Original Article

Effects of Ethinyl Estradiol in Combined Oral Contraceptives on Cell Proliferation and Apoptosis in Ectopic Endometrial Tissue: A Randomized Controlled Study

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Abstract

Objective: Since endometriosis is an estrogen-dependent disease; therefore, combined oral contraceptives (COCs) may not be the best choice for the treatment of endometriosis. The objective of the present study was to investigate the effects of ethinyl estradiol (EE) and desogestrel (DSG) in COCs on cell proliferation and apoptosis in ectopic endometrial tissue as compared to DSG alone.

Materials and methods: Forty-five women of reproductive age with at least one endometriotic cyst were recruited into this single-blind randomized controlled trial study and randomly divided equally into three groups. EE-DSG and DSG groups received EE (0.03 mg) and DSG (0.15 mg) or DSG alone daily for 28-35 days before surgery. The control group was prescribed nothing. Endometriotic cyst tissues were collected during ovarian cystectomy for immunohistochemistry.

Results: Levels of Ki-67 positive cells in the ectopic endometrial tissue of the EE-DSG group were significantly higher than the DSG group (median [IQR]; 1.4[1.2] vs 0.6 [0.7], P <0.016). There were significantly more TUNEL-positive cells in the EE-DSG group compared to the DSG group (median [IQR]; 2.8[0.7] vs 1.8[1.4], P < 0.016, respectively). Moreover, the number of TUNEL-positive cells in the EE-DSG and DSG groups were significantly higher than the control (median [IQR]; 2.8[0.7] vs 0.2[0.2] and 1.8[1.4] vs 0.2[0.2], P <0.016). The levels of cells that positively stained for *Bcl2* were not different among all groups.

Conclusion: Progestin alone increased cell apoptosis in ectopic endometria. However, concurrent EE in COCs enhanced proliferation and promoted a greater apoptotic effect in ectopic endometria compared to progestin alone.

Keywords: Ethinyl Estradiol; Desogestrel; Apoptosis; Endometriosis; Ovarian Cysts

Introduction

Endometriosis is a common gynecologic disorder that affects 10% of women of reproductive age (1). Although the precise causes of endometriosis are not well known, the possible mechanisms contributing to the development of endometriosis include apoptosis resistance (2), progesterone resistance (3), and local

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estradiol production (4). Additionally, there are several other factors involved in the pathogenesis of this disease including inflammation, hormonal regulation, genetic, and immunologic factors (5, 6).

Proliferation and apoptosis of cells in the endometrium play an important role in the menstrual cycle. Apoptosis is a normal mechanism that occurs in endometrial or eutopic tissues in order to eliminate dysfunctional cells and leads to cyclical endometrial bleeding. Previous studies have demonstrated that ectopic endometrial cells from women with endometriosis have enhanced rates of proliferation and an increased ability to implant and survive in ectopic locations. Moreover, apoptosis resistance in ectopic endometrial tissue reduces the ability of the endometrium to undergo cyclical changes and contributes to abnormal implantation and growth of endometrial tissue at ectopic sites (7).

Combined oral contraceptive pills (COCs) containing ethinyl estradiol (EE) and progestins have been widely used for the treatment of endometriosis and prevention of endometriosis recurrence after surgical management (8, 9). However, a recently published review has posited that COCs may not be the best option for treatment of endometriosis since it is an estrogen-dependent disease (10). Likewise, clinical data have suggested that COCs are not completely effective for treatment of endometriosis. Patients with endometriosis treated with COCs do not exhibit a decrease in non-menstrual pelvic pain and dyspareunia (11). Moreover, the risk of developing deep infiltrating endometriosis was greatly increased in patients who had taken COCs in the past (12). As such, progestin might be a suitable first step of treatment for endometriosis (13).

Previous studies have shown that both progestin and COCs affect apoptosis and proliferation of ectopic and eutopic endometrial tissue in women with endometriosis (14-17). Furthermore, estrogen in COCs may enhance the severity of the disease (13). However, no previous studies have examined the effects of EE in COCs on endometriosis. The objective of the present study was to investigate the effects of EE in COCs on cell proliferation and apoptosis on ectopic endometrial tissue of women with endometriosis. Patients received COCs containing both EE and progestin, pills containing progestin only, or did not receive any treatment.

Materials and methods

Patients: Forty-five women of reproductive age with at least one endometriotic cyst were recruited in a single-blind randomized controlled trial study between July 2014 and June 2016 (Figure 1). All patients were admitted to the outpatient gynecologic clinic of the Reproductive Endocrinology and Infertility Unit, Department of Obstetrics & Gynecology, Ramathibodi hospital, Bangkok, Thailand.

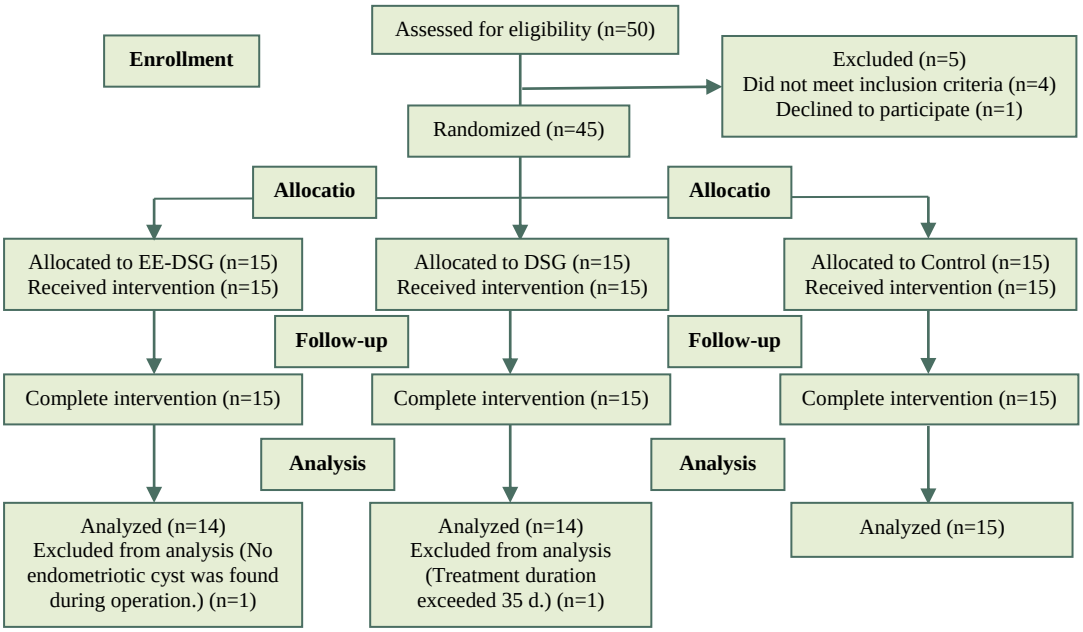

Figure 1: The study flow chart

The study was approved by the Ethical Clearance Committee on Human Related to Researches Involving Human Subjects and Faulty of Medicine Ramathibodi Hospital, Mahidol University (MURA2014/205) and was registered on the Thai Clinical Trial Registry (TCTR). This study was conducted in accordance with the 1964 Helsinki Declaration. Informed consent were obtained from all individual participants.

All patients had at least one endometriotic cyst. Endometriotic cysts were diagnosed when, on two different hospital visits at least 1-2 months apart, patients presented at least one cyst (>3 cm) based on detection of diffuse homogenous low-level internal echoes by ultrasonography with acoustic enhancement. The other inclusion criteria were patients who had not received any hormonal treatment in the previous three months or depot-medroxyprogesterone acetate (DMPA) or gonadotropin releasing hormone agonist in the previous nine months. The exclusion criteria included breast cancer, active liver disease, cardiovascular disease, and intolerability to side effects of EE or DSG, such as vaginal spotting and hypermenorrhea. Patients who had contraindications to EE or DSG were also excluded.

The patients were randomly assigned to one of three groups (n=15) by means of a computer-generated randomization scheme using opaque sealed envelopes in a ratio of 1:1 that randomized patients based on order of entry into the study. The randomization scheme was performed by one research nurse. Two gynecologists (AS and ST) enrolled and assigned participants to interventions. There were fifteen patients in each of the three groups that were or were not treated daily for 28-35 days before surgery: 1) EE (0.03 mg) and DSG (0.15 mg) (Marvelon®, NV Organon, Oss, the Netherlands); 2) DSG (0.15 mg) (two tablets of Cerazette®, NV Organon, Oss, the Netherlands); and 3) control group, which received no treatment. Oral medication was started during the first seven days of each patient's menstrual period. The recruited patients were then scheduled for either laparoscopic or laparotomy ovarian cystectomy. Endometriotic cyst wall tissues were obtained during laparoscopic or laparotomy ovarian cystectomy. Tissue samples were prepared with 10% buffered formaldehyde overnight. The primary goal was to identify the number of cells that were positively stained for apoptosis. The secondary goal was to identify the number of cells undergoing proliferation and the number of anti-apoptotic positive cells.

The ideal sample sizes for each treatment group for optimal statistical power were calculated by comparing the two means of apoptotic cells using a normally distributed formula. The expected number was 15 patients for each group. The means of apoptotic positive cells derived from the pilot study were 3.5, 1, and 6 cells per 100 cells for EE-DSG, DSG, and control, respectively, with a common standard deviation equal to 2, considering a data loss of 10%, alpha of 0.05, and power of 80% (β=0.8).

$$n = \frac{2(Z_{1-\frac{\alpha}{2}} + Z_{1-\beta})^2}{(\frac{\mu_1 - \mu_2}{\sigma})^2}$$

Immunohistochemistry: Immunohistochemistry (IHC) was conducted for Ki-67, a marker for cellular proliferation, and B-cell lymphoma 2 (Bcl-2), a regulator of apoptosis. Monoclonal mouse anti-human Ki-67 antibody (M7240) (DakoCytomation, Carpinteria, CA, USA) was used at a 1:50 dilution and the monoclonal mouse anti-human Bcl-2 antibody (124) (Cell Marque, Rocklin, CA) was used at a 1:2000 dilution. Immunohistochemical analyses were performed using the Bond Polymer Refine Detection kit (Leica Microsystems). Briefly, sections were deparaffinized, incubated, and treated with Bond Dewax Solution. Epitope retrieval was performed by incubating the slides in Bond Epitope Retrieval Solution 2 for 20 minutes at 100°C. Primary antibody was applied for 45 minutes at room temperature followed by consecutive rinses with Bond Wash Solution. Peroxide block (3% hydrogen peroxide) was then applied for 5 minutes. Post primary polymer was applied before rinsing with Bond Wash Solution. Polymer Poly-HRP IgG was applied before the diaminobenzidine chromogen was applied. Slides were counterstained with hematoxylin for 5 minutes. The 30 representative fields in each slide were counted using a microscope at 400x magnification by only one observer that was blinded to the treatment group. The data were expressed as the median of positive cells /100 cells

Apoptotic detection system: Apoptosis was assessed using terminal deoxynucleotidyl transferase (TdT) – mediated dUTP digoxygenin nick-end labeling (TUNEL) with the DeadEnd™ Colorimetric TUNEL System (Promega, Madison, WI, USA). Paraffin-embedded sections were deparaffinized and then were permeabilized with 20 µg/ml proteinase K solution. TdT reaction mix was added to the deparaffinized tissue slides. Slides were bound with 100 µl streptavidin HRP and incubated for 30

minutes. Finally, slides were stained with 3 - diaminobenzidine tetrahydrochloride. Positive control slides were prepared by treating a sample with DNase I to cause DNA fragmentation. The apoptotic cells were counted the same way as the IHC method.

Statistical analysis: Demographic data were analyzed using the one-way analysis of variance (ANOVA) and Chi-square tests for normally distributed continuous and discrete data, respectively. The Kruskal-Wallis One-way ANOVA was used for the comparison of three groups with non-normally distributed continuous variables. The Mann-Whitney U Test was used for subgroup comparison of non-normally distributed continuous data. The outcomes were analyzed by per-protocol analysis. All statistical analyses were performed by SPSS for Microsoft Windows Version 21. The level of statistical significance was set at $P < 0.016$ for the Kruskal-Wallis One-way ANOVA analysis and at $P < 0.05$ for other statistical tests.

Results

The 45 recruited patients were randomly divided into three groups. Results from two participants were not analyzed. No endometriotic cysts were found in one patient from the EE-DSG group. The other patient was from the DSG group and was excluded since medication lasted longer than 35 days. The characteristics of both the EE-DSG and EE groups were not significantly different in age, body mass index, duration of medication, type of operation, American Society of Reproductive Medicine (ASRM) classification, and side effects ($P>0.05$) (Table 1). No severe adverse effects were found (data not shown).

Cell proliferation in ectopic endometrium (Ki-67 IHC): The median and interquartile range [IQR] of cell proliferation in cells from ectopic endometrial tissue in the EE-DSG group was significantly higher than that in DSG group (1.4 [1.2] vs 0.6 [0.7], $P < 0.016$) (Figure 2).

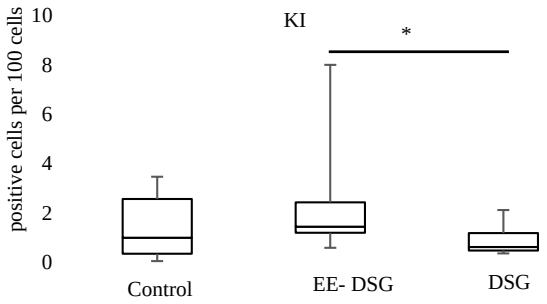

Figure 2: Effect of a combination of ethinyl estradiol and desogestrel, and desogestrel only compared with control on cell proliferation in ectopic endometrial tissue. The data were expressed as median and quartile of cell proliferation/100 cells.
*P<0.016 was considered statistically significant.
Note: EE, ethinyl estradiol; DSG, desogestrel.

Apoptosis in ectopic endometrium (TUNEL assay): There was significant difference between the medians and IQRs of apoptosis in cells from ectopic endometrial tissue between the EE-DSG, DSG, and control groups (2.8 [0.7] vs 1.8 [1.4] vs 0.1 [0.2], respectively, $P < 0.016$ for each pair) (Figure 3).

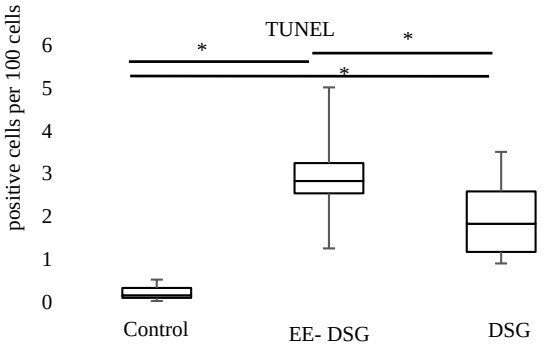

Figure 3: Effect of a combination of ethinyl estradiol and desogestrel, and desogestrel only compared with control on apotosis in ectopic endometrial tissue.
The data were expressed as median and quartile of apoptotic cells/100 cells.
*P<0.016 was considered statistically significant.
Note: EE, ethinyl estradiol; DSG, desogestrel.

Table 1: Characteristics of the participants

	EE-DSG (n =15)	DSG (n =15)	Control (n =15)
Age (yrs) (mean ± SD)	30.6 ± 4.9	34.1 ± 7.1	34.6 ±6.1
BMI (kg/m^2) (mean ± SD)	22.3 ± 3.9	20.8 ± 3.3	22.2 ± 3.0
Duration of medication (days) (mean ± SD)	31.1 ± 2.7	32.7 ± 6.4	-
Side-effects (n, %)	7 (46.7%)	8 (53.3%)	0 (0%)
Recurrent endometriosis (n, %)	1 (6.7%)	1 (6.7%)	2 (13.3%)
Type of operation (n, %)			
Laparoscopy	12 (80%)	9 (60%)	9 (60%)
Laparotomy	3 (20%)	6 (40%)	6 (40%)
ASRM classification (n, %)			
Stage III	9 (60%)	6 (40%)	8 (53.3%)
Stage IV	6 (40%)	9 (60%)	7 (46.7%)

EE, ethinyl estradiol; DSG, desogestrel; BMI, body mass index; ASRM, American Society of Reproductive Medicine. No statistical significance between groups was demonstrated by ANOVA.

Expression of Bcl-2: No significant differences between the medians and IQRs of Bcl-2/pro-apoptosis positive cells from ectopic endometrial tissue in the EE-DSG, DSG and control groups were found (Figures 4 and 5).

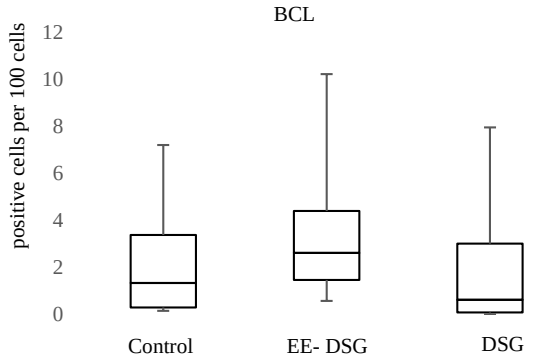

Figure 4: Effect of a combination of ethinyl estradiol and desogestrel, and desogestrel only compared with control on apotosis in ectopic endotetrial tissue.
The data were expressed as median and quartile of apoptotic cells/100 cells.
*P<0.016 was considered statistically significant.
Note: EE, ethinyl estradiol; DSG, desogestrel.

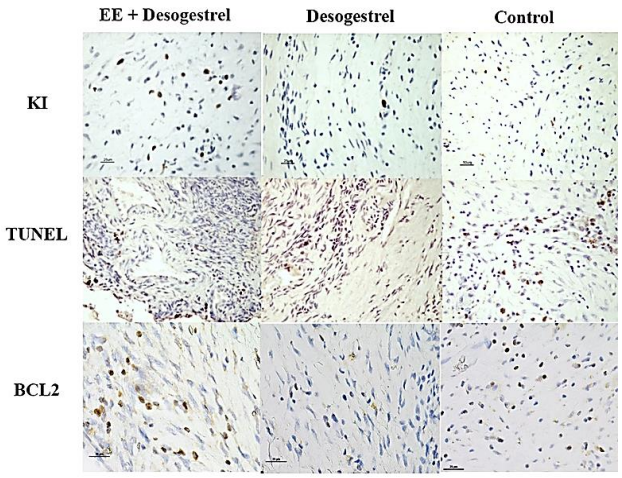

Figure 5: Representative photomicrograph of immunohistochemical resections for KI and BCL2 and TUNEL analysis from endometriotic cyst wall in EE-DSG, DSG and control groups. The cells expressing immunoreactivity for Ki-67 were determined by counting all brown nuclear staining cells at 400x magnification in 30 representative fields.

Discussion

The development and growth of endometriosis are directly dependent on estrogen. In regards to the concurrent estrogenic effect of oral COCs, one treatment option for endometriosis has recently been published. Our single-blind randomized controlled trial compared the effects of treating patients with daily treatments of DSG only or COCs (a combination of EE and DSG) over a course of 28-35 days with patients receiving no treatment. We demonstrated that patients receiving COCs exhibited rates of cell proliferation that were higher than patients given only DSG, but they were not higher than patients receiving no treatment. DSG alone increased cell apoptosis but did not affect cell proliferation. Although both COCs and DSG alone increased cell apoptosis in ectopic endometrial tissue, COCs caused higher rates of cell apoptosis. Therefore, concurrent EE in COCs enhanced both cell proliferation and apoptosis in ectopic endometrial tissue. These results likely explain why the clinical outcomes of patients treated for endometriosis with COCs are not largely different from patients receiving progestin alone.

The characteristics of eutopic endometrium are different from ectopic endometrium. Apoptosis or programmed cell death is a key factor in endometrial cell cycle dynamics and impacts the process of cyclical endometrial shedding during menstruation (18). Increased proliferation but reduction of apoptosis of ectopic endometrium as well as changes in enzyme activity and steroid hormone receptor levels were found in ectopic endometrium compared to the eutopic endometrium of healthy women (19, 20). Reduction in apoptosis of ectopic endometrium can be explained by apoptosis resistance that reduces the endometrium's ability to undergo cyclical changes (18). This abnormality is caused by the up-regulation of anti-apoptotic factors and down-regulation of pro-apoptotic factors (21). Moreover, progesterone resistance is caused from reduced progesterone receptor B expression that inhibits cyclical changes and decreases apoptosis in ectopic endometria. The enhanced proliferation of ectopic endometrium could be explained by local estradiol production that is caused by an increase of aromatase enzyme and decrease of 17 β hydroxysteroid dehydrogenase type 2 (22, 23).

Progestin has been demonstrated to have anti-proliferative effects on endometrial tissue *in vitro*, in animal models, and clinical studies. Minami et al and Miyashita et al (2015) treated endometriotic and eutopic stromal cells *in vitro* with progestins (i.e., norethisterone (NET) or drospirenone) (15, 16). They demonstrated that progestins inhibit cell proliferation in both endometriotic and eutopic stromal cells (15,

16). Similar to Minami et al and Miyashita et al, Bo et al demonstrated that the progestin Duphaston decreased the proliferation of isolated endometriotic cells in the mouse model of endometriosis (24). Clinical studies performed by Miyashita et al (2014), Gomes et al, and Tingthanatikul et al have also demonstrated the anti-proliferative effect of progestins on ectopic and eutopic endometrial tissue (17, 25, 26). However, in our current study, progestin did not have anti-proliferative effects on ectopic endometrial tissue. There are many possible reasons for why our results are not in accordance with the previously mentioned studies. For example, it may have been due to the different types of progestin used, sample sizes, and objectives of each study. The progestin used in our study exerted less progestogenic effects (DSG) than the progestins used in the studies of Miyashita et al (i.e., dienogest), Gomes et al (i.e., LNG-IUS), and Tingthanatikul et al (i.e., DMPA). Furthermore, the ideal sample sizes that were calculated for our current study to enhance the statistical power of our results were calculated based on the differences in apoptosis positive cells between the treatment and control groups.

Although the role of progestin in promoting apoptosis in endometrial tissue is controversial, there are a number of studies that have demonstrated the apoptotic effects of progestin in women with endometriosis. In the *in vitro* study by Minami et al, they found that endometriotic stromal cells (ESCs) treated with norethisterone (NET) exhibited increased levels of apoptotic cells, but ESCs treated with progesterone did not (15). However, in the *in vivo* studies by Miyashita et al (2014) and Tingthanatikul et al, they found that treatment with dienogest or DMPA increased apoptosis in endometrial tissue and eutopic endometria in women with endometriosis (17, 26). The results from our study were comparable to most previous studies, but the *in vitro* study by Miyashita et al (2015) found that ESCs treated with drospirenone demonstrated no increase in apoptosis (16). Thus, the different types of progestin used would be the most likely cause for this different outcome.

Our study demonstrated that high doses of estrogen in COCs stimulated cell proliferation and counteracted the effects of progestin by enhancing apoptosis of ectopic endometrial tissue. We compared COCs containing supraphysiologic doses of estrogen with the same type and dose of progestin alone. Very few studies have focused on how estrogen in COCs may impact the effects of progestin on endometriosis. Bono et al cultured and treated immortalized epithelial cell lines with NET, LNG, and dienogest alone or in combination with EE. Their results demonstrated that EE enhanced the effects in progestin on growth suppression in epithelial cells via estrogen receptor α and by promoting expression of progesterone (PR)-B (27). However, since our study did not investigate the mechanisms of the pro-apoptotic effects in terms of steroidal receptors of concurrent estrogen in COCs, further research is needed.

There is no clear consensus on how COCs affect proliferation in ectopic endometrium. Our results demonstrated that COCs enhance cell proliferation. In contrast, Meresman et al conducted a clinical trial in which they examined eutopic endometria of patients before and after treatment that either was treated with COCs (DSG 0.15 mg + Ethinyl E2 30 μg daily for 30days) or received no treatment (14). They found that cell proliferation was significantly decreased in eutopic endometrial tissue after treatment (14). While Meresman et al observed effects of COCs on endometrial tissue that was different from what we observed, they also examined different time points (i.e., pre and post-treatment) and tissues (i.e., eutopic vs ectopic) than we did.

The apoptotic effects of DSG alone and COCs containing DSG likely do not act through through the anti-apoptosis Bcl2 protein. Apoptotic pathways such as extracellular signal-regulated kinase (ERK1/2) phosphorylation and serine/threonine protein kinase B Akt phosphorylation, among others, may be involved (18). In line with our study, a previous study demonstrated no correlation between *Bcl2* expression and number of apoptotic cells (28). However, further research is needed to elucidate the mechanism of action by which DSG promotes apoptosis.

This is the first study to evaluate the effects of EE in COCs on cell proliferation and apoptosis in ectopic endometrial tissue *in vivo*. Nevertheless, we were not able to assess cell proliferation and apoptosis before and after treatment for each person. Another limitation of our study is that the period of hormonal therapy was relatively short. Further studies are needed to elucidate the mechanism of action of EE alone and in combination with progestin in endometriosis. In addition, future studies should examine EE dose responses and the effects of EE on ER and PR expression.

Conclusion

In conclusion, EE in COCs enhances both

proliferation and apoptosis in ectopic endometrium. However, COC promoted more apoptotic cells effect than progestin alone. The pro-apoptotic effects of progestin and COCs were not exerted through *Bcl2*.

Conflict of Interests

Authors have no conflict of interests.

Acknowledgments

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