface. Gene expression analyzes of tissues were performed as *HIF-1a*, *ATG5*, *ATG12*, *Beclin2*, *Beclin1*, *LC3BII*, *CateninB*, *GSK3b*, *TCF*, *WNT2*, *WNT7a*, and *WNT10a* gene analyzes. Drug effects were examined by histological examination. HIF1a and WNT2 protein expressions were examined immunohistochemically. Gene expression coefficients of control, metformin day 1 (Met1g), metformin day 7 (Met7g), ganirelix day 1 (Gnx1g), and ganirelix day 7 (Gnx7g) groups are shown in tables. Data are presented as mean and standard error.

Results: *Beclin2* gene expression coefficients of metformin 1st day, metformin 7th day, ganirelix 1st day, and general 7th day groups were found to have significantly decreased compared with the control group coefficient. *Beclin1* gene expression coefficients of metformin 1st day, metformin 7th day, ganirelix 1st day, and genirelix 7th day groups were found to have significantly decreased compared with the control group coefficient. *LC3BII* gene expression coefficients of metformin 1st day and metformin 7th day groups were found to have significantly decreased compared with *LC3BII* gene expression coefficients of control, genirelix 1st day, and genirelix 7th day groups. These findings were supported by histological and immunohistochemical staining.

Conclusion: These genes are actively involved in the autophagy pathway, and we think that the use of metformin in endometriosis might create an autophagy-based suppression mechanism.

Keywords: Endometriosis, ganirelix, metformin, subcutaneous endometriosis, mouse model

Öz

Amaç: Bu çalışmanın amacı, deneysel bir fare modelinde oluşturulan deri altı endometriotik dokular üzerindeki metformin ve genireliksin etkinliğini araştırmaktır.

PRECIS: We aimed to to investigate the efficacy of metformin and ganirelix on subcutaneous endometriotic tissues created in an experimental mouse model might create an autophagy-based suppression mechanism.

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Gereç ve Yöntemler: Her grupta 8 hayvan olacak şekilde 5 grup oluşturuldu. Gruplardan biri kontrol grubu olarak belirlendi. Endometriotik lezyonlar, 40 adet farenin otolog endomyometrial dokusunun fare deri altı dokusuna vasküler bir yüzeye nakledilmesiyle oluşturuldu. Dokuların gen ekspresyon analizleri *HIF1a*, *ATG5*, *ATG12*, *Beclin2*, *Beclin1*, *LC3BII*, *CateninB*, *GSK3b*, *TCF*, *WNT2*, *WNT7a*, *WNT10a* gen analizleri olarak yapıldı. Histolojik inceleme ile ilaç etkileri incelendi. İmmünohistokimyasal olarak HIF1a and WNT2 protein ifadelenmeleri incelendi.

Bulgular: Metformin 1. gün, metformin 7. gün, genireliks 1. gün, genireliks 7. gün gruplarının *Beclin2* gen ifadelenme katsayıları kontrol grubu katsayısı ile karşılaştırıldığında anlamlı olarak azalmış bulundu. Metformin 1. gün, metformin 7. gün, genireliks 1. gün, genireliks 7. gün gruplarının *Beclin1* gen ifadelenme katsayıları kontrol grubu katsayısı ile karşılaştırıldığında anlamlı olarak azalmış bulundu. Metformin 1. gün, metformin 7. gün gruplarının *LC3BII* gen ifadelenme katsayıları kontrol, genireliks 1. gün ve genireliks 7. gün gruplarının *LC3BII* gen ifadelenme katsayıları ile karşılaştırıldığında anlamlı olarak azalmış bulundu. Histolojik ve immünohistokimyasal boyamalar ile bu bulgular desteklendi.

Sonuç: Araştırma kapsamına alınan genler aktif olarak otofaji yolunda yer almaktadır, araştırma bulguları endometrioziste etkin kabul edilen genireliks ile aynı araştırma düzeneğinde incelenen metforminin otofajiye dayalı bir baskılama mekanizması oluşturarak endometriotik odakları baskıladığını desteklemektedir.

Anahtar Kelimeler: Endometriozis, genirelix, metformin, subkütan endometriozis, fare modeli

Introduction

Endometriosis is a chronic inflammatory gynecological condition characterized by the implantation and estrogen-dependent growth of endometrial glands and stromal structures outside the uterine cavity[1]. It usually causes chronic pelvic pain, infertility, menstrual irregularity, and dyspareuni[1]. It is a common disease affecting 7-11% of women of reproductive age and 40% of women undergoing infertility treatment worldwide[2]. It brings a serious economic burden because of a delay in diagnosis, misdiagnosis, and not providing effective treatments despite its high prevalence.

Although the pathophysiology of the disease is not fully known, a wide variety of genetic and environmental factors are thought to be effective[3]. Possible mechanisms such as retrograde menstruation, altered immunity, celomic metaplasia, and metaplastic distribution are considered likely factors in the disease pathophysiology. Although endometriosis is a benign disease, it possesses migration and invasive features similar to cancer[4]. Many studies have reported many factors contributing to the development of this disease, including inflammation, angiogenesis, vascularization, oxidative stress, apoptosis resistance, and immunological dysregulation[5].

The human endometrium is a dynamic tissue undergoing monthly cyclic changes such as proliferation, differentiation, and degeneration. These regular changes, due to alterations in sex hormone concentrations, cause ischemic necrosis in the functional layer of the endometrium, usually because of the contraction of the spiral arteries[6]. Recent studies have reported that apoptosis, a form of programed cell death, is observed in endometrial epithelial cells in the late secretory and menstrual phases and is reduced during the proliferative phase or the beginning of the secretory phase[7]. During the late secretory and menstrual phases of the menstrual cycle, aging endometrial cells are detached from their functional layer by apoptosis[8]. However, non-apoptotic forms of programed cell death have also been observed in human endometrial tissues[8].

Apoptosis and autophagy are two tightly regulated biological processes that play an important role in maintaining tissue homeostasis and cell development. Studies have classified cell death into three forms; apoptosis, autophagic cell death, and necrosis. Autophagy is a double-edged sword with protective and harmful effects[9]. Autophagy is a conservative lysosome-dependent intracellular catabolic process that degrades aged or dysfunctional proteins to regenerate energy and intracellular structures to maintain cellular homeostasis under various stress conditions such as nutrient deprivation, energy depletion, and hypoxia[10]. Autophagy, characterized by the accumulation of autophagic vacuoles, is considered a type II programed cell death known as autophagic cell death[11]. However, uncontrolled autophagy may play a harmful role in cell survival by promoting autophagic cell death[11]. Autophagy plays a significant role in the normal development and maintenance of homeostasis in various tissues, including the female reproductive system[7]. Recent studies have shown the abnormal autophagic activity in endometriosis[11].

In a study investigating Beclin 1 (Becn1) expression in human adenomyosis and its relation to clinical features, it was revealed that Becn1 mRNA and protein expression were significantly decreased in the eutopic endometrium of women with adenomyosis. Besides, Beclin 1 was negatively correlated with serum CA125 and pelvic pain[7]. According to current findings, Becn1 contributes to the initiation and progression of endometriosis. Becn1 is the autophagy protein required for autophagy. Initially, Beclin 1 was identified as a tumor suppressor inducing autophagy and was the first natural link identified between autophagy and carcinogenesis[12]. Beclin 2 (Becn2) has been recently identified as a Becn1 homolog with both autophagy-dependent and independent functions targeting G protein-coupled receptors (GPCRs) for degradation by the endosomal-lysosomal pathway[12]. The LC3BII protein also plays an active role in the autophagy pathway[13].

Ganirelix is a third - generation GnRH antagonist and is released in a dose-dependent and sustained manner. It stops the release of FSH and LH from the pituitary by binding to GnRH receptors. It is used to treat pain due to endometriosis[24]. Recent studies have shown that metformin has several effects and is responsible for autophagic changes in this context[14]. Since the autophagic biomarkers Beclin1, Beclin2 and LC3BII are actively involved in the autophagy pathway, we think that the use of metformin in endometriosis may constitute an

autophagy-based suppression mechanism. Our study aimed to examine the changes in these biomarkers when metformin was used together with ganirelix, a GnRH inhibitor, in a mouse endometriosis model.

Materials and Methods

Study Design

Ethics committee approval of the study was obtained from Sivas Cumhuriyet University (SCU) Animal Experiments Local Ethics Committee (decision no: 65202830-050.04.04-306, date: 28.08.2019). In this study, 40 mice weighing between 20 and 30 g were used and all experiments were performed in the Laboratory of Experimental Animals of SCU Faculty of Medicine. During the experiment, the mice were kept in an environment with ad libitum access to food (standard rat feed) at a constant temperature of 22°C, with a 12-h day and 12-h night cycle. The animals to be used in the experiment were fasted 12 h before and were allowed to drink only water.

Five groups were formed, with 8 animals in each group. One of the groups was set as the control group. Endometriotic lesions were created by transplanting 40 mouse autologous endomyometrial tissues into the mouse subcutaneous tissue to a highly vascular surface. Metformin was started to be administered to one group by adding it to the drinking water of the animals 24 h after the operation and continued until the end of the study. In another group, Ganirelix application was continued until the end of the study at a dose of 10 mg/kg, starting 24 h after the operation. Metformin was added to drinking water 7 days after the operation in one group and continued until the end of the study. Ganirelix at a dose of 10 mg/kg was started 7 days after the operation in one group and continued until the end of the study. The second operation was performed 2 weeks after the first operation, and the endometriotic lesions were evaluated. The degree of shrinkage in endometriotic lesions was recorded. The lesion regression rates in the treatment groups were compared with the control group. The treatments were discontinued after the second operation, and a necropsy operation was performed to evaluate their condition. Biopsies were taken from endometrial lesions in all operations for histopathological score evaluation. For genetic analysis, tissue pieces were placed in pre-sterilized and labeled 1.5 mL Eppendorf tubes and 1 mL of Ribo Saver (Gene All, Seoul, Korea) solution was added to them. Tissue samples were stored at -80 °C until total RNA isolation.

Gene Expression Analyzes

The GeneAll® Hybrid-RTM Kit was used for total RNA isolation (GeneAll® Hybrid-RTM - Seoul, Korea. Cat. No: 305-101- Lot. No: 30519L09056) and procedures were performed according to the manufacturer's instructions. The concentration and purity control of the obtained samples were measured spectrophotometrically (Denovix DS nanodrop). Care was taken to keep the total RNA in the range of approximately 20-40 ng for each sample. WizScript™ cDNA Synthesis Kit (South Korea) was used to obtain cDNA. In the cDNA synthesis stage, the reaction content was created from 10X reaction buffer, 20X dNTP mix, random hexamer, WizScript™ RTase, RNase inhibitor, and RNase free water components, and the kit procedure was applied.

Applied Biosystem Step One Plus real-time PCR (USA) device was used for gene expression analysis. GeneAll Real Amp TM SYBR master mix (Seoul, Korea) was used in Real Time qPCR stage. SYBR Green and passive reference dye ROX were used for quantitative analysis of the synthesized cDNA samples. In the Real-Time qPCR stage, the reaction medium was prepared in 10 µL, including Master mix (2X), ROX (50X), forward and reverse primers (10pmol each), nuclease-free water, and cDNA. For RT-qPCR, reaction conditions of 40 cycles of 10 min of initial denaturation at 95 °C, 15 s of denaturation at 95°C, and 1-minute annealing/extension/melting at 60 °C were used. The primers used for the genes whose expressions were investigated in this study are given below (Table 1). *ACTB* gene was used as a housekeeping gene in the real time qPCR stage. The delta delta Ct Method (ΔΔCt) was used for relative quantification. ΔΔCt values were taken as 2-ΔΔCt, and the fold change of expression level was calculated.

Histopathological Evaluation

The endometriotic cysts were fixed in 10% buffered neutral formalin for 30-36 hours. After routine histological processing, the cyst walls were cut into 5-µm thick sections and stained with hematoxylin-eosin (H&E) for glandular tissue and stromal tissue evaluation in the cyst walls. Glandular and stromal tissues were histopathologically evaluated and scored as described previously[15]. Briefly, while determining the average percentage of stromal tissue-containing areas in each of the 10 high-power areas (HPF), glandular tissue scoring was performed by the average of the gland numbers in these 10 HPF. Then, the score was evaluated as follows: score 0, no stromal and glandular tissue; score 1, <25% stromal tissue and 1 glandular tissue; score 2, 25% to 50% stromal tissue and 2-3 glandular tissue; and score 3, >50% stromal tissue and ≥4 glandular tissue.

The implants were excised and fixed with 10% formalin solution for histological examination. After routine histological follow-ups with alcohol, xylene, and paraffin, the tissues were embedded in paraffin blocks. Serial sections of 5-micrometer thickness were taken from endometriotic foci. After routine deparaffinization, sections from each sample (four sections from each sample) were stained with hematoxylin and eosin, and routine histopathological evaluations were made under a light microscope (Olympus BX50). In control and experimental groups;

Endometrial surface epithelium in each cyst was evaluated as a- well preserved, b- moderately preserved, c- poorly preserved epithelial layer, and d- absence of epithelial cells.

Glandular tissue amount was evaluated as 0 if there is no gland, 1 if there is one gland, 2 if there are one to three glands, and 3 if

Table 1. Primer information used for expression analysis

Primer Name	Primer Sequence
LC3BII-F	TTATAGAGCGATACAAGGGGGAG
LC3BII-R	CGCCGTCTGATTATCTTGATGAG
Beclın1-F	ATGGAGGGGTCTAAGGCGTC
Beclın1-R	TCCTCTCCTGAGTTAGCCTCT
ATG5-F	AGCCAGGTGATGATTCACGG
ATG5-R	GGCTGGGGGACAATGCTAA
ATG12-F?	TCCCCGGAACGAGGAACTC
ATG12-R?	TTCGCTCCACAGCCCATTTC
Beclın2-F	TCAGCCGGAGACTCAAGGT
Beclın2-R	CACAGCGGGTGATCCACATC
HIF1a-F	ACCTTCATCGGAAACTCCAAAG
HIF1a-R	ACTGTTAGGCTCAGGTGAACT
CateninB-F	ATGGAGCCGGACAGAAAAGC
CateninB-R	CTTGCCACTCAGGGAAGGA
GSK3b-F	TGGCAGCAAGGTAACCACAG
GSK3b-R	CGGTTCTTAAATCGCTTGTCCTG
TCF-F	CGCACCAGCAGTACAGATGAG
TCF-R	CAGCTTGGTCTGCGCCTTA
WNT7a-F	TCAGTTTCAGTTCCGAAATGGC
WNT7a-R	CCCGACTCCCCACTTTGAG
WNT10a-F	GCTCAACGCCAACACAGTG
WNT10a-R	CGAAAACCTCGGCTGAAGATG
WNT2-F	CTCGGTGGAATCTGGCTCTG
WNT2-R	CACATTGTCACACATCACCCT
ACTB-F	GGCTGTATTCCCCTCCATCG
ACTB-R	CCAGTTGGTAACAATGCCATGT

there are four or more glands after examining the cyst wall with 20X and 40X magnification.

Stromal tissue amount; After examining the cyst wall with 20X and 40X magnifications, endometrial stroma intensity was evaluated semiquantitatively and scored from 0 to 3.

The presence and severity of inflammation were evaluated as absent, mild, moderate, and severe according to the intensity of inflammatory cells (macrophages, lymphocytes, plasma cells, and eosinophils) in the cyst wall and periphery.

The scoring system used in the evaluation of the examined parameters is summarized in Table 2.

Immunohistochemical Evaluation

Wnt2 and Hif1a expressions were immunohistochemically evaluated in cyst walls. For immunohistochemistry, after

Table 2. Histopathological scoring

Score	Epithelium	Gland Amount	Stroma Amount	Inflammation
0	Absent	No gland	Not observed	Absent
1	Poorly	Decreases: 1	Sparse	Mild
	Preserved	Gland		
2	Moderately	Moderate: 2-3	Moderate	Moderate
	Preserved	Glands		
3	Well	Abundant: ≥4	Abundant	Severe
	Preserved	Glands		

deparaffinization and rehydration, the slides were immersed in boiling 0,01M sodium citrate buffer (pH 6.0) for 10 min, and then incubated in 3% hydrogen peroxide to deactivate endogenous peroxidase. After incubation with ultra V block (Thermo Fisher Scientific), the slides were incubated with primary antibodies (Wnt2, 1:100, BTLAB and BT-AP00075 and Hif1a, 1:100, BTLAB and SKNDRANTK) overnight at 4 °C. The next day, after washing in PBS, sections were incubated with Primary Antibody Enhancer Solution for 20 min (TL-015-PB, Thermo Fisher Scientific, USA) followed by horseradish peroxidase (HRP) Polymer TL-015-PH, Thermo Fisher Scientific, USA) for 30 min at room temperature and subsequently treated with 3,3-diaminobenzidine (DAB) solution for color development. The sections were counterstain with Harris' hematoxylin and evaluated under a microscope (Olympus BX50). The slides were scored as described previously[16]. Wnt2 and HIF1a immunostaining were scored based on intensity and percentage of positive staining cells. The intensities were scored as follows: 0, negative; 1, weak; 2, intermediate; 3, strong. The percentage of positive staining cells was scored as follows: 0, no staining; 1% to 10%; 2, 1% to 50%; 3, >50%. The final immunoreactive score was calculated by multiplying the staining intensity and the percentage of positive staining cells and the final staining score ranged from 0 to 9.

Tissue sections taken from the blocks used in the histopathological examination on the adhesive (silanized) slides with a thickness of 5 micrometers were incubated at 37 °C for 12 h, then deparaffinized in xylol, dehydrated in alcohol, and lowered into the water. Before anti-Wnt2 and Hif1a antibody application, an "antigen retrieval" procedure was applied. Trisodium citrate buffer solution (antigen retrieval solution) prepared as 0.01M (pH 6.0) was used for this procedure. Sections were placed in citrate solution and boiled in a microwave oven at 750 W, 500 W, respectively, for 4.5 min at each stage. It was then treated with 0.3% H_2O_2 for 15 min at room temperature to suppress the peroxidase activity. Before primary antibody application, Ultra V Block solution (Thermo Fisher Scientific) was dripped onto the sections and blocked for 5 min, then primary antibodies Wnt2 (1:100,

BRAND and CATALOG number) and Hif1a (1:100, BRAND and CATALOG number) were applied to the sections without washing and incubated at +4 °C overnight. The next day, the sections were washed with PBS (phosphate buffered saline) and left for 20 min by dripping Lab Vision Primary Antibody Enhancer (Thermo Fisher Scientific) solution and washed with PBS again. Sections washed with PBS 30 min after the HRP Polymer (Thermo Fisher Scientific) solution of Lab Vision was dropped and kept in diaminobenzidine (DAB, Thermo Fisher Scientific) for 3 min as a chromogen to ensure the visibility of the reaction. Harris hematoxylin was used for background staining. Finally, the sections passed through alcohol, and xylol was closed with entellan.

Statistical Analysis

The analyzes were conducted using the Statistical Package for Social Sciences (SPSS) version 23 (IBM Corp., Armonk, NY, USA). The data are presented as mean and standard error. A comparison of gene expression coefficients was performed using the ANOVA test and then the Tukey test. A p-value of <0.05 was considered significant.

Results

The *Beclin2* gene expression coefficients of the metformin day 1, metformin day 7, ganirelix day 1, and ganirelix day 7 groups were found to be significantly decreased compared to the control group. (1.19±0.25 versus 0.42±0.12, 0.23±0.04, 0.50±0.08, and 0.39±0.04, respectively). The *Beclin1* gene expression coefficients of metformin day 1, metformin day 7, ganirelix day 1, and ganirelix day 7 groups were found to be significantly lower than the control group (2.39±0.51 versus 0.95±0.10; 1.03±0.20; 0.68±0.11 and 0.88±0.30, respectively) (Figure 1).

The *LC3BII* gene expression coefficients of the metformin day 1 and metformin day 7 groups were found to be significantly decreased compared to the control, ganirelix day 1, and ganirelix day 7 groups *LC3BII* gene expression coefficients (0.45±0.10 to 0.24±0.05 versus 1.29±0.38, 0.68±0.11 and 1.27±0.32, respectively; p<0.05).

The *CateninB* gene expression coefficients of metformin day 1, metformin day 7, ganirelix day 1, and ganirelix day 7 groups were significantly lower than the gene expression coefficient of the control group (2.53±1.24 vs. 0,17±0.03;0.14±0.06; 0.47±0.09 and 0.55±0.10, respectively).

The *TCF* gene expression coefficient of the ganirelix day 1 group was lower than the gene expression coefficients of the control, metformin day 1, metformin day 7, and ganirelix day 7 groups. (0.41±0.07 versus 1.30±0.31; 0.83±0.34; 0.49±0.10 and 0.59±0.22, respectively).

The *WNT2* gene expression coefficient of the metformin day 7 group was significantly higher than the gene expression coefficients of the control, metformin day 1, ganirelix day 1, and ganirelix day 7 groups (22.17±11.57 vs 1.27±0.27; 3. 32±1.21; 0.93±0.16 compared to 1.35±0.29).

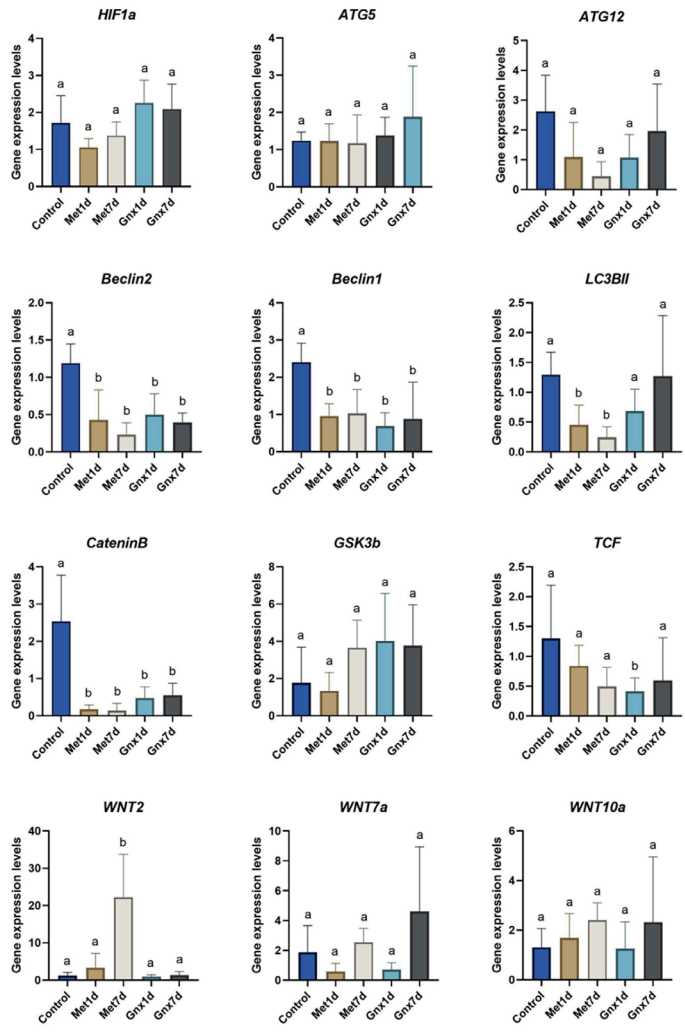

Figure 1. *HIF1a*, *ATG5*, *ATG12*, *Beclin2*, *Beclin1*, *LC3BII*, *CateninB*, *GSK3b*, *TCF*, *WNT2*, *WNT7a*, and *WNT10a* gene expressions. Data are presented as median with interquartile range

Considering endometriosis histomorphology data, the epithelium score of the ganirelix day 7 group was significantly lower than that of the control (denoted by a; p<0.05), and the amount of gland score of the ganirelix day 7 group was significantly lower than that of the control group (denoted by b; p<0.05). Metformin caused a partial decrease, but this difference did not reach significance (p>0.05). Considering immunohistochemical staining data regarding WNT2 and HIF1a (Figures 2 and 3), under the effects of study drugs, the expressions of the study provided some changes, but they did not reach statistical significance and are not conclusive (p>0.05).

Discussion

In this animal model of endometriosis, we tested the effects of ganirelix and metformin and the relationship of their effects with the expression of autophagy-related genes. Under the

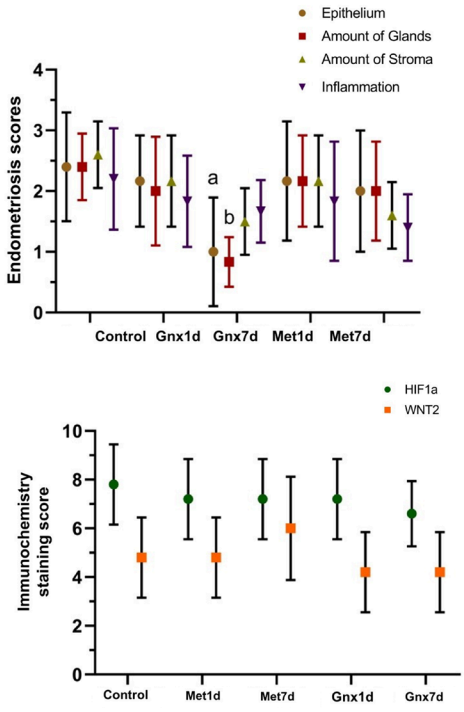

Figure 2. Endometriosis scores and immunochemistry staining scores for all control, gnx1d, gnx7d, met1d, and met7d groups. Data are presented as median with interquartile range. Considering endometriosis histomorphology data, the epithelium score of the gnx7d group was significantly lower than that of the control (denoted by a; $p<0.05$), and the amount of gland score of the gnx7d group was significantly lower than that of the control group (denoted by b; $p<0.05$). Considering immunohistochemical staining data regarding WNT2 and HIF1a

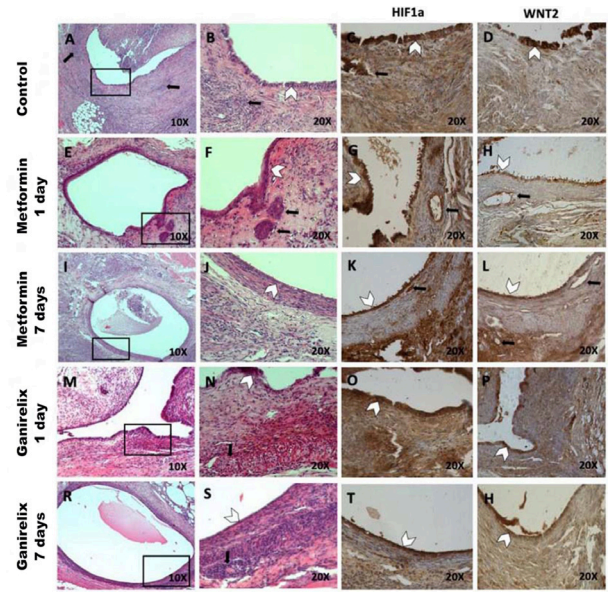

Figure 3. *HIF1a* and *WNT2* gene immunohistochemical evaluation of the control, metformin day 1, metformin day 7, ganirelix day 1, ganirelix day 7 groups. Hif1a immunoreactivity in the control group (C); intensely positive areas in the epithelium, glands, and stroma (immunohistochemistry score: 9, DAB). Wnt2 immunoreactivity in the control group (D); strong positive areas in the epithelium and glands, and medium positive areas in the stroma (immunohistochemistry score: 6, DAB). Metformin day 1 (E-F); endometrial glands in the endometriosis cyst wall with normal epithelium (Epithelial score; 3, Gland score: 3, Stroma score: 2). Hif1a immunoreactivity in the metformin day 1 group (G); strong positive areas in the epithelium and glands and medium positive areas in the stroma (immunohistochemistry score: 6, DAB). Wnt2 immunoreactivity in the metformin day 1 group (H); medium - positive areas in the epithelium and glands, and weak - positive areas in the stroma (immunohistochemistry score: 3, DAB). Metformin day 7 (I-J); endometrial glands in the endometriosis cyst wall with moderately preserved epithelium (Epithelial score; 2, Gland score:1, Stroma score:1). Hif1a immunoreactivity (K) in the metformin day 7 group; strong positive areas in the epithelium and glands and medium positive areas in the stroma (immunohistochemistry score: 6, DAB). Wnt2 immunoreactivity (L) in the metformin day 7 group; strong positive areas in the epithelium and glands and medium positive areas in the stroma (immunohistochemistry score: 6, DAB). Ganirelix day 1 (M-N); endometrial glands in the endometriosis cyst wall with a well-preserved epithelium (Epithelial score; 3, Gland score: 2, Stroma score: 2). Hif1a immunoreactivity (O) in the Ganirelix day 1 group; strong positive areas in the epithelium, glands, and stroma (immunohistochemistry score: 9, DAB). Wnt2 immunoreactivity (P) in the Ganirelix day 1 group; medium-positive areas in the epithelium, glands, and stroma (immunohistochemistry score: 6, DAB). Ganirelix day 7 group (R-S); endometrial glands in the endometriosis cyst wall with poorly preserved epithelium (Epithelial score; 1, Gland score:1, Stroma score:1). Hif1a immunoreactivity (T) in the Ganirelix day 7 group; medium positive areas in the epithelium, glands, and stroma (immunohistochemistry score: 6, DAB). Wnt2 immunoreactivity (U) in the Ganirelix day 7 group; weakly positive areas in the epithelium, glands, and stroma (immunohistochemistry score: 3, DAB). (Arrows: Endometrial glands, arrowheads: epithelium) (H&E, x200)

effects of study agents, *Beclin2* and *Beclin1*, and *CateninB* gene expressions presented a meaningful decrease; however, *LC3BII* gene expression presented a decrease with only metformin. *TCF* and *WNT2* gene expression revealed no strong decrease related to ganirelix and metformin, respectively. Overall, considering the immunohistochemical findings, these gene expression data support that these study drugs can inhibit the growth of endometriotic tissue and they can use autophagy-related pathways.

Although medical treatment methods for endometriosis have increased considerably in recent years, an agent with definite therapeutic efficacy has not yet been developed. The agents in use prevent the implantation, invasion, vascularization, and development of the endometriotic focus by both hormonal and anti-inflammatory mechanisms. The most important goal for treating endometriosis is to reduce or prevent disease-related pain[17]. Combined oral contraceptives (OCS) and progestins are the first-line therapy for treating deep endometriosis, which causes severe pain in women[17]. However, due to the side effects of the drugs in use, new drug searches have emerged.

Subcutaneous endometriosis cases have been frequently recently, affecting the social lives of patients. Increased cesarean

section operations, surgeries related to infertility in recent years, surgeons performing laparoscopic surgeries more comfortably due to the increase in technology, and endometriosis surgeries performed more easily are among the reasons for its emergence. With the increase in intra-abdominal surgeries, endometriotic foci can be moved to different parts of the body and cause disease[18]. Subcutaneous endometriosis cases are therefore more common. Patients may present with complaints of pain, tenderness, and a palpable mass at the incision site after surgery[18]. This situation disrupts the social comfort of people, and the feeling of constant pain can even cause workforce loss. Metformin is an inexpensive and effective treatment option used in many diseases such as polycystic ovary syndrome. Our study aims to evaluate the results of metformin and ganirelix (frequently used, inexpensive, quickly available, and used in the control and treatment of different diseases) in cases of subcutaneous endometriosis, which are common recently. Although rats are seen as more suitable animals for creating an endometriosis model in the literature, endometriosis animal models can be created with mice when suitable conditions are provided. Autologous endometrial pieces were implanted into their peritoneal cavities by autotransplantation, and endometriosis foci similar to those in humans were formed. Thus, in these models, information about the pathophysiology of endometriosis can be obtained and the effectiveness of new treatment agents can be evaluated.

The endometriosis animal model created by Oner et al.[19] has shown that while there was no regression on endometriotic implants in the control group, a statistically significant regression of endometriotic foci in the groups receiving metformin and letrozole. They found the effect of metformin on endometriotic tissues to be at least as significant as letrozole[19]. Yilmaz et al.[20] created a model of endometriosis in rats and randomly divided the animals into groups. Oral metformin 25 mg/kg/day was given to one group, oral metformin 50 mg/kg/day to the other group, and saline to another group. Endometriotic foci were more reduced in groups receiving metformin than in those not taking it. Takemura et al.[21] suggested that metformin, which increases insulin sensitivity and is widely used for diabetes, is effective for treating endometriosis because it has both anti-inflammatory properties and a modulating effect on ovarian steroid production. To determine the efficacy of metformin for treating endometriosis, they evaluated the effects of this agent on the inflammatory response, estradiol production, and proliferation of endometriotic stromal cells (ESCs). ESCs derived from ovarian endometriomas were cultured with varying concentrations of metformin. They measured IL-8 production, mRNA expression and aromatase activity, and the incorporation of 5-bromo-2'-deoxyuridine into ESCs. Metformin dose-dependently suppressed IL-1beta-induced IL-8 production, cAMP-induced mRNA expression and aromatase activity, and the incorporation of 5-bromo-2'-deoxyuridine into ESCs. Thus with these results, they argued that further research is needed on metformin's unique therapeutic potential as an anti-endometriotic drug. Zhou et al.[22] reported that metformin was able to inhibit PGE2-induced CYP19A1 mRNA expression and aromatase activity in human ESCs through AMPK activation and inhibition of CREB to CYP19A1 PII, and they argued that metformin may have unique therapeutic potential as an anti-endometriotic drug in the future. Zhang et al.[23] investigated the effect of metformin on the crosstalk of stromal-epithelial cells in endometriosis. Metformin regulates stromal-epithelial cell communication in endometriosis via Wnt2/β-catenin signaling[24].

Conclusion

In this animal model of endometriosis, ganirelix, and metformin affected the expression of autophagy-related genes and provided an anti-endometriotic effect. In the search for new drugs for the treatment of endometriosis, it seems useful to include genirelix and metformin, which have been shown in our study to cause changes in gene expressions involved in important molecular mechanisms such as autophagy, in the scope of further research.

Ethics

Ethics Committee Approval: Ethics committee approval of the study was obtained from Sivas Cumhuriyet University (SCU) Animal Experiments Local Ethics Committee (decision no: 65202830-050.04.04-306, date: 28.08.2019).
Informed Consent: Not necessary.
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Authorship Contributions

Surgical and Medical Practices: G.S.Ü., Y.A., Ç.Y., Concept: G.S.Ü., Y.A., Ç.Y., M.Ç., A.Ç., Design: G.S.Ü., Y.A., M.Ç., S.D.D., E.G., A.Ç., Data Collection or Processing: G.S.Ü., Y.A., S.D.D., E.G., Analysis or Interpretation: G.S.Ü., N.Y., S.D.D., E.G., A.Ç., Literature Search: G.S.Ü., N.Y., A.Ç., Writing: G.S.Ü., N.Y., A.Ç.

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