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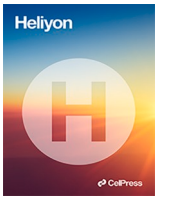

Therapeutic effects of Huayu Jiedu formula on endometriosis via downregulating GATA 6 expression

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ABSTRACT

Background: Endometriosis (EMs) is a common chronic inflammatory disease which is characterized by multiple clinical symptoms and high recurrence rate due to the absence of effective therapies. Huayu Jiedu Formula (HYJDF), is a traditional Chinese medicine prescription with five major herbs. It has been used as traditional medicine to treat EMs for more than twenty years and exerted a good therapeutic effect. However, the underlying mechanism is unclear. Here we aim to observe the effects of HYJDF on EMs and investigate the therapeutic mechanism.

Methods: The extract components of HYJDF were identified and quantified by an UHPLC-QE-MS method. Network pharmacology was used to obtain the core targets of HYJDF for the treatment of EMs and the specific biologic processes involved. A total of 68 EMs cases were randomly divided into control (gestrinone) and observation (HYJDF) groups. The overall effectiveness, pain scores, cyst-size changes, serum CA125 levels, quality-of-life scores, safety, and adverse events were evaluated before and after treatment. For the mechanism research, DNA methylation-chip analysis was performed to determine the differential genes. EMs mice models and human ectopic stromal cells (ESCs) were treated with HYJDF and its pharmaceutical serum, respectively. The ectopic foci was measured via H&E staining while the expressions of the target genes were verified by real-time PCR and Western blot analysis. The inflammatory cytokine levels in the peritoneal fluid of mice were detected by ELISA. The proliferative potential of cells was analyzed by MTS whereas the apoptosis and cell cycle were determined through flow analysis.

Results: The total number of components detected in positive and negative ion modes was 839 and 597, respectively. Network pharmacology suggested that HYJDF treated EMs through DNA methylation. We found that HYJDF and gestrinone exerted good therapeutic effect with no obvious difference, but the HYJDF treatment group had fewer side effects. GATA 6, which was hypomethylated and abundant in endometriotic cells, potently induced inflammatory response. This finding indicated the important role of GATA 6 in EMs development. Moreover, HYJDF

Abbreviations: EMs, endometriosis; HYJDF, Huayu Jiedu Formula; VAS, Visual Analogue Scales; EHP-5, Endometriosis Health Profile −5; DAVID, Database for Annotation, Visualization, and Integrated Discovery; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GO, Gene Ontology; TGF-β, transforming growth factor-β; PGE2, Prostaglandin E2; COX-2, Cyclooxygenase −2; IL-1β, Interleukin-1β.

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ameliorated inflammatory response (i.e., reduced the levels of IL-1β and PGE2 in peritoneal fluid), suppressed ESCs proliferation, and increased cell apoptosis by down-regulating GATA 6 expression.

Conclusion: We demonstrated that HYJDF has anti-inflammation activity and increased cell apoptosis through the reduction of GATA 6 expression in ectopic tissues, which showed good therapeutic effect without any obvious side effects. These findings suggest that HYJDF may be a new and efficient traditional Chinese medicine for the treatment of EMs.

1. Introduction

Endometriosis (EMs) is an estrogen-dependent disease in which functional endometrial tissue is present outside the uterus. EMs is characterized by multiple symptoms, extensive disease, invasiveness, and recurrence [1]. Currently, drug therapy and surgery are available for the treatment of EMs patients. But even after the removal of endometriosis lesions, it can still cause persistent pain, which seriously affects the life quality of reproductive women. Medical hormonal treatment should be the first-line therapeutic option, especially for symptomatic patients who have not an immediate desire to become pregnant. Gestrinone, as a common drug, is a synthetic triene hormone with anti-progesterone, moderate anti-estrogen, and anti-gonadal effects. Its therapeutic effects have been confirmed in clinical practice. However, the treatment is often stopped because of its adverse reactions (e.g., abnormal uterine bleeding, ponderal growth, voice change, and hirsutism), and high rate of relapse, thus affecting the clinical application [2,3].

Therefore, there is an urgent need for new drugs that can effectively treat EMs with acceptable side effects. EMs is recorded as *Zheng Jia* in traditional Chinese medicine (TCM). Because there are masses and nodules in the pelvic cavity in most patients, and they are fixed and can't be moved, which accords with the characteristics of blood stasis syndrome of TCM, promoting blood circulation and removing stasis is the main treatment principle of EMs. Considerable number of research studies have been conducted to determine the role of TCM in pain alleviation, fertility promotion, and relapse prevention [4–6]. The research group has long been dedicated to the exploration of TCM for EMs treatment. Huayu Jiedu Formula (HYJDF) is an empirical formula for the clinical treatment of EMs [7–10].

However, the lack of mechanism research limits the extensive use of TCM. Studies have confirmed that inflammatory response provides a microenvironment for local tissue survival, invasion, and angiogenesis; and such response is an important factor for promoting the EMs development [11,12]. Indeed, the mechanism of inflammation in EMs has become a research hotspot. The GATA transcription factor family is one of the most important regulatory factors discovered to be involved in inflammatory response [13,14]. Previous studies have shown that compared with normal endometrial tissues, abnormal methylation modification of GATA promoter exists in ectopic tissues, which simultaneously stimulates multiple signaling pathways and inflammatory cytokines [15–17].

Accordingly, we used ultra-high-performance liquid chromatography (UHPLC) Q-Exactive (QE) mass spectrometry (MS) analysis and network pharmacology to analyze the core components and targets for EMs, and designed the whole experiment. The therapeutic effects of HYJDF on EMs were evaluated in clinical research and *in vivo/vitro* studies. DNA methylation chips were used to determine the methylation level, and the regulation effect of HYJDF on GATA 6 (GATA family transcription factor) was also studied.

2. Materials and methods

2.1. Preparation of HYJDF and application

All the traditional Chinese medicine materials used to prepare the HYJDF were obtained from the TCM Pharmacy Department of the First Affiliated Hospital of Naval Medical University (Shanghai, China). HYJDF consists of five herbs, including *Typha angustifolia* L (*Pu Huang* in Chinese) (10 %), *Sargentodoxae caulis* (*Da Xue Teng* in Chinese) (30 %), *Patrinia scabiosaefolia* (*Bai Jiang Cao* in Chinese) (30 %), *Epimrdii Herba* (*Yin Yang Huo* in Chinese) (15 %) and *Faeces Togopteri* (*Wu Lin Zhi* in Chinese) (15 %). All herbs are traditionally used to treat patients in China. The daily dose of herbs was soaked in pure water for 30 min, then boiled over high heat for a while and simmered for 40 min, then taken at 30 min after food intake twice a day for 3 months.

2.2. Quantitative analysis of HYJDF by UHPLC-QE-MS

The chemical components of HYJDF were identified by Ultra-high performance liquid chromatography-mass spectrometry (UHPLC-QE-MS). 100 mg of HYJDF sample was added to 500 μL of extracted solution containing 1 μg/mL of internal standard, then the samples were homogenized at 45 Hz for 4 min and sonicated for 1 h in ice-water bath. After placing 1 h in −20 °C, the samples were centrifuged at 12000 rpm for 15 min at 4 °C. Finally, 300 μL of the supernatant was carefully filtered through a 0.22 microporous membrane and put in a fresh 2 mL tube for LC-MS/MS analysis. LC-MS/MS analysis was performed on an UHPLC Agilent 1290 system with an UPLC BEH C18 column (1.7 μm 2.1*100 mm) (Waters, USA). The flow rate was set at 0.4 mL/min and the sample injection volume was set at 5 μL. The mobile phase consisted of 0.1 % formic acid in water (A) and 0.1 % formic acid in acetonitrile (B). The multi-step linear elution gradient program was as follows: 0–3.5 min, 95–85 % A; 3.5–6 min, 85 - 70 % A; 6–6.5, 70 - 70 % A; 6.5–12 min, 70 - 30 % A; 12–12.5 min, 30 - 30 % A; 12.5–18 min, 30 - 0 % A; 18–25 min, 0 - 0 % A; 25–26 min, 0–95 % A; 26–30 min, 95 - 95 % A. An Q Exactive Focus mass spectrometer coupled with an Xcalibur software was employed to obtain the MS and MS/MS data based on the IDA acquisition mode. During each acquisition cycle, the mass range was from 100 to 1500, and the top three of every cycle were

screened and the corresponding MS/MS data were further acquired. Sheath gas flow rate: 45 Arb, Aux gas flow rate: 15 Arb, Capillary temperature: 400 °C, Full ms resolution: 70000, MS/MS resolution: 17500, Collision energy: 15/30/45 in NCE mode, Spray Voltage: 4.0 kV (positive) or −3.6 kV (negative).

2.3. Network pharmacology

Based on the plasmatic analysis, the information corresponding to each component of HYJDF was obtained using TCMSP (https://tcmspw.com), a systematic pharmacological database and analytical platform for Chinese medicine. The main active ingredients of HYJDF were obtained by limiting the pharmacokinetic parameters according to OB $\geq$ 30 % and DL $\geq$ 0.18. The data were imported into Cytoscape 3.9.1 to construct a "herbal-component" network, and the results were presented in a more intuitive network diagram. TCMSP was used to collect the potential targets of each active ingredient, and the common names of the above target proteins were retrieved using the Uniprot database (https://www.uniprot.org). The generic gene names corresponding to the target proteins were obtained. Obtain EMs disease-related genes using the GeneCards database (https://www.genecards.org/). Obtain HYJDF potential targets for EMs treatment using the online Venn diagram tool. Enter the genes of potential targets of HYJDF for EMs treatment in the STRING database (https://string-db.org/) and the Euclid platform database (https://cloud.oebiotech.com/) to obtain relevant KEGG pathway information and GO information. The data were imported into the Euclid database to construct bubble and bar charts [18].

2.4. Clinical research

EMs patients who meet the inclusion criteria were divided into HYJDF group and control group according to the random number table. A total of 68 EMs patients were included in the clinical study. A total of 43 patients in the HYJDF group excluded one patient who stopped taking medicine and two patients who withdrew from laparoscopic surgery. A total of 25 cases in the control group excluded four patients who stopped taking medicine and one patient who withdrew from laparoscopic surgery. The characteristics of recruited subjects are listed in Supplementary Table 1. In general, no significant difference was noted between the two groups with regard to the clinical characteristics. The treatment group was given HYJDF and the control group was given gestrinone. All patients were treated for three menstrual cycles. The overall effectiveness, visual analogue scales (VAS), cyst-size changes, serum CA125 levels, endometriosis health profile (EHP-5) scores, safety and adverse event status before and after treatment were evaluated. This study was approved by the Ethic Committee of Changhai Hospital of Shanghai (No: CHEC2016-109). Gestrinone was purchased from China Resources Zizhu Pharmaceutical Co., Ltd.

2.5. DNA methylation analysis based on whole genome methylation chip

Four patients with laparoscopically confirmed EMs and four healthy women who underwent tubal infertility who were free of EMs were recruited for the study. All subjects had no adenomyosis (AM), no record of pregnancy, and no hormonal treatments within three months prior to sample acquisition. Tissue procurement, processing, genomic DNA extraction and storage were conducted in accordance to manufacturer's protocol. Bisulfite conversion was done using the EZ DNA methylation Kit (Zymo research, USA) based on the manufacturer's protocol. All samples passed all quality controls (QCs) and were further assayed by the quantitative Illumina Human Methylation 850k chip, which determines DNA methylation levels at 853 307 CpG sites. The images were analyzed using Genome Studio software and all DNA methylation values were scored as β-values [β = Signal B/(Signal A + Signal B+100)] and ranged from 0 to 1 (from no methylation to complete methylation, respectively).

2.6. Real-time PCR and Western blot analysis

The endometriotic tissues were collected and the key genes were selected, then GATA 6 was confirmed by real-time PCR using the QuantiNova™ SYBR® Green PCR Kit (Qiagen, Hilden, Germany). Primer design of GATA 6 and GAPDH was shown in Supplementary Table 2. The real-time PCR was performed in triplicate and the relative expression was calculated with the comparative CT method. The proteins of cleaved-caspase 3, caspase 3, GATA 6 and COX-2 of the tissues or cells were detected as described previously [19]. Antibodies against cleaved-caspase 3, caspase 3, GATA 6 and COX-2 were purchased from Abcam (Beverly, MA, USA). Antibody against GAPDH was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.7. Establishment of EMs mouse model, drug intervention, and serum biochemical assessments

According to our previous studies [20], the EMs mouse model was successfully established and randomly divided into the following four groups: model group, gestrinone group, HYJDF group, and sham operation group. The model and sham operation group mice were treated orally with 0.2 mL of normal saline (NS) daily, and the gestrinone group mice were treated orally with the gestrinone (dissolved in NS) at a dose of 0.325 mg/kg/day every three days. The HYJDF group mice were treated orally with HYJDF at a dose of 13 g/kg/day every day. All mice were administrated between 9:00 a.m. and 10:00 a.m. daily for 15 days and then underwent surgery in the anestrus phase of the estrous cycle. The histological identification of the ectopic focus was first performed by HE, and the number and size of the ectopic foci were analyzed and compared. The IL-1β and PGE2 levels in the peritoneal fluid of mice after treatment were detected by ELISA kits (R&D Systems). All animal procedures were approved by the Ethics Committee of Naval Medical University. 17-β-Estradiol, progestin, and sesame oil (for injection) were purchased from Sigma.

2.8. Preparation of pharmaceutical serum

Male Kunming mice were administered by gavage with the HYJDF or NS (0.6 mL/g/d) twice a day for 7 days, about 1 h after the final dose, the blood was collected and centrifuged at 3000 rpm for 15 min at 4 °C, then the serum was collected and incubated in a water broth at 56 °C for 30 min for inactivation. After filter, pharmaceutical serums were subpackaged and stored at −80 °C before use.

2.9. Ectopic endometrial stromal cells isolation and culture

Ectopic endometrial tissues were obtained from patients with ovarian endometriosis who underwent laparoscopic surgery. All patients enrolled in this study had regular menstrual cycles and had been independent of hormonal treatment for at least 3 months before surgery. And written informed consent was obtained from each patient. All specimens were collected, ESCs were isolated, cultured and assessed of purity as described previously elsewhere [21].

2.10. Cell proliferation, apoptosis, and cell cycle assay

ESCs were plated in appropriate cell plates at a density of 4×10^4 cells/mL. After 24 h, ESCs were treated with various concentrations of pharmaceutical serum of HYJDF and NS.

The proliferative potential of cells was analyzed according to the MTS protocol (Promega, USA).

The apoptotic cells were detected by Guava Nexin Reagent (Millipore, USA) according to the manufacturer's protocol.

For cell cycle assays, after treatment cells were trypsinized, collected and fixed in 70 % cold ethanol, incubated with propidium iodide, and then analyzed by FACS (Miltenyi, Germany).

2.11. Statistical analysis

The statistical analysis was performed using SPSS 21.0 software package. Data were assessed for normality and homogeneity of variance and then presented as the mean ± standard deviation (s.d.). Student's *t*-test and non-parametric test were used to assess the differences between the groups. Chi-squared test was used to assess the therapeutic efficacy between the groups. The differences were considered statistically significant at p value < 0.05.

3. Results

3.1. Total ion flow chromatogram of HYJDF

The typical total ion flow chromatograms (TIC) of HYJDF were plotted using ionic strength as the ordinate and time as the abscissa, as shown in Supplementary Fig. 1A (positive ion modes) and 1B (negative ion modes). The results show that the peak retention time and signal intensity of the TIC spectrum in this study overlap well, indicating the stability of the instrument. The retention time and

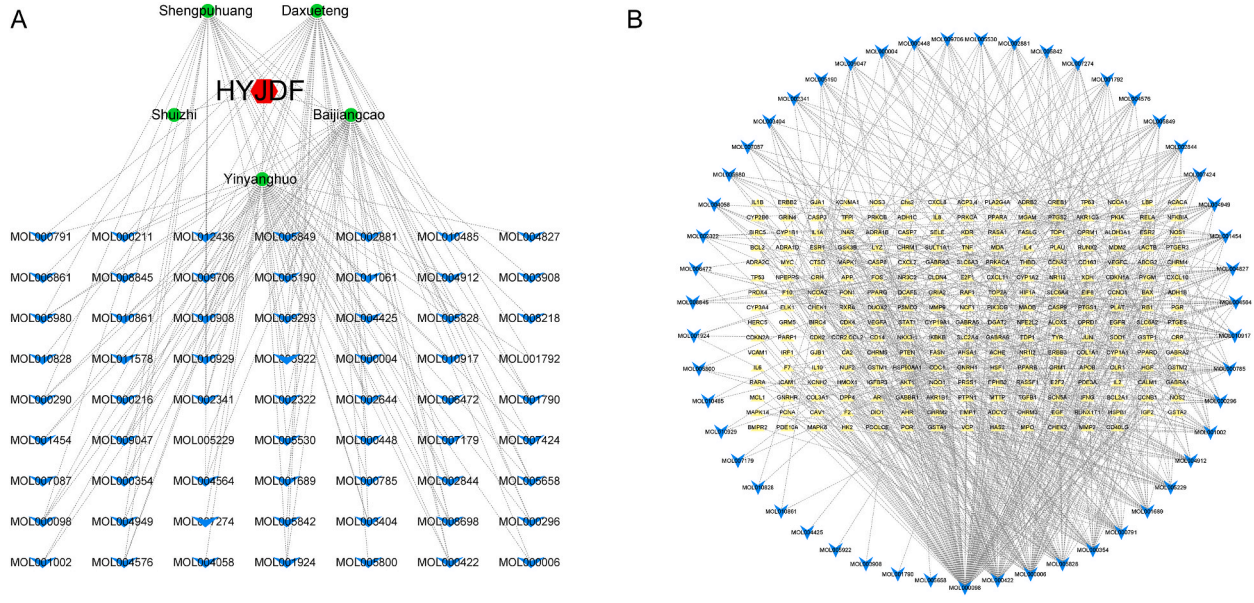

Fig. 1. (A) HYJDF composition. (B) Corresponding target of HYJDF.

response strength of the internal standard in the sample are very stable, indicating that the instrument data collection stability is very good. In the detection of single drug samples, the number of *Epimrdii Herba* compounds was the highest, which reached 450 and 328 species under the positive and negative ion modes, respectively, while the number of *Typha angustifolia L* compounds was the lowest, which was 303 and 227 species under the positive and negative ion modes, respectively. The total number of components detected in positive and negative ion modes was 839 and 597, respectively.

3.2. Results of network pharmacology

After combining the positive and negative ion data of the mass spectrometry results, each component was compared and queried by the TCMSP database to obtain the unique molecular ID number and the corresponding target information. After eliminating duplicate components and components without corresponding IDs, a total of 344 components with unique molecular ID numbers of HYJDF were finally obtained. Further, 55 main active ingredients were screened according to OB $\geq$ 30 % and DL $\geq$ 0.18, as shown in Fig. 1A. 238 targets corresponding to the ingredients were obtained according to the target database of TCMSP, as shown in Fig. 1B. A total of 145 targets were intersected with EMs disease, accounting for 60.66 % of all targets, as shown in Fig. 2A, and the specific target names are shown in Fig. 2B. After GO and KEGG analysis of the intersecting genes, GO analysis suggested that HYJDF could interfere with EMs through DNA methylation, as shown in Fig. 3A, while KEGG results suggested that the targets involved pathways including NF-kappa B signaling pathway, NOD-like receptor signaling pathway, GnRH signaling pathway, Estrogen signaling pathway and other inflammatory and hormone-related pathways, as shown in Fig. 3B.

3.3. Clinical therapeutic effects

With 12 markedly effective and 21 effective cases, the total efficacy of the HYJDF treatment group was 82.5 %. With 6 markedly effective and 12 effective cases, the gestrinone treatment group had the total efficacy of 90 %. There was no obvious differences between the two groups. In both groups, the VAS and EHP-5 score as well as clinical signs (the size of endometriotic cysts and serum CA125 level) were significantly reduced after treatment (Table 1).

Regarding side effects, different adverse reactions were observed in the gestrinone treatment group (menolipsis 40 %, abnormal uterine bleeding 35 %, ponderal increase 10 %). Meanwhile, HYJDF showed fewer adverse reactions for clinical application and good patient compliance (Table 2).

3.4. Abnormal methylation modification and gene expression of GATA 6 in ectopic endometrial tissues

A total of four endometriotic tissues from patients with ovarian EM (case group) who underwent laparoscopic surgery and four normal endometrial tissue samples from EM-free women (control group) were used for the DNA methylation analysis and qRT-PCR.

After data normalization and quality control steps, we identified 58 779 different methylated CpG sites between endometriotic tissues and control endometria (on the basis of adjusted p value < 0.01), which includes 24 458 hypermethylated sites and 34 321 hypomethylated sites that were primarily distributed in gene bodies. Combined with bioinformatics analysis, we manually selected genes from a list of differentially methylated genes. Compared with the normal group, the hypopethylated genes in the disease group were ESR2, GATA6, WNT5A, DNMT3B, CCL24, PIK3CG, and VCAM1, and the hypermethylated genes include ESR1, PTEN, c8orf4, NR5A1, CYP19A1, GATA2 and HOXA10, etc. Some of these differentially methylated genes (DMGs) were hierarchically clustered and

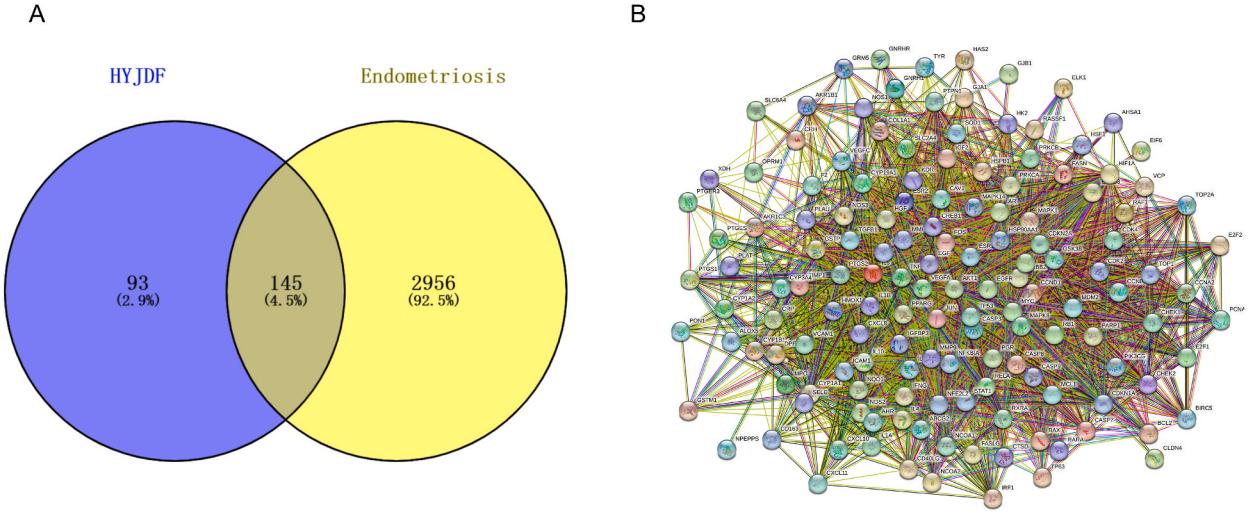

Fig. 2. (A) Venn diagram of EMs targets treated by HYJDF. (B) PPI network of EMs targets treated by HYJDF.

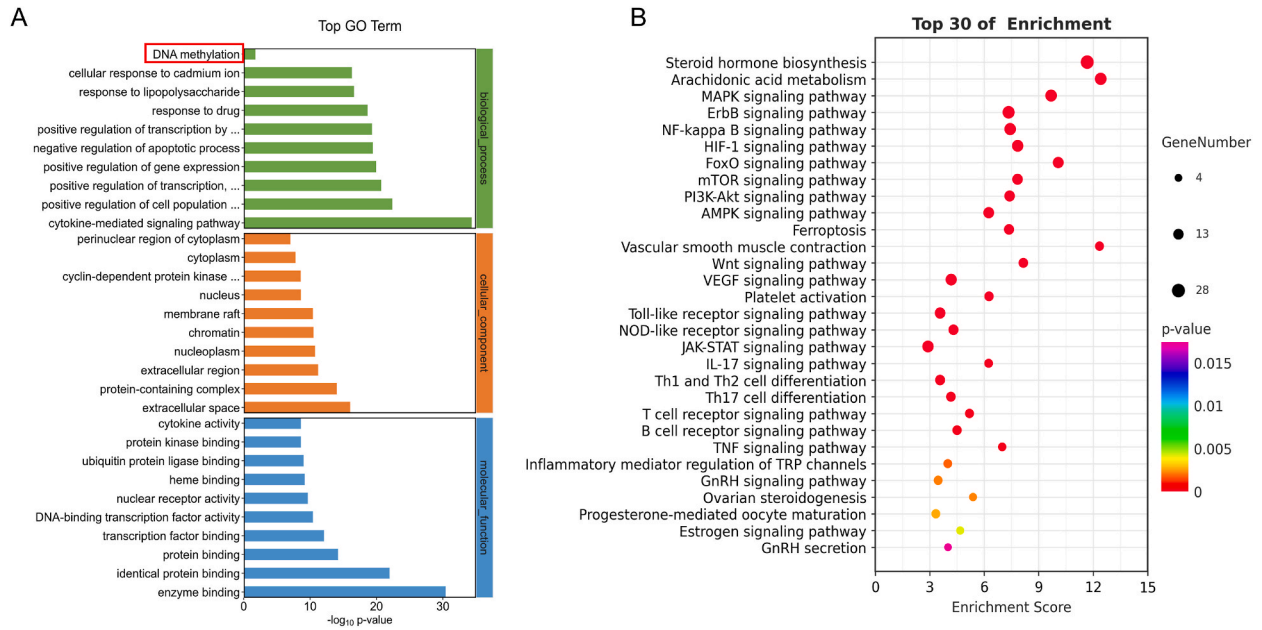

Fig. 3. (A) Therapeutic target GO map. (B) Therapeutic target KEGG map.

Table 1
Clinical therapeutic effects (Mean ± SD).

Evaluation indicator	NYF Treatment (n = 40)		Gestrinone Treatment (n = 20)	
	Before	After	Before	After
VAS	6.07 ± 2.73	3.30 ± 1.09	6.13 ± 2.33	0.73 ± 1.03[b]
EHP-5 score	19.40 ± 7.68	14.08 ± 4.92[a]	19.80 ± 7.17	12.90 ± 3.39[a]
Maximum diameter (mm)	42.97 ± 17.02	33.30 ± 19.42[a]	37.3 ± 13.26	22.18 ± 18.77[a]
CA125 (U/mL)	52.88 ± 18.16	36.17 ± 14.96[a]	69.3 ± 28.44	39.03 ± 18.50[c]
Therapeutic efficiency	82.50 %		90 %	

[a] $P < 0.05$ VS pre-treatment.
[b] $P < 0.01$ VS pre-treatment.
[c] $P < 0.01$ VS Gestrinone treatment.

Table 2
Summary of side effects.

Item	NYF Treatment		Gestrinone Treatment	
	Number of case	Rate	Number of case	Rate
Menolipsis	0	0 %	8	40 %
Prolonged bleeding	0	0 %	1	5 %
Abnormal uterine bleeding	0	0 %	7	35 %
Ponderal increase	0	0 %	2	10 %
Acne	0	0 %	0	0 %
Mood change	0	0 %	1	5 %
Hot flush	0	0 %	2	10 %
Diarrhea	2	5 %	0	0 %

visualized in a heat map (Fig. 4A). Furthermore, the expressions of GATA 6 whose differences of methylation were significant were verified by real-time PCR and Western blot analysis. Results showed that GATA 6 expression in ectopic tissue is abnormally higher than that in eutopic tissue (Fig. 4B and C). After validation of the reliability of the profile data, the biological processes involving these DMGs were identified by GO enrichment analysis using the DAVID tool. The pathogenesis of these genes related to EMs is known or suspected to be involved in epigenetic regulation, which includes cell adhesion, regulation of apoptotic process, cell migration, inflammatory response, angiogenesis, and hormone receptor reactivity. According to the enrichment scores, GATA 6 was a significantly important gene in the GO term of negative regulation of apoptotic process (As shown in Supplementary Table 3). Meanwhile, siRNA experiment showed that knockdown of GATA 6 expression could induce the apoptosis of the ESCs (Fig. 4D and E).

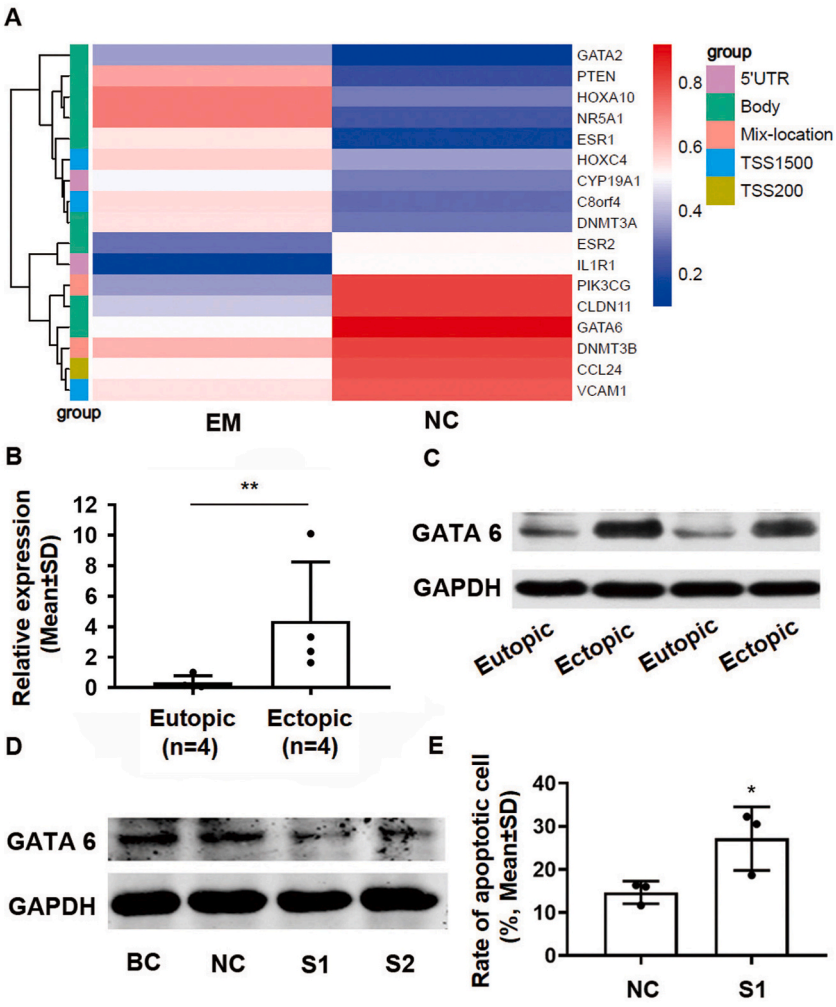

Fig. 4. Abnormal methylation modification and gene expression of GATA 6 in ectopic endometrial tissues. (A) A heat map of hierarchically clustered and visualized DMGs, which were upregulated or downregulated in ectopic endometrial tissues vs normal endometrial tissues. (B) Real-time PCR detected the gene expression of GATA 6 in ectopic tissues and eutopic tissues. (C) Western blot analysis of GATA 6 in ectopic tissues and eutopic tissues. (D) Western blot analysis demonstrated an absent of GATA 6 after treatment of siRNA sequences (BC: blank control; NC: treated with negative sequence; S1 and S2: siRNA sequence 1 and 2). (E) SiRNA sequence increased the rate of apoptotic cells. *: $p < 0.05$; **: $p < 0.01$.

3.5. *HYJDF regressed endometriotic lesions and reduced inflammation in a mouse model*

Laparotomy was performed after successful modeling. Ectopic lesions were mostly located in abdominal wall, intestinal wall, adipose tissue, and mesentery, where blood vessel formation and adhesion could be observed in the surrounding areas (Fig. 5A). Compared with human endometrial tissue section, ectopic lesion tissue section of model group showed similar structure that contained endometrial glands and stroma in the photos of tissue HE staining (Fig. 5A).

After respective intervention for 15 days (about three normal estrous cycles), all mice ($n = 15$ each group) underwent laparotomy. The number of ectopic foci were significantly less in the HYJDF group than that in the model group (Fig. 5B); but the size of ectopic foci showed no significant difference between HYJDF group and model group (Fig. 5C).

Meanwhile, the IL-1β and PGE-2 levels in peritoneal fluid were tested. As results showed that the IL-1β and PGE-2 levels elevated in the model group compared with sham-operated group and HYJDF group; such finding indicated that a high level of inflammatory cytokines in the model group and HYJDF treatment could significantly reduce the level of inflammation (Fig. 5D and E).

To further confirm the regulatory effect of HYJDF to the GATA 6 gene expression, the level of GATA 6 mRNA and protein in ectopic tissues of all groups were determined by real-time PCR, Western blot analysis, and IHC. The results showed that GATA 6 expression was obviously downregulated by HYJDF compared with that in the model group (Fig. 5F–H). In addition, the expression of inflammatory response related gene Cox-2 in ectopic tissues were tested through Western blot analysis, which showed that the Cox-2 expression was obviously lower in the HYJDF group compared with that in the model group (Fig. 5G). The effects of HYJDF in ectopic tissue apoptosis were also tested by Western blot analysis, which showed that the Cleaved Caspase-3 expression in the ectopic foci was obviously higher

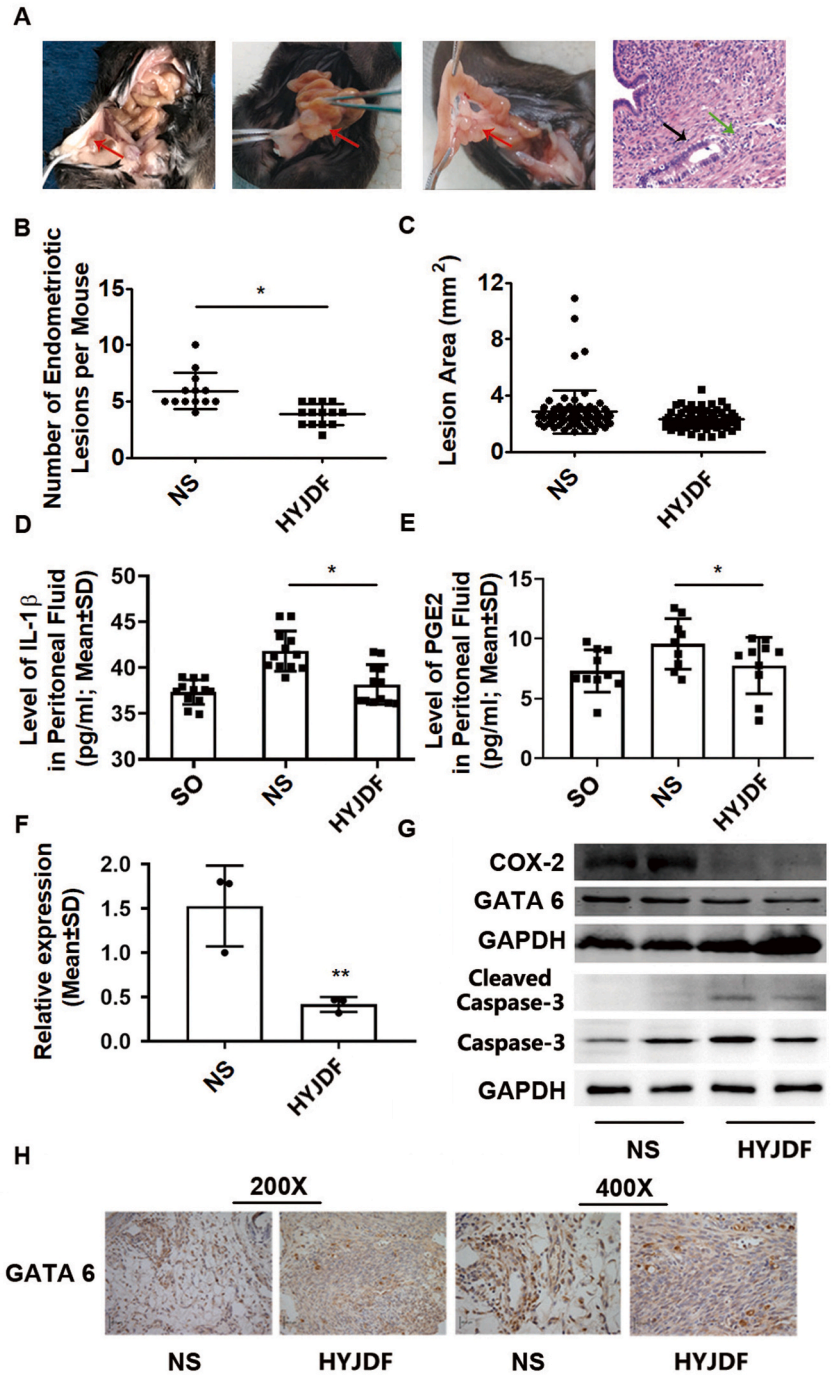

Fig. 5. HYJDF regressed endometriotic lesions in a mouse model. (A) The locations of ectopic foci (Red arrows: abdominal wall, intestine, and mesentery) and the hematoxylin eosin staining of ectopic foci (black arrow: endometrial glandular epithelial cells; green arrow: endometrial stromal cells). The number (B) and area (C) of ectopic foci after treatment with NS or HYJDF. The IL-1β (D) and PGE-2 (E) levels in the peritoneal fluid after treatment with NS or HYJDF. (F) Real-time PCR detected the GATA 6 gene expression in the ectopic foci. (G) Western blot analysis of COX-2, GATA 6 and Cleaved Caspase-3 in the ectopic foci (The uncropped images seen in Supplementary Fig. 3). (H) Immunohistochemistry staining of GATA 6 in the ectopic foci. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

in the HYJDF group compared with that in the model group (Fig. 5G).

3.6. HYJDF inhibited the proliferation and induced apoptosis via downregulating GATA 6 expression in ESCs

MTS method was used to detect cell proliferation ability at different times after various concentration interventions. Compared with the control group, no significant difference was observed in other groups except the pharmaceutical serum of 20 % HYJDF. The inhibitory effect of cell proliferation gradually became significant after 48 h of application ($P < 0.05$) (Fig. 6A). The results showed that the inhibitory effect of HYJDF on cell proliferation was concentration-dependent and time-dependent, and 20 % HYJDF pharmaceutical serum had the strongest inhibitory effect.

ESCs treated with HYJDF pharmaceutical serum were analyzed with Annexin V/PI double staining. As shown in Fig. 6B and C, after treatment with 20 % HYJDF pharmaceutical serum, apoptotic cells appeared significantly increased ($P < 0.05$).

We next analyzed whether the treatment with 20 % HYJDF pharmaceutical serum reduced the expression of GATA 6. As expected, after 48 h treatment, the results showed that the GATA 6 level was lower in 20 % HYJDF pharmaceutical serum group than those in the control and 20 % NS groups (Fig. 6D).

4. Discussion

EMs is a common chronic inflammatory disease and it's mainly linked with severe and chronic pelvic pain, dysmenorrhea, and deep dyspareunia, as well as reproductive issues which seriously affects the quality of women's life and needs long-term management [22]. As a commonly used drug for EMs in clinical, gestrinone is a synthetic 19-nortestosterone derivative, which can reduce the levels of sex hormone binding globulin and estrogen in the blood. However, because of its androgen-like effect and liver damage, the patient's compliance is poor and it can't be used for a long time. TCM has been regarded as the most important therapeutic method in China for more than 2000 years. EMs belongs to the categories of "dysmenorrhea", "*Zheng Jia*" and "infertility" in TCM. EMs ectopic lesions appear periodic bleeding and necrosis under the action of estrogen and progesterone, and the blood from ectopic lesions is called "*Li Jing Zhi Xue*" in TCM which means the blood does not follow the normal path, and can't be discharged from the body, then deposited in the pelvic cavity or other parts, blood stasis accumulates in the body which blocks the uterine vessels, *Chong* meridian and *Ren* meridian. It is easy to turn heat into toxin after blood stasis for a long time, followed by pelvic inflammation and even fibrosis which causes structural changes. Therefore, the basic treatment principle is to promote blood circulation and remove blood stasis, clearing heat and detoxification. Based on the holistic concept, TCM is often used in a combined form that is usually composed of multiple chemical components from several herbs, which usually could strengthen the therapeutic effects or minimize the potential adverse effects of certain components.

With long-term clinical studies, we found that HYJDF treatment significantly reduced the score of pelvic symptoms, especially dysmenorrhea, and clinical signs, e.g., the size of endometriotic cysts and serum CA125 level, of EMs patients. In the present study,

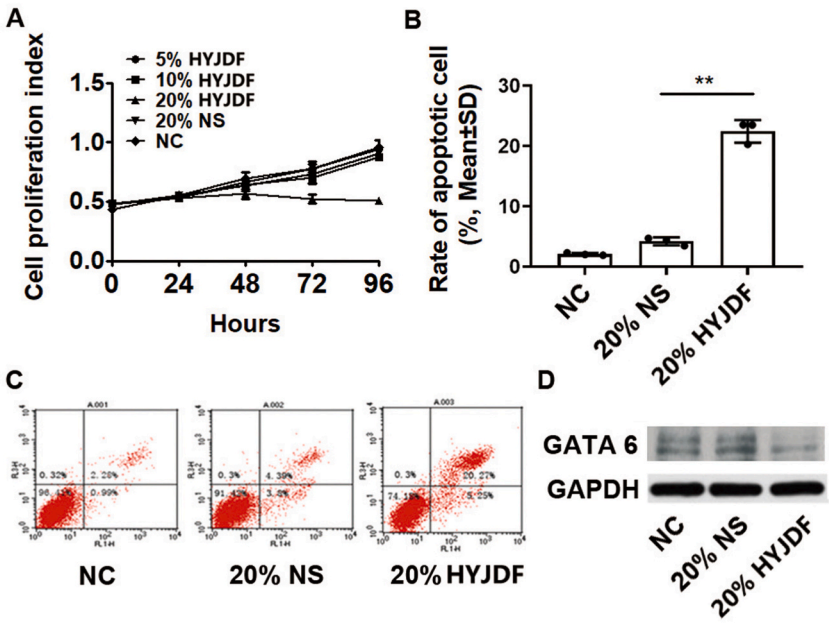

Fig. 6. HYJDF inhibited the proliferation and induced apoptosis in ESCs. (A) Growth of ESCs treated with different concentrations of HYJDF pharmaceutical serum. (B and C) 20 % HYJDF pharmaceutical serum increased the rate of apoptotic cells. (D) Western blot analysis of GATA 6 in the different groups.

both HYJDF and gestrinone showed good therapeutic effect with no obvious difference. Although the effect on VAS of HYJDF was a little lower than that of gestrinone, gestrinone treatment group reported more cases of side effects, which indicated that HYJDF might be a promising treatment method for EMs for long-term management.

DNA methylation serves as a critical regulator of gene expression, and global differences in DNA methylation affect multiple aspects of diseases [23,24]. Recent studies have found that aberrant DNA methylation may play an essential role in the pathogenesis of EMs [25,26].

To clarify the aberrant DNA Methylation of EMs, whole genome methylation-chip analysis was used in the current study. Abnormal DNA methylation in EMs affects the expression of several genes, which include *homeobox A10 (HOXA10), estrogen receptor beta (ESR2), steroidogenic factor 1 (NR5A1),* and *aromatase (CYP19A1)*, and were critically involved in EMs development [27–29]. In addition, previous research found that GATA 6, which is a transcription factor of GATA family, potently blocked hormone sensitivity and transformed an endometrial stromal cell to an endometriotic phenotype [30,31]. The results of genome methylation-chip and siRNA experiments validated that GATA 6 was hypomethylated and abundant in ectopic tissue and ESCs. The gene expression of *GATA 6* in ectopic tissue is abnormally higher than that in eutopic tissue. Furthermore, knockdown *GATA 6* expression could induce the apoptosis of the ESCs and growth inhibition of EMs tissue. Ectopic endometrial cells have the growth characteristics of tumor cells, so EMs is called "benign cancer". According to the enrichment scores, GATA 6 was a significantly important gene in the GO term of negative regulation of apoptotic process, which indicated that GATA 6 played a highly important role in EMs development.

EMs is often considered to be an inflammatory disease [32–34]. An increasing number of studies suggest that the co-existence of high levels of pro-inflammatory and anti-inflammatory cytokines (e.g., interleukin-1β, PGE2, TNF-α, and transforming growth factor-β1) are present in peritoneal fluid and ectopic lesions of women with EMs, these cytokines play indispensable roles in the EMs progression through promotion of survival, growth, invasion, differentiation, angiogenesis, and immune escape of the EMs lesions [12, 35,36]. In the present study, we found that the levels of IL-1β and PGE2 in peritoneal fluid and the expression of Cox-2 in ectopic lesions were significantly higher in the EMs model group, which was consistent with the previous results.

HYJDF inhibited inflammatory response through reduction of IL-1β and PGE2 levels in the peritoneal fluid as well as Cox-2 expression in ectopic lesions and suppressed the development of ectopic lesions in mice with EMs via downregulation of GATA 6 expression. *In vitro* test also confirmed these findings, which showed that HYJDF pharmaceutical serum significantly inhibited cell proliferation and increased cell apoptosis of ESCs via downregulation of GATA 6 expression.

The shortcoming of this research is that although we identified the ingredients using UHPLC-QE-MS and network pharmacology, which component plays the most central role, and how it interacts with GATA 6, we haven't explained yet, and we will go into more details in future studies. To extend its usage in China and worldwide, the active ingredients of HYJDF and pharmaceutical serum will be explored in the following research studies. Meanwhile, the more extensive mechanism of HYJDF will also be further researched.

5. Conclusion

In summary, our findings provided the first evidence that HYJDF had anti-inflammation activity and promoted cell apoptosis through the reduction of GATA 6 expression in ectopic tissues, which showed good therapeutic effect without any obvious side effects. These findings strongly suggest that HYJDF may be a new and efficient traditional Chinese medicine for the treatment of EMs during clinical application.

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Ethics approval and consent to participate

All of the study processes were approved by the Ethic Committee of Changhai Hospital of Shanghai (No: CHEC2016-109). Animal experiments were carried out at the Central Laboratory of Changhai Hospital and approved by the Experimental Animal Ethics Review Committee (Registration no. CHEC (AE) 2022–002).

Consent for publication

Not applicable.

CRediT authorship contribution statement

Wen Cheng: Writing - original draft, Investigation, Funding acquisition, Formal analysis, Data curation. **Jing Shan:** Writing - original draft, Methodology, Investigation, Formal analysis, Data curation. **Jie Ding:** Writing - review & editing, Software, Methodology, Investigation. **Yiqun Liu:** Investigation. **Shuai Sun:** Investigation. **Lianwei Xu:** Supervision, Project administration. **Chaoqin Yu:** Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Wen Cheng and Chaoqin Yu reports financial support was provided by National Natural Science Foundation of China. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e23149.

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