Identification of Potential Active Ingredients and Mechanisms of Cattail Pollen for Treating Infertile Patients With Endometriosis Based on Bioinformatics and Molecular Docking

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Abstract

Introduction: Cattail Pollen is a commonly used Chinese medicine for promoting blood circulation and removing blood stasis in infertility patients with endometriosis, but its ingredients and mechanism of action are still unclear. The aims of this study were to explore the potential active ingredients, targets and mechanisms of Cattail Pollen in treating infertility patients with endometriosis based on bioinformatics and molecular docking. Methods: The GSE120103 dataset was downloaded from the Gene Expression Omnibus (GEO) database to screen out differentially expressed genes. Cytoscape software was constructed to construct the protein–protein interaction network and screen for hub proteins, and molecular docking was performed to identify the binding activity of Cattail Pollen active ingredients and infertility genes. Then, DAVID software was used to perform gene ontology (GO) functional analysis and KEGG pathway enrichment analysis on differentially expressed genes. Results: There were 1320 differentially expressed genes in patients with endometriosis. ADCY5, RLN3, and ADCY6 proteins encoded by genes that were upregulated in infertile patients with endometriosis. Eight active ingredients of Cattail Pollen were obtained from the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform: naringenin ((2R)-5,7-dihydroxy-2-(4-hydroxyphenyl)chroman-4-one), arachidonic acid, isorhamnetin, β-sitosterol, kaempferol, Testosterone palmitate, kaempferol-3-O-α-L-rhamnosyl(1→2)-β-D-glucoside and quercetin. Molecular docking identified that the binding activity of arachidonic acid with ADCY5, RLN3, and ADCY6; and testosterone palmitate with ADCY5 and ADCY6 was strong. GO analysis suggested that differentially expressed genes were involved in multiple biological processes, cellular components, and molecular functions. KEGG enrichment analysis found that differentially expressed genes were enriched in neuroactive ligand–receptor interaction, cytokine–cytokine receptor interaction, chemokine signaling pathway, and Jak-STAT signaling pathway. Conclusions: This study discovered the differentially expressed genes of naturally conceived and infertile patients with endometriosis, and clarified the effective ingredients, targets, and potential signaling pathways of Cattail Pollen in the treatment of infertility patients with endometriosis.

Keywords
bioinformatics, Chinese medicine, infertility, fertility, genes, pathways

Received: September 9th, 2021; Accepted: July 1st, 2022.
Introduction

Endometriosis is the endometrial tissue including stroma and glands, which appear outside the uterus. Its main symptoms are dysmenorrhea, lower abdominal pain, painful intercourse, and even infertility, causing a psychological and physical burden on the patient. It has been reported that the infertility rate of patients with endometriosis is 50%.

However, the etiology and mechanism of endometriosis-related infertility are still unclear. Previous studies have found that in addition to inflammatory cytokines, growth and angiogenesis factors, abnormally expressed genes may also contribute to endometriosis-related infertility disease. For example, a study of Chinese Han females found that the CYP19 rs700518AA genotype was significantly associated with an increased risk of endometriosis-related infertility.

Coincidentally, a study in Taiwanese women also showed that MUC17 polymorphisms were associated with the development of endometriosis and associated infertility. Therefore, it is of great significance to analyze and discover the relevant targets of endometriosis-related infertility and then explore their potential pathogenesis or treatment mechanism. At present, gene chip data can reveal the background of genetic information on a large scale. Bioinformatics analysis is often used to mine potential information related to gene expression, gene products, gene function, and cell function to identify key factors in pathogenesis, predict potential therapeutic targets, and lay a solid foundation for the development of precision medicine. Therefore, this study screened the gene chip data of naturally fertile and infertile endometriosis patients through the GEO database to identify differentially expressed genes, and then gene ontology (GO) analysis, KEGG analysis, and gene expression product analysis were performed to identify the biological process (BP), cellular component (CC), molecular function (MF), and the potential signaling pathways and pivot protein targets of differentially expressed genes.

Currently, treatment of endometriosis-related infertility focuses on improving fertility by removing or reducing ectopic endometrial implants and restoring normal pelvic anatomy. Common strategies include expectant management, surgery, assisted reproductive technology, and medication. Chinese medicine is also widely used in endometriosis as a complementary and alternative medicine method. According to Chinese Medicine theory, the main pathogenesis of endometriosis is blood stasis blocking the uterus, so promoting blood circulation to dissipate blood stasis is the main method of treatment. Our previous studies have confirmed that Chinese medicine for promoting blood circulation to dissipate blood stasis could increase the incidence of pregnancy in endometriosis patients and reduce the rate of spontaneous abortion and recurrence. Moreover, our data mining analysis found that Cattail Pollen is a commonly used Chinese medicine for promoting blood circulation and removing blood stasis in the treatment of endometriosis. However, the potential active ingredients, targets and mechanisms of Cattail Pollen in treating infertility patients with endometriosis are still unclear.

Therefore, solving this problem is of great significance to promoting the clinical application of Cattail Pollen and provides direction for further experimental verification in the later stage.

The Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP) is a unique and widely used systematic pharmacology platform for Chinese herbal medicine that provides data on the pharmacokinetic properties of natural compounds, such as oral bioavailability, drug likeness, to facilitate the identification and screening of active ingredients of Chinese herbal medicine, as well as subsequent mechanistic research. Molecular docking technology matches potential active ingredients with protein targets and evaluates their binding power to find effective ingredients and binding sites for disease treatment. For example, Lin et al. discovered the potential active ingredient luteolin of Yinqiao powder in the treatment of COVID-19 through TCMSP and found that it has good binding activity with ACE2 through molecular docking. In addition, we previously discovered 5 ingredients of Curcumae Radix through TCMSP and found that the depression-related protein PTGS2 has good binding activity to oxyurecumenol through molecular docking, which may have an antidepressant effect through calcium, estrogen, PI3K-Akt, and ErkB signaling pathways. Therefore, in this study, 8 active ingredients of Cattail Pollen were screened according to their pharmacokinetic properties through the TCMSP database, namely naringenin, arachidonic acid, isorhamnetin, β-sitosterol, kaempferol, testosterone palmitate, kaempferol-3-O-α-L-rhamnosyl(1→2)-β-D-glucoside, and quercetin. We hypothesize that these active ingredients of Cattail Pollen can reduce the occurrence of infertility by combining with the hub proteins of differential gene regulation in patients with endometriosis-related infertility, which are reported as follows.

Materials and Methods

The Source and Screening of Differentially Expressed Gene Data

The GEO database is a public database managed and maintained by the National Center for Biotechnology Information and is currently the largest public database of gene expression profiles in the world. The GEO (https://www.ncbi.nlm.nih.gov/geo/) database was used to find and download the endometriosis-related gene data set GSE120103, and 5 fertile women and 5 infertile patients with endometriosis gene sample data were selected. The GEO2R tool was used for analysis, and a corrected P-value<.01, logFc>2.5 or logFc<-2.5 was set to screen the differentially expressed genes of natural pregnancy and infertility patients with endometriosis.

Construction and Module Analysis of the Interaction Network of Proteins Encoded by Differentially Expressed Genes

The String (https://string-db.org/) database is a database containing known and predicted protein–protein interactions, including data obtained from experimental testing and
predicted by bioinformatics methods. The String database was used to analyze the protein interactions of the proteins encoded by the differentially expressed genes, and Cytoscape software was used to perform topological analysis of the network. The top 6 proteins in terms of degree value were used as the key proteins of the protein interaction network. The degree value refers to the number of relationships between a gene and surrounding genes in the network. The greater the degree, the more genes that interact with it.

**Screening of Active Ingredients of Cattail Pollen**

TCMSP (https://old.tcmsp-e.com/tcmsp.php) is a Chinese herbal medicine system pharmacology platform, which contains 499 herbs and the compound components of each herb (more than 29,000 in total), provides comprehensive evaluation data of human absorption, distribution, metabolism, and excretion properties for each compound, such as oral bioavailability, drug-likeness (DL), intestinal epithelial permeability, and blood–brain barrier. This has stimulated new interest in finding drug candidates in various Chinese herbal medicines and provided a new platform for studying the mechanism of Chinese medicines at the system level. The active ingredients of Cattail Pollen were obtained from the TCMSP (http://lsp.nwu.edu.cn/, version 2.3). Oral availability (OB) ≥ 30%, DL ≥ 0.18 was used to screen the active ingredients of Cattail Pollen. The mol2 format structure of the active ingredients was downloaded.

**Molecular Docking**

The Surfex-dock program in the Sybyl-X2.1 software was used to molecularly dock the active ingredients of Cattail Pollen with the hub protein in infertility patients with endometriosis and to screen the potential active ingredients and targets of Cattail Pollen for the treatment of infertility patients with endometriosis. It is generally believed that the total score of molecular docking greater than 3.0 indicates that the molecule has good binding activity to the target and that a score greater than 5.0 indicates that it has strong binding activity.\(^1\)

**GO Function Enrichment of Differentially Expressed Genes**

DAVID (https://david.ncifcrf.gov/) software was used for functional enrichment analysis of differentially expressed genes, and a count>2 and \(P<.05\) were set as the screening conditions. GO analysis provides functional annotations on 3 aspects: BP, CC, and MF. BP refers to the process or location where genes and their products function. CC refers to the locations where genes and their products function. MF refers to the molecular activities of genes and their products.

**KEGG Pathway Enrichment of Differentially Expressed Genes**

The KEGG pathway (https://www.kegg.jp/kegg/pathway.html) database was used to conduct enrichment analysis of differentially expressed genes and explore the signaling pathways of differentially expressed genes to clarify the potential mechanism of Cattail Pollen in treating infertility of endometriosis.

**Results**

**Screening Results of Differentially Expressed Genes**

Five patients with endometriosis who could conceive naturally and 5 infertile endometriosis patients were included. There was no significant difference between the 2 groups at baseline (Figure 1). Using GEO2R analysis, the differentially expressed genes were screened under the conditions of corrected \(P\)-value<.01, log\(Fc\)>2.5, or log\(Fc\)<−2.5. There were 1320 differentially expressed genes, including 269 upregulated genes and 1051 downregulated genes (Supplemental Table 1).

**Interaction Network of Differentially Expressed Genes Encoding Proteins**

The differentially expressed genes between naturally pregnant patients with endometriosis and infertile patients with endometriosis were mapped to the String software, and the protein
interaction network information encoded by the differentially expressed genes was obtained and imported into Cytoscape for labelling. The degree value indicates the importance of the protein in the network. The greater the degree value, the more important the protein. The top 6 hub proteins with degree values were KNG1, GNG7, ADCY5, RLN3, ADCY8, and ADCY6. KNG1, ADCY5, RLN3, and ADCY8 were the proteins encoded by downregulated genes, GNG7 and ADCY6 were the proteins encoded by upregulated genes (Figure 2). As shown in Figure 3, KNG1 was significantly highly expressed in naturally conceived patients with endometriosis, and ADCY5, RLN3, and ADCY6 were significantly highly expressed in infertile patients with endometriosis.

Screening of Active Ingredients in Cattail Pollen

Thirty-three active ingredients of Cattail Pollen were obtained from the TCMSP database, and 8 active ingredients were screened out according to the OB and DL of the active ingredients, namely, naringenin, arachidonic acid, isorhamnetin, β-sitosterol, kaempferol, Testosterone palmitate, kaempferol-3-O-α-L-rhamnosyl(1→2)-β-D-glucoside, and quercetin (Table 1).

Molecular Docking

The hub proteins in infertile patients with endometriosis (ADCY5, RLN3, ADCY6) and related active ingredients of Cattail Pollen were molecularly docked (Table 2). It is generally believed that the total score of molecular docking greater than 3.0 indicates that the ingredients have good binding activity to the targets, and that greater than 5.0 indicates that it has strong binding activity. The molecular docking results showed that there were 5 (20.83%) docking scores greater than 5.0, 13 (54.17%) scores between 3.0 and 5.0, and 6 (25%) scores less than 3.0. The results suggested that the binding activity of arachidonic acid with ADCY5, RLN3,
Figure 3. Hub proteins screened by topological analysis. (A. KNG1; B. GNG7; C. ADCY5; D. RLN3; E. ADCY8; F. ADCY6; The light blue boxes represent 5 patients with endometriosis-related infertility, and the pink boxes represent 5 patients with endometriosis who can conceive naturally.)
and ADCY6; and testosterone palmitate with ADCY5 and ADCY6 was strong.

GO Function Enrichment Analysis of Differentially Expressed Genes

The GO functional enrichment analysis of differentially expressed genes showed that in terms of BP, the G protein coupled receptor signaling pathway involved 62 genes, the nervous system development involved 32 genes, and the transport involved 31 genes (Figure 4). In terms of CC, the integral component of the membrane involved 307 genes, the plasma membrane involved 265 genes, and the extracellular region involved 149 genes. In terms of MF, 56 genes were involved in calcium ion binding, 22 genes were involved in transport activity, and 19 genes were involved in growth factor activity.

KEGG Pathway Enrichment Analysis of Differentially Expressed Genes

Count>2 and \( P < .05 \) were used as the screening conditions of KEGG pathway enrichment analysis to screen the signaling pathways involved in differentially expressed genes, and it was found that 17 common signaling pathways were involved (Figure 5). Neuroactive ligand–receptor interaction involved 36 genes (14.94%), cytokine–cytokine receptor interaction involved 25 genes.

Table 1. The Main Active Ingredients of Cattail Pollen.

| No. | Molecular ID | Molecular name                  | Chemical formula | Structure          | OB (%) | DL  | Pubchem Cid |
|-----|--------------|---------------------------------|------------------|--------------------|--------|-----|-------------|
| 1   | MOL001040    | Naringenin                      | C15H12O5         | ![structure](image) | 42.36  | 0.21| 667495      |
| 2   | MOL001439    | Arachidonic acid                | C20H32O2         | ![structure](image) | 45.57  | 0.2 | 444899      |
| 3   | MOL000354    | Isorhamnetin                    | C16H12O7         | ![structure](image) | 49.6   | 0.31| 5281654     |
| 4   | MOL000358    | β-sitosterol                    | C29H50O          | ![structure](image) | 36.91  | 0.75| 222284      |
| 5   | MOL000422    | Kaempferol                      | C15H10O6         | ![structure](image) | 41.88  | 0.24| 5280863     |
| 6   | MOL006111    | Testosterone palmitate          | C35H58O3         | ![structure](image) | 34.14  | 0.71| 164765      |
| 7   | MOL006115    | Kaempferol-3-O-α-L-rhamnosyl(1→2)β-D-glucoside | C16H12O5 | ![structure](image) | 62.87  | 0.24 | NA          |
| 8   | MOL000098    | Quercetin                       | C15H10O7         | ![structure](image) | 46.43  | 0.28| 5280343     |

Abbreviations: OB, oral availability; DL, drug-likenes; NA, not available.
genes (10.37%), chemokine signaling pathway involved 19 genes (7.88%), and Jak-STAT signaling pathway involved 16 genes (6.64%).

Discussion

In this study, the GSE120103 gene chip data set in the GEO database was analyzed, and 1320 differentially expressed genes of naturally conceived and infertile patients with endometriosis were discovered. The top 6 hub proteins encoded by differentially expressed genes were KNG1, GNG7, ADCY5, RLN3, ADCY8, and ADCY6. Among them, KNG1 was highly expressed in patients with endometriosis who can conceive naturally. KNG1 is positively correlated with progesterone levels and progesterone can upregulate the expression of KNG1.\(^{16}\) ADCY5, RLN3, and ADCY6 were obviously highly expressed in infertile patients with endometriosis. It has been reported that the genetic variation in ADCY5 is related to low birth and lower placental weight, which leads to asymmetric fetal growth restriction.\(^{17}\) Li et al found that ADCY5 may affect the fertility of dairy cows by regulating the process of ovarian development.\(^{18}\) However, RLN3 and ADCY6 have not been experimentally confirmed in endometriosis.

Eight active ingredients of Cattail Pollen were screened by the TCMSP database, all of which were flavonoids, except arachidonic acid, β-sitosterol, testosterone palmitate. These findings were consistent with the study by Wang.\(^{19}\) Flavonoids have been reported as the main active compounds for promoting blood circulation and removing blood stasis.\(^{20}\) The combined strategy of metabolomics and chemometrics further confirmed the direct inhibition of thrombin by Cattail Pollen.\(^{21}\) Further molecular docking with highly expressed protein targets related to infertility revealed that the binding activity of arachidonic acid with ADCY5, RLN3, and ADCY6; and testosterone palmitate with ADCY5 and ADCY6 was strong. These may be the potential active ingredients and targets of Cattail Pollen in the treatment of infertility patients with endometriosis. The continuous oxygenation of arachidonic acid leads to the formation of lipoxin, which participates in the anti-inflammatory, immune regulation and pregnancy processes of endometriosis.\(^{22}\) In addition, arachidonic acid can synthesize prostaglandin F (2α), promote the secretion of progesterone in the luteal body, and improve the chance of conception.\(^{23}\) Addo et al found that the level of testosterone palmitate is closely related to infertility.\(^{24}\) However, the effects of arachidonic acid, testosterone palmitate, and ADCY5, RLN3, ADCY6 have not been further verified by experiments.

GO analysis revealed that the differentially expressed genes involved in BP were the G protein-coupled receptor signaling pathway, nervous system development, and transport. G protein-coupled receptors are widely present in estrogen-dependent diseases and can regulate the rapid signal transduction of estrogen.\(^{25}\) For example, G protein-coupled receptor 30 has been shown to participate in the maturation of...
endometriotic follicles and macrophages, and participate in the effect of estrogen on target cells in a variety of ways. The nervous system is closely related to endometriosis-related pain. Su et al have shown that peripheral inflammation and central pain-generating factors, central nervous system structural changes, and endocrine changes may all lead to endometriosis-related pain. Transport is related to the characteristics of endometriosis dissemination, and ectopic implantation can occur. The differentially expressed genes involved in CC were integral components of the membrane, plasma membrane, and extracellular region. Liang found that the adhesion and invasion of endometrial cells into the peritoneal mesothelial cell layer is one of the key steps in the occurrence of pelvic endometriosis. In addition, it also involves the degradation of the extracellular matrix and basement membrane. The differentially expressed genes involved in MF were calcium ion binding, transport activity, and growth factor activity. The expression of the calcium channel Cav1.3 protein in
endometriosis tissues is higher than that in eutopic hyperplasia endometrial tissue, and it plays an important role in the formation of endometriosis lesions. Transport activity is related to the invasion and metastasis characteristics of endometriosis. Vascular growth factor is closely related to the formation of endometriotic lesions. Xi et al have found that acupuncture can reduce the vascular endothelial growth factor in the ectopic endometrium and abdominal cavity microenvironment, which hinders ectopic angiogenesis.

Enrichment of the KEGG pathway revealed that the differentially expressed genes were mainly distributed in the neuroactive ligand–receptor interaction. Neuroactive ligand–receptor interactions are a collection of related receptors on the cell membrane, constituting a signaling pathway inside and outside the cell, and are physiologically related to neural function. The symptoms of dysmenorrhea and lower abdominal pain in patients with endometriosis were closely related to this pathway. The second largest number of differentially expressed genes enrichment was cytokine–cytokine receptor interaction. Liu et al also found that IL-1β may contribute to endometriosis through cytokine–cytokine receptor interactions. Differentially expressed genes were also significantly enriched in the chemokine signaling pathway and Jak-STAT signaling pathway. Previous studies have reported that a variety of chemokines and immune cells are involved in the invasion, adhesion, and neovascularization of ectopic endometrial tissue. In addition, the Jak-STAT signaling pathway was also found to be involved in the proliferation, migration, invasion, apoptosis, and angiogenesis of endometrial cells.

Conclusions
This study screened 1320 differentially expressed genes in patients with endometriosis that can conceive naturally and infertility through bioinformatics. Among them, ADCY5, RLN3, and ADCY6 were significantly expressed in infertile patients, and molecular docking identified that the active
ingredients of Cattail Pollen, including arachidonic acid and testosterone palmate, have strong binding potential with the proteins regulated by the above genes, which may be the potential active ingredients for the treatment of infertility patients with endometriosis. Enrichment of KEGG pathways revealed 17 potential signaling pathways for Cattail Pollen to treat infertility patients with endometriosis. This will provide new drugs, targets, and directions for the treatment of infertility patients with endometriosis in the future, which needs to be further verified by experiments.

Author Contributions
Conceptualization: Wang X.T. and Lin H. Data curation: Wang X.T., Lu H., Wen L., Chen H., Wang X., Li L., and Lin H. Formal analysis: Wang X.T., Lu H., Wen L., Chen H., Wang X., Li L., and Lin H. Methodology: Wang X.T., Lu H., Wen L., Chen H., Wang X., Li L., and Lin H. Project administration: Lin H. Supervision: Lin H. Validation: Wang X.T. Visualization: Lin H. Writing original draft: Wang X.T. and Lin H. Writing review & editing: Wang X.T., Lu H., Wen L., Chen H., Wang X., Li L., and Lin H. All authors contributed constructive comments on the paper.

Availability of Data and Materials
The data and materials generated or analyzed during this study are available from the corresponding author on reasonable request.

Declaration of Conflicting Interests
The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding
The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the Natural Science Foundation of Ningxia Hui Autonomous Region (grant number 2022AAC03398, 2022AAC03407), Scientific research project of Ningxia Hui Autonomous Region Health Committee (2021-NW-037).

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Supplemental Material
Supplemental material for this article is available online.

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