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STUDY ON ASIATICOSIDE INHIBITING 17B-ESTRADIOL-INDUCED EPITHELIAL-MESENCHYMAL TRANSITION BY UP-REGULATING NUMB EXPRESSION AND DOWN-REGULATING NOTCHL PATHWAY

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Abstract

Objective: Endometriosis is a benign disease with malignant biological manifestations of metastasis and invasion. The present experiment explored the effect of Asiaticoside on EMT of glandular epithelial cells in endometriosis, and further investigated the signaling pathways that may be involved.

Methods: The endometrial tissues of 60 fertile women were obtained. Immunohistochemical analysis was performed to detect the expressions of Notchl/Numb signaling pathway and EMT-related indicators in normal endometrial tissue and endometriosis eutopic endometrial tissue. The eutopic endometrium of normal patients and endometriosis patients were subjected to primary isolation and culture of glandular epithelial cells, and the effect of epithelial cell proliferation was detected by the CCK-8 method. Transwell test was conducted to detect the influence of epithelial cell migration and invasion. Western blot method was applied to detect the Notchl/Numb signaling pathway and EMT-related proteins.

Results:The expression levels of Notchl, Slug, Snail and N-cadherin proteins in the eutopic endometrial tissue of endometriosis were significantly increased, while the expression levels of E-cadherin and Numb proteins presented a decreasing trend. Asiaticoside and Notch pathway specific inhibitors significantly inhibited the proliferation, migration, invasion and expression of EMT-related indicators of normal and endometriotic eutopic endometrial gland epithelial cells.

Conclusion: Abnormal Notchl/Numb signaling pathway and its EMT exist in endometriosis. Asiaticoside inhibits the epithelial-mesenchymal transition induced by 17β-estradiol through up-regulating the expression of Numb and down-regulating the activity of the Notchl pathway.

Keywords: Endometriosis; Asiaticoside; Notchl/Numb; EMT; 17β-estradiol

Centella asiatica contains a variety of triterpenoids of a-aromatic alcohol type, of which asiaticoside is the most widely used and studied. In recent years, studies have found that asiaticoside has a variety of effects, not only can promote the proliferation and repair of skin mucous membrane so as to promote wound healing, and can also inhibit the excessive proliferation of fibroblasts. It is widely used in inhibiting scar hyperplasia and breast hyperplasia. The latest research has also found that it has a certain effect in the central nervous system and tumor treatment [1-2].

In this study, Asiaticoside is hypothesized to be involved in the EMT regulation process in endometriosis. The paper explored the effect of Asiaticoside on the migration and proliferation of normal endometrial and endometriotic endometrial epithelial cells, and on the expressions of EMT-related proteins, and also investigated possible molecular signaling pathways that may involve in.

Methods

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1. Extraction and grouping of cells

Normal eutopic endometrium and eutopic endometrium of patients with endometriosis are taken from fresh tissue specimens obtained during surgery under sterile conditions for immunohistochemical analysis. The normal endometrium was obtained from 30 patients who were pathologically diagnosed as having no endometriosis after surgery. Endometriosis eutopic endometrial lesions were obtained from 30 patients with endometriosis who had undergone complete or partial hysterectomy. The study has excluded patients with malignant diseases, and neither endometriosis patients nor normal patients have other diseases. All patients had not received any hormone therapy within 3 months before surgery. The endometrial cycle is judged by the patient's last menstruation, intraoperative findings and histological observation under the microscope.

Both the normal eutopic endometrium and the eutopic endometrium of patients with endometriosis were obtained from fresh tissue specimens under sterile conditions during surgery for the primary cell culture. The normal endometrium was obtained from 6 patients with no endometriosis diagnosed pathologically after surgery. Endometriosis eutopic endometrial lesions were obtained from 6 patients with endometriosis who had undergone complete or partial hysterectomy. The inclusion and exclusion criteria are the same as above.

Cell experiment grouping are as follows: A, the Control group; B, the 10 nM estrogen group; C, the 40 μM Asiaticoside group; D, the 10 nM estrogen + 40 μM Asiaticoside group; E, the 10 μM DAPT group; and F, the 10 μM DAPT + 10 nM estrogen group.

2. Immunohistochemical staining.

Following the instructions, the staining effect of the tissue section was observed under the light microscope, and the LeicaDM4000B microscope image acquisition system was applied to record the staining situation. The positive cell staining ratio and staining intensity was determined in each view field.

3. CCK detecting cell proliferation

The CCK8 experiment was performed to detect cell proliferation under different treatment conditions. Normal endometrial and endometriosis eutopic glandular epithelial cells were seeded in 96-well plates, and the corresponding concentrations of Asiaticoside, DAPT and 17β-Estradiol were added to the cells respectively in the medium containing 2% FBS for 72 h treatment. The microplate reader was used to detect the absorbance value of 50 nM wavelength.

4. Transwell migration and invasion experiment

A Transwell chamber with an aperture of 8 nm was used for the experiment, and pictures were taken under a microscope with a field of view of ×200 times.

5. Western blotting to detect the expression of various proteins

The protein was detected according to the Western blotting procedure, and the ImageLab gel imaging system was used for visualization and photographing.

6. Statistical analysis

SPSS19.0 software was used for statistical analysis of the experimental data. The data is expressed in the form of mean ± standard deviation ($X \pm SD$). One-way analysis of variance (ANOVA) or *t* test was used to compare the means between samples. $P<0.05$ was considered statistically significant.

Results.

1. Asiaticoside inhibited cells proliferation

The CCK8 kit was used to test the effects of different grouping treatments on the proliferation of EEC and NEC. The results showed that at 72 h, 17β-estradiol significantly promoted the growth of EEC and NEC cells. DAPT, as an inhibitor of Notch signaling pathway, significantly inhibited the growth of NEC and EEC. Meanwhile, DAPT can also counteract the cell growth effect induced by 17β-estradiol. In the CCK8 experiment, Asiaticoside significantly inhibited the growth of NEC and EEC at 72 h. Asiaticoside can also offset the growth of the two types of cells induced by 17β-estradiol, as shown in Figure 1.

2. Asiaticoside inhibited the migration and invasion

Transwell test was performed to detect the migration and invasion capabilities of EEC and NEC under different treatment conditions. In the migration experiment, the migration ability of EEC and NEC cells was significantly increased when treated with 17β-estradiol. DAPT significantly inhibited the migration of EEC and NEC, and at the same time inhibited the migration of the two cells induced by 17β-estradiol. In the cell invasion experiment, similar results to the migration experiment were observed, see Figures 2, 3, 4, and

5.

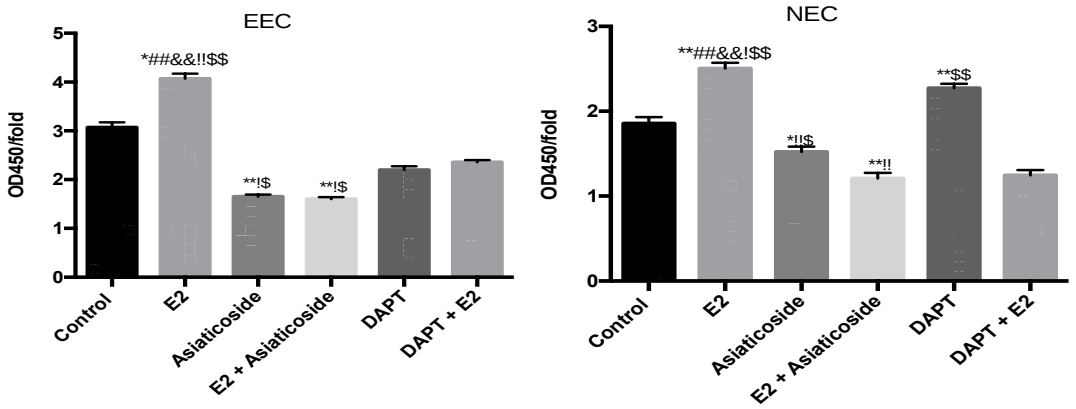

Figure 1. **Asiaticoside inhibited cell proliferation induced by 17β-estradiol in EEC and NEC.** ** p < 0.01 vs Control * p < 0.05 vs Control ## p < 0.01 vs Asiaticoside && p < 0.01 vs E2 + Asiaticoside & p < 0.05 vs E2 + Asiaticoside !! p < 0.01 vs DAPT ! p < 0.05 vs DAPT $$ p < 0.01 vs DPPT + E2 $ p < 0.05 vs DPPT + E2

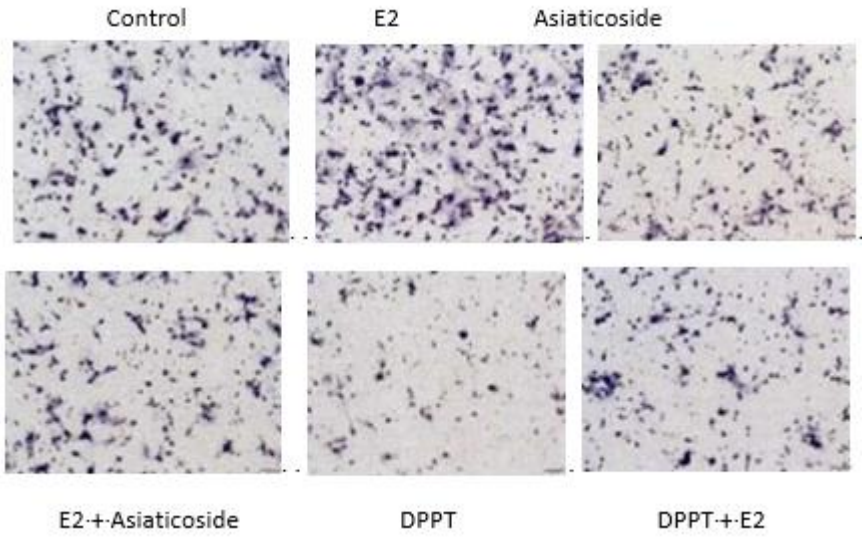

Figure 2. **The effect of Asiaticoside on cell migration induced by 17β-estradiol in EEC**

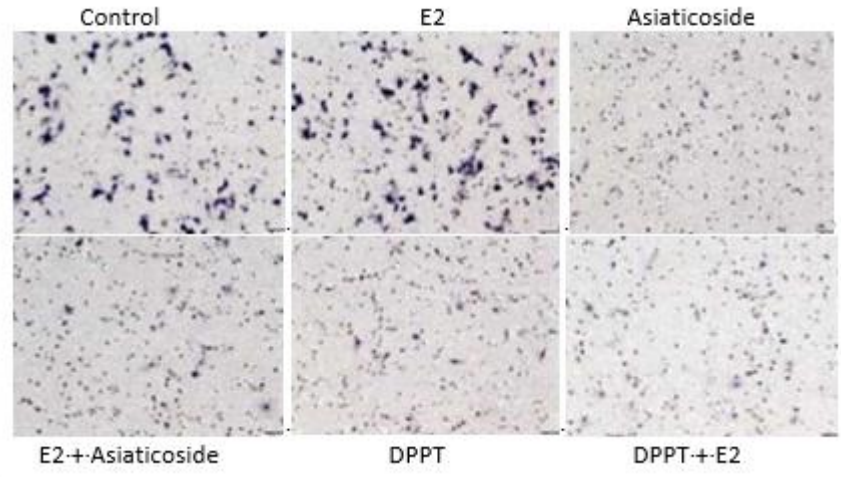

Figure 3. **The effect of Asiaticoside on cell migration induced by 17β-estradiol in NEC**

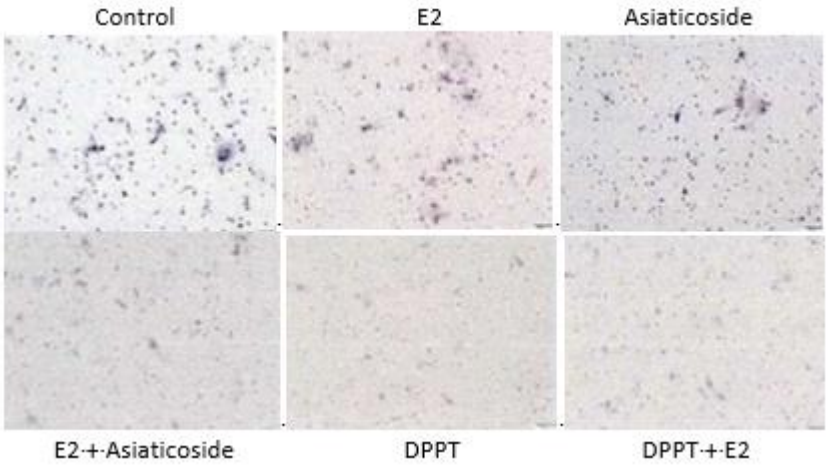

Figure 4. **The effect of Asiaticoside on cell invasion induced by 17β-estradiol in EEC**

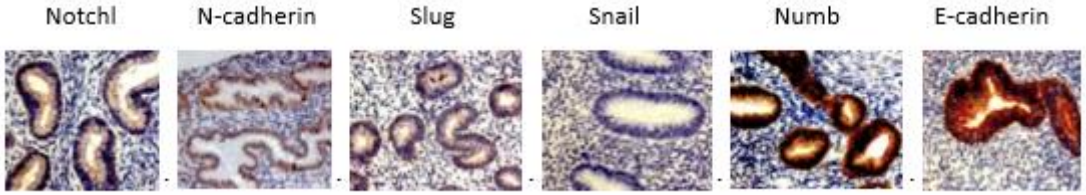

Figure 5 **The effect of Asiaticoside on 17β-estradiol-induced cell invasion in NEC**

3. Notch/Nurab signaling pathway and EMT-related proteins were abnormally expressed in the endometrium of endometriosis.

In normal endometrium, the expressions of Notchl, N-cadherin, Snail and Slug are weakly positive to positive, and the immunostaining is pale yellow to yellow, mainly distributed in the endometrium glandular epithelial cell membrane. In the mesenchymal cell membrane, Notchl, N-cadherin, Snail and Slug are weakly expressed.

In the eutopic endometrium of endometriosis, the protein expression is strongly positive. The proteins mainly concentrated in the cell membrane of glandular epithelial cells, the immunostaining is brown, and the expression in mesenchymal cells is weak. Compared with the normal endometrium, the eutopic endometrium of endometriosis exhibited higher expressions of Notchl, N-cadherin, Snail and Slug proteins, and lower Numb and E-cadherin expression, as shown in Figure 6.

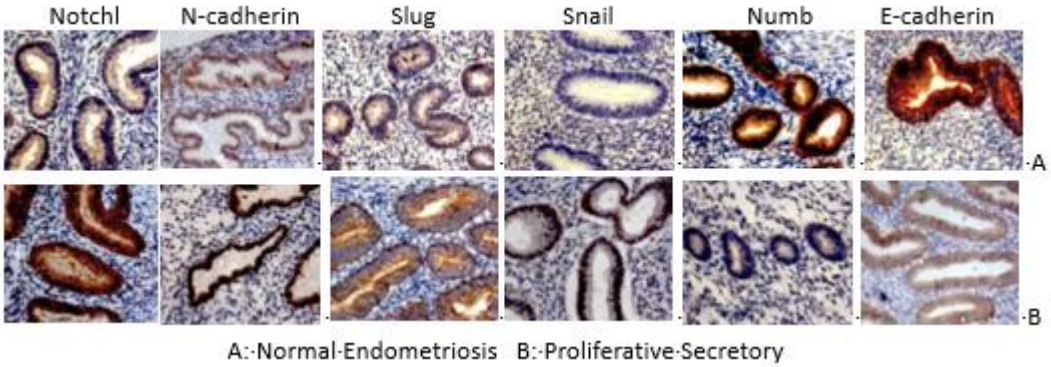

Figure 6. **The expressions of Notch/Nurab signaling pathway and EMT-related proteins in the endometrium of endometriosis.**

4. Asiaticoside inhibited 17β-estradiol-induced EMT in endometriosis endometrial epithelial cells

In Western blot experiments, Asiaticoside significantly reduced the expressions of Notch (NICD), Vimentin, Slug and Snail proteins in EEC cells, while enhancing the expression of E-cadherin

protein. In NEC cells, Asiaticoside promoted Numb protein expression and affected the expressions of Notch, Vimentin, N-cadherin, E-cadherin, Slug and Snail proteins, as shown in Figure 7.

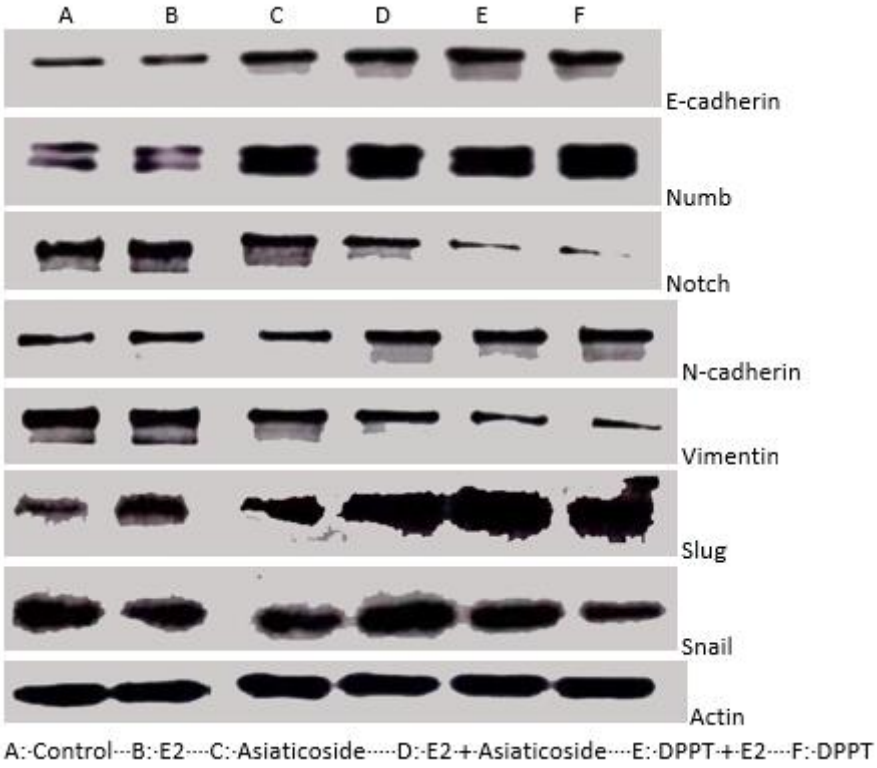

Figure 7. **The effect of Asiaticoside on various proteins.**

Discussion

Endometriosis refers to the appearance of functional endometrial glands and stroma outside the mucosa covering the surface of the uterine cavity. It is a common and frequently-occurring disease in women of childbearing age and is estrogen-dependent [3]. In the process of epithelial-mesenchymal transition, the loss of epithelial polarity and cell connections can enhance the metastasis, invasion, and resistance to apoptosis of cells. This process is also considered to be a key mechanism for the pathogenesis and progression of endometriosis [4]. Key zinc finger protein transcription factors including Snail, Slug and Twist mediate the EMT process. The Snail and Slug can inhibit the transcription, production and cleavage of E-cadherin by binding to the promoter region of the E-cadherin gene, and promote the enhancement of the expression of interstitial phenotype-related proteins, such as N-cadherin, Vimentin and so on [5-7].

The Notch signaling pathway is a key regulatory pathway of the EMT process and is involved in the progression of a series of diseases. The hNotch signaling pathway can promote the TGF-β-induced EMT process through mediating the Snail gene [8-9]. In a variety of disease models, the activation of the Ntotch signaling pathway mediated by Jagged-1 can enhance the Snail and Slug proteins, thereby inhibiting the expression of E-cadherin protein [10]. As a negative feedback regulator of the Notchl signaling pathway, Numb plays a role by promoting the ubiquitination and degradation of the intracellular segment of Notchl [11].

The up-regulation of epithelial phenotypic marker protein expression and the down-regulation of mesenchymal phenotype marker protein in endometriosis indicated the existence of EMT process in this disease. In addition, as EMT-inducing genes, Snail and Slug proteins are up-regulated in the eutopic endothelial tissue of endometriosis, suggesting that EMT may also be involved in the pathogenesis and progression of endometriosis. Immunohistochemical analysis showed that in eutopic endometrial epithelial tissues of endometriosis, the expression of Notchl protein was up-regulated and Numb protein expression was down-regulated, indicating that the Notchl/Numb signaling pathway may be involved in the regulation of EMT in the eutopic endometrium of

endometriosis. Besides, in endometriosis, there is no significant difference in the expression of each protein in the proliferative and secretory phases. In previous studies, the expressions of Numb, Slug, and E-cadherin proteins exhibited no significant differences, while the expressions of Snail, N-cadherin and Notchl proteins displayed periodic changes. These results indicate that in endometriosis, the secretion of sex hormones may be abnormal.

Endometriosis is an estrogen-dependent disease [12]. A large number of biochemical data indicate that in the endometrial tissue of endometriosis, aromatase activity and P450 aromatase mRNA expression are abnormal [13], suggesting that endometriosis lesions can locally produce estrogen. Studies have reported that the estrogen receptor signaling pathway can regulate the expression of E-cadherin protein and the process of EMT through the Slug gene, and estrogen has been confirmed to be involved in the regulation of EMT [14]. In human ovarian cancer and breast cancer cells, 17β-estradiol induces the occurrence of EMT by activating the PI3K/AKT signaling pathway and enhancing the expression of Snail and Slug proteins [15]. In prostate epithelial cells, the enhancement of estrogen effect mediated by estrogen receptor is the key inducer of epithelioid effect, which in turn promotes the activation of EMT program [16].

In endometriosis, the effect of estrogen on EMT is rarely reported. It is found that estrogen can promote the migration, invasion and mesenchymal phenotype of normal endometrial and endometrial glandular epithelial cells, suggesting that estrogen may play a role in regulating EMT of endometrial glandular epithelial cells. What's more, 17β-estradiol can activate the Notch pathway, which is a key signaling pathway that regulates EMT, and it can also down-regulate the expression of Numb protein, which is a negative regulator of the Notch pathway. DAPT, as an inhibitor of the Notch pathway, can eliminate the effects of 17β-estradiol in endometrial glandular epithelial cells, suggesting that the Notch pathway may be involved in the increase of migration and invasion ability, and the change of EMT-related proteins caused by 17β-estradiol.

In this study, the results showed that specific inhibitors of Notch signaling pathway can inhibit the proliferation, migration and invasion of normal endometrium and endometrial glandular epithelial cells, indicating that Notch signaling pathway may have an impact on EMT in endometriosis. The drug can inhibit the activity of Notchl signaling pathway in endometriotic gland epithelial cells by weakening the expression of NICD, which is not found in normal endometrium. This study demonstrates that abnormal Notchl / numb signaling pathway and EMT exist in eutopic endometrium of endometriosis, and provides basic experimental support for Asiaticoside as a potential treatment for endometriosis. In addition, we found that Notch signaling pathway may be involved in the development of endometriosis. The effect of Asiaticoside on Notch signaling pathway in endometriosis needs further study,suggests that Notch signaling pathway may be a potential therapeutic target for endometriosis.

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