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Original Research Article

Cirsilineol inhibits the proliferation and migration of endometriotic cells

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Abstract

Purpose: *To determine the effect of cirsilineol on the proliferation and migration of endometriotic cells.*
Methods: *Human endometriotic epithelial cells 12Z were treated with 5, 10 and 20 μM cirsilineol, respectively. Cell proliferation was evaluated using BrdU incorporation assay whereas wound healing assay was used to investigate cell migration, and cell invasion was assessed by Transwell assay. Cell cycle was investigated using flow cytometry while protein expression and phosphorylation levels of p65 and IκBα were evaluated by Western blot.*
Results: *Treatment with cirsilineol at either 10 μM or 20 μM resulted in a significant reduction in cell proliferation and cell cycle arrest at the G2/M phase ($p < 0.05$). Moreover, cirsilineol weakened cell migration and invasion in 12Z cells, but did not alter the expression of p65. However, it significantly downregulated the phosphorylation of p65 and increased the expression of IκBα, while attenuating the phosphorylation level of IκBα ($p < 0.05$). Furthermore, 5 μM cirsilineol did not show any significant effects on these cellular activities, suggesting the activity of cirsilineol in 12Z cells is dose-dependent.*
Conclusion: *Cirsilineol inhibits cell proliferation, induces cell cycle arrest, suppresses migration and invasion of endometriotic epithelial cells by regulating NF-κB pathway. Thus, cirsilineol might be a potential therapeutic candidate for endometriosis treatment.*

Keywords: *Cirsilineol, Cell migration, Cell proliferation, Endometriotic cells, NF-κB pathway*

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INTRODUCTION

Endometriosis is a challenging condition marked by the occurrence of endometrial cells located outside the confines of uterine cavity. There are at least 176 million women with this disorder worldwide. It often leads to pelvic pain, and up to 50 % of patients also experience fertility issues [1]. Despite its prevalence, the exact cause and underlying mechanisms of endometriosis remain unknown. While the leading hypothesis attributes the condition to retrograde menstruation transporting endometrial fragments into the pelvic cavity, other factors also contribute to the progress of ectopic lesions. These factors involve processes that alter the viability, proliferation,

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adhesion, neovascularization, and migration of reversed endometrial fragments and cells; hence the need further research to fully comprehend the molecular mechanisms [2].

A potential factor in endometriosis pathogenesis is the constitutive activation of nuclear factor kappa B (NF-κB) observed in endometriotic cells [3]. Specifically, the expression of NF-κB p65 is increased when women experiencing advanced endometriosis, suggesting a critical role for NF-κB signaling in the disease. NF-κB is known to regulate various genes associated with cell proliferation, apoptosis, inflammation, migration, invasion, and angiogenesis [4].

In the realm of traditional medicine, the herbal remedies from Chinese and African traditions are gaining increased attention. Cirsilineol, a flavonoid bioactive compound found in *Artemisia vestita* wall, has shown potent properties such as antioxidant, antibacterial, hypnotic, antineoplastic, and sedative effects. It has also exhibited cytotoxicity against various cancer cells and has been found to attenuate particulate matter 2.5 (PM 2.5)-induced lung injury in mice [5]. Additionally, cirsilineol exerts inhibitory effects on the migration and invasion of prostate cancer cells, and has been observed to suppress NF-kB signaling protein expression, thereby exhibiting anti-inflammatory properties [6,7].

The role of cirsilineol in endometriosis however remains unclear, and therefore. requires further investigation. Studies on its potential effects on endometriosis pathophysiology would shed light on new therapeutic avenues for managing this complex and enigmatic condition.

EXPERIMENTAL

Cell culture

Epithelial endometriotic cell line 12Z was maintained in Dulbecco's Modified Eagle Medium, and added to 10 % fetal bovine serum (FBS) in a 37 °C incubator in an atmosphere of 5 % CO_2. Approximately 10^6 cells were seeded in a 6-well plate; 12 h later, the cells were treated with cirsilineol (PHL82500, Millipore Sigma, Burlington, MA, USA) at concentrations of 5, 10 and 20 μM, respectively.

5-bromo-2'-deoxyuridine (BrdU) proliferation assay

The 12Z cells treated with 5, 10 and 20 μM cirsilineol were labeled with 50 μM BrdU for 30 min in 96-well plate. the cells fixed with 4 % paraformaldehyde (PFA) for 15 min and permeabilized with 0.5 % Triton X-100 for 15 min at room temperature. Next, the cells were incubated with blocking buffer (1 % bull serum albumin in PBS) for 1 h, followed by anti-BrdU antibody (ab8152, Abcam, Cambridge, UK; 1:200) for 1 h. After washing twice with PBS, the cells were incubated with horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (31160, Thermo Fisher, Waltham, MA, USA; 1:1000) for 1 h at room temperature. Furthermore, the washed cells were incubated with 100 μl TMB substrate at room temperature for 5 min and then replaced with 100 μl stop solution. The absorbance of each well was measured under a microplate reader (Microplate Reader 550; Bio-Rad, Hercules, CA, USA) at 450 nm.

Cell cycle analysis

Flow cytometry was performed to quantify the cell counts at G1, G2/M and S phases. The 12Z cells treated with 5, 10 and 20 μM cirsilineol were harvested for cell cycle analysis using flow cytometry. The single cell suspension was centrifuged at 200 g for 5 min, and the cell pellet was fixed with 4 % PFA for 30 min. The cell samples were incubated with 20 μg/ml RNaseA at 37 ℃ for 30 min, washed once with PBS and then stained with 50 μg/ml propidium iodide (PI) for 30 min at room temperature. The PI-stained cells were analyzed using flow cytometry (BD LSR II; BD Bioscience, Franklin Lakes, NJ, USA) and the cell cycle was analyzed using FlowJo software.

Transwell assay

The 12Z cells treated with 5, 10 and 20 μM cirsilineol were seeded in the upper chamber above the membrane inserts of Transwell plates (PI8P01250; Merck, Darmstadt, Germany). Matrigel (E1270; Sigma Aldrich, Shanghai, China) was applied to the upper compartment for invasion assay. The lower chamber contained medium with 10 % FBS, while the upper chamber was filled with serum-free medium. After incubation at 37 ℃ for 48 h, cells that migrated through the membrane were fixed with 4 % PFA and stained with 1 % crystal violet. At least 10 randomly selected fields were assessed for each sample, and the mean counts of stained cells were quantified using ImageJ software (National Institutes of Health, USA).

Wound healing assay

The 12Z cells treated with 5, 10 and 20 μM cirsilineol were seeded into a 6-well plate, and after the cells were attached to the bottom, the cell layer was scratched using a pipette tip as

previously described [8]. The cells were imaged at the scratching scope, and the width of the scratch line was measured using ImageJ software.

Western blot assay

The total protein of 5, 10 and 20 μM cirsilineol-treated 12Z cells were homogenized in lysis buffer (25 mM Tris-HCl pH 7.5, 200 mM NaCl, 50 mM KCl, 0.5 % sodium deoxycholate, 10 % Glycerol, 0.1 % SDS, 1 % NP-40). After 10,000 g centrifuging at 4 for 10 min, the supernatant was mixed with loading buffer and boiled for 10 min. The samples were subjected SDS-PAGE gel separation and transferred to nitrocellulose membrane. Then the membrane was incubated with the primary antibodies at 4 ℃ overnight.

The antibodies used are p65 (10745-1-AP, ProteinTech, IL, USA; 1:500), phosphorylated p65 (p-p65) (82335-1-RR, ProteinTech; 1:4000), phosphorylated IκBα (p-IκBα) (80455-01-RR, ProteinTech; 1:2000), IκBα (10268-1-AP, ProteinTech; 1:8000) and β-actin (82349-1-RR, ProteinTech; 1:5000). They were then incubated with HRP-labeled with anti-rabbit IgG (B900210, ProteinTech; 1:5000) at room temperature for 1 h. The target bands were visualized with ECL reagents (Solarbio, Beijing, China), and the relative intensity was determined by ImageJ software and normalized to β-actin [9].

Quantification and statistical analysis

Data of three biological replicates are presented as mean ± standard deviation (SD), the figures were drawn using GraphPad Prism 8.0 (Dotmatics, Boston, MA, USA) and the differences between any two groups were calculated by unpaired t-test. $P < 0.05$ was considered significant differences.

Cirsilineol inhibits proliferation of endometriotic epithelial cells

The chemical structure of cirsilineol is shown in Figure 1 A. The BrdU proliferation assay in 12Z cells revealed that cirsilineol negatively regulated cell proliferation. There was a reduction in absorbance in 5 μM cirsilineol-treated cells ($p < 0.05$). When the concentration of cirsilineol increased to 10 or 20 μM, cell growth significantly decreased compared to the cirsilineol-free group (0 μM cirsilineol; Figure 1 B). Therefore, cirsilineol attenuated the proliferation of endometriotic epithelial cells in a dose-dependent manner.

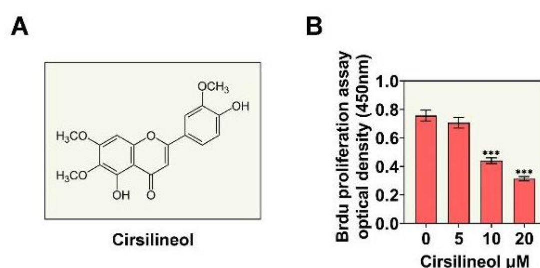

Figure 1: Cirsilineol inhibits the proliferation of endometriotic epithelial cells. (A) Chemical structure of cirsilineol; (B) Effect of cirsilineol on the proliferation of 12Z cells. Error bar, mean ± SD (n = 3); ***$p < 0.001$

Cirsilineol induced cell cycle arrest in endometriotic epithelial cells

Cell cycle is tightly involved in cell proliferation regulation, and hence it was necessary to study the cell cycle in cirsilineol-treated cells. Specifically, the variation of cell cycle mainly occurred at G1 and G2/M phases, but no difference was observed at S phase. Compared to control group (0 μM cirsilineol), no significant difference was observed in 12Z cells treated with 5 μM cirsilineol ($p < 0.05$). When the cells were treated with 10 or 20 μM cirsilineol, there was a significant reduction in cell counts at G1 phase, and increase at G2/M phase (Figure 2). The data showed that cirsilineol arrested cell cycle at G2/M phase in a dose-dependent manner.

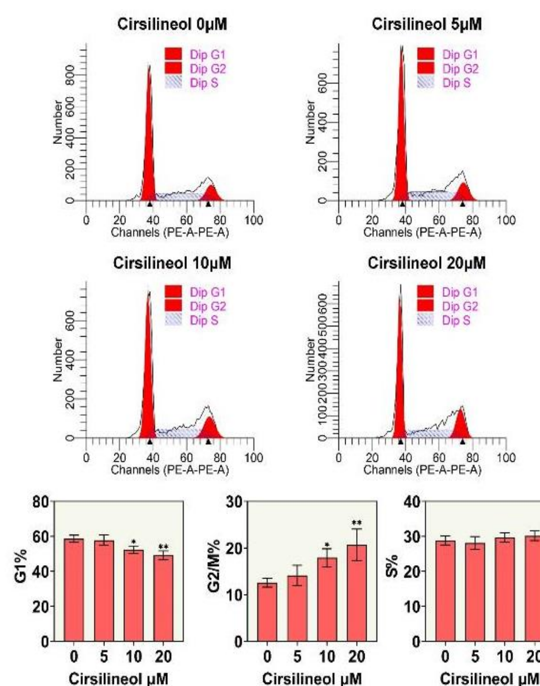

Figure 2: Cirsilineol induces cell cycle arrest in endometriotic epithelial cells. Cell counts at G1, G2/M and S. Error bar, mean ± SD (n = 3); * $p < 0.05$; ** $p < 0.01$

Cirsilineol suppresses the migration and invasion of endometriotic epithelial cells

The wound healing assay revealed larger scratches were left in 12Z cells treated with 10 or 20 μM Cirsilineol than the control group, indicating that Cirsilineol weakened cell migration (Figure 3 A). The 5 μM cirsilineol did not change the scratch width, indicating that cirsilineol effect on cell migration was dose-dependent (Figure 3 B). The number of 12Z cells that passed through the membrane insert was reduced in 10 or 20 μM cirsilineol-treated cells, but no reduction was observed in 5 μM cirsilineol-treated cells (Figure 3 C). There was no significant difference in the 5 μM cirsilineol group compared to the control group, but the difference was significant in the 10 or 20 μM cirsilineol group (Figure 3 D; $p < 0.05$). Thus, cirsilineol inhibited the migration and invasion of endometriotic epithelial cells.

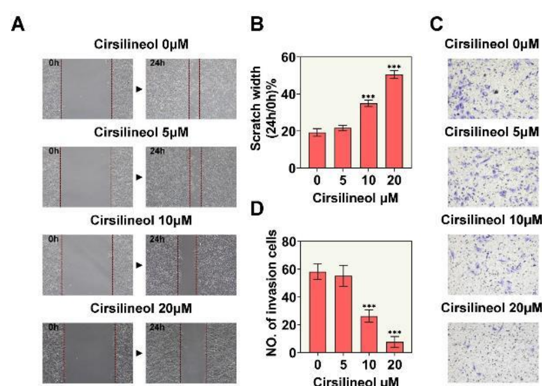

Figure 3: Cirsilineol suppressed migration and invasion of endometriotic epithelial cells. (A) Remaining scratch width in cirsilineol-treated 12Z cells; (B) Scratch width; (C) Invasion of 12Z cells treated with cirsilineol; (D) Invasion cells. Error bar, mean ± SD (n =3); *** $p < 0.001$

Cirsilineol inhibits NF-κB pathway

The results obtained showed that 10 or 20 μM cirsilineol downregulated the phosphorylation of p65, but did not affect p65 expression. Furthermore, cirsilineol at the concentration of (10 or 20 μM concentration) enhanced the expression of IκBα, while decreasing IκBα phosphorylation level (Figure 4). Therefore, cirsilineol inhibited NF-κB pathway by reducing p65 and IκBα phosphorylation.

DISCUSSION

Endometriosis is a chronic and often debilitating gynecological disorder affecting millions of women worldwide.

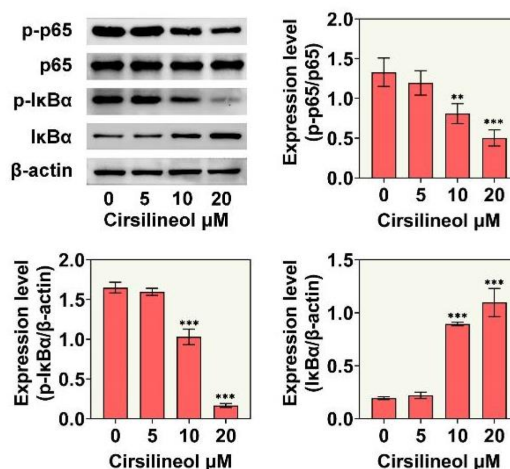

Figure 4: Cirsilineol inhibits NF-κB pathway. Protein expression and phosphorylation level of p65 and IκBα in cirsilineol-treated 12Z cells. Error bar, mean ± SD; ** $p < 0.01$, *** $p < 0.001$

The pathogenesis of endometriosis involves the proliferation of endometriotic epithelial cells, leading to the formation and growth of endometriotic lesions [1]. Cirsilineol has been recognized for its anti-inflammatory, antioxidant, and anti-cancer properties in various studies [6]. While the efficacy of cirsilineol in treating endometriosis is still to be determined, this study utilized BrdU assay to evaluate the proliferation of 12Z cells. The results support the conclusion that cirsilineol attenuated the proliferation of endometriotic epithelial cells.

Cell cycle progression is a meticulously controlled mechanism overseeing both cell growth and division. Dysregulation of cell cycle is a hallmark of various diseases, including endometriosis. In this disorder, endometriotic epithelial cells exhibit abnormal cell cycle activity, resulting in rapid proliferation, and the development of ectopic tissue [1]. Therefore, identifying compounds restoring cell cycle is of considerable interest in developing effective treatments for endometriosis. Recently, cirsilineol has emerged as a potential candidate for modulating cell cycle progression in several cells. Cirsilineol inhibits *in vitro* and *in vivo* the growth of esophageal squamous cell carcinoma by targeting tyrosine kinase 2 [10]. Cirsilineol also enhances reactive oxygen species (ROS)-mediated apoptosis, and this inhibits the proliferation of lung squamous cell carcinoma [11,12]. In malignant melanoma cells, cirsiliol inhibits the transition from epithelial to mesenchymal by regulating PI3K/Akt/NF-κB pathway [13]. In this work, flow cytometry data showed that cirsilineol-treated 12Z cells were arrested in G2/M phase of the cell cycle. When exposed to cirsilineol, these cells illustrated a

significant dose-dependent decrease in cell proliferation rates.

Inhibition of migration and invasion are critical aspects of controlling endometriotic lesion growth and spread. Uncontrolled migration and invasion of endometriotic epithelial cells into surrounding tissues are responsible for the formation of ectopic lesions, leading to chronic inflammation, pain, and infertility in the affected women [14]. The findings of this study demonstrate that cirsilineol curtailed the migration and invasion of endometriotic epithelial cells, offering a potential strategy for countering the progression of endometriotic lesions. The inhibitory effects of cirsilineol on cellular movement and invasion are crucial in preventing the establishment of new lesions and restricting the expansion of existing ones [15].

In this study, cirsilineol's inhibitory effects on the migration and invasion of endometriotic epithelial cells present some evidence for its potential as a novel therapeutic approach for endometriosis. By impeding cellular motility and invasion, cirsilineol offers a means of controlling the progression of endometriotic lesions and alleviate the burden of this chronic condition on affected individuals. However, further preclinical and clinical investigations are necessary to fully explore the molecular mechanisms involved, and to determine the safety and efficacy of Cirsilineol as a potential treatment for endometriosis.

NF-κB is reported to be closely associated with cell proliferation, apoptosis, migration, invasion, and angiogenesis. NF-κB activating proteins enhance the proliferation, migration, invasion, and reduction in radiation sensitivity of gastric cancer cells [16]. NF-κB signaling pathway was deactivated in endometriosis, which functions by inhibiting the proliferation, migration, invasion and inflammation of endometrial stromal cells [17]. Prior research suggests the persistent activation of NF-κB in endometriotic cells. Elevated nuclear expression of NF-κB p65 is observed in the eutopic endometrium of women experiencing advanced endometriosis, suggesting a critical role for NF-κB signaling in the disease [18]. The western blot data in this work suggested that the protein expression or phosphorylation level of two key molecules, p65 and IκBα, were reduced in cirsilineol-treated cells. This demonstrates that NF-κB pathway is regulated by cirsilineol. While the precise molecular mechanisms through which cirsilineol inhibits NF-κB pathway need further investigation, understanding these mechanisms would provide valuable insights into the compound's mode of action and aid in optimizing its potential therapeutic applications.

CONCLUSION

Cirsilineol inhibits cell proliferation, induces cell cycle arrest, and suppresses migration and invasion in endometriotic epithelial cells by regulating NF-κB pathway. Thus, these findings indicate that cirsilineol is a promising therapeutic candidate for the management of endometriosis.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Xumeng Gu and Wenpeng Wu designed the study and carried them out; Xumeng Gu, Tianchan Zhang, Tongtong Liu and Huifang Cong supervised the data collection, analyzed and interpreted the data, and Xumeng Gu and Wenpeng Wu prepared the manuscript for publication and reviewed the draft of the manuscript. All authors read and approved the manuscript.

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