creative commons
COMMONS DEED

저작자표시-비영리-변경금지 2.0 대한민국

이용자는 아래의 조건을 따르는 경우에 한하여 자유롭게

- 이 저작물을 복제, 배포, 전송, 전시, 공연 및 방송할 수 있습니다.

다음과 같은 조건을 따라야 합니다:

저작자표시. 귀하는 원저작자를 표시하여야 합니다.

비영리. 귀하는 이 저작물을 영리 목적으로 이용할 수 없습니다.

변경금지. 귀하는 이 저작물을 개작, 변형 또는 가공할 수 없습니다.

- 귀하는, 이 저작물의 재이용이나 배포의 경우, 이 저작물에 적용된 이용허락조건을 명확하게 나타내어야 합니다.
- 저작권자로부터 별도의 허가를 받으면 이러한 조건들은 적용되지 않습니다.

저작권법에 따른 이용자의 권리는 위의 내용에 의하여 영향을 받지 않습니다.

이것은 이용허락규약(Legal Code)을 이해하기 쉽게 요약한 것입니다.

Disclaimer

dCollection

연세대학교
YONSEI UNIVERSITY

Formononetin Inhibits Progression of Endometriosis via Regulation of p27, pSTAT3, and Progesterone Receptor: In Vitro and In Vivo Studies

Yunjeong Park

Department of Medicine

The Graduate School, Yonsei University

연세대학교
YONSEI UNIVERSITY

Formononetin Inhibits Progression of Endometriosis via Regulation of p27, pSTAT3, and Progesterone Receptor: In Vitro and In Vivo Studies

Directed by Professor SiHyun Cho

The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Medical Science

Yunjeong Park

December 2023

연세대학교
YONSEI UNIVERSITY

This certifies that the Doctoral Dissertation of
Yunjeong Park is approved.

Thesis Supervisor: SiHyun Cho

Thesis Committee Member#1: Young Sik Choi

Thesis Committee Member#2: Jung-Ho Shin

Thesis Committee Member#3: Jong-Baeck Lim

Thesis Committee Member#4: Hak-Joon Sung

The Graduate School
Yonsei University
December 2023

연세대학교
YONSEI UNIVERSITY

ACKNOWLEDGEMENTS

I would like to take this opportunity to express my sincere gratitude to all those who have supported and encouraged me throughout my doctoral journey.

연세대학교
YONSEI UNIVERSITY

<TABLE OF CONTENTS>

ABSTRACT ····· iv

I. INTRODUCTION ····· 1
II. MATERIALS AND METHODS ····· 2
1. Study Population and Ethical Consideration ····· 2
2. Formononetin ····· 2
3. Primary Cell Culture ····· 3
4. Cell Counting Kit-8 Assay (CCK 8 Assay) ····· 3
5. Western Blot Analysis ····· 4
6. Fluorescence-Activated Cell Sorting (FACS) ····· 4
7. Wound Healing Assay ····· 4
8. Mouse Model of Endometriosis and Ethical Consideration ····· 5
9. Immunohistochemistry (IHC) ····· 5
10. Statistical Analysis ····· 5
III. RESULTS ····· 6
1. Formononetin Affected Endometrial Cell Viability ····· 6
2. Formononetin Decreased Expression of p27 and pSTAT3 on Eutopic Endometrium with Endometriosis and p27 of Ectopic Endometrium with Endometriosis in Dose-Dependent Manner ····· 6
3. Formononetin Did Not Affect Apoptosis in Eutopic Endometrium with and without Endometriosis ····· 8
4. Formononetin Inhibits Cell Migration in Eutopic Endometrium with and without Endometriosis ····· 10
5. Formononetin Decreased Expression of Progesterone Receptor-B (PR-B) on Eutopic Endometrium with Endometriosis in Dose-Dependent Manner ····· 11
6. Mouse Model of Endometriosis ····· 12
IV. DISCUSSION ····· 16

연세대학교
YONSEI UNIVERSITY

V. CONCLUSION ····· 18

REFERENCES ····· 19
APPENDICES ····· 22
ABSTRACT(IN KOREAN) ····· 23
PUBLICATION LIST ····· 25

연세대학교
YONSEI UNIVERSITY

LIST OF FIGURES

Figure 1. CCK8 assay showed decreased cell viability in eutopic endometrium without endometriosis ····· 6
Figure 2. Band of Western blot of BAX, p53, pAKT, pERK, and p27 in eutopic endometrium without endometriosis ····· 7
Figure 3. Formononetin did not affect apoptosis on eutopic endometrial cells with and without endometriosis ····· 9
Figure 4 Formononetin affects cell migration in eutopic endometrial cells with and without endometriosis, but has more anti-proliferative effect in eutopic endometrium with endometriosis in wound healing assay ····· 10
Figure 5. Relative expression levels of androgen receptor (AR), estrogen receptor (ER), progesterone receptor (PR), ERβ/α, and PR-B/A in eutopic endometrium with endometriosis had decreasing tendency, but there is statistical significance only in PR-B ····· 11
Figure 6. Mouse endometriosis model results ····· 13
Figure 7. H&E staining of the endometriotic implants of each treatment group ····· 14
Figure 8. Immunohistochemistry staining for p27 expression of the endometriotic implants of each treatment group ····· 15

연세대학교
YONSEI UNIVERSITY

ABSTRACT

Formononetin Inhibits Progression of Endometriosis via Regulation of p27, pSTAT3, and Progesterone Receptor: In Vitro and In Vivo Studies

Yunjeong Park

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor SiHyun Cho)

Objectives: Formononetin is one of the phytoestrogens that functions like a selective estrogen receptor modulator (SERM). In this study, we evaluated the effects of formononetin on endometriosis progression in vitro and in vivo.

Materials and methods : After pathological confirmation, 10 eutopic and ectopic endometria were collected from patients with endometriosis. Ten eutopic endometria samples were collected from patients who did not have endometriosis. To determine the cytotoxic dose and therapeutic dose of formononetin, the concentration of 70% of the cells that survived after formononetin administration was estimated using a Cell counting kit-8 (CCK 8) assay. Western blot analysis was used to determine the relative expression levels of BAX, p53, pAKT, ERK, pERK, p27, and pSTAT3 in the eutopic endometria without endometriosis, eutopic endometria with endometriosis, and ectopic endometria with endometriosis as the formononetin concentration was increased. We confirmed the effect of formononetin on apoptosis and migration in endometriosis using fluorescence-activated cell sorting (FACS) and wound healing assays, respectively. A mouse model of endometriosis was prepared using a non-surgical method, as previously described. The mice were intraperitoneally administered formononetin for four weeks after dividing them into control, low-dose formononetin (40 mg/kg/day) treatment, and high-dose (80 mg/kg/day) formononetin treatment groups. All the mice were euthanized after formononetin treatment. Endometriotic lesions were retrieved and confirmed using hematoxylin and eosin (H&E) staining. Immunohistochemical (IHC) staining of p27 was

연세대학교
YONSEI UNIVERSITY

performed.

Results: We set the maximum concentration of formononetin administration to 80 μM through the CCK8 assay. Based on formononetin concentration, the expression levels of BAX, p53, pAKT, ERK, pERK, p27, and pSTAT3 proteins were measured using Western blot analysis (N = 4 per group). The expression level of pERK, p27, and pSTAT3 in eutopic endometrium with endometriosis tended to decrease with increasing formononetin concentration, and a significant decrease was noted at 80 μM. The expression of p27 in ectopic endometrium with endometriosis was also significantly decreased at 80 μM of formononetin. FACS analysis revealed that formononetin did not significantly affect apoptosis. In the wound healing assay, formononetin treatment revealed a more significant decrease in the proliferation of the eutopic endometrium in patients with endometriosis than in the eutopic endometrium without endometriosis. Relative expression of sex hormone receptors decreased with increasing formononetin doses. Although no significant differences were observed in the ER, PR-A, ERβ/ERα, and PR-B/PR-A, significant down-regulation of PR-B expression was noted after formononetin treatment at 80 μM. In the in vivo study, endometriotic lesions in the formononetin-treated group significantly decreased compared to those in the control group. The relative expression of p27 using IHC was highest in the control group and lowest in the high-dose formononetin treatment group.

Conclusion: Formononetin treatment was shown to inhibit the proliferation of eutopic and ectopic endometria in patients with endometriosis through the regulation of p27, pSTAT3, and PR-B. In an endometriosis mouse model, formononetin treatment significantly reduced the number of endometriotic lesions with decreased p27 expression. The results of this study suggest that formononetin may be used as a non-hormonal treatment option for endometriosis.

Keywords : formononetin; endometriosis; kip1 protein; p27; stat3 transcription factor; endometriosis mouse model

연세대학교
YONSEI UNIVERSITY

Formononetin Inhibits Progression of Endometriosis via Regulation of p27, pSTAT3, and Progesterone Receptor: In Vitro and In Vivo Studies

Yunjeong Park

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor SiHyun Cho)

I. INTRODUCTION

Endometriosis is a chronic gynecologic disease that pathologically defines the endometrial stromal and glandular tissue outside the uterus[1]. Endometriosis is found in approximately 10% of reproductive-aged women and is known to account for 25–40% of female infertility.[2 3] The etiology of endometriosis remains indefinite, but it is well known to be related to high estrogen levels and progesterone resistance[4 5].

Management of endometriosis requires consideration of subjective factors, such as patient symptoms, individual preferences and compliance, previous treatment, medical history, and family planning. Surgical treatment can destroy endometriotic lesions and dissect adhesive lesions, resulting in pain and subfertility. However, surgical treatment may not provide complete relief of symptoms, and it may induce a reduction in ovarian reserve[6].

Traditionally, hormonal treatment has been the primary treatment option for endometriosis. The main therapeutic strategy is to lower local or systemic estrogen levels. Progestins and oral contraceptives, as well as gonadotropin-releasing hormone (GnRH) agonists, are widely used to treat endometriosis-associated pain and prevent the recurrence of the disease after surgery[7]. Recently, GnRH antagonists have also been approved as a new treatment option for endometriosis[8]. Aromatase inhibitors, especially in post-menopause women[9], and COX inhibitors are another treatment option[10]. Approximately 50% of women diagnosed with endometriosis experience a recurrence of symptoms within 5 years despite proper treatment[11]. New treatments for endometriosis are being sought for various reasons, such as the recurrence of endometriosis despite appropriate treatment.

Phytoestrogens have received attention as potential therapeutic candidates for endometriosis. Phytoestrogens are compounds from plants that act as selective estrogen modulators in a tissue-specific manner[12]. Phytoestrogens can offer anti-proliferative, anti-inflammatory, antioxidant, and analgesic properties[13]. Specifically, formononetin, a phytoestrogen derived from *Pongamia pinnata*, *Astragalus membranaceus*, *Ononis angustissima*, and *Trifolium pratense* (*T. pratense*)[14], has been shown to have anti-tumor

연세대학교
YONSEI UNIVERSITY

effects such as anti-proliferative, apoptosis-inducing, anti-cell migration, and anti-invasion effects on the breast, prostate, colon, nasopharyngeal, and lung cancer cells in vitro. Additionally, similar anti-tumor effects have been demonstrated under various tumor conditions in vivo experiments as well[15].

To date, no studies have reported the effects of formononetin on endometriosis pathogenesis. In this study, we evaluated the effects of formononetin on endometriosis and investigated its potential as a therapeutic agent for endometriosis using in vitro and in vivo experiments.

II. MATERIALS AND METHODS

1. Study Population and Ethical Consideration

This study included 20 women. The patients underwent laparoscopic surgery for benign ovarian cysts at Gangnam Severance Hospital (Seoul, Republic of Korea) between 1 January 2018 and 31 December 2019. After pathological confirmation, patients were assigned to the study group. Ten patients were assigned to the endometriosis group. Eutopic and ectopic endometrial tissue samples were collected from patients with endometriosis. Ten patients without endometriosis diagnosed with benign ovarian cysts were assigned to the control group. Eutopic endometrial tissue samples without endometriosis were collected from patients of the control group.

This study was approved by the Institutional Review Board (IRB) of the Gangnam Severance Hospital, Yonsei University College of Medicine, Seoul, Republic of Korea (IRB approval number: 2021-0854-001). All patients in this study provided written informed consent, and the hospital's ethics committees approved enrollment.

2. Formononetin

Formononetin has a molecular formula as follows: $C_{16}H_{12}O_4$ (CAS no:485-72-3, IUPAC:7-hydroxy-3-(4-methoxyphenyl) chromen-4-one) and a molecular weight of 268.268 g/mol. It can be extracted from *Pongamia pinnata*, *Astragalus membranaceus*, *Ononis angustissima*, and *Trifolium pratense* (*T. pratense*)[14]. Formononetin was purchased from Sigma-Aldrich (St. Louis, MO, USA).

3. Primary Cell Culture

Each tissue sample was finely minced in Hank's balanced salt solution containing hydroxyethyl piperazine ethane sulfonic acid (HEPES) (2 mmol/mL), penicillin (100 U/mL)/streptomycin (100 μg/mL), and collagenase (1 mg/mL, 15 U/mg). After 1 h of incubation at 37 °C, the dispersed cells were pelleted, washed, and resuspended in Dulbecco's modified Eagle's medium/Ham´s Nutrient Mixture F12 (DMEM/F-12) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. After filtering using a 40 μm mesh cell strainer (Falcon, Corning, NY, USA), the cells were plated on 75 cm^2 Falcon tissue culture flasks (BD Biosciences, Bedford, MA, USA). The analyses included cultured cells at 3–5 passages.

연세대학교
YONSEI UNIVERSITY

4. Cell Counting Kit-8 Assay (CCK 8 Assay)

After cell culture, eutopic endometrial cells from patients with and without endometriosis were assessed for cell viability and cytotoxicity using a cell counting kit 8 (CCK8) (Sigma-Aldrich). The cells in the exponential growth phase were seeded in 96-well plates at a density of 1×10^3 cells/100 μL. After 24 h of culture at 37 °C, 0, 20, 40, 80, 100, and 200 μL of formononetin was added to each well. A total of 20 μL of CCK8 solution (CA1210-100, Beijing Solarbio Science & Technology, Co., Ltd., Beijing, China) was added to each well, including empty wells as controls. The optical density (OD) of each well was measured at 450 nm using a microplate reader (Bio-Rad 680; Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 0.5, 1, 2, and 4 h after adding the CCK8 solution. The resulting OD was calculated by subtracting the control OD from the measured OD. The cell proliferation curves were plotted using these values.

5. Western Blot Analysis

Extraction of protein was carried out using radio-immunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, Rockford, IL, USA) and protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Rockford, IL, USA). Extracted protein concentration was measured using a bicinchoninic acid assay kit (Thermo Scientific, Waltham, MA, USA). A 20 μg protein sample was mixed with sodium dodecyl sulfate–polyacrylamide gel (SDS-PAGE) loading buffer (Biosesang, Seongnam, Gyeonggi, Republic of Korea). After heating at 95 °C for 5 min, protein sample was separated by SDS-PAGE. Then, protein sample was electrotransferred to a polyvinylidene fluoride membrane (Millipore, Burlington, MA, USA) using a transblot apparatus (Bio-Rad, Hercules, CA, USA). The polyvinylidene fluoride membranes (Millipore, Burlington, MA, USA) were blocked by 5% non-fat skim milk in Tris-buffered saline solution (10 mmol/L Tris-HCl (pH 7.4) and 0.5 mol/L NaCl) and 0.1% (*v/v*) Tween-20. The blots were probed with primary antibodies against the following proteins: phosphorylated protein kinase B (pAKT) (1:1000; Cell Signaling Technology, Danvers, MA, USA), BCL2 associated X (BAX) (1:1500, Cell Signaling Technology, Danvers, MA, USA) extracellular signal-regulated kinase (ERK) (1:1500 with 5% skim milk; Cell Signaling Technology, Danvers, MA, USA), phosphorylated ERK (pERK) (1:1000 with 5% skim milk; Cell Signaling Technology, Danvers, MA, USA), phosphorylation signal transducer and activator of transcription 3 (pSTAT3) (1:2000; Cell Signaling Technology, Danvers, MA, USA), Cyclin-dependent kinase inhibitor 1B ($p27^{Kip1}$) (1:1000; Cell Signaling Technology, Danvers, MA, USA), p53 (1:1200, Cell Signaling Technology, Danvers, MA, USA), androgen receptor (AR) (1:400, Santa Cruz Biotechnology, Palo Alto, CA, USA), estrogen receptor (ERα) (1:100, Santa Cruz Biotechnology, Palo Alto, CA, USA), estrogen receptor (ERβ) (1:100, Santa Cruz Biotechnology, Palo Alto, CA, USA), progesterone receptor (PR-A) (1:200, Santa Cruz Biotechnology, Palo Alto, CA, USA), progesterone receptor (PR-B) (1:200, Santa Cruz Biotechnology, Palo Alto, CA, USA), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:1000, Abcam, Cambridge, UK).

연세대학교
YONSEI UNIVERSITY

For secondary antibody, a horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody (1:2000; Thermo Fisher Scientific) was used. Enhanced chemiluminescence (Santa Cruz Biotechnology, Dallas, TX, USA) was used for protein detection. All experiments were performed in triplicates. Relative OD of blot was measured by ImageQuant LAS 4000 (GE Healthcare, Little Chalfont, UK), and the blot images were analyzed using ImageJ software (https://imagej.nih.gov/ij/, version 1.53k; Java 1.8.0_172, Wayne Rasband, US National Institutes of Health) accessed on 1 March 2022.

6. Fluorescence-Activated Cell Sorting (FACS)

FACS (BD Cell Quest® version 3.3) was carried out using an Annexin V-Fluorescein isothiocyanate (FITC) Early Apoptosis Detection Kit (Cell Signaling Technology, Inc.). This kit included Annexin V-FITC and propidium iodide (PI) to confirm changes in the apoptotic populations of eutopic endometrial cells from patients with and without endometriosis treated with formononetin. Eutopic endometrial cells from patients with and without endometriosis were cultured and treated with 0, 80 μM formononetin for further 24 h. The plate on which the cells adhered was rinsed with Dulbecco's phosphate-buffered saline (DPBS), and cells were detached using trypsinization. The cells underwent resuspension in 1X binding buffer (BD Biosciences). An amount of 1 μL Annexin V-FITC and 12.5 μL PI were added to 96 μL cell suspension. After incubation for 15 min at 37 °C, each apoptotic cell population was analyzed by the BD Cell Quest® version 3.3. software (Becton Dickinson and Company, Franklin Lakes, NJ, USA).

7. Wound Healing Assay

Eutopic endometrial cells from patients with and without endometriosis were seeded in 6-well culture plates (5.0×10^5 cells/well) containing DMEM/F12, 10% FBS, and antibiotics with 0 or 80 μM formononetin. The culture plates were placed in a humidified atmosphere containing 5% CO_2/95% air at 37 °C for 24 h. A linear wound was made by a sterile 200 μL pipette tip. After washing the debris with phosphate-buffered saline (PBS) and adding culture media, the culture plates were incubated for 24 h at 37 °C with 5% CO_2. The images obtained by EVOS inverted microscope (Advanced Microscopy Group, Mill Creek, WA, USA) were analyzed using ImageJ software with the following calculation: [(mean wound width × mean remaining width)/mean wound width × 100 lrb%].

8. Mouse Model of Endometriosis and Ethical Consideration

All procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. This in vivo study was approved by the Institutional Animal Care and Use Committee of Gangnam Severance Hospital, Yonsei University College of Medicine, Seoul, Republic of Korea (Protocol number: 2021-0220). Healthy female C57BL/6 mice (seven weeks of age) were imported from Orient Bio Inc. (Seongnam, Gyeonggi, Republic of Korea). The mice were randomly assigned. They were housed in standard clear plastic cages, with five mice in each cage under a 12:12 h light/dark cycle at 21 °C with ad libitum access to water and food. Euthanasia was induced

연세대학교
YONSEI UNIVERSITY

by CO_2 inhalation, and an effort was made to minimize the suffering of the animals in all procedures. A mouse model of endometriosis was established using a non-surgical method. Uterine tissues were obtained from 15 donor mice that had grown endometria by subcutaneous injection of 3 mg estradiol benzoate, sliced to <1 mm in diameter, and injected intraperitoneally into 30 recipient mice, as previously described[16].
One week after endometriosis induction, the mice were randomly assigned to three groups: control (0 mg/kg/day) group (N = 10), low-dose formononetin (40 mg/kg/day) group (N = 10), and high-dose formononetin (80 mg/kg/day) group (N = 10). The dose of formononetin administered in this study was set with reference to the dose administered in previously published in vivo tumor-bearing animal models[15]. Formononetin was injected intraperitoneally daily. After four weeks of formononetin treatment, no difference was observed in the general appearance of all three groups. All 30 mice were euthanized for the confirmation and retrieval of endometriotic lesions. Endometriotic lesions included implants and cysts. These values were based on the length and width of each lesion. The implants were retrieved, fixed in 10% formalin-acetic acid, and embedded in paraffin. Hematoxylin and eosin staining of paraffin-embedded sections was carried out to confirm endometriosis. Immunohistochemical staining was performed to confirm p27 expression.

9. Immunohistochemistry (IHC)

After deparaffinization with xylene and dehydration with an alcohol gradient, 3% H_2O_2 was applied for 10 min to inactivate endogenous peroxidase activity. After incubation with 10% normal goat serum in PBS for 1 h at room temperature to block non-specific binding, the slides were incubated with primary antibody p27 overnight at 4 °C, followed by biotinylated goat anti-rabbit IgG (Sigma, MO, USA) for 1 h at room temperature. A streptavidin–biotin–peroxidase complex assay was carried out. The expression of p27 was analyzed by ImageJ software with the IHC Image Analysis Toolbox. Briefly, IHC toolbox identified the chromogen-stained areas and assigned them to the region of interest (ROI). The IHC-stained ROI was darker and had values of <255, and unstained or white areas in the RGB images had a maximum value of 255.

$$\text{IHC staining intensity} = [(255 - \text{ROI stain score}) \times \%\text{ROI (ROI pixels/total image pixels} \times 100)].$$

10. Statistical Analysis

Data are presented as means ± standard deviation (SD). Data from in vitro experiments were analyzed using Kolmogorov–Smirnov or Shapiro–Wilk tests to investigate whether they were normally distributed. Student's t-test or the Mann–Whitney U test was used to evaluate the difference between groups. The in vivo data were analyzed using the one-way analysis of variance to investigate differences between three groups, followed by Bonferroni's post hoc analysis. All statistical analyses were performed using SPSS 25.0 software (SPSS Inc., Chicago, IL, USA). A p-value < 0.05 was defined as statistically significant.

연세대학교
YONSEI UNIVERSITY

III. RESULTS

1. Formononetin Affected Endometrial Cell Viability.

The CCK8 assay was carried out to investigate the cytotoxicity of formononetin in eutopic endometrial cells from patients with and without endometriosis. Formononetin decreased the cell viability in a dose-dependent manner in both endometrial cell lines. The decrease in cell viability was statistically significant over 80 μM in eutopic endometrial cells from patients with endometriosis (1 vs. 0.73 ± 0.02, $p = 0.013$) and over 200 μM in eutopic endometrial cells without endometriosis (1 vs. 0.37 ± 0.15, $p = 0.02$) (**Figure 1**). In this study, we set the maximum dose at 80 μM with a cell viability of 70% when formononetin was used.

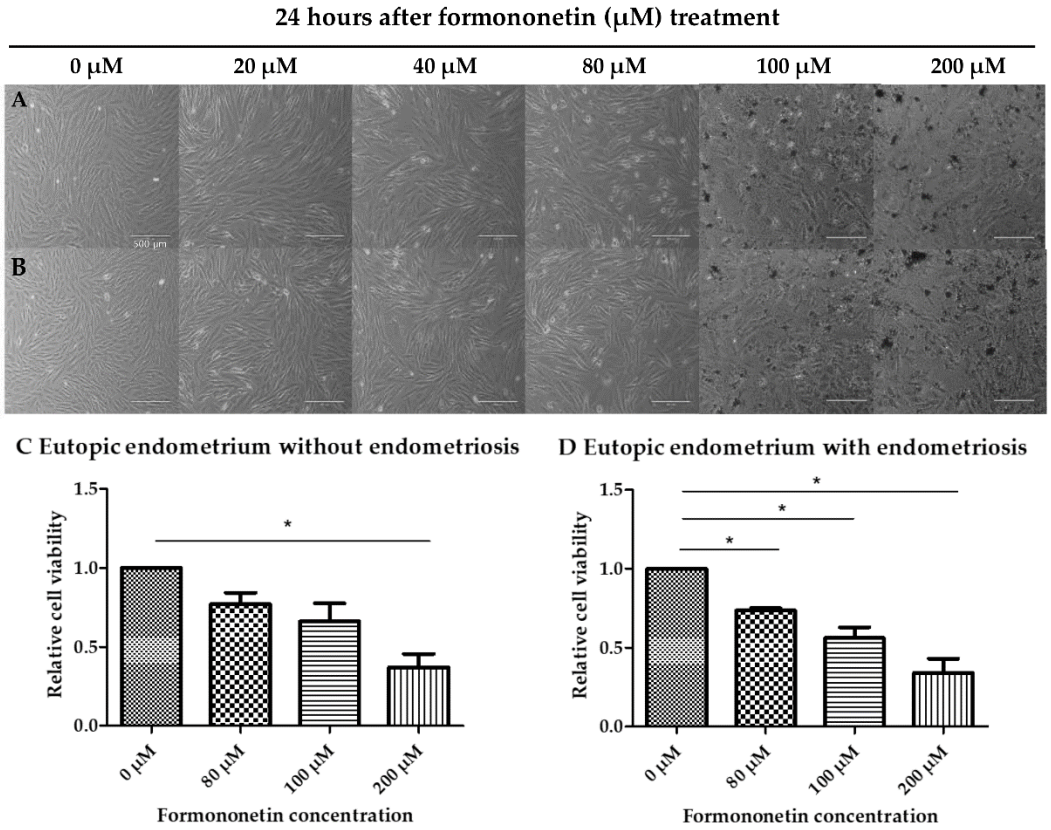
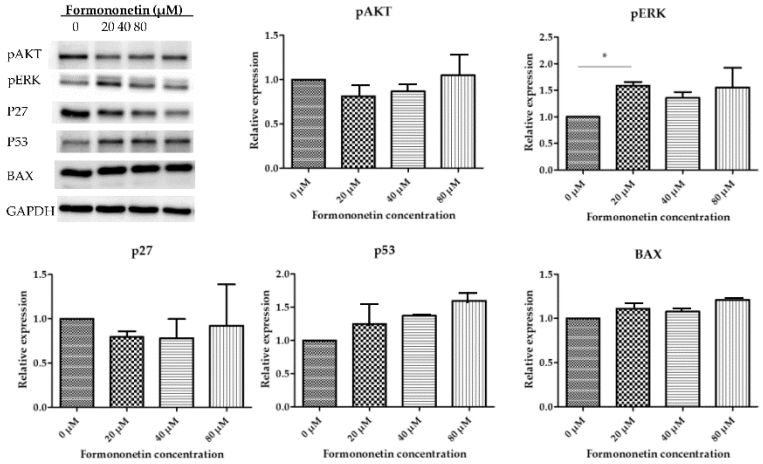


Figure 1. CCK8 assay showed decreased cell viability in eutopic endometrium without endometriosis (**A**,**C**) and eutopic endometrium with endometriosis (**B**,**D**) by increasing dose of formononetin (×40 magnification, bar scale 500 μm, * $p < 0.05$, $N = 3$ per group).

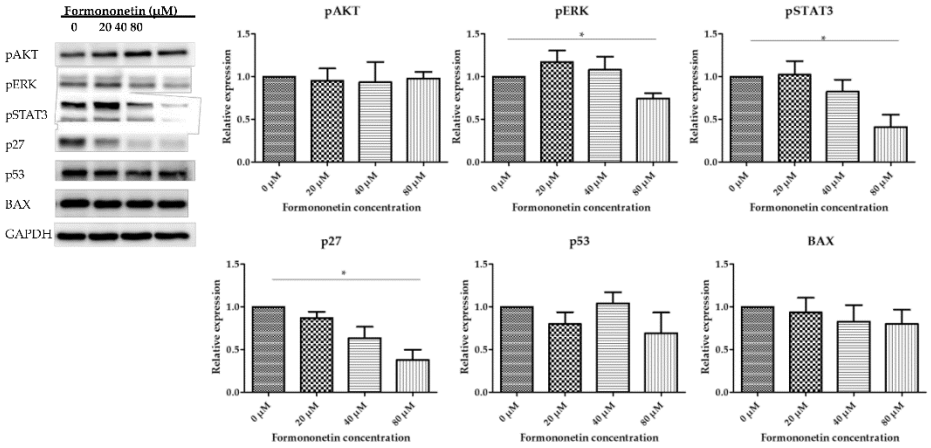
2. Formononetin Decreased Expression of p27 and pSTAT3 on Eutopic Endometrium with Endometriosis and p27 of Ectopic Endometrium with Endometriosis in Dose-Dependent Manner

연세대학교
YONSEI UNIVERSITY

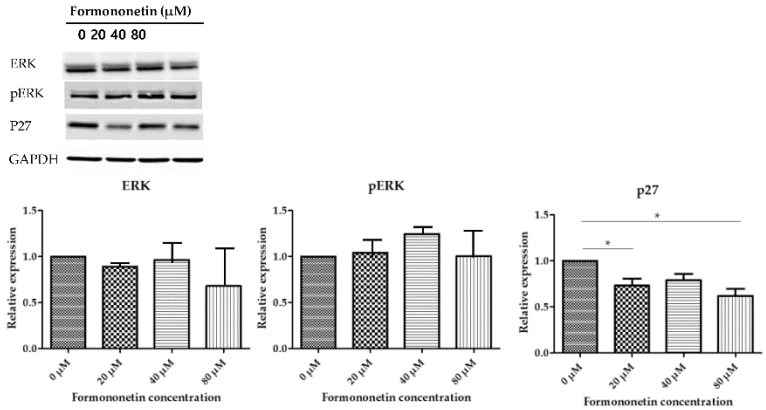
A Eutopic endometrium without endometriosis



B Eutopic endometrium with endometriosis



C Ectopic endometrium with endometriosis



연세대학교
YONSEI UNIVERSITY

Figure 2. Band of Western blot of BAX, p53, pAKT, pERK, and p27 in eutopic endometrium without endometriosis (**A**), BAX, p53, pAKT, pERK, p27, and pSTAT3 in eutopic endometrium with endometriosis (**B**), and ERK, pERK, and p27 in ectopic endometrium with endometriosis (**C**). Relative expression level of BAX, p53, pAKT, ERK, pERK, p27, and pSTAT3 was described in a box plot. Relative expression levels of BAX, p53, pAKT, and ERK with increased formononetin concentration did not show a statistically significant trend in 3 groups. Relative expression level of p27 decreased statistically significantly in dose-dependent manner in eutopic endometrium and ectopic endometrium with endometriosis, not in eutopic endometrium without endometriosis (* $p < 0.05$, $N = 4$ per group).

Figure 2 shows the relative expression levels of BAX, p53, pAKT, ERK, pERK, p27, and pSTAT3 in eutopic endometria with and without endometriosis, and in ectopic endometria with endometriosis, after formononetin treatment.
In eutopic endometria without endometriosis, no significant differences were observed in the expression of BAX, p53, pAKT, or p27 after formononetin treatment. Only pERK showed higher expression levels in the 20 μM treatment group than in the control group with statistical significance (1 vs. 1.6 ± 0.11, $p = 0.01$).
In the eutopic endometrium of patients with endometriosis, no significant differences were observed in the expression of BAX, p53, or pAKT after formononetin exposure. However, the expressions of pERK, pSTAT3, and p27 showed a tendency to decrease with increasing formononetin concentration, and statistical significance had reached 80 μM compared with the control group (1 vs. 0.74 ± 0.12, $p = 0.02$, 1 vs. 0.41 ± 0.29, $p = 0.03$, and 0.38 ± 0.24, $p = 0.01$, respectively).
The relative expression of ERK and pERK in ectopic endometria with endometriosis did not show any increasing or decreasing trend with increasing formononetin treatment dose. However, the relative expression of p27 significantly decreased in the 20 μM and 80 μM treatment groups than in the no treatment group (1 vs. 0.73 ± 0.15, $p = 0.04$, and 1 vs. 0.62 ± 0.15, $p = 0.01$, respectively).

3. Formononetin Did Not Affect Apoptosis in Eutopic Endometrium with and without Endometriosis

Annexin V- and PI-based apoptosis and necrosis discrimination assays were performed on eutopic endometria with and without endometriosis. The results showed that formononetin did not significantly induce apoptosis in eutopic endometrial cells with or without endometriosis in a dose-dependent manner (**Figure 3**).

연세대학교
YONSEI UNIVERSITY

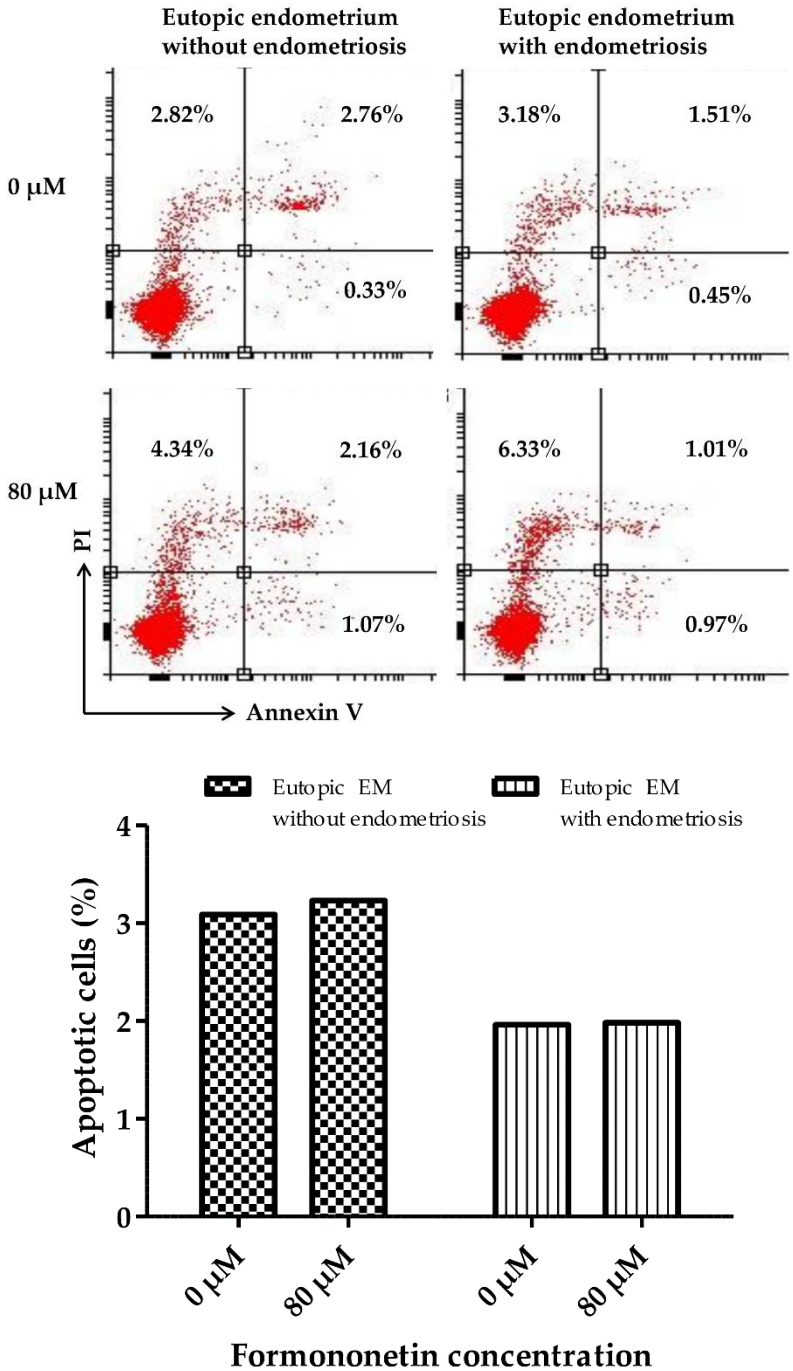


Figure 3. Formononetin did not affect apoptosis on control and eutopic endometrial cells

연세대학교
YONSEI UNIVERSITY

4. Formononetin Inhibits Cell Migration in Eutopic Endometrium with and without Endometriosis

A wound healing assay was performed to investigate the anti-migratory effect of formononetin. In eutopic endometria without endometriosis, administration of formononetin induced a significant decrease in wound closure (21.62 ± 6.27 vs. 13.72 ± 5.99, p = 0.047). In eutopic endometrium with endometriosis, the reduction in wound closure was more prominent than control endometrium with statistical significance (34.37 ± 8.61 vs. 16.28 ± 8.45, p = 0.001) (**Figure 4**).

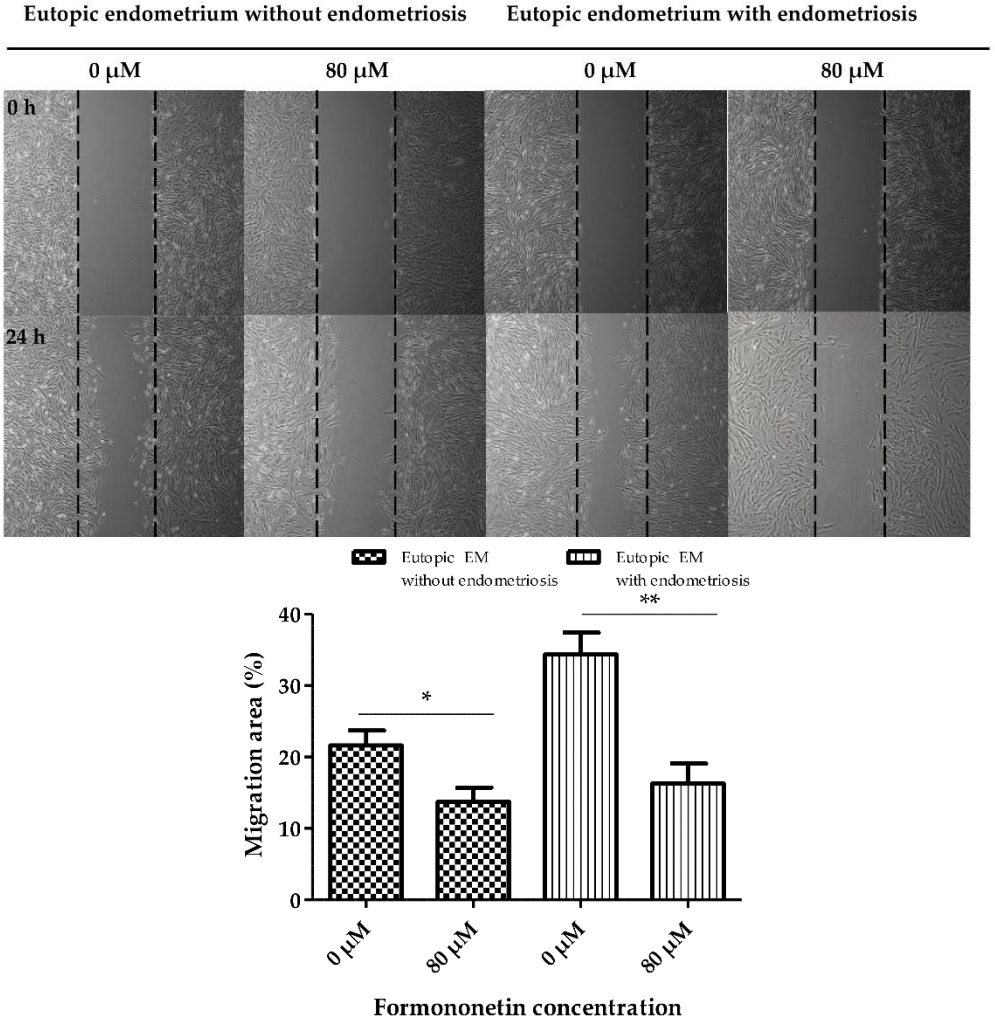


Figure 4. Formononetin affects cell migration in eutopic endometrial cells with and without endometriosis, but has more anti-proliferative effect in eutopic endometrium with endometriosis in wound healing assay (×40 magnification, * $p < 0.05$, ** $p < 0.01$, $N = 3$ per group).

연세대학교
YONSEI UNIVERSITY

5. Formononetin Decreased Expression of Progesterone Receptor-B (PR-B) on Eutopic Endometrium with Endometriosis in Dose-Dependent Manner

Figure 5 shows the difference in the relative expression level of sex hormone receptors, including androgen receptor (AR), estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), progesterone receptor-A (PR-A), progesterone receptor-B (PR-B), ERβ/ERα, and PR-B/PR-A with increased formononetin concentration in eutopic endometrium with endometriosis. Although the relative expression of each hormone receptor tended to decrease with increasing formononetin, no significant difference was observed in AR, ERα, ERβ, PR-A, ERβ/ERα, and PR-B/PR-A. In contrast, significantly decreased expressions of PR-B were noted after formononetin treatment at 80 μM (0.36 ± 0.21, $p = 0.03$).

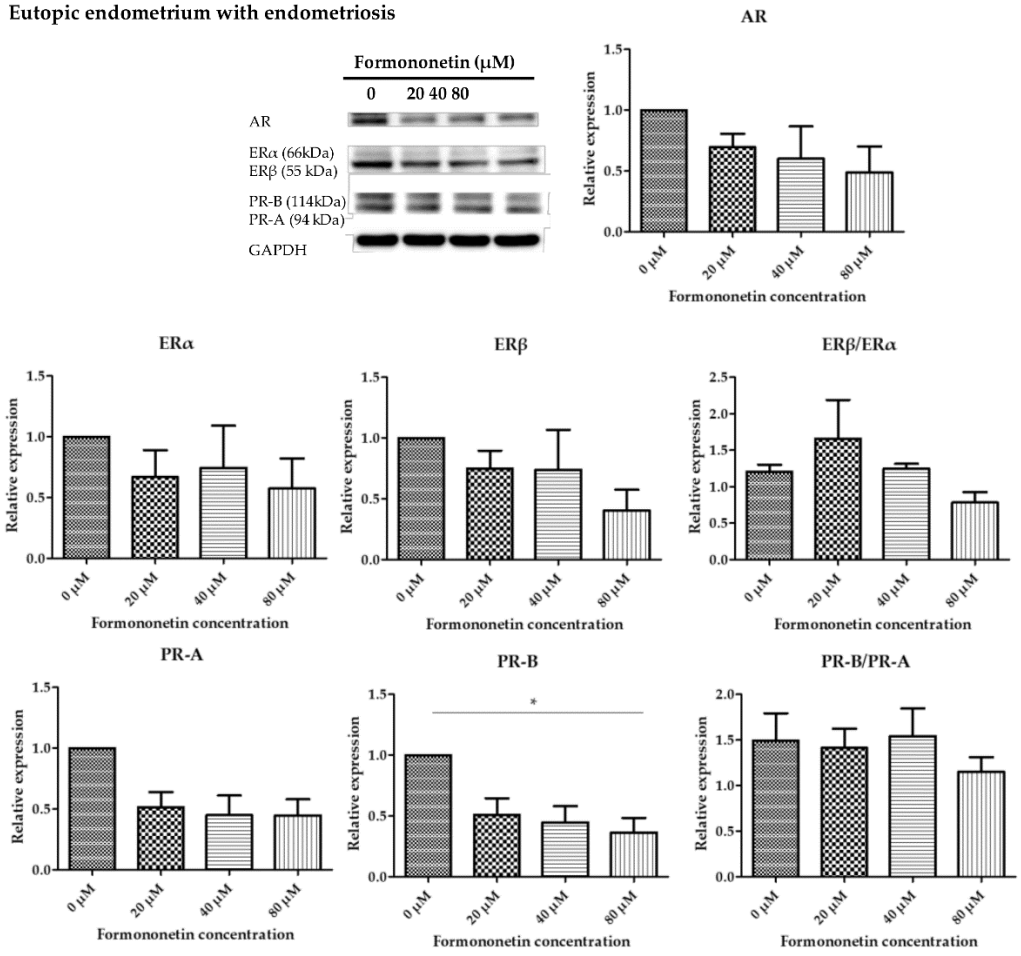


Figure 5. Relative expression levels of androgen receptor (AR), estrogen receptor (ER), progesterone receptor (PR), ERβ/α, and PR-B/A in eutopic endometrium with endometriosis had decreasing tendency, but there is statistical significance only in PR-B (* $p < 0.05$, $N = 3$ per group).

연세대학교
YONSEI UNIVERSITY

6. Mouse Model of Endometriosis

After formononetin treatment, 30 recipient mice were euthanized to confirm and collect endometriotic lesions. The endometriotic lesions were evaluated based on the number of implants (ea) rather than the area (ex. mm2) because all three groups had similar endometriotic implants (4 mm width × 2 mm length). An average of 3.2 ± 0.4 (1–5) endometriosis implants (ea) occurred in the control group not treated with formononetin. In the low-dose group, an average of 1.7 ± 0.4 (0–4) endometriotic implants were found in the low-dose group, which was significantly lower than that in the control group. In the high-dose group, an average of 2.0 ± 0.5 (0–6) endometriotic implants were identified in the high-dose group, which was significantly lower than that in the control group; however, it did not differ significantly from the low-dose group. The high-dose group showed greater heterogeneity than the other groups, ranging from zero to six implants. Furthermore, the formation of a characteristic chocolate-colored fluid containing an endometriotic cyst measuring 4 mm × 4 mm was also confirmed in one case (**Figure 6**). Retrieved endometriotic implants from the three groups were stained with H&E to confirm the presence of endometrial glands and stroma. The endometrial glands and stroma were observed in all three groups (**Figure 7**).

We performed IHC staining to investigate the expression of p27, which showed a statistically significant trend with increasing formononetin concentrations in vitro. The relative expression of p27 was the highest in the control group and lowest in the high-dose formononetin treatment group (0.36 ± 0.04 vs. 0.23 ± 0.05 vs. 0.20 ± 0.05, control vs. low dose; $p = 0.03$, control vs. high dose; $p = 0.002$) (**Figure 8**).

연세대학교
YONSEI UNIVERSITY

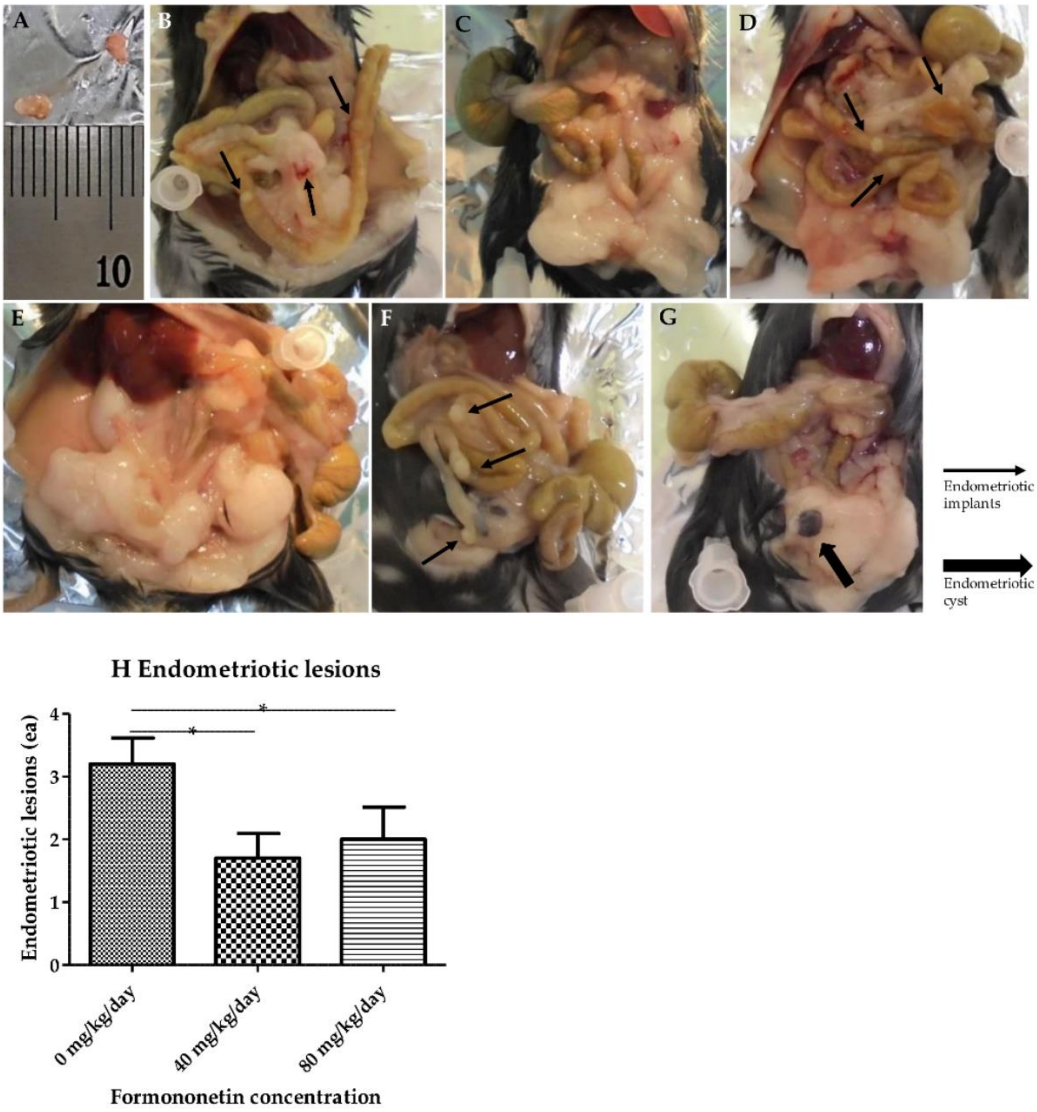


Figure 6. Mouse endometriosis model results (**A–H**). Photographs showing endometriotic implants in mouse model in control, low dose (40 mg/kg/day), and high dose (80 mg/kg/day) of formononetin for 4 weeks. (**A**) The size of endometriotic implants of 3 groups was similar (4 mm × 2 mm). (**B**) Control group with endometriotic implants (narrow arrow). (**C**) Low-dose (40 mg/kg/day) group without endometriotic implants. (**D**) Low-dose group with endometriotic implants (narrow arrow). (**E**) High-dose (80 mg/kg/day) group without endometriotic implants. (**F**) High-dose group with endometriotic implants (narrow arrow). (**G**) High-dose group with endometriotic cysts (4 mm × 4 mm) (wide arrow). (**H**) Endometriotic lesions (implants and cysts) (* $p < 0.05$, N = 10 per group).

연세대학교
YONSEI UNIVERSITY

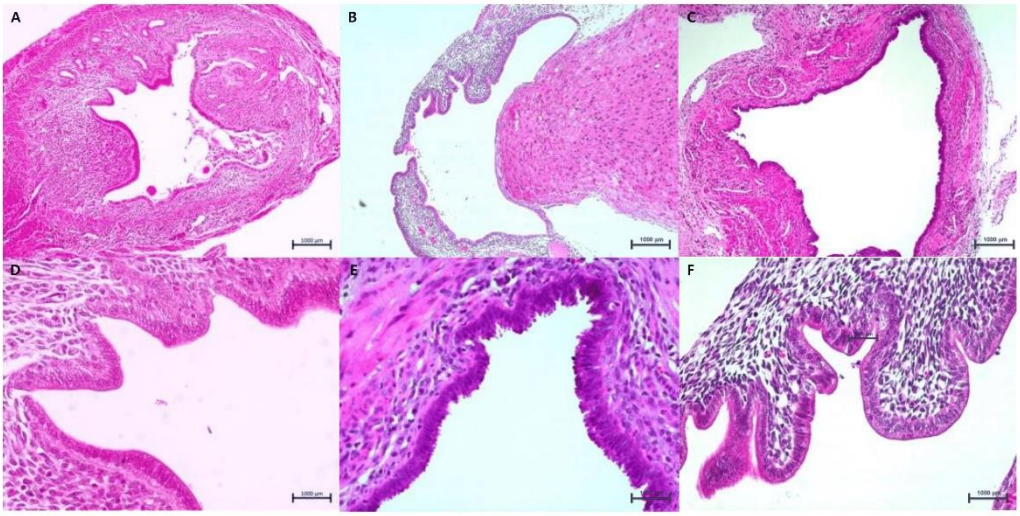


Figure 7. H&E staining of the endometriotic implants of each treatment group ((**A–C**): ×10 magnification, (**D–F**): ×400 magnification). Dilated glands lined a single layer of columnar cells with surrounding stroma were confirmed in all three groups. (**A**,**D**) control group, (**B**,**E**) low-dose formononetin treatment group (40 mg/kg/day), (**C**,**F**) high-dose formononetin treatment group (80 mg/kg/day).

연세대학교
YONSEI UNIVERSITY

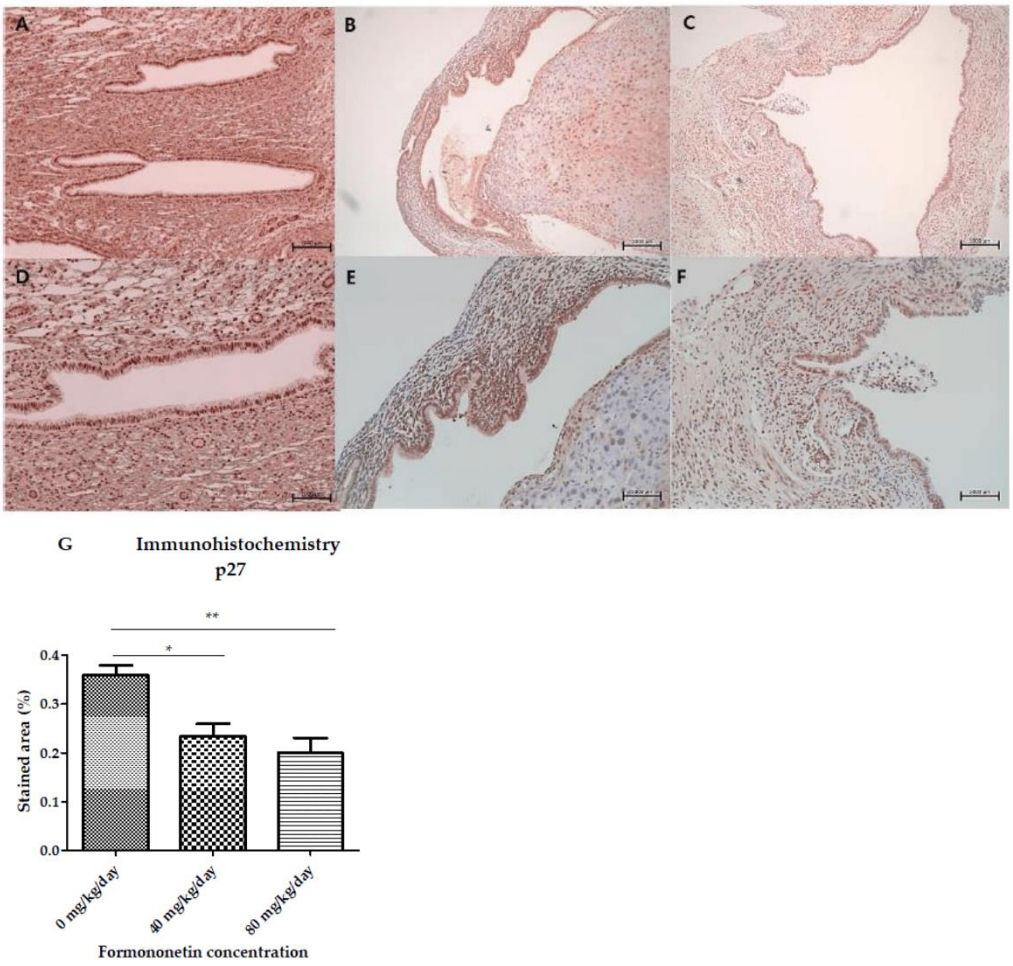


Figure 8. Immunohistochemistry staining for p27 expression of the endometriotic implants of each treatment group: (**A**,**D**) control group, (**B**,**E**) low-dose group (40 mg/kg/day), (**C**,**F**) high-dose group (80 mg/kg/day). ((**A–C**): ×100 magnification, (**D–F**): ×200 magnification). (**G**) The relative expression of p27 was highest in the control group and lowest in the high-dose formononetin treatment group (* $p < 0.05$, ** $p < 0.01$).

연세대학교
YONSEI UNIVERSITY

IV. DISCUSSION

This study aimed to evaluate the effects of formononetin on endometriosis and explore its potential as a therapeutic agent for endometriosis using in vitro and in vivo experiments. In this study, we demonstrated that formononetin treatment effectively suppressed the proliferation of endometrial cells in endometriosis at appropriate concentrations, which appeared to be mediated through the downregulation of p27, pSTAT3, and PR, especially PR-B expression.

In this study, we set the maximum concentration of formononetin to 80 μM in vitro using the CCK8 assay. Western blot was performed to identify the expression of pAKT, p27, and pSTAT3 in relation to proliferation and BAX, ERK, pERK, and p53 in relation to apoptosis using previous cancer studies as reference[17]. When treated with formononetin, the expression of p27 in the eutopic and ectopic endometria of patients with endometriosis significantly decreased in a dose-dependent manner. The expression of pERK showed a significant decrease in eutopic endometrium with endometriosis at 80 μM formononetin treatment but did not show a specific trend in ectopic endometrium with endometriosis. On the other hand, the expression of p27 showed a significant reduction in both eutopic and ectopic endometria with endometriosis at 80 μM formononetin treatment. This means that ectopic endometrium with endometriosis may act in some different molecular pathways than eutopic lesions in the process of engraftment outside the uterus. Interestingly, the expression levels of hormone receptors also changed significantly following formononetin treatment. The expressions of ER, PR, and AR, as well as ERβ/ERα and PR-B/PR-A ratios, all decreased with increasing formononetin concentration, with a statistically significant decrease in PR-B expression. Our findings in the wound healing assay indicate that formononetin may have an anti-migratory effect on the eutopic endometrium in patients with endometriosis, although the flow cytometry results show that its effect on apoptosis is minor. In the in vivo study, formononetin reduced endometriotic lesions compared to those in the non-treated control group. When the relative expression levels of p27 were evaluated, the highest formononetin dose treatment group had the lowest expression, followed by the low-dose formononetin treatment group and the non-treated group.

The CDKN1B-encoded protein p27, also known as KIP1, binds to cyclin-dependent kinases (Cdks) and regulates G0 to S phase transition[18]. It is not a classic tumor suppressor like p53, but has been examined in various tumor types[19]. This protein may positively or negatively influence cell proliferation, cell motility, and apoptosis[20]. Deregulated cytoplasmic p27 could activate cell motility and invasion, and cause tumor progression[21]. p27 is also known as a key regulator of endometriosis[22]. However, a previous study reported significantly decreased levels of p27 in endometrial cells in patients with endometriosis compared to those without the disease. The expression of p27 in the endometriosis group increases according to the severity of the disease[23]. Additionally, 57.7% of patients with endometriosis showed p27 expression, compared to 50.0% of patients without endometriosis[24]. Moreover, increased expression of p27 contributes to the progression of ectopic lesions such as peritoneal endometriosis[25]. Therefore, we believe that the effect of formononetin on the reduction in endometriotic lesions in a mouse model was achieved via

연세대학교
YONSEI UNIVERSITY

the reduction in increased expression of p27 in endometriosis.

Our in vitro results indicated that pSTAT3 expression was significantly reduced by formononetin treatment. STAT3, a member of the STAT (signal transducer and activator of transcription pathway) family, was phosphorylated by Janus kinase (JAK), and it could promote proliferation, angiogenesis, and regulate tumor microenvironment[26 27]. Regardless of the proliferative or secretory phase, eutopic endometrium with endometriosis was found to increase pSTAT3 expression more than eutopic endometrium without endometriosis[28]. Tofacitinib, a JAK inhibitor, showed potential as a therapeutic agent for endometriosis by reducing pSTAT3 expression and the volume of endometriosis lesions in the endometriosis mouse model[29]. Interestingly, formononetin has potential effects in endometriosis via the alteration of pSTAT3 expressions. Notably, our results indicate a possible link between p27 and STAT3 in endometriosis. To the best of our knowledge, no study has evaluated the relationship between p27 and pSTAT3 expression in endometriosis. Previous studies have indicated the possible roles of p27 and STAT3 in malignant conditions. In a study of immortalized human mammary epithelial MCF-12A cell lines, p27 upregulated STAT3, promoting cell migration[30]. Endometriosis is not a malignant condition, but it can form ectopic lesions like cancer. Therefore, cell migration is thought to be important in the progression of endometriosis[2]. Similarly, our results showed that formononetin treatment reduced p27 expression in the eutopic and ectopic endometrium and pSTAT3 expression in the eutopic endometrium of patients with endometriosis, thereby explaining the potential therapeutic effects of formononetin in endometriosis.

Our study also revealed an intriguing effect of formononetin on sex steroid hormone receptors. Phytoestrogen may serve as a selective progesterone receptor modulator (SPRM), operating as an agonist or antagonist depending on the target tissue, though its precise impact and mechanism on certain tissues are still poorly known[31]. To our knowledge, this is the first study to evaluate the effects of formononetin on sex hormone receptors in the human endometrium. Our findings suggest that formononetin functions as an anti-progestin agent in the eutopic endometrium of patients with endometriosis. Formononetin effectively reduced PR-B expression in the eutopic endometria of patients with endometriosis. These results contrast previous research on dienogest (17a-cyanomethyl-17b-hydroxyestra-4,9-dien3-one), which is frequently used to treat endometriosis and alleviate progesterone resistance by elevating PR-B, PR-B/PR-A ratio and lowering ERβ/ERα ratio[32]. We believe that formononetin has a different mechanism of action on progesterone receptors in endometriosis.

These results may be secondary to a decrease in estrogen receptor expression. A relatively high level of ERβ in endometriosis is known to inhibit the induction of estradiol-dependent PR by suppressing Erα[33]. Notably, PR-B undergoes post-translational modification and has a rapid turnover, making PR-B difficult to detect[34]. The ERβ level of endometriosis stromal cells was 142-fold higher than normal stromal cells, while the ERα level of endometriosis stromal cells was 9-fold lower than normal stromal cells. An abnormally high ERβ/ERα ratio reduces PR expression by derangement by estradiol induction. Progesterone resistance indicates low PR expression[35 36].

연세대학교
YONSEI UNIVERSITY

Phytoestrogens are known to act through estrogen receptors because of their similar chemical structures. They have a lower affinity than estrogen for binding to both ERα and ERβ. Estradiols show similar affinity to ERα and ERβ, but phytoestrogens show higher affinity to ERβ than to Erα[37]. Several studies were conducted to investigate the effect of phytoestrogen on endometriosis treatment, including a total of seven human studies. One study found no change in endometriosis pain, one reported a reduced risk of endometriosis, and five studies reported endometriosis pain relief[38].

Female mice in the reproductive period are known to have periodic fluctuations in hormone levels[39]. The action of hormone receptors varies between species, and it is known that hormone receptor mutations are more critical in humans than in rodents[40]. Therefore, the experiment for the hormone lab and their receptor expression was not carried out in the in vivo study. If the hormone measurements had been taken multiple times per individual mouse, we might find that formononetin treatment affects hormonal levels.

There were fewer endometriotic implants in the formononetin 40 mg/kg group than in the 80 mg/kg group, but the difference between the two groups was not statistically significant. This study was initiated in the absence of a reference value for formononetin in an endometriosis animal model. In the future study, it will be necessary to confirm by fine-tuning whether the corresponding concentration range is the threshold that has the therapeutic effect for endometriosis.

V. CONCUSION

In conclusion, this study is the first to evaluate the effects of formononetin on endometriosis. Formononetin revealed an anti-proliferative effect in endometriosis in the in vitro study. It may have been due to the regulation of p27, pSTAT3, and PR-B. In a mouse model of endometriosis, formononetin reduced the number of endometriotic lesions with decreased p27 expression. Our results suggest that formononetin may have therapeutic potential in endometriosis. Further studies are warranted to elucidate the underlying therapeutic mechanisms of formononetin and its effects in clinical settings.

연세대학교
YONSEI UNIVERSITY

REFERENCES

1. Sampson JA. Metastatic or Embolic Endometriosis, due to the Menstrual Dissemination of Endometrial Tissue into the Venous Circulation. *Am J Pathol* 1927;3(2):93-110.43. [published Online First: 1927/03/01]
2. Giudice LC. Clinical practice. Endometriosis. *N Engl J Med* 2010;362(25):2389-98. doi: 10.1056/NEJMcp1000274 [published Online First: 2010/06/25]
3. Ozkan S, Murk W, Arici A. Endometriosis and infertility: epidemiology and evidence-based treatments. *Ann N Y Acad Sci* 2008;1127:92-100. doi: 10.1196/annals.1434.007 [published Online First: 2008/04/30]
4. Han SJ, Jung SY, Wu SP, et al. Estrogen Receptor β Modulates Apoptosis Complexes and the Inflammasome to Drive the Pathogenesis of Endometriosis. *Cell* 2015;163(4):960-74. doi: 10.1016/j.cell.2015.10.034 [published Online First: 2015/11/07]
5. Patel BG, Rudnicki M, Yu J, et al. Progesterone resistance in endometriosis: origins, consequences and interventions. *Acta Obstet Gynecol Scand* 2017;96(6):623-32. doi: 10.1111/aogs.13156 [published Online First: 2017/04/20]
6. Horne AW, Daniels J, Hummelshoj L, et al. Surgical removal of superficial peritoneal endometriosis for managing women with chronic pelvic pain: time for a rethink? *Bjog* 2019;126(12):1414-16. doi: 10.1111/1471-0528.15894 [published Online First: 2019/07/31]
7. Dunselman GA, Vermeulen N, Becker C, et al. ESHRE guideline: management of women with endometriosis. *Hum Reprod* 2014;29(3):400-12. doi: 10.1093/humrep/det457 [published Online First: 2014/01/18]
8. Taylor HS, Giudice LC, Lessey BA, et al. Treatment of Endometriosis-Associated Pain with Elagolix, an Oral GnRH Antagonist. *N Engl J Med* 2017;377(1):28-40. doi: 10.1056/NEJMoa1700089 [published Online First: 2017/05/20]
9. Takayama K, Zeitoun K, Gunby RT, et al. Treatment of severe postmenopausal endometriosis with an aromatase inhibitor. *Fertil Steril* 1998;69(4):709-13. doi: 10.1016/s0015-0282(98)00022-3 [published Online First: 1998/04/21]
10. Daniels S, Robbins J, West CR, et al. Celecoxib in the treatment of primary dysmenorrhea: results from two randomized, double-blind, active- and placebo-controlled, crossover studies. *Clin Ther* 2009;31(6):1192-208. doi: 10.1016/j.clinthera.2009.06.003 [published Online First: 2009/08/22]
11. Becker CM, Gattrell WT, Gude K, et al. Reevaluating response and failure of medical treatment of endometriosis: a systematic review. *Fertil Steril* 2017;108(1):125-36. doi: 10.1016/j.fertnstert.2017.05.004 [published Online First: 2017/07/03]
12. Kurzer MS, Xu X. Dietary phytoestrogens. *Annu Rev Nutr* 1997;17:353-81. doi: 10.1146/annurev.nutr.17.1.353 [published Online First: 1997/01/01]
13. Kolahdouz Mohammadi R, Arablou T. Resveratrol and endometriosis: In vitro and animal studies and underlying mechanisms (Review). *Biomed Pharmacother* 2017;91:220-28. doi: 10.1016/j.biopha.2017.04.078 [published Online First: 2017/05/02]

연세대학교
YONSEI UNIVERSITY

14. Oh JS, Kim TH, Park JH, et al. Formononetin induces apoptotic cell death through the suppression of mitogen-activated protein kinase and nuclear factor-κB phosphorylation in FaDu human head and neck squamous cell carcinoma cells. *Oncol Rep* 2020;43(2):700-10. doi: 10.3892/or.2019.7432 [published Online First: 2020/01/03]
15. Tay KC, Tan LT, Chan CK, et al. Formononetin: A Review of Its Anticancer Potentials and Mechanisms. *Front Pharmacol* 2019;10:820. doi: 10.3389/fphar.2019.00820 [published Online First: 2019/08/14]
16. Fattori V, Franklin NS, Gonzalez-Cano R, et al. Nonsurgical mouse model of endometriosis-associated pain that responds to clinically active drugs. *Pain* 2020;161(6):1321-31. doi: 10.1097/j.pain.0000000000001832 [published Online First: 2020/03/07]
17. Ong SKL, Shanmugam MK, Fan L, et al. Focus on Formononetin: Anticancer Potential and Molecular Targets. *Cancers (Basel)* 2019;11(5) doi: 10.3390/cancers11050611 [published Online First: 2019/05/06]
18. Polyak K, Kato JY, Solomon MJ, et al. p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. *Genes Dev* 1994;8(1):9-22. doi: 10.1101/gad.8.1.9 [published Online First: 1994/01/01]
19. Slingerland J, Pagano M. Regulation of the cdk inhibitor p27 and its deregulation in cancer. *J Cell Physiol* 2000;183(1):10-7. doi: 10.1002/(sici)1097-4652(200004)183:1<10::Aid-jcp2>3.0.Co;2-i [published Online First: 2000/03/04]
20. Chu IM, Hengst L, Slingerland JM. The Cdk inhibitor p27 in human cancer: prognostic potential and relevance to anticancer therapy. *Nat Rev Cancer* 2008;8(4):253-67. doi: 10.1038/nrc2347 [published Online First: 2008/03/21]
21. Besson A, Dowdy SF, Roberts JM. CDK inhibitors: cell cycle regulators and beyond. *Dev Cell* 2008;14(2):159-69. doi: 10.1016/j.devcel.2008.01.013 [published Online First: 2008/02/13]
22. Gonçalves GA. p27(kip1) as a key regulator of endometriosis. *Eur J Obstet Gynecol Reprod Biol* 2018;221:1-4. doi: 10.1016/j.ejogrb.2017.11.026 [published Online First: 2017/12/08]
23. Schor E, da Silva ID, Sato H, et al. P27Kip1 is down-regulated in the endometrium of women with endometriosis. *Fertil Steril* 2009;91(3):682-6. doi: 10.1016/j.fertnstert.2007.12.070 [published Online First: 2008/03/07]
24. Kim TH, Lee HH, Chung SH, et al. Expression of p27 and Jun activation domain-binding protein 1 in endometriosis. *Arch Gynecol Obstet* 2015;292(2):377-81. doi: 10.1007/s00404-015-3642-0 [published Online First: 2015/02/11]
25. Matsuzaki S, Canis M, Murakami T, et al. Expression of the cyclin-dependent kinase inhibitor p27Kip1 in eutopic endometrium and peritoneal endometriosis. *Fertil Steril* 2001;75(5):956-60. doi: 10.1016/s0015-0282(01)01752-6 [published Online First: 2001/05/04]
26. Heinrich PC, Behrmann I, Haan S, et al. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochem J* 2003;374(Pt 1):1-20. doi:

연세대학교
YONSEI UNIVERSITY

10.1042/bj20030407 [published Online First: 2003/05/30]
27. Carro MS, Lim WK, Alvarez MJ, et al. The transcriptional network for mesenchymal transformation of brain tumours. *Nature* 2010;463(7279):318-25. doi: 10.1038/nature08712 [published Online First: 2009/12/25]
28. Kim BG, Yoo JY, Kim TH, et al. Aberrant activation of signal transducer and activator of transcription-3 (STAT3) signaling in endometriosis. *Hum Reprod* 2015;30(5):1069-78. doi: 10.1093/humrep/dev050 [published Online First: 2015/03/10]
29. Kotlyar AM, Mamillapalli R, Flores VA, et al. Tofacitinib alters STAT3 signaling and leads to endometriosis lesion regression. *Mol Hum Reprod* 2021;27(4) doi: 10.1093/molehr/gaab016 [published Online First: 2021/03/12]
30. Zhao D, Besser AH, Wander SA, et al. Cytoplasmic p27 promotes epithelial-mesenchymal transition and tumor metastasis via STAT3-mediated Twist1 upregulation. *Oncogene* 2015;34(43):5447-59. doi: 10.1038/onc.2014.473 [published Online First: 2015/02/17]
31. Chabbert-Buffet N, Meduri G, Bouchard P, et al. Selective progesterone receptor modulators and progesterone antagonists: mechanisms of action and clinical applications. *Hum Reprod Update* 2005;11(3):293-307. doi: 10.1093/humupd/dmi002 [published Online First: 2005/03/26]
32. Hayashi A, Tanabe A, Kawabe S, et al. Dienogest increases the progesterone receptor isoform B/A ratio in patients with ovarian endometriosis. *J Ovarian Res* 2012;5(1):31. doi: 10.1186/1757-2215-5-31 [published Online First: 2012/11/02]
33. Kim JJ, Kurita T, Bulun SE. Progesterone action in endometrial cancer, endometriosis, uterine fibroids, and breast cancer. *Endocr Rev* 2013;34(1):130-62. doi: 10.1210/er.2012-1043 [published Online First: 2013/01/11]
34. Hagan CR, Lange CA. Molecular determinants of context-dependent progesterone receptor action in breast cancer. *BMC Med* 2014;12:32. doi: 10.1186/1741-7015-12-32 [published Online First: 2014/02/21]
35. Xue Q, Lin Z, Cheng YH, et al. Promoter methylation regulates estrogen receptor 2 in human endometrium and endometriosis. *Biol Reprod* 2007;77(4):681-7. doi: 10.1095/biolreprod.107.061804 [published Online First: 2007/07/13]
36. Bulun SE, Cheng YH, Pavone ME, et al. Estrogen receptor-beta, estrogen receptor-alpha, and progesterone resistance in endometriosis. *Semin Reprod Med* 2010;28(1):36-43. doi: 10.1055/s-0029-1242991 [published Online First: 2010/01/28]
37. Kuiper GG, Lemmen JG, Carlsson B, et al. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology* 1998;139(10):4252-63. doi: 10.1210/endo.139.10.6216 [published Online First: 1998/09/29]
38. Bartiromo L, Schimberni M, Villanacci R, et al. Endometriosis and Phytoestrogens: Friends or Foes? A Systematic Review. *Nutrients* 2021;13(8) doi: 10.3390/nu13082532 [published Online First: 2021/08/28]
39. Devillers MM, Mhaouty-Kodja S, Guigon CJ. Deciphering the Roles & Regulation of

연세대학교
YONSEI UNIVERSITY

Estradiol Signaling during Female Mini-Puberty: Insights from Mouse Models. *Int J Mol Sci* 2022;23(22) doi: 10.3390/ijms232213695 [published Online First: 2022/11/27]

40. Antonson P, Apolinário LM, Shamekh MM, et al. Generation of an all-exon Esr2 deleted mouse line: Effects on fertility. *Biochem Biophys Res Commun* 2020;529(2):231-37. doi: 10.1016/j.bbrc.2020.06.063 [published Online First: 2020/07/25]

APPENDICES

연세대학교
YONSEI UNIVERSITY

ABSTRACT(IN KOREAN)

Formononetin이 p27, pSTAT3, 그리고 프로게스테론 수용체를 통해 자궁내막증의 증식을 억제함: In Vitro 및 In Vivo 연구

<지도교수 조시현>

연세대학교 대학원 의학과

박윤정

목적: Formononetin은 선택적 에스트로겐 수용체 조절제로 기능하는 것으로 알려진 식물성 에스트로겐이다. 이 연구에서 in vitro 및 vivo 실험을 통해 자궁내막증의 증식에 대한 formononetin의 효과를 평가하고자 한다.

재료 및 방법: 자궁내막증을 진단받은 10명의 환자로부터 eutopic 및 이소성 자궁내막 조직 샘플을 얻었다. 또한 자궁내막증이 없는 10명의 환자로부터 eutopic 자궁내막 조직샘플을 얻었다. Formononetin의 치료 용량을 결정하기 위해 CCK8 분석을 통해 formononetin 투여시 70%의 세포가 생존한 농도를 구했다. 해당 농도까지 formononetin 농도를 증가시키면서 대조군, eutopic 자궁내막, 이소성 자궁내막 세 그룹에서의 BAX, p53, pAKT, ERK, pERK, p27 및 pSTAT3의 상대적 발현 수준을 웨스턴 블롯으로 측정했다. Formononetin이 세포자연사에 미치는 영향을 확인하기 위해 유세포분석을 시행하였고, 자궁내막증 증식에 미치는 영향을 확인하기 위해 상처회복분석을 시행하였다. 자궁내막증 쥐 모델을 비수술적 방법으로 준비하였다. 대조군, 저용량 formononetin 투여군 (40 mg/kg/day), 고용량 formononetin 투여군 (80 mg/kg/day)으로 나누어 4주동안 매일 formononetin을 복강내 투여하였다. 모든 쥐들로부터 회수한 자궁내막 병변을 헤마톡실린&에오신 염색 및 p27의 면역조직화학염색을 시행하였다.

결과: 우리는 CCK8 분석을 통해 formononetin 투여의 최대 농도를 80 μM로 설정했다. Formononetin 농도에 따라 BAX, p53, pAKT, ERK, pERK, p27, pSTAT3

연세대학교
YONSEI UNIVERSITY

단백질의 상대적 발현량을 웨스턴 블롯으로 측정하였다. (그룹당 N=4) 자궁내막증이 있는 Eutopic 자궁내막에서 pERK, p27, pSTAT3의 상대적 발현량은 formononetin 농도가 증가함에 따라 감소하는 경향을 보였고, 80 μM에서 유의하게 감소하였다. 이소성 자궁내막에서 p27의 발현 수준 또한 80 μM에서 유의하게 감소하였다. 유세포 분석으로 확인된 세포자연사에 대한 formononetin 투여에 유의한 효과는 없었다. 그러나 상처회복분석에서 formononetin은 자궁내막증이 있는 eutopic 자궁내막에서의 증식을 더 유의하게 감소시켰다. 각 호르몬 수용체의 상대적인 발현은 formononetin 농도 증가에 따라 감소하는 경향을 보였으나, ER, PR-A, ERβ/ERα, and PR-B/PR-A 에서는 유의한 차이가 나타나지 않았다. 그러나 프로게스테론 수용체 B(PR-B)의 경우 80 μM의 formononetin 처리시 그 발현이 유의하게 감소하였다.
In vivo 연구에서 대조군보다 formononetin 투여군에서 자궁내막증 병변의 유의하게 감소하였다. 면역조직화학염색을 이용한 p27의 상대적인 발현 또한 대조군에서 가장 높았고, 고용량 formononetin 투여군에서 가장 낮았다.

결론: Formononetin 치료는 p27, pSTAT3, 그리고 프로게스테론 수용체 B의 조절로 자궁내막증의 증식을 억제하는 것으로 보인다. 자궁내막증 쥐 모델에서도 formononetin 치료는 자궁내막증 병변의 수를 유의하게 감소시켰다. 이 연구의 결과는 formononetin이 자궁내막증에 대한 비호르몬 치료 옵션으로 사용될 수 있음을 시사한다.

핵심되는 말 : formononetin, 자궁내막증, p27, pSTAT3, 프로게스테론 수용체, 자궁내막증 쥐 모델

연세대학교
YONSEI UNIVERSITY

PUBLICATION LIST

Park Y, Choo SP, Jung GS, Kim S, Lee MJ, Im W, Park H, Lee I, Lee JH, Cho S, et al. Formononetin Inhibits Progression of Endometriosis via Regulation of p27, pSTAT3, and Progesterone Receptor: In Vitro and In Vivo Studies. Nutrients. 2023; 15(13):3001. https://doi.org/10.3390/nu15133001