의학박사 학위논문

자궁내막증에서 디에노게스트의 에스트라디올 및 염증 싸이토카인 작용 저해를 통한 자궁 내막 세포의 생존능과 증식 감소 효과 검증: 세포배양 및 동물실험 연구

Dienogest may reduce endometrial cell viability and proliferation by inhibition of the action of estradiol and inflammatory cytokine in endometriosis: A cell culture- and mouse model-based study

울 산 대 학 교 대 학 원

의 학 과

김 현 진

Dienogest may reduce endometrial cell viability and proliferation by inhibition of the action of estradiol and inflammatory cytokine in endometriosis: A cell culture- and mouse model-based study

지 도 교 수 김 성 훈

이 논문을 의학박사학위 논문으로 제출함

2022 년 2 월

울 산 대 학 교 대 학 원

의 학 과

김 현 진

김현진의 의학박사학위 논문을 인준함

심사위원 채 희 동 (인)

심사위원 김 성 훈 (인)

심사위원 김 대 연 (인)

심사위원 조 시 현 (인)

심사위원 이 사 라 (인)

울 산 대 학 교 대 학 원

2022 년 2 월

국문 요약

디에노게스트는 경구 합성 프로게스틴 제제로, 자궁내막증의 장기 치료에 효과적으로 알려져 있다. 그러나 디에노게스트의 작용 기전과 자궁내막 세포에 대한 억제 효과, 그리고 이후의 생물학적 반응에 대해서는 아직 제한적인 연구결과가 존재한다. 따라서 본 연구에서는 생체 내 및 체외 실험을 통하여 디에노게스트가 인간 자궁내막 세포에서 어떤 생물학적 변화를 일으키는지 알아보고자 하였다. 이를 확인하기 위해, 인간 자궁내막 기질 세포를 이용하여 에스트라디올 단독 처리 혹은 디에노게스트와 함께 처리하였을 때 세포 생존능과 세포 침투성, 그리고 자궁내막증 발병 기전과 연관이 있다고 알려진 몇 가지 인자들을 평가하였다. 또한 종양괴사인자 알파와 인터루킨 1 베타 및 32 알파/감마를 동일한 방식으로 실험하여, 디에노게스트의 잠재적 억제 효과를 알아보고자 하였다. 이에 더하여, 생쥐 실험을 통하여 디에노게스트 섭취 유무에 따른 생쥐 내 이식된 자궁내막 조직의 크기를 비교하고, 면역조직화학 염색을 통하여 증식세포 핵항원, 단백질 Ki-67, 에스트로겐 수용체 알파/베타, 프로게스테론 수용체, 그리고 크루펠 유사 인자 9 의 표현을 확인하였다. 실험 결과, 자궁내막 기질 세포를 에스트라디올과 디에노게스트와 함께 처리하였을 때 세포 생존능, 단백질 인산화효소 B 의 인산화, p21 활성화 키나아제 4 와 혈관내피생성인자의 표현이 통계적으로 유의하게 감소함을 확인할 수 있었다. 종양괴사인자 알파와 디에노게스트를 처리하였을 때는 세포 생존능과 단백질 인산화효소 B 의 인산화, 그리고 증식세포 핵항원의 표현이 유의하게 감소하였으며, 인터루킨 1 베타, 인터루킨 32 알파/감마 각각과 디에노게스트를 함께 처리하였을 때는 순서대로 세포 생존능 만이, 혹은 이와 함께 증식세포 핵항원의 표현이 통계적으로 유의하게 감소함을 확인하였다. 생쥐실험에서, 이식된 자궁내막 조직의 크기는 디에노게스트를 먹인 생쥐 군에서 통계적으로 유의하게 그 크기가 감소함을 확인할 수 있었으며, 이에 대한 면역조직화학 염색에서 증식세포 핵항원의 발현이 감소되어 있었다. 결론적으로, 이번 실험을 통하여 디에노게스트가 에스트라디올, 종양괴사세포 알파, 인터루킨 1 베타와 32 을 통하여 유도된 세포 생존능과 증식을 감소시키고, 증식세포 핵항원의 발현을 감소시킴으로 자궁내막증의 발병을 억제할 수 있으리라 생각된다.

목차

Ⅰ. Introduction

Endometriosis is a disorder characterized by the presence of the endometrial stroma and glands outside the uterine cavity [1]. It affects women of reproductive age, causing several symptoms, including pelvic pain, dysmenorrhea, dyspareunia and infertility, and considerably affects women's quality of life [2-4]. In general, the prevalence of endometriosis is reported to be 2–10 % in women; however, it is reported to be up to 50 % in infertile women [5, 6]. One of the conventional treatments for endometriosis includes surgical excision of the entire lesion, followed by postoperative medical treatment, owing to a high recurrence rate. However, surgical excision can be associated with complications and disease recurrence; a considerable number of patients do not respond to treatment or ultimately require multiple surgeries [7-10].

Gonadotropin-releasing hormone (GnRH) analogs represent the most effective medical treatment for endometriosis. These analogs induce hypoestrogenism, provide effective pain relief, and suppress the progression of endometriotic implants [11]. However, GnRH agonists are limited in that they cannot be used for long-term treatment because of their severe hypoestrogenic effects. In contrast, progestins, which are synthetic progesterone, reduce serum estrogen levels by preventing ovulation, without causing hypoestrogenism [12]. Progestins also exert progestogenic effects on an estrogen-primed endometrium [13]. Dienogest (DNG), a progestin and a 19-nortestosterone derivative, was first approved for the treatment of endometriosis in Europe in 2009. It is highly selective for progesterone receptors (PGRs) and restricts serum estrogen levels by preventing ovulation, which indirectly controls endometriosis. In addition, it promotes apoptosis and reduces the proliferation of endometriotic cells [14, 15].

Although many studies have been conducted, the etiologies and mechanism of endometriosis remain unclear. It is widely accepted that endometriosis is an estrogen-dependent chronic inflammatory condition, and several proinflammatory cytokines may play an important role in its pathophysiology. Tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β) are proinflammatory factors, which have been associated with the progression of endometriosis. Several studies have shown that TNF-α levels are significantly elevated in the peritoneal fluid of women with endometriosis, and it stimulates the proliferation of endometriotic stromal cells (ESCs) [16, 17]. Interleukin 32 (IL-32) is a proinflammatory cytokine and an emerging factor that may play a potential role in the pathophysiology of endometriosis. This was identified in our previous study, owing to high levels of IL-32 in the peritoneal fluid of

patients with endometriosis, using cell culture and a mouse model [18]. Additionally, the mechanism of action of DNG remains unclear. We decided to use these proinflammatory factors, along with estradiol, to investigate the effect of DNG on human ESCs.

The aim of this study was to investigate the effect of DNG on the biological changes occurring in human endometrial cells, through *in vitro* and *in vivo* experiments. To ascertain this, we evaluated cell viability, cell invasion, and several markers associated with the pathogenesis of endometriosis, using estradiol and other proinflammatory factors alone or in combination with DNG treatment in ESCs. Furthermore, we compared the size of the implanted endometrial tissues between mice with and without the administration of DNG, and evaluated the expression of cell proliferation factors and hormone receptors by immunohistochemistry.

Ⅱ. Materials and Methods

1) Sample collection and primary cell isolation and culture

Endometrial samples were obtained from women undergoing laparoscopic or transabdominal hysterectomies with a diagnosis of uterine leiomyomas. Women with endometrial abnormalities, adenomyosis or pelvic endometriosis (including ovarian endometriomas), and those who were taking hormonal medication in the preceding three months were excluded from the study. The samples were placed in Hank's balanced salt solution and transported to the laboratory for the isolation and culture of ESCs. Written informed consent was obtained from each patient using consent forms, and the protocols were approved by the Institutional Review Board for Human Research of the Asan Medical Center (2014-1165).

Cell isolation and culture processing were performed using a previously described protocol [19]. After the first passage, ESCs were assayed immunocytochemically, using specific cell-surface markers; we have earlier shown that the purity of isolated ESCs is above 95 % [19, 20]. In all the experiments involving ESCs, cells were utilized only after the first passage.

2) Experimental setups for Estradiol, TNF-α, IL-1β, IL-32, and DNG treatment

In each experiment, separate passages of ESCs from different patients were used. After growing the ESCs to 70 % confluence, they were incubated with serum-free, phenol red-free medium (Sigma-Aldrich, St Louise, MO, USA) for 24 h, followed by treatment with estradiol (Sigma-Aldrich) alone, or estradiol and DNG (Sigma-Aldrich) for 24, 48, and 72 h. Similarly, ESCs were treated with TNF-α (R&D Systems. Minneapolis, MN, USA), IL-1β (R&D Systems), and IL-32 α / γ (R&D Systems). Cells were treated independently with the vehicle (dimethyl sulfoxide, Sigma-Aldrich; DMSO, control), estradiol (10^{-8} M), TNF-α (10 ng/mL), IL-1β (10 ng/mL), or IL-32 α / γ (25 ng/mL), or each of these with DNG (10^{-6} M) treatment.

3) Cell viability assay

Cell survival was analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay (Cell Titer 96 Aqueous Cell Proliferation Assay kit; Promega). Absorbance was assessed using a microplate reader at a wavelength of 450 nm, and the results were expressed as a percentage of the absorbance observed in control (DMSO) cells. Cell viability assay was repeated six times with estradiol, five times with TNF-α, eight times with IL-32α and six times with IL-32γ.

4) Invasion assay

Cell invasion was analyzed using a 96-well transwell plate containing 8 μm pore size inserts (Corning, Corning, NY, USA) and coated with Cultrex basement membrane extract (Trevigen, Gaithersburg, MD, USA). After starvation of the cells in serum-free Dulbecco's Modified Eagle's medium (DMEM) for 18 h, 50 μL of the cell suspension (50,000 cells/well) was seeded in the top chamber and 100 μL of serum-free DMEM with DMSO, DMSO + Estradiol (10^{-8} M) or Estradiol (10^{-8} M) + DNG (10^{-6} M) was added to the bottom chamber. The chambers were incubated in a humidified atmosphere containing 5 % CO2 at 37 °C for 24 h. After 24 h of incubation, the top chamber inserts were washed with washing buffer, to remove non-invading cells, and the inserts were transferred to an assay chamber plate to analyze the number of invaded cells. The invaded cells were labeled with 5 μg/mL calcein-AM (Trevigen) in cell dissociation solution at 37 °C for 1 h. The process was repeated six times. The same approach was employed for the assay with TNF- α (10 ng/mL, repeated seven times), and IL-32 α / γ (25 ng/mL, repeated six times). Cell invasion was assessed by measuring the absorbance of the samples at 485 nm (excitation) and 520 nm (emission)—while maintaining the same parameters each time—using a Victor X3 multilabel plate reader (PerkinElmer, Waltham, MO, USA).

5) Enzyme-Linked Immunosorbent Assay (ELISA)

Vascular epithelial growth factor (VEGF) expression in the cell supernatants and cell lysates was quantified using an ELISA kit (R&D Systems), according to the manufacturer's instructions. Absorption of the color produced in response to the streptavidin-horseradish peroxidase reaction was measured at 450 nm, and the optical density was corrected at 570 nm. The optical density was compared with serial dilutions of recombinant human VEGF (standard). The expression of IL-1β / 17 / 32 was also quantitated using the same approach.

6) Western Blot Analysis

The cells were resuspended in lysis buffer (Cell Signaling Technology, Danvers, MA, USA), containing a protease inhibitor cocktail (complete mini tablet, Roche, Indianapolis, IN, USA). The membranes were blocked by incubation for 1 h at room temperature with 25 °C prior to overnight incubation with primary antibodies raised against proliferating cell nuclear antigen (PCNA), p21-activated kinase 4 (Pak4), protein kinase B (AKT), extracellular-signal-regulated kinase (ERK), and β-actin at 4 ºC. After three washes, the membranes were further incubated with a secondary horseradish peroxidase-conjugated anti-immunoglobulin G antibody (Invitrogen, Carlsbad, CA, USA) and visualized using a Pierce chemiluminescent substrate (Life Technologies, Carlsbad, CA, USA). Densitometric quantification of the bands was performed using the Multi Gauge Software (Version 2.3., Fujifilm, Tokyo, Japan).

7) Allotransplantation of endometrial tissues in C57BL/6 mice

Thirty number of all five-week-old C57BL/6 mice (SLC Inc., Shizuoka, Japan) were divided into the donor (six mice) and recipient (twenty-four mice) groups, housed in sterile cages with laminar flow-filtered hoods, and provided *ad libitum* access to rodent laboratory chow (Purina) and reverse osmosis water. All the mice were ovariectomized and administered estrogen pellets, containing 3.2 μg of 17β-estradiol. After seven days of recovery, the mice were anesthetized by inhalation of Alfaxan (100 mg/kg), xylazine (10 mg/kg), and isoflurane (2 %). Endometrial tissues were obtained from the uterus of the mice in the donor group. We divided the uterus of the mice into the right and left horn, and two identical pieces of 3 x 3 mm^2 endometrial tissues cut from the same horn were separately implanted onto the peritoneal wall of one mouse in the control (n=12) and one mouse in DNG administered groups (n=12), to make a pair. After two weeks of allotransplantation, the mice in the DNG treatment group were administered DNG (1 mg/kg) for two weeks; following this, all 12 pairs of recipient mice were euthanized. The lesions were photographed, removed, and measured before fixation for immunohistochemical staining. The study protocol was approved by Institutional Animal Care and Use Committee of the Asan Medical Center (2021-12-116).

8) Immunohistochemistry

Sections from formalin-fixed, paraffin-embedded tissue blocks of endometriotic lesions were cut (3 μm thick) and mounted on glass slides. Immunohistochemistry was performed in accordance with a method described in a previous study [19]. Sections of the lesions were stained using antibodies against PCNA (1:500, Abcam, Cambridge, UK), Ki-67 (1:400, Abcam), Krüppel-like factor 9 (KLF9) (1:400, LSBio, Seattle, WA, USA), estrogen receptor (ER)

α (1:400, Abcam), ER β (1:1000, Invitrogen), and PGR (1:400, Bioss Inc., Woburn, MA, USA). Positive signals were amplified using ultra-VIEW copper, and sections were counterstained with hematoxylin and blue reagent. The intensity of immunoreactivity for each marker was evaluated and analyzed semi-quantitatively, and expressed as an H score, which was used during analysis with the inForm Advanced Image Analysis software (version 2.2., PerkinElamer).

9) Statistical analysis

Normal distribution of the data was assessed using the Kolmogorov–Smirnov test. If the data were normally distributed, continuous variables were compared using the Student's *t*-test (two groups) or analysis of variance and Fisher's least significant difference post hoc test for pairwise comparisons (three groups). If the data were not normally distributed, they were compared using the Mann–Whitney U test (two groups) or the Kruskal–Wallis test, followed by the Mann–Whitney U test with Bonferroni correction (three groups). Statistical computations were conducted using the Statistical Program for the Social Sciences software (version 14.0, IBM). $P < 0.05$ was considered significant.

Ⅲ. Results

1) Changes in ESCs in response to treatment with estradiol alone or a combination of estradiol and DNG

To evaluate the effect of DNG in comparison to the estradiol alone treatment, cell viability, AKT phosphorylation, and Pak4 and VEGF expression were analyzed in ESCs (Fig. 1(a-d)). Cell viability increased after 48 and 72 h of treatment with estradiol alone, and decreased significantly after 48 h of treatment with a combination of DNG and estradiol. The p-AKT/AKT ratio and Pak4 expression also decreased after DNG and estradiol treatment for 48h, and increased after treatment with estradiol alone. In addition, ELISA of estradiol-treated ESCs increased VEGF expression, which significantly decreased after DNG and estradiol treatment.

Similarly, cell invasion and expression of several ILs were also evaluated to determine the effect of DNG treatment. A similar trend was observed in cell invasion; however, the difference was not significant. The expressions of IL-1β, 17, and 32 were also not significantly different (Fig. 1(e-g)).

2) Changes in ESCs in response to treatment with each TNF- α & IL-1β alone or in combination with DNG

We performed a similar analysis after treatment with TNF-α and IL-1β alone, which are the representative cytokines associated with endometriosis, and in combination with DNG. ESCs showed increased cell viability when treated with TNF-α alone, which significantly decreased on treatment with both DNG and TNF-α; similar results were obtained with IL-1β (Fig. 2(a) and 3(a)). Both AKT phosphorylation and PCNA expression showed a significant increase, with a significant decrease on treatment with both DNG and TNF-α. However, there were no significant changes in AKT phosphorylation and PCNA expression based on IL-1β treatment. We evaluated cell invasion and Bcl-2 expression on treatment with TNF-α and IL-1β, respectively, and found were no significant changes (Fig. 2 and 3).

3) Changes in ESCs in response to treatment with IL-32 α / γ alone or in combination with DNG

To evaluate the inhibitory effects of DNG in ESCs with IL-32 treatment, we cultured the cells and treated them with IL-32 α and γ separately. Cell viability significantly increased after treatment with IL-32 α and γ, and

decreased with both IL-32 α and γ, in combination with DNG. When treated with IL-32α, PCNA expression also showed a significant decrease, 24 h after treatment with DNG; however, no significant differences were observed with IL-32γ. Cell invasion, phosphorylation of AKT and ERK, and VEGF expression were analyzed after treatment with IL-32 α/γ alone or in combination with DNG; no significant differences were observed (Fig. 4).

4) Comparison of endometrial tissue implants in mice fed with and without DNG treatment

Endometrial tissues were allotransplanted into 12 pairs of C57BL/6 mice. The results were obtained from nine pairs of mice (total 18 mice), and the size of the lesion was compared between the control and DNG administered groups. The lesion size of the implanted endometrial tissues in mice treated with DNG was significantly reduced than that in the control mice from 53.70 mm^3 to 21.46 mm^3 (Fig. 5(a)). In addition, we evaluated the inhibition of DNG as a percentage of direct pairs, which shared the same horn of the uterus. The inhibitory effect of DNG showed a more significant decrease in lesion size (61.42 % when regarding the control as 100 %) and was statistically significant (Fig. 5(b)).

5) Expression of several markers in endometrial tissues implanted in mice with and without DNG treatment

Using the mouse model experiment, we performed immunohistochemical staining of the implanted endometrial tissues using several markers, already known as endometriosis-related factors. The expression level of PCNA, a cell proliferative factor, was significantly higher in glandular and whole lesions of mice treated with DNG than those of control mice. Ki-67 and KLF9 expression showed a similar pattern of decrease with PCNA expression in glandular and whole lesions of mice treated with DNG; however, the difference was not statistically significant. The expression of ER α/β and its ratio, as well as the expression of PGR increased in both the groups. However, the ER and PGR expression all showed no significant changes (Fig. 6).

Ⅳ. Discussion

The present study showed that under *in vitro* conditions, DNG induced a decrease in cell viability and proliferation with increasing levels of estradiol, TNF-α, IL-1β, and IL-32. Furthermore, our mouse model demonstrated that the size of the implanted endometrial tissues was smaller in mice treated with DNG than in mice without DNG treatment, along with decreased expression of PCNA, without any significant changes in the expression of hormone receptors. These *in vitro* and *in vivo* data showed the effects of DNG on human ESCs and its anti-inflammatory characteristics, as well as the changes in lesions occurring in endometriosis.

The etiology of endometriosis is still unclear, and many researchers have attempted to determine the mechanism underlying endometriosis development. Diverse cytokines, chemokines, and pathways have been shown to be involved in the development of endometriosis. However, it is assumed that endometriosis could potentially be related to a chronic inflammatory situation and an estrogen-dependent disease [21-24]. When ESCs were treated with estradiol, we observed an increase in viability, AKT phosphorylation, and Pak4 and VEGF expression. Upon treatment with both estradiol and DNG, we observed decreased viability, AKT phosphorylation, and Pak4 and VEGF expression, which indicates the inhibitory effect of DNG in human ESCs. However, the same was not true for the expression of interleukins (IL-1β/17/32), whose levels are known to be elevated in women with endometriosis or ectopic lesions [18, 25, 26]. In addition, no unified results were observed based on the time of treatment, but the most significant results were obtained after 24 or 48 h of treatment. We assumed that this might be because of a difference in the experimental setup, wherein we subjected human ESCs to treatment with both estradiol and DNG, in contrast to using direct ectopic lesions or the peritoneal environment. Additionally, with time, human ESCs can become refractory to treatment of estradiol or inflammatory cytokines used in this study.

The inflammatory cytokines, TNF-α and IL-1β, are known to play a crucial role in triggering the inflammatory pathway and have been detected at high levels in the peritoneal fluid of women with endometriosis. They promote ESC proliferation and induce the upregulation of nerve growth factor, which could be the main mechanism by which DNG relieves endometriosis-associated pain. Additionally, they affect nuclear signaling, which may play an important role in the pathogenesis of endometriosis [16, 27-29]. Our results showed decreased cell viability,

AKT phosphorylation, and PCNA expression, when ESCs were treated with both DNG and TNF-α. The data showed a significant decrease in cell viability after IL-1β treatment. This suggests the importance of TNF-α in the mechanism of action of DNG (in comparison to IL-1β).

IL-32 is also a proinflammatory cytokine that is known to be a potent inducer of other cytokines, including TNF-α and a controller of immune function, host defense and cell death [30-33]. It is involved in several chronic inflammatory diseases, such as chronic rhinosinusitis, ankylosing spondylitis, and inflammatory bowel diseases [34-36]. IL-32 levels have been reported to be elevated in the serum and peritoneal fluid of patients with endometriosis [37]. We have previously shown a significant increase in the viability of ESCs, following treatment with IL-32 α and γ [18]. In this study, we investigated the same in the background of DNG treatment, to determine the factors or pathways associated with its inhibitory effects. We observed a significant decrease in cell viability and PCNA expression, particularly with IL-32α, whereas other factors such as cell invasion, AKT/ERK phosphorylation, and VEGF expression did not exhibit a significant change. These results suggest IL-32 might play an important role in the pathogenesis and/or pathophysiology of endometriosis; however, it is not closely related to the mechanism of action of DNG.

The present study used a mouse model to confirm the lesion–reducing effect of DNG. As mentioned, DNG is now the first regimen for endometriosis treatment, i.e., to inhibit the growth of proliferative lesions and to manage the associated symptoms. A 2mg per day dose of DNG is the standard that demonstrates significant efficacy with respect to lesion and pain reduction, (superior to the placebo, but equivalent to the GnRH analog) [38-42]. We observed a gross reduction in lesion size (Fig. 6), and the reduction was significant when compared between paired mice, which shared the same horn and uterus of the donor mouse. The dose of DNG administered was considerably higher than the daily dose of DNG recommended for use in human; however, a higher dose was used with the aim of more precisely confirming the lesion reduction effect.

DNG is a synthetic progestin and a selective PGR agonist. Pharmacodynamically, it has moderate affinity for PGR and does not activate either ER α or β [43, 44]. The pathogenesis of endometriosis is still unclear, but hormonal influences and genetic/epigenetic factors in individual patients with endometriosis are expected to justify the impairment of mechanisms. It is known that estrogen-mediated alterations may play a role in the pathophysiology of endometriosis, especially alterations in ER activity and an increased ER β/ER α ratio in endometriotic cells, thereby

triggering proinflammatory factors and cell proliferation [45, 46]. We performed immunohistochemistry to check the expression of ER α and β in the allotransplanted endometrial tissue of mice treated with DNG; there was no reverse effect of DNG on the ER β/ER α ratio, which did not decrease significantly. Hayashi et al. [47] showed a decrease in ER β/ER α ratio with DNG treatment; however, they used human endometrial cells and treated the *in vitro* culture, which differs from our study using a mouse model. In addition, increased expression of ER β in the endometriotic tissue resulted in the downregulation of the PGRs [46, 48]. However, immunohistochemistry did not reveal any significant difference in PGR expression between lesions with and without DNG treatment, even though the HSCORE was higher than that of the control. Our results regarding hormone receptors may not support the direct hormonal effect of DNG on endometriosis. We also analyzed the expression of PCNA, Ki-67, and KLF9 using immunohistochemistry; however, the expression of only PCNA was significant. The transcription factor KLF9 has emerged as a potent player in reproductive dysfunctions associated with aberrant ER and/or PGR signaling, and several studies have investigated its relationship with endometriosis [49-51]. Heard et al. [52] have shown that endometrial KLF9 deficiency promotes the establishment of endometriotic lesion, but the whole and glandular HSCOREs of KLF9 were decreased in mice treated with DNG, without any significance. In this study, we evaluated the factors that are known to be associated with the pathogenesis of endometriosis. Future studies may address the issue of finding anti-inflammatory targets for investigating the mechanism underlying the development of endometriosis and its treatment with DNG.

This study has successfully gathered extensive data—from cell culture to mouse model-based research—and confirmed the association between several endometriosis-associated factors and the therapeutic mechanism of DNG. However, there are several limitations of this study that warrant discussion. First, we evaluated several factors associated with inflammation and endometriosis, but could not define the specific factor closely related to the pathophysiology of endometriosis or the mechanism of action of DNG. This limitation can be justified by the fact that inflammation is not a linear process but a more complicated webbed pathway, and it is difficult to identify a single most critical factor. Second, cell culture and immunohistochemistry finding from the mouse model showed limited data, i.e., only PCNA expression showed a significant change in the whole and glandular regions. This may be caused by the different ecologies between humans and mice and the high dose of DNG used. In addition, the results could be influenced by unknown differences between the subjects, such as disease activity, type of the lesions, menstrual phase and genetic diversity. Lastly, this study evaluated the effects of DNG alone and did not consider other types of progestins, such as medroxyprogesterone acetate or levonorgestrel. Importantly, further studies are needed to

investigate the inhibitory effect of DNG based on distinguishable characteristics, in addition to experiments using other progestins.

In conclusion, the study findings suggest that DNG may reduce cell viability and proliferation induced in response to treatment with estrogen, TNF-α, IL-1β, or IL-32, and inhibit the pathogenesis of endometriosis by decreasing PCNA expression.

Ⅴ. Figures

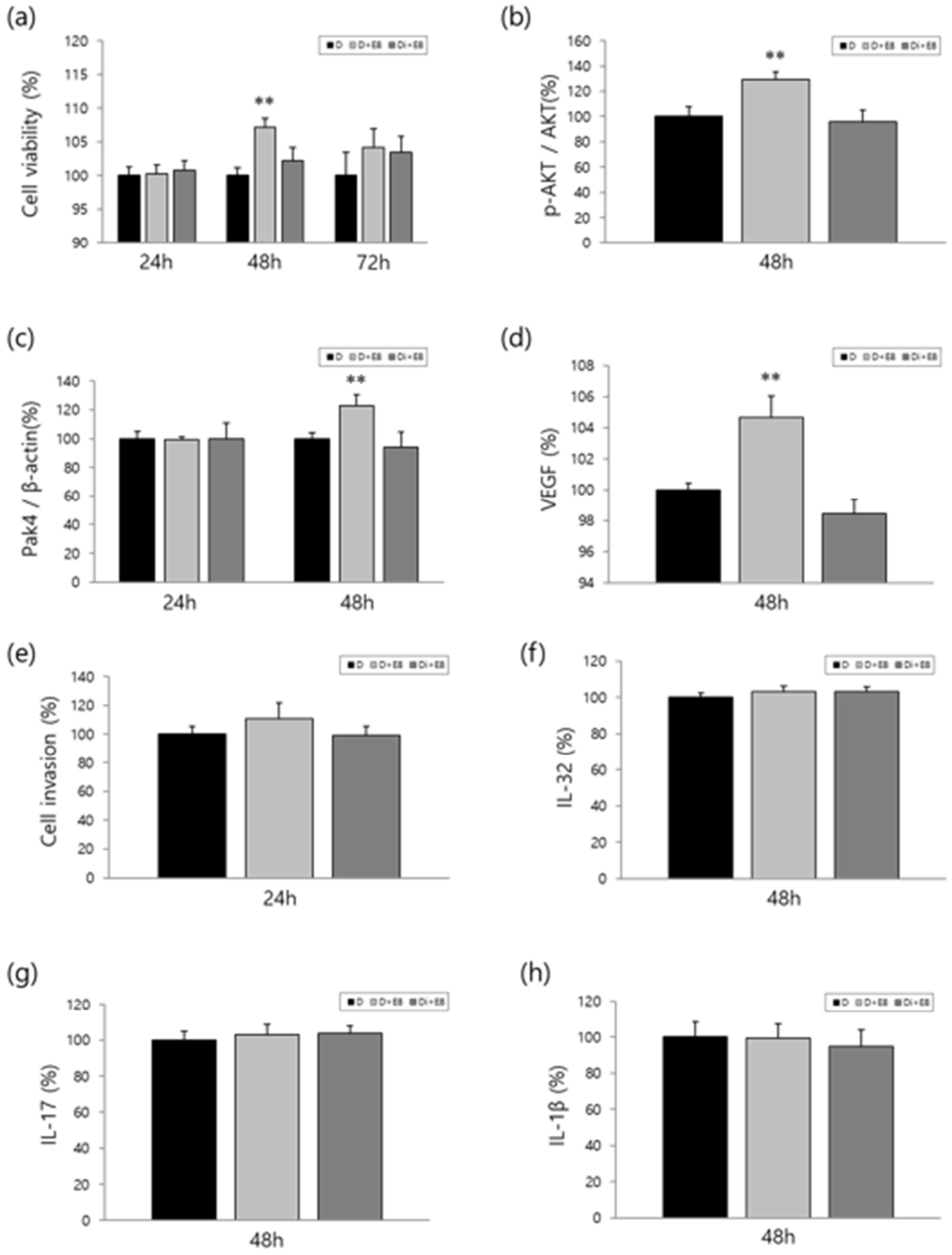

(a)
Cell viability (%)
120
115
110
105
100
95
90
**
24h
48h
72h
(b)
p-AKT / AKT(%)
160
140
120
100
80
60
40
20
0
**
48h
(c)
Pak4 / β-actin(%)
140
120
100
80
60
40
20
0
**
24h
48h
(d)
VEGF (%)
108
106
104
102
100
98
96
94
**
48h
(e)
Cell invasion (%)
140
120
100
80
60
40
20
0
24h
(f)
IL-32 (%)
120
100
80
60
40
20
0
48h
(g)
IL-17 (%)
120
100
80
60
40
20
0
48h
(h)
IL-1β (%)
120
100
80
60
40
20
0
48h

Figure 1. Effect of Dienogest (DNG) with estradiol in human endometrial stromal cells (ESCs). The graphs show time-dependent variation evaluated in the four groups for the following factors: (a) Cell viability, (b) AKT phosphorylation, (c) Pak4 expression, (d) VEGF expression, (e) Cell invasion, (f) IL-32 expression, (g) IL-17 expression, and (h) IL-1β. Error bars show the mean ± standard error of mean (SEM). *$P < 0.05$ versus control, **$P < 0.05$ versus control as well as treatment with both DNG and estradiol in ESCs. D = ESCs in dimethyl sulfoxide solution (DMSO) (control); D + E8 = treated with estradiol 10^{-8} M; Di + E8 = treated with DNG 10^{-6} M and estradiol 10^{-8} M. Data are expressed as a percentage, wherein cells treated with the vehicle are normalized to 100 %

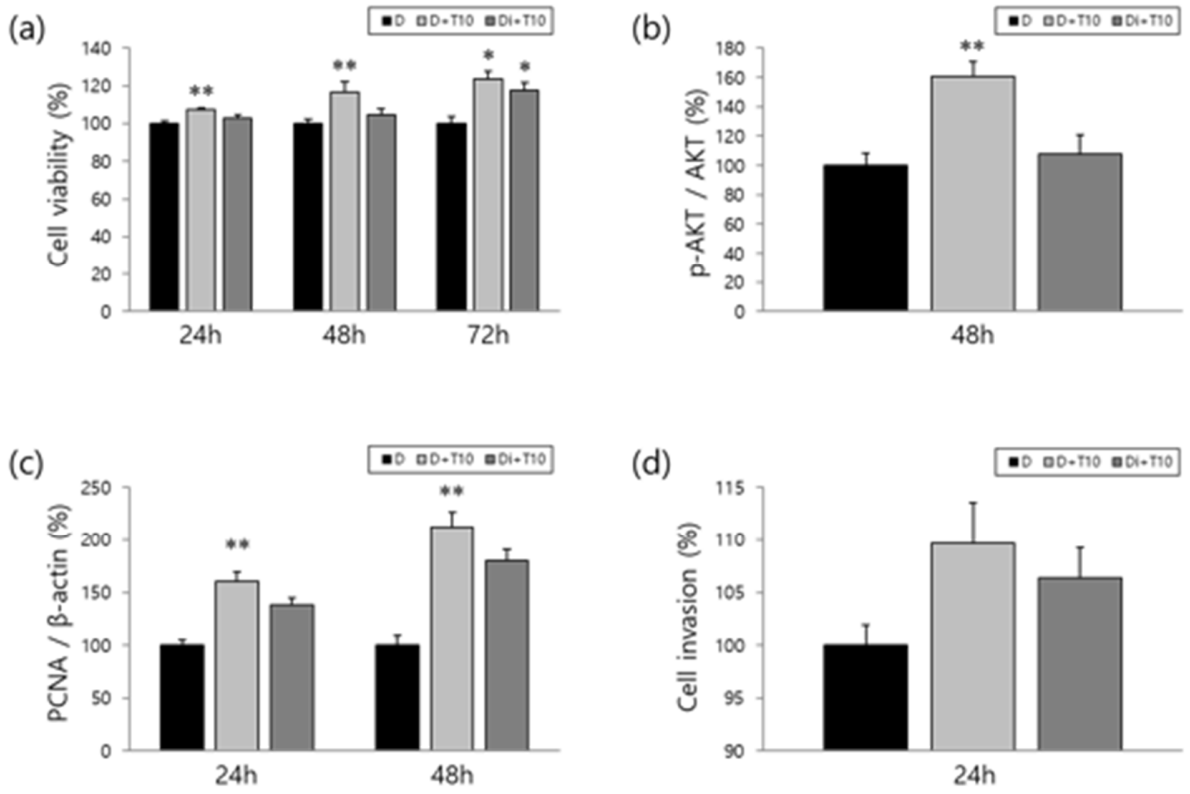


Figure 2. Effect of DNG with tumor necrosis factor-alpha (TNF-α) in human ESCs. The graphs show time-dependent variation evaluated in the three groups for the following factors: (a) Cell viability, (b) AKT phosphorylation, (c) expression of PCNA, and (d) cell invasion. Error bars show the mean ± SEM. *P < 0.05 versus control, **P < 0.05 versus control as well as treatment with both DNG and TNF-α in ESCs. D = ESCs in DMSO solution (control); D +

T10 = treated with TNF-α 10 ng/mL; Di + T10 = treated with DNG 10^{-6} M and TNF-α 10 ng/mL. Data are expressed as a percentage, wherein cells treated with the vehicle are normalized to 100 %.

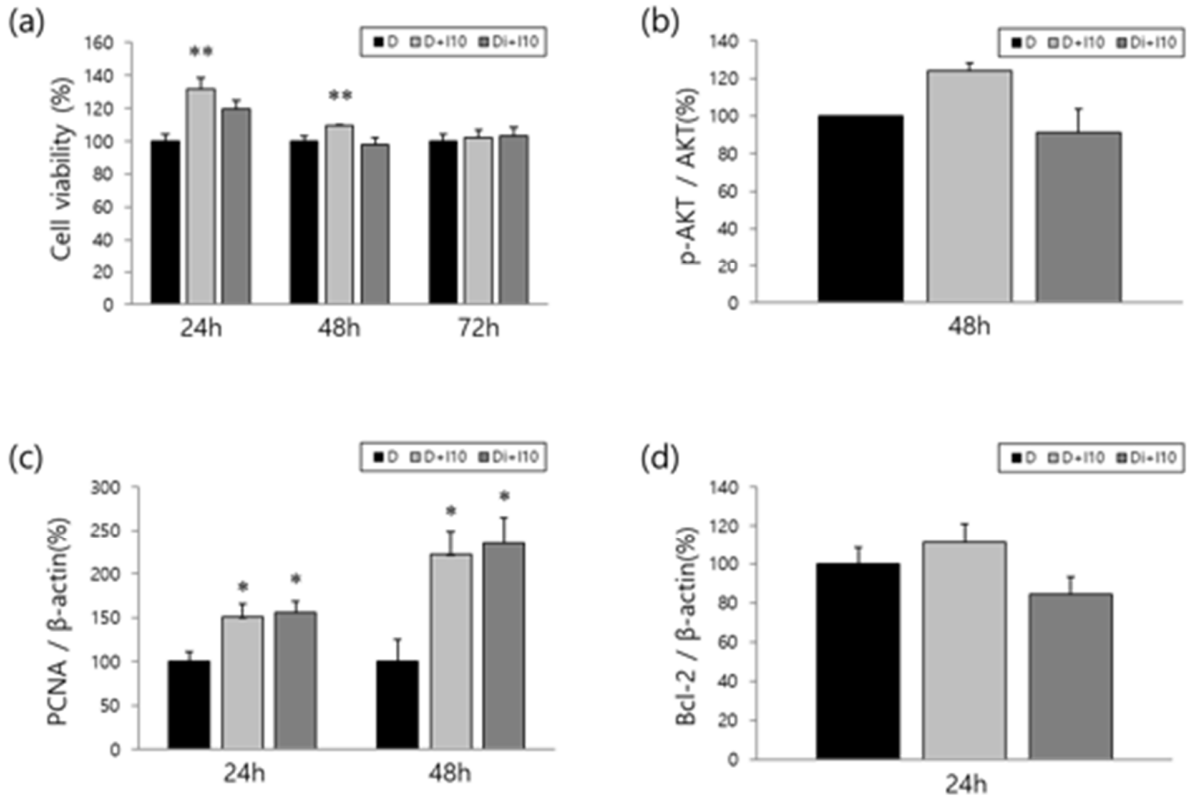
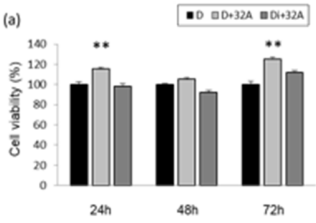
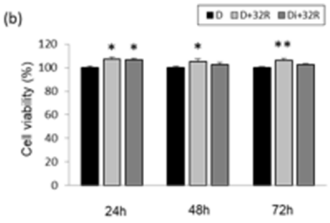
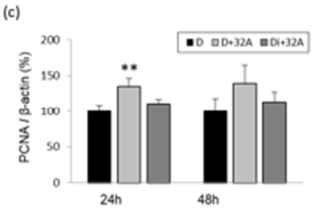
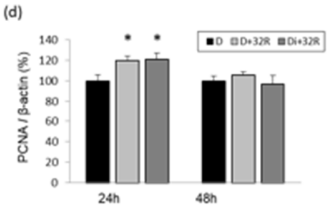


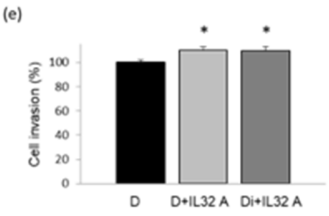
Figure 3. Effect of DNG with interleukin (IL)-1β in human ESCs. The graphs show time-dependent variation evaluated in the three groups for the following factors: (a) Cell viability, (b) AKT phosphorylation, (c) PCNA expression, and (d) Bcl-2 expression. Error bars show the mean ± SEM. *$P < 0.05$ versus control, **$P < 0.05$ versus control as well as treatment with both DNG and IL-1β in ESCs. D = ESCs in DMSO solution (control); D + I10 = treated with IL-1β 10 ng/mL; Di + I10 = treated with DNG 10^{-6} M and IL-1β 10 ng/mL. Data are expressed as a percentage, wherein cells treated with the vehicle are normalized to 100 %.

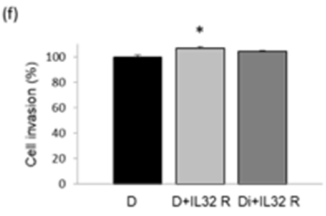

(a)
D D+32A Di+32A
Cell viability (%)
24h
48h
72h

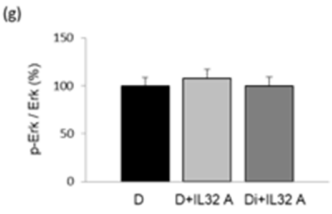

(b)
D D+32R Di+32R
Cell viability (%)
24h
48h
72h

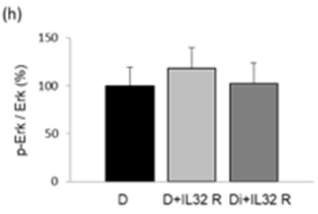

(c)
D D+32A Di+32A
PCNA / β-actin (%)
24h
48h

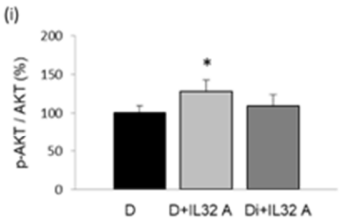

(d)
D D+32R Di+32R
PCNA / β-actin (%)
24h
48h

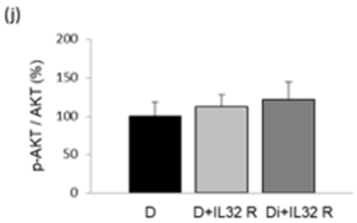

(e)
Cell invasion (%)
D
D+IL32 A
Di+IL32 A

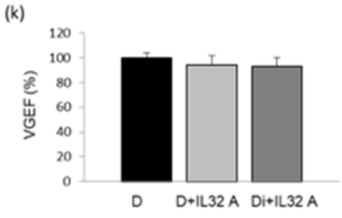

(f)
Cell invasion (%)
D
D+IL32 R
Di+IL32 R


(g)
p-Erk / Erk (%)
D
D+IL32 A
Di+IL32 A


(h)
p-Erk / Erk (%)
D
D+IL32 R
Di+IL32 R


(i)
p-AKT / AKT (%)
D
D+IL32 A
Di+IL32 A


(j)
p-AKT / AKT (%)
D
D+IL32 R
Di+IL32 R


(k)
VGEF (%)
D
D+IL32 A
Di+IL32 A

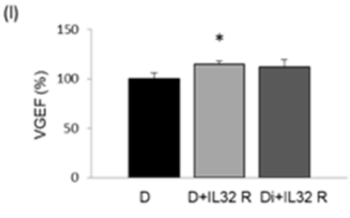

(l)
VGEF (%)
D
D+IL32 R
Di+IL32 R

Figure 4. Effect of DNG treatment with IL-32 alpha and gamma in human ESCs. Cell viability, expression of PCNA, cell invasion, ERK and AKT phosphorylation, and VEGF expression are shown with (a, c, e, g, i, k): IL-32α treatment, and (b, d, f, h, j, l): IL-32γ treatment alone or with DNG in ESCs. Error bars show the mean ± SEM. *$P < 0.05$ versus control, **$P < 0.05$ versus control as well as with both DNG and IL-32 α/γ treatment in ESCs. D = ESCs in DMSO solution (control); D + 32A / D + 32R = treated with IL-32α / IL-32γ (25 ng/mL); Di + 32A / Di + 32R = treated with DNG 10^{-6} M and IL-32α / IL-32γ (25 ng/mL). Data are expressed as a percentage, wherein cells treated with the vehicle are normalized to 100 %.

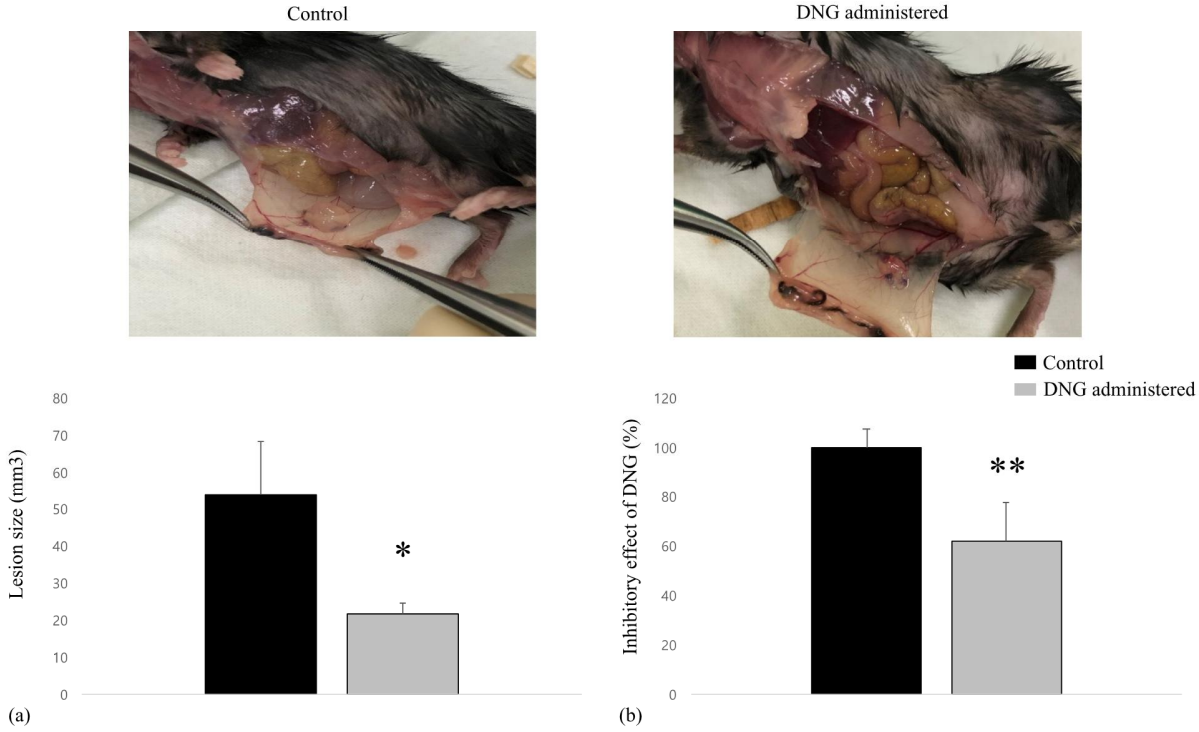
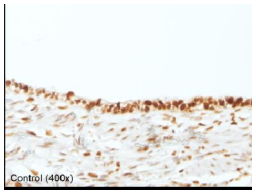
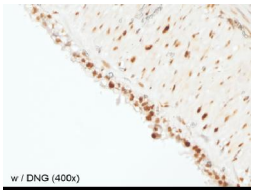
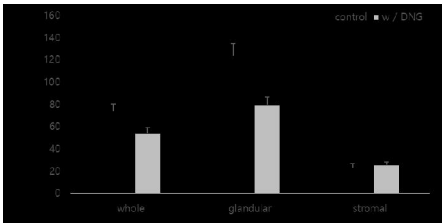


Figure 5. Photographs and (a) graph showing ectopic endometrial tissues in control mice and DNG-treated mice. (b) The size change of the implanted tissues and paired comparison allotransplanted from the same uterus of a mouse, showing the inhibitory effect of DNG. *$P = 0.049$ vs. control. **$P = 0.019$ vs. control.

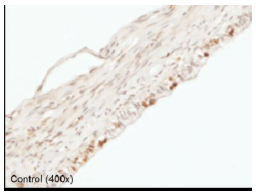
(a) PCNA

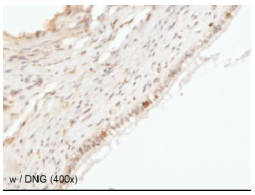


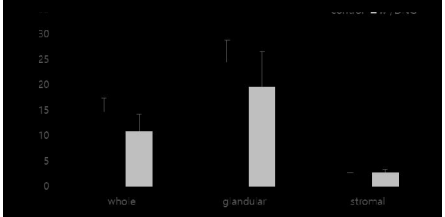




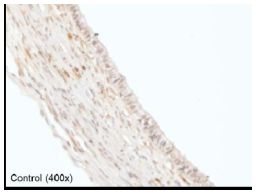
(b) Ki-67

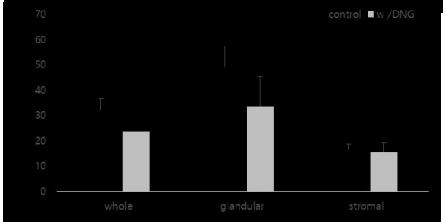




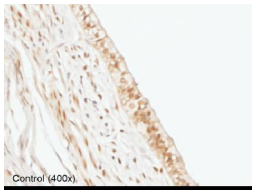


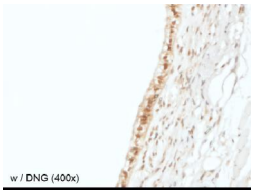
(c) KLF9

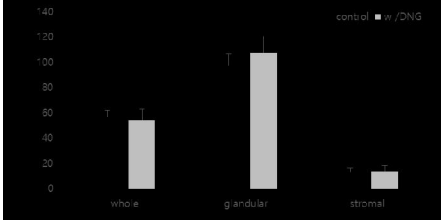




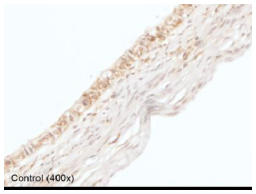
(d) ER α

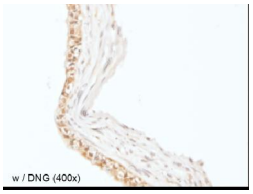


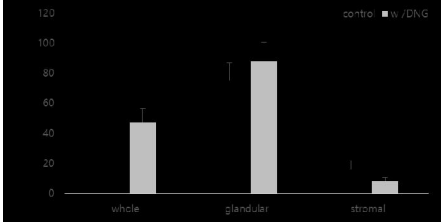


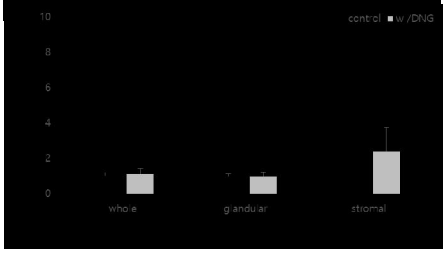


(e) ER β

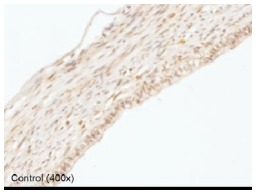


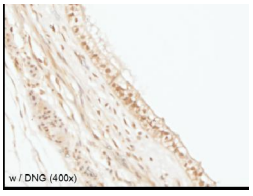






(f) PGR





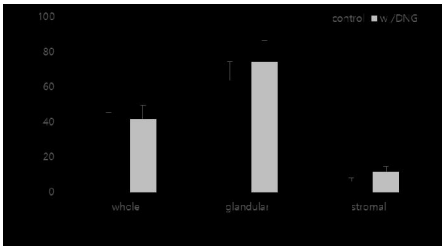


Figure 6. Representative micrographs (x400) and HSCOREs of immunostainings for (a) proliferating cell nuclear antigen (PCNA), (b) Ki-67, (c) Krüppel-like factor 9 (KLF9), (d) estrogen receptor (ER) α, (e) ER β, and ER β / ER α ratio and (f) progesterone receptor (PGR) in allotransplanted endometrial tissues to mice fed with and without DNG. Values are expressed as the mean ± SEM. *P < 0.05 vs. control.

Ⅵ. References

1. Wu M-H, Hsiao K-Y, Tsai S-J. Endometriosis and possible inflammation markers. Gynecology and Minimally Invasive Therapy. 2015;4(3):61-7.
2. Eshre. Management of women with endometriosis: Guideline of the European Society of Human Reproduction and Embryology. 2013. doi: 10.1093/humrep/det457.2.(September):1-97.
3. Mounsey AL, Wilgus A, Slawson DC. Diagnosis and management of endometriosis. American family physician. 2006;74(4).
4. Sinaii N, Plumb K, Cotton L, Lambert A, Kennedy S, Zondervan K, et al. Differences in characteristics among 1,000 women with endometriosis based on extent of disease. Fertility and sterility. 2008;89(3):538-45.
5. Eskenazi B, Warner ML. Epidemiology of endometriosis. Obstetrics and gynecology clinics of North America. 1997;24(2):235-58.
6. Pritts EA, Taylor RN. An evidence-based evaluation of endometriosis-associated infertility. Endocrinology and metabolism clinics of North America. 2003;32(3):653-67.
7. Zanelotti A, Decherney AH. Surgery and Endometriosis. Clinical obstetrics and gynecology. 2017;60(3):477-84.
8. Abbott J, Hawe J, Hunter D, Holmes M, Finn P, Garry R. Laparoscopic excision of endometriosis: a randomized, placebo-controlled trial. Fertility and sterility. 2004;82(4):878-84.
9. Guo SW. Recurrence of endometriosis and its control. Human reproduction update. 2009;15(4):441-61.
10. Kondo W, Bourdel N, Tamburro S, Cavoli D, Jardon K, Rabischong B, et al. Complications after surgery for deeply infiltrating pelvic endometriosis. BJOG : an international journal of obstetrics and gynaecology. 2011;118(3):292-8.
11. Brown J, Pan A, Hart RJ. Gonadotrophin-releasing hormone analogues for pain associated with endometriosis. The Cochrane database of systematic reviews. 2010;2010(12).
12. Irahara M, Harada T, Momoeda M, Tamaki Y. Hormonal and histological study on irregular genital bleeding in patients with endometriosis during treatment with dienogest, a novel progestational therapeutic agent. Reproductive medicine and biology. 2007;6(4):223-8.

13. Schweppe KW. Current place of progestins in the treatment of endometriosis-related complaints. Gynecological endocrinology : the official journal of the International Society of Gynecological Endocrinology. 2001;15 Suppl 6(SUPPL. 6):22-8.

14. Shimizu Y, Takeuchi T, Mita S, Mizuguchi K, Kiyono T, Inoue M, et al. Dienogest, a synthetic progestin, inhibits the proliferation of immortalized human endometrial epithelial cells with suppression of cyclin D1 gene expression. Molecular human reproduction. 2009;15(10):693-701.

15. Miyashita M, Koga K, Takamura M, Izumi G, Nagai M, Harada M, et al. Dienogest reduces proliferation, aromatase expression and angiogenesis, and increases apoptosis in human endometriosis. Gynecological endocrinology : the official journal of the International Society of Gynecological Endocrinology. 2014;30(9):644-8.

16. Iwabe T, Harada T, Tsudo T, Nagano Y, Yoshida S, Tanikawa M, et al. Tumor Necrosis Factor-Promotes Proliferation of Endometriotic Stromal Cells by Inducing Interleukin-8 Gene and Protein Expression. The Journal of Clinical Endocrinology & Metabolism. 2000;85(2):824-9.

17. Horie S, Harada T, Mitsunari M, Taniguchi F, Iwabe T, Terakawa N. Progesterone and progestational compounds attenuate tumor necrosis factor alpha-induced interleukin-8 production via nuclear factor kappaB inactivation in endometriotic stromal cells. Fertility and Sterility. 2005;83(5):1530-5.

18. Lee MY, Kim SH, Oh YS, Heo SH, Kim KH, Chae HD, et al. Role of interleukin-32 in the pathogenesis of endometriosis: In vitro, human and transgenic mouse data. Human Reproduction. 2018;33(5):807-16.

19. Kim SH, Lee HW, Kim YH, Koo YH, Chae HD, Kim CH, et al. Down-regulation of p21-activated kinase 1 by progestin and its increased expression in the eutopic endometrium of women with endometriosis. Human Reproduction. 2009;24(5):1133-41.

20. Lee SR, Kim SH, Lee HW, Kim YH, Chae HD, Kim CH, et al. Increased expression of glutathione by estradiol, tumor necrosis factor-alpha, and interleukin 1-beta in endometrial stromal cells. American journal of reproductive immunology (New York, NY : 1989). 2009;62(6):352-6.

21. Fakih H, Baggett B, Holtz G, Tsang KY, Lee JC, Williamson HO. Interleukin-1: a possible role in the infertility associated with endometriosis. Fertility and Sterility. 1987;47(2):213-7.

22. Eisermann J, Gast MJ, Pineda J, Odem RR, Collins JL. Tumor necrosis factor in peritoneal fluid of women undergoing laparoscopic surgery. Fertility and sterility. 1988;50(4):573-9.

23. Suen JL, Chang Y, Chiu PR, Hsieh TH, Hsi E, Chen YC, et al. Serum level of IL-10 is increased in patients with endometriosis, and IL-10 promotes the growth of lesions in a murine model. The American journal of pathology. 2014;184(2):464-71.

24. Bulun SE, Yilmaz BD, Sison C, Miyazaki K, Bernardi L, Liu S, et al. Endometriosis. Endocrine Reviews: Endocrine Society; 2019. p. 1048-79.

25. Katsuki Y, Takano Y, Futamura Y, Shibutani Y, Aoki D, Udagawa Y, et al. Effects of dienogest, a synthetic steroid, on experimental endometriosis in rats. European journal of endocrinology. 1998;138(2):216-26.

26. Ahn SH, Edwards AK, Singh SS, Young SL, Lessey BA, Tayade C. IL-17A Contributes to the Pathogenesis of Endometriosis by Triggering Proinflammatory Cytokines and Angiogenic Growth Factors. Journal of immunology (Baltimore, Md : 1950). 2015;195(6):2591-600.

27. Dai SM, Matsuno H, Nakamura H, Nishioka K, Yudoh K. Interleukin-18 Enhances Monocyte Tumor Necrosis Factor α and Interleukin-1β Production Induced by Direct Contact with T Lymphocytes: Implications in Rheumatoid Arthritis. Arthritis and Rheumatism. 2004;50(2):432-43.

28. Mita S, Shimizu Y, Sato A, Notsu T, Imada K, Kyo S. Dienogest inhibits nerve growth factor expression induced by tumor necrosis factor-α or interleukin-1β. Fertility and Sterility. 2014;101(2).

29. Kim SH, Ihm HJ, Oh YS, Chae HD, Kim CH, Kang BM. Increased Nuclear Expression of Nuclear Factor Kappa-B p65 Subunit in the Eutopic Endometrium and Ovarian Endometrioma of Women with Advanced Stage Endometriosis. American Journal of Reproductive Immunology. 2013;70(6):497-508.

30. Kim SH, Han SY, Azam T, Yoon DY, Dinarello CA. Interleukin-32: A cytokine and inducer of TNFα. Immunity. 2005;22(1):131-42.

31. Li W, Sun W, Liu L, Yang F, Li Y, Chen Y, et al. IL-32: a host proinflammatory factor against influenza viral replication is upregulated by aberrant epigenetic modifications during influenza A virus infection. Journal of immunology (Baltimore, Md : 1950). 2010;185(9):5056-65.

32. Bai X, Kim S-H, Azam T, McGibney MT, Huang H, Dinarello CA, et al. IL-32 is a host protective cytokine against Mycobacterium tuberculosis in differentiated THP-1 human macrophages. Journal of immunology (Baltimore, Md : 1950). 2010;184(7):3830-40.

33. Joosten LAB, Heinhuis B, Netea MG, Dinarello CA. Novel insights into the biology of interleukin-32. Cellular and molecular life sciences : CMLS. 2013;70(20):3883-92.

34. Soyka MB, Treis A, Eiwegger T, Menz G, Zhang S, Holzmann D, et al. Regulation and expression of IL-32 in chronic rhinosinusitis. Allergy. 2012;67(6):790-8.

35. Ciccia F, Rizzo A, Accardo-palumbo A, Giardina AR, Bombardieri M, Guggino G, et al. Increased expression of interleukin-32 in the inflamed ileum of ankylosing spondylitis patients. Rheumatology (Oxford, England). 2012;51(11):1966-72.

36. Shioya M, Nishida A, Yagi Y, Ogawa A, Tsujikawa T, Kim-Mitsuyama S, et al. Epithelial overexpression of interleukin-32alpha in inflammatory bowel disease. Clinical and experimental immunology. 2007;149(3):480-6.

37. Choi YS, Kim S, Oh YS, Cho SH, Hoon Kim S. Elevated serum interleukin-32 levels in patients with endometriosis: A cross-sectional study. American Journal of Reproductive Immunology. 2019;82(2):e13149-e.

38. Petraglia F, Hornung D, Seitz C, Faustmann T, Gerlinger C, Luisi S, et al. Reduced pelvic pain in women with endometriosis: efficacy of long-term dienogest treatment. Archives of gynecology and obstetrics. 2012;285(1):167-73.

39. Köhler G, Faustmann TA, Gerlinger C, Seitz C, Mueck AO. A dose-ranging study to determine the efficacy and safety of 1, 2, and 4mg of dienogest daily for endometriosis. International journal of gynaecology and obstetrics: the official organ of the International Federation of Gynaecology and Obstetrics. 2010;108(1):21-5.

40. Strowitzki T, Faustmann T, Gerlinger C, Seitz C. Dienogest in the treatment of endometriosis-associated pelvic pain: a 12-week, randomized, double-blind, placebo-controlled study. European journal of obstetrics, gynecology, and reproductive biology. 2010;151(2):193-8.

41. Strowitzki T, Faustmann T, Gerlinger C, Schumacher U, Ahlers C, Seitz C. Safety and tolerability of dienogest in endometriosis: pooled analysis from the European clinical study program. International journal of women's health. 2015;7:391-401.

42. B C, Jw R, J P, K J, Th K, Ys K, et al. Safety and Effectiveness of Dienogest (Visanne®) for Treatment of Endometriosis: A Large Prospective Cohort Study. Reproductive sciences (Thousand Oaks, Calif). 2020;27(3):905-15.

43. Harada T, Taniguchi F. Dienogest: a new therapeutic agent for the treatment of endometriosis. Women's health (London, England). 2010;6(1):27-35.

44. Sasagawa S, Shimizu Y, Kami H, Takeuchi T, Mita S, Imada K, et al. Dienogest is a selective progesterone receptor agonist in transactivation analysis with potent oral endometrial activity due to its efficient pharmacokinetic profile. Steroids. 2008;73(2):222-31.

45. Vannuccini S, Clemenza S, Rossi M, Petraglia F. Hormonal treatments for endometriosis: The endocrine background. Reviews in Endocrine and Metabolic Disorders: Springer; 2021.

46. Bulun SE, Monsavais D, Pavone ME, Dyson M, Xue Q, Attar E, et al. Role of estrogen receptor-β in endometriosis. Seminars in reproductive medicine. 2012;30(1):39-45.

47. Hayashi A, Tanabe A, Kawabe S, Hayashi M, Yuguchi H, Yamashita Y, et al. Dienogest increases the progesterone receptor isoform B/A ratio in patients with ovarian endometriosis. Journal of Ovarian Research. 2012;5(1):31-.

48. Ichioka M, Mita S, Shimizu Y, Imada K, Kiyono T, Bono Y, et al. Dienogest, a synthetic progestin, down-regulates expression of CYP19A1 and inflammatory and neuroangiogenesis factors through progesterone receptor isoforms A and B in endometriotic cells. Journal of Steroid Biochemistry and Molecular Biology. 2015;147:103-10.

49. Pabona JMP, Simmen FA, Nikiforov MA, Zhuang DZ, Shankar K, Velarde MC, et al. Krüppel-like factor 9 and progesterone receptor coregulation of decidualizing endometrial stromal cells: implications for the pathogenesis of endometriosis. The Journal of clinical endocrinology and metabolism. 2012;97(3).

50. Simmen RCM, Eason RR, McQuown JR, Linz AL, Kang TJ, Chatman L, et al. Subfertility, uterine hypoplasia, and partial progesterone resistance in mice lacking the Kruppel-like factor 9/basic transcription element-binding protein-1 (Bteb1) gene. The Journal of biological chemistry. 2004;279(28):29286-94.

51. Lee B, Du H, Taylor HS. Experimental murine endometriosis induces DNA methylation and altered gene expression in eutopic endometrium. Biology of reproduction. 2009;80(1):79-85.

52. Heard ME, Simmons CD, Simmen FA, Simmen RC. Krüppel-like factor 9 deficiency in uterine endometrial cells promotes ectopic lesion establishment associated with activated notch and hedgehog signaling in a mouse model of endometriosis. Endocrinology. 2014;155(4):1532-46.

Abstract

Dienogest (DNG), an orally active progestin, has been shown to be effective in the long-term management of endometriosis. However, limited data are available regarding the mechanism of action of DNG, its effect on endometrial cells, and the consequent biological changes. Using in vivo and in vitro systems, we aimed to investigate whether treatment with DNG causes significant biological changes in human endometrial cells. To ascertain this, we evaluated cell viability, invasion, and several markers related to the pathogenesis of endometriosis in endometrial stromal cells (ESCs), using estradiol treatment alone and a combination of estradiol and DNG. We further treated ESCs with tumor necrosis factor alpha (TNF-α), interleukin 1β (IL-1β), and interleukin 32 alpha / gamma (IL-32 α/γ), using the same approach, and DNG was used to evaluate the potential inhibitory effect. In addition, we compared implanted endometrial tissues between C57BL/6 mice with and without the administration of DNG. The size of implanted endometrial lesions was measured, and the expression of proliferating cell nuclear antigen (PCNA), Ki-67, estrogen receptor (ER) α/β, progesterone receptor (PGR), and Krüppel-like factor 9 (KLF9) was analyzed using immunohistochemistry. We observed a significant decrease in cell viability, protein kinase B (AKT) phosphorylation, and the expression of p21-activated kinase 4 (Pak4) and vascular endothelial growth factor (VEGF) in ESCs treated with estradiol and DNG. Cell viability, AKT phosphorylation, and PCNA expression also decreased significantly on treatment with both TNF-α and DNG. On treatment with IL-1β or IL-32 α/γ, cell viability only and PCNA expression both were significantly decreased respectively with DNG treatment. The size of the implanted endometrial tissue also significantly decreased in mice treated with DNG, a phenomenon that was accompanied by a significant decrease in the expression of PCNA (immunohistochemistry data). In summary, these findings suggest that DNG may reduce cell viability and proliferation induced by the action of estrogen, TNF-α, IL-1β, and IL-32, and inhibit the pathogenesis of endometriosis by decreasing PCNA expression.