DMPA Suppresses Cell Proliferation and Enhances Cell Apoptosis of Eutopic Endometrium in Women with Endometriosis: A Randomized Controlled Study

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Background: *Although Depo-medroxyprogesterone acetate (DMPA), an injectable contraceptive progestin, is very effective for pain relief and prevention of recurrence in women with endometriosis, there is no report on the mechanism of this medication about cell proliferation and apoptosis.*
Objective: *To investigate the effects of DMPA on cell proliferation and apoptosis in the eutopic endometrium of women with endometriosis.*
Material and Method: *A randomized controlled study was conducted in 28 women with endometriosis. The DMPA-treated group included 14 women who were scheduled to undergo laparoscopic surgery after 150 mg of DMPA injections. The control group included 14 women who were scheduled to undergo the surgery without DMPA injection. The endometrial tissue was obtained from each woman by endometrial aspiration before surgery. The ELISA formats of PCNA and the quantitative colorimetric analysis of TUNEL were used for estimating cell proliferation and apoptosis of the eutopic endometrium.*
Results: *There were no differences in the women characteristics between the two groups. The relative level of cell proliferation was significantly less in the DMPA than the control groups (1.08±0.57 vs. 1.73±0.50, p = 0.014). Whereas the relative level of cell apoptosis was greater in the DMPA group than that in the control group (1.12±0.36 vs. 0.82±0.39, p = 0.034).*
Conclusion: *Three months of 150 mg DMPA treatment could suppress cell proliferation and enhance cell apoptosis of the eutopic endometrium of women with endometriosis.*

Keywords: *Apoptosis, Cell proliferation, Depo-medroxyprogesterone, Endometriosis, Eutopic endometrium*

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Endometriosis is a common disease that is defined by the growth of endometrium-like glandular and stromal tissues outside the uterus[1]. The main clinical manifestations include chronic pelvic pain, dysmenorrhea, infertility, and endometrioma[1]. The most widely accepted theory on the pathogenesis of endometriosis is retrograde menstruation[2]. A recent study showed that the eutopic endometrial cells from women with endometriosis have a greater proliferative activity[3] than in women without endometriosis. Interestingly, a reduction of apoptosis of both eutopic and ectopic endometrial samples of women with endometriosis has been demonstrated[4,5]. As a result, the endometrial cells could survive on the pelvic peritoneum and develop the disease.

Many hormonal regimens have been used to treat endometriosis including progestins, oral contraceptive pill (OCP), danazol, and gonadotropin-releasing hormone agonists (GnRH-a). These medications have high efficacies on pain relief and on prevention of the disease recurrence[6-8]. Progestins and OCP should be considered as the first-line therapy based on their safety, tolerability, and cost[7].

Depo-medroxyprogesterone acetate (DMPA) is a progestin that has been used as a contraceptive for a long time, and it was shown to have a high efficacy

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on pain relief in women with endometriosis[7]. Furthermore, DMPA appears to be effective and safe for using in long-term treatment in order to prevent recurrence of endometriosis[9]. To date, the mechanism of DMPA action on endometriosis has not yet been studied, while other progestins including levonorgestrel-releasing intrauterine system (LNG-IUS) and OCP could significantly reduce cell proliferation and increase the apoptosis in the eutopic endometrium of women with endometriosis[10-12]. Therefore, in the present study we have investigated the effect of DMPA on cell proliferation and apoptosis in the eutopic endometrium of women with endometriosis.

Material and Method

A randomized control trial (RCT) was conducted between September 2011 and February 2012, which was approved by the Ethical Clearance Committee on Human Related to Researches Involving Human Subjects, Faulty of Medicine, Ramathibodi Hospital, Mahidol University. An informed consent was obtained from each woman.

Subjects

Women with endometriosis were recruited in the present study. These women were scheduled to undergo a laparoscopic surgery at the Department of Obstetrics and Gynecology, Faculty of Medicine, Ramathibodi Hospital. The inclusion criteria were 1) age 25-45 years old, 2) having regular menstrual cycle (21-35 days interval) at least three months, and 3) having received no OCP for at least three months, nor DMPA and GnRH-a for nine months before recruitment.

These women were randomly allocated into two groups. A randomization with a block of four, and at study to control ratio of 1:1 was run by a computer-generated random number list. The allocation sequence was concealed in sequentially numbered, opaque, sealed, and stapled envelopes. This process was prepared by a statistician from the Clinical Epidemiology Unit of Research Center. The corresponding envelope was opened by a nurse after the woman was enrolled. In the first group (DMPA group), each woman received a single dose of 150 mg DMPA by intramuscular injection. In the second group, the women did not receive DMPA (control group). A laparoscopic surgery was scheduled to be performed in the mid to late proliferative phase of the menstrual cycle (seventh to fourteenth day) for the control group and within 12±2 weeks after DMPA injection for the DMPA group.

The eutopic endometrium was obtained by a vacuum aspirator (Endocell®, Wallach surgical devices, CT, USA) after anesthesia and before starting the surgery. The specimens obtained were kept at -80°C for assessing cell proliferation and apoptosis. The revised classification of American Fertility Society (rAFS)[13] was used to evaluate the severity of the disease. The endometriotic tissues including cyst walls were sent for the pathological diagnosis.

The exclusion criteria were 1) women denied to participate, 2) no the eutopic endometrial tissue was obtained, and 3) the pathological diagnoses of diseases other than endometriosis.

Sample size was calculated according to measurement outcomes for both cell proliferation and apoptosis. From a pilot study with alpha of 0.05, beta of 0.80, and suspecting 10% loss, a number of samples were 16 and 28. Therefore, 14 women for each group were included in the present study.

Assessment of cell proliferation

To evaluate cell proliferation the enzyme-link immunosorbent assay (ELISA) for proliferating cell nuclear antigen (PCNA) (TREVIGEN, Gaithersburg, MD, USA) was used. The 96 wells plate was coated with 100 µl coating buffer pH 9.6 containing 1.25 µg/ml of each tissue and incubated at 4°C in a moist chamber overnight. The plate was then gently shaken for 10 minutes and washed three times with 0.01 M of phosphate-buffered saline/tween (PBST) for 1 minute each. The non-specific binding was blocked by incubating with 100 µl/well blocking solution (10% skim milk in PBST). After three washings with PBST, the coated plate was incubated with anti PCNA at dilutions 1:500 for two hours at 37°C in moist chamber. After three washings with PBST, the coated plate was incubated with 100 µl of secondary antibody, goat anti-rabbit IgG HRP (Southern Biotech, AL, USA), in PBS at dilution 1:5,000 for 1 hour at 37°C. The color was developed with TMB substrate (SureBlueTM TMB microwell peroxidase substrate) containing 3,3', 5,5'-tetra-methylbenzidine in an acidic buffer (KPL, Gaithersburg, MD, USA), then incubated for 10 minutes in a dark chamber. After incubation, the reaction was stopped by adding 100 µl of 1 N HCL, then the optical density (OD) was measured at 450 nm using a spectrophotometer to determine the levels of antibody.

Assessment of cell apoptosis

The quantitative colorimetric analysis of terminal deoxynucleotidyl transferase dUTP nick end

labeling (TUNEL) was used to evaluate cell apoptosis with HT titer TACSTM Assay kit (TREVIGEN, Gaithersburg, MD, USA) that is a highly sensitive and specific assay for apoptosis. Briefly, Genomic DNA from tissue was extracted by using DNAzol reagent (Invitrogen, CA, USA). After centrifuged at 10,000 g, 4°C, the supernatant was collected and transferred to a fresh tube. The DNA was then precipitated and washed by using 100% and 75% ethanol, respectively. After washing, the DNA was dried and re-suspended with deionized water. One microgram of DNA from each tissue was diluted in coating buffer (1.5 M NaCl, 0.3 M Tris and 0.3 M $MgCl_2$, pH 8.0) and placed in 96-well plate. After incubation at 4°C overnight, DNA was then washed by washing buffer (1 M NaCl, 0.1 M Tris, 2 mM $MgCl_2$, 0.1% Tween -20, pH 9.3). The endogenous peroxidase was eliminated by incubating 3% H_2O_2 in 100% methanol for five minutes. at room temperature, and then incubated with the labeling reaction mix containing 1xTdT labeling buffer, Mn^{2+}, dNTP mixed, and TdT enzyme to label the fragmented DNA. Streptavidin-HRP and substrate, TACS-Sapphire (TREVIGEN, Gaithersburg, MD, USA), were added to the wells to detect apoptosis. The reaction was then stopped by adding 0.2 N HCl, and absorbance was read at 450 nm with and ELISA plate reader.

Statistical analysis

Statistical analysis was performed by using STATA version 12. The data were presented as mean and standard deviation. To compare the relative level of cell proliferation and apoptosis as detected by PCNA and TUNEL, respectively, the student's t-test or the quartile regression analysis was used where appropriate. Statistical significance was considered with $p<0.05$.

Results

Endometriosis was confirmed histologically for all 28 women. The endometrial tissue of all women could be obtained and no sample was excluded. The baseline characteristics were presented in Table 1. There were no significant differences between groups in age, BMI, and staging of endometriosis. The mean age and BMI were 32.3±5.3 years and 25.8±3.4 kg/m^2 in the DMPA group and 33.7±5.5 years and 23.2±3.9 kg/m^2 in the control group, respectively. Twelve women (85%) in the DMPA group and eleven women (78%) in the control group were classified as the endometriosis stage IV.

The eutopic endometrium obtained from women who received DMPA showed a significant lower the relative level of cell proliferation than that from the women without DMPA (1.08±0.57 vs. 1.73±0.50; $p = 0.014$). The relative level of cell apoptosis, assessed by the quantitative colorimetric analysis for TUNEL, was significantly greater in women with DMPA than those without DMPA (1.12±0.36 vs. 0.82±0.39, $p = 0.034$) (Table 2).

Discussion

The eutopic endometrium of women with endometriosis has some characteristics that are different from those without endometriosis. For examples, an increased cell proliferation and a decreased apoptosis have been reported in women with endometriosis[3-5]. These conditions can facilitate a survival and growth of the tissues in some ectopic sites that may progress to the peritoneal endometriosis, which supports the retrograde theory[1].

The current study has shown the effect of DMPA on suppression of cell proliferation of the eutopic endometrium in women with endometriosis. The finding supported a study that demonstrated anti-proliferative effect of DMPA on cell proliferation in vitro[12]. In addition, this observation was similar to the suppressive effect when other progestins were used. A few studies have also shown that OCP and LNG-IUS can reduce cell proliferation of the eutopic glands and stromas in women with endometriosis[10,14].

Table 1. Baseline characteristics

	DMPA group (n = 14)	Control group (n = 14)	*p*-value
Age (year)	32.3±5.3	33.7±5.5	NS
BMI (kg/m^2)	25.8±3.4	23.2±3.9	NS
Stage of disease (rAFS 1998)			NS
Moderate	2 (15)	3 (22)	
Severe	12 (85)	11 (78)	

DMPA = depo-medroxyprogesterone acetate; BMI = body mass index; rAFS = revised American Fertility Society
Data were presented as mean ± SD and n (%)

Table 2. Comparison of cell proliferation and apoptosis between the women with and without DMPA

	DMPA group (n = 14)	Control group (n = 14)	*p*-value
Cell proliferation	1.08±0.57	1.73±0.50	0.014
Cell apoptosis	1.12±0.36	0.82±0.39	0.034

DMPA = depo-medroxyprogesterone acetate
Data were presented as mean ± SD

The increase of cell apoptosis in the endometrium by DMPA observed in the present study is similar to the data reported in recent studies that showed that OCP and LNG-IUS induced apoptosis of the endometrial cells in women with endometriois[10,14]. In addition, in women without endometriosis, there has been a report of a significant increase in apoptosis of the stromal cells after treatment with medroxyprogesterone acetate (MPA)[15].

It is possible that DMPA acts on cell proliferation and apoptosis by the same mechanisms as other progestins. Progesterone and OCP can inhibit prostaglandin E2 (PGE2) synthesis, and suppress aromatase expression that results in the decrease of estradiol production in endometrial epithelial cells[16]. Since PGE2 and estradiol are key factors in inducing endometrial cell proliferation[17,18], this activity are therefore decreased after progesterone and OCP treatments. Furthermore, progestins can cause cell apoptosis by exerting their effects on either the mitochondrial pathway involving Bax, Bcl-2 proteins and gene expressions[11] or the death receptor pathway involving the Fas-Fas ligand (FasL) system[19], both pathways play roles in cell apoptosis[20,21]. As a result, progestins can increase the cell apoptosis.

The ELISA and the quantitative colorimetric analysis techniques used for estimating cell proliferation and apoptosis in the present study could only demonstrate the action of DMPA on the whole endometrium, but these methods cannot specify the cell types, i.e., the glandular or the stromal cells of the endometrium. Previously, there have been reports on the selective effects of progestins on cell proliferation and apoptosis of the glandular and stromal cells[4,10,14]; therefore, a further study with specific techniques, such as immunohistochemistry, is needed to elucidate the specific action of DMPA on endometrial cells.

Limitation of the present study was that it could not compare the data between pre- and post-DMPA treatment due to ethical consideration and inconvenience to the patients. Alternatively, we have conducted the RCT that demonstrated that the effect of DMPA on both apoptosis and proliferation rates before treatment were similar to those without treatment[14]. Therefore, the results from the DMPA-treated group compared with the control group could represent the real effect of DMPA.

In conclusion, 12 weeks of DMPA administration enhanced cell apoptosis and suppressed cell proliferation in the eutopic endometrium of women with endometriosis. However, the ELISA technique used in the present study could not differentiate the specific action of DMPA on various types of endometrial cells, and this will be investigated in our future study.

What is already known on this topic?

A review of the literature found a recent study showing that the eutopic endometrial cells from women with endometriosis have a greater proliferative activity and a reduction of apoptosis of both eutopic and ectopic endometrium.

Many hormonal regimens have been used to treat endometriosis such as progestins, OCP, danazol, and GnRH-a.

DMPA is a progestin that has been used as a contraceptive for a long time, and it was shown to have a high efficacy on pain relief in women with endometriosis. To date, the mechanism of DMPA action on cell proliferation and apoptosis of treated endometriosis patient has not yet been studied, while other progestins including LNG-IUS and OCP could significantly reduce cell proliferation and increase the apoptosis in the eutopic endometrium of women with endometriosis.

What this study adds?

The present study showed that DMPA could suppress endometrial cell proliferation and enhance cell apoptosis after 12 weeks administration. It was shown that the number of endometrial cell proliferation in stromal and glands, which was evaluated by ELISA for PCNA, was significantly decreased. In addition, the number of apoptosis cells, which assessed by the quantitative colorimetric analysis of TUNEL, had increased significantly is stromal (but not gland) when compared with pre-treated endometrium. Our finding corresponding with previews studies on human endometrium with outs endometriosis.

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Potential conflicts of interest

None.

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ยา DMPA ยับยั้งการแบ่งเซลล์และเร่งกระบวนการเซลล์แตกตายของเซลล์เยื่อบุมดลูกในสตรีที่มีภาวะเยื่อบุมดลูกเจริญผิดที่: การศึกษาแบบสุ่ม

ญาดา ติงธนาธิกุล, โยโกะ ทาวาราชูมิดา, ศรีเธียร เลิศวิกูล, แอนนา วงษ์กุหลาบ, อารีย์พรรณ โสภณสฤษฎ์สุข, เสวก วีระเกียรติ, มรกต สร้อยระย้า, ปิยะฉัตร จันทร์เสละ, นรินทร์ ช่างกลึงเหมาะ, สินีนาฏ ทรงคุ้มครอง, จารุวรรณ ผลเจริญ, ประเสริฐ โศภน

ภูมิหลัง: *การฉีดยาคุมกำเนิดชนิด DMPA ช่วยลดความเจ็บปวดและป้องกันการกลับเป็นซ้ำของภาวะเยื่อบุมดลูกเจริญผิดที่ได้ แต่ยังไม่มีรายงานกลไกของยา DMPA เป็นต้นว่าการแบ่งเซลล์และกระบวนการเซลล์แตกตายของเซลล์*

วัตถุประสงค์: *เพื่อศึกษาผลของ DMPA ต่อการแบ่งเซลล์และกระบวนการเซลล์แตกตายของเซลล์เยื่อบุมดลูกในสตรีที่มีภาวะเยื่อบุมดลูกเจริญผิดที่*

วัสดุและวิธีการ: *การศึกษาแบบสุ่มในสตรีที่มีภาวะเยื่อบุมดลูกเจริญผิดที่จำนวน 28 ราย กลุ่มศึกษาจำนวน 14 ราย ได้รับการรักษาด้วยการฉีด DMPA 150 มก. ส่วนกลุ่มควบคุมจำนวน 14 ราย ไม่ได้รับการฉีด DMPA อาสาสมัครทั้งหมดได้รับการดูดชิ้นเนื้อเยื่อบุมดลูกก่อนทำการผ่าตัดส่องกล้อง เพื่อศึกษาการแบ่งเซลล์โดยใช้ PCNA ด้วยวิธี ELISA และกระบวนการเซลล์แตกตาย (TUNEL) ด้วยวิธี the quantitative colorimetric analysis ของเซลล์เยื่อบุมดลูก*

ผลการศึกษา: *ลักษณะทั่วไปของสตรีทั้งสองกลุ่มไม่มีความแตกต่างกัน การแบ่งเซลล์ของเซลล์เยื่อบุมดลูกในกลุ่มที่ได้รับ DMPA มีน้อยกว่ากลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ (1.08±0.57 กับ 1.73±0.50, p = 0.014) แต่กระบวนการเซลล์แตกตายของกลุ่มที่ได้รับ DMPA มีมากกว่ากลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ (1.12±0.36 กับ 0.82±0.39, p = 0.034)*

สรุป: *การฉีด DMPA 150 มก. ก่อนการผ่าตัด 3 เดือน สามารถยับยั้งการแบ่งเซลล์และเร่งกระบวนการเซลล์แตกตายของเซลล์เยื่อบุมดลูกในสตรีที่มีภาวะเยื่อบุมดลูกเจริญผิดที่ได้*