Abstract. Marrubiin can improve blood and lymph microcirculation disturbance, and has pharmacological effects in myocardial protection, anti-inflammation and anti-oxidation. The aim of the present study was to evaluate the protective effects of marrubiin on endometriosis through suppression of the expression of regulated on activation, normal T cell expressed and secreted (RANTES). Endometriotic cells were implanted into the peritoneal cavity of mice, and these mice were injected estradiol benzoate (30 µg/kg) once each day for 14 days. The mice with endometriosis were then treated with 12, 25 or 50 mg/kg marrubiin. Reverse transcription-quantitative polymerase chain reaction was used to assess the mRNA expression of RANTES, and western blot analysis was used to analyze the protein expression of RANTES, TNF-α and PGE2. Inflammation factors were measured by ELISA. Treatment with marrubiin effectively improved lesion regression and inhibited toxicity in the mouse model of endometriosis. Marrubiin significantly inhibited endometrial lesions and monocyte chemotaxis in the mice with endometriosis, and reduced U937 cell migration. Calcium mobilization, levels of tumor necrosis factor-α and the secretion of RANTES were effectively suppressed by marrubiin treatment. The calcium levels were effectively induced, whereas the protein expression of prostaglandin E2 (PGE2) and formation of thromboxane B2 (TXB2) were effectively inhibited by marrubiin treatment. These findings indicated that the protective effect of marrubiin improved endometriosis in the mice through the suppression of inflammation and downregulation of the expression of RANTES, followed by mediation of the levels of calcium, PGE2 and TXB2.

Introduction

Endometriosis refers to the appearance of endometrial tissue with growth function, including glands and mesenchyme, in the endometrium-coated inner membrane and other regions outside the muscle layers of the uterus body (1). As one of the common diseases in gynecology and obstetrics, endometriosis is usually found in women of childbearing age and seriously affects the quality of life of patients (2). This disease is predominantly found in women aged 25-45 years old with an incidence of 10-15% in China, and incidence is significantly increasing with a trend towards younger individuals (3). Although endometriosis is benign in histopathology, it features hyperplasia, infiltration, metastasis, recurrence and other malignant activity, with a malignant transformation rate of 0.7-1.0% (4). It has various clinical manifestations and the primary symptoms include dysmenorrhea, chronic pelvic pain, sexual intercourse pain, infertility and menstrual changes (5). At present, endometriosis is treated medicinally and/or through surgery, however, there is no ideal radical treatment, with the exception of radical surgery, for pharmaceutical therapy or conservative surgery, for which the recurrence rate remains high (6).

Endometriosis is one of the most common diseases in women of childbearing age; however, its pathogenesis remains to be fully elucidated (7). In previous years, it has been shown that chemotaxis is important in the development of epithelial-mesenchymal transition (EMT) (7). The overexpression of regulated on activation, normal T cell expressed and secreted (RANTES) in peritoneal fluid and ectopic foci is found in patients with endometriosis (8). RANTES, as an activating and regulatory factor, is formed on the expression and secretion of normal T cells, has specific chemotactic properties on memory T cells, monocytes and other immune cells, and is involved in the development of EMT though regulation of the immune response and interactions with other cellular factors, including ovarian hormones (8,9).

Widely distributed all over China, whiteflower lagopsis is a herb of Lagopsis, lamiales, also known as small motherwort, floralwhite motherwort, foralwhite horebound and lantern tree (10). It tastes bitter and is neural in nature with marginal toxicity. With functions, including the promotion of blood circulation, removal of blood stasis and regulation of menstruation, it is a form of gynecological medicine predominantly used to treat irregular menstruation, hemiplegia, amenorrhea due to
stagnation of blood, anemia dizziness and other diseases (11). In previous years, investigations on its chemical constitu- ents have shown that the herb of whiteflower lagopsis predominantly contains labdane-type diterpene, flavonoids, phenethyl alcohol and other compounds (11). A previous study indicated that marrubiin has several pharmacological activities, including improving disturbances in blood and lymph microcirculation, myocardial protection, anti-inflammation and anti-oxidation (12). The aim of the present study was to evaluate the protective effects of marrubiin on endometriosis, via suppression of inflammation and downregulation of the expression of RANTES.

Materials and methods

Animal endometriosis model. All experiments in the present study were approved by the experimental animal committee of Nanhua Hospital Affiliated to Nanhua University (Hengyang, China). Severe combined immunodeficiency (SCID) female mice (20±2 g, 6-7 weeks) were purchased from the Animal Laboratory of Nanhua University and housed under a 12 h light-dark cycle at 22-24°C, with food and water available ad libitum in pathogen-free conditions. Endometriotic tissue was collected from the endometrium of the mice under anesthesia and placed into sterile PBS, cut into coarse fragments and suspended. The endometriotic cells (10⁶ cell/ml) were intraperitoneally implanted into peritoneal cavity of mice, and these mice were injected with estradiol benzoate (30 µg/kg) once a day for 14 days.

Groups and treatments. All SCID mice were randomly divided into the following groups (n=12 per group): Sham group, endometriosis model group, endometriosis model+12 mg/kg marrubiin treatment, endometriosis model+25 mg/kg marrubiin treatment, endometriosis model+50 mg/kg marrubiin treatment. After treatment with marrubiin, mice were sacrificed using decollation under 30 mg/kg pentobarbital anesthesia.

Extraction. Marrubiin was extracted from Marrubium vulgare and Leonotis leonurus using a procedure described previ- ously with modifications (13). Acetone was added to the plant material (10 ml/g) for organic extraction, and extraction was performed for 1 h at 37°C. Subsequently, the mixture was filtered through Whatman No. 1 filter paper. A rotary evaporator was used to remove solvent, and distilled water was used to concentrate the extract at 60°C.

Cell treatment. U937 cells were acquired from American Type Culture Collection (Manassas, VA, USA) and cultured with RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA USA) and 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) supplemented 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a 5% CO₂ humidified atmosphere.

Hematoxylin and eosin (H&E) staining. The implanted endo- metrium was fixed in 4% paraformaldehyde for 1-2 days and embedded into paraffin. Histological material was cut into 4 µm sections and stained with H&E, which were visualized using an upright microscope (E600FN; Nikon, Tokyo Japan).

Monocyte chemotaxis. Blood was obtained via cardiac puncture and resuspended in 2 ml mouse monocyte isolation buffer. The layer containing enriched monocytes was carefully removed following centrifugation at 1,500 x g for 10 min at 4°C and washed with Hank’s solution. Monocyte purity was determined using flow cytometric experiments as ≥95%.

Western blot analysis. The implanted endometrium was collected and homogenized in lysis buffer with 1% protease inhibitor cocktail and centrifuged for 15 min at 12,000 x g at 4°C. The protein concentrations were determined using a BCA protein assay kit, and 50 mg total proteins were separated using 10-12% SDS-PAGE and transferred onto nitrocellulose membranes. Following soaking in 5% skim milk powder in TBS-Tween (0.1%), the membrane was incubated with the following primary antibodies: Anti-RANTES (sc-20731; 1:300; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-TNF-α (sc-8301; 1:5,00; Santa Cruz Biotechnology, Inc.), anti-prostaglandin E2 (PGE2; sc-514224; 1:2,00; Santa Cruz Biotechnology, Inc.) and β-actin (sc-7210; 1:4,00; Santa Cruz Biotechnology, Inc.) overnight at 4°C. The membrane was developed using the anti-rabbit or anti-mouse HRP-linked secondary antibody (sc-2004 or sc-2005; 1:5,000, Santa Cruz Biotechnology, Inc.) at 37°C for 1 h and a chemiluminescent detection system.

ELISA. Expression levels of RANTES (E-EL-H0023c and E-EL-M0009c; Elabscience Biotechnology Co., Ltd., Bethesda, MD, USA), interleukin (IL)-6 and IL-1β (EM004-96 and EM001-96, both from ExCell Bio Co., Ltd., Taicang, China) were determined by ELISA, according to the manufacturer’s protocol. Expression levels were measured using a Tecan microplate reader (Safire2; Tecan, Männedorf, Switzerland).

Calcium mobilization assay. Blood was resuspended in Tyrode’s buffer containing no calcium at a density of 3x10⁸ platelets/ml, into which Fura2-AM was added (4 M final concentration) and the mixture was incubated at 37°C for 30 min. Calcium mobilization was measured using a Tecan microplate reader (Safire2; Tecan).

Thromboxane B2 (TXB2) assay. TXB2 was assayed using a commercial enzyme immunoassay kit according to the manu- facturer’s protocol (E-EL-M1144c, Elabscience Biotechnology Co., Ltd., Bethesda, MD, USA), interleukin (IL)-6 and IL-1ß (EM004-96 and EM001-96, both from ExCell Bio Co., Ltd., Taicang, China) were determined by ELISA.

Statistical analysis. All data are expressed as the mean ± standard error of the mean, unless otherwise indicated. The results were evaluated using the Mann-Whitney test. P<0.05 was considered to indicate a statistically significant difference using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA).

Results

Protective effect of marrubiin inhibits endometrial lesions. The endometrial lesions in each group were verified using H&E staining, the results of which are shown in Fig. 1. The endometrial lesions in the endometriosis model were more marked, compared with those in the control group. Treatment
with 25 or 50 mg/kg marrubiin significantly reduced endometrial lesions in the mice with endometriosis (Fig. 1).

**Protective effect of marrubiin inhibits monocyte chemotaxis.** As shown in Fig. 2, there was a marked increase in monocyte chemotaxis in the endometriosis model, compared with the sham group. Treatment with 25 or 50 mg/kg marrubiin significantly inhibited monocyte chemotaxis in the mice with endometriosis (Fig. 2).

**Protective effect of marrubiin inhibits U937 migration.** Compared with U937 migration in the control group, marrubiin inhibited U937 migration in a dose-dependent manner. In particular, treatment with 10 or 20 µM significantly inhibited U937 migration (Fig. 3).

**Protective effects of marrubiin inhibit the expression of RANTES.** To examine the protective effects of marrubiin on the mRNA expression of RANTES, RT-qPCR analysis was used to determine the mRNA expression of RANTES in the SCID mice and U937 cells. As shown in Fig. 4A, the expression of RANTES in the endometriosis model group was higher, compared with that in the sham group. Marrubiin significantly inhibited the expression of RANTES in the SCID mice and U937 cells (Fig. 4A and B).

**Protective effects of marrubiin inhibit the protein expression of RANTES.** To examine the protective effects of marrubiin on the protein expression of RANTES, western blot analysis was used to detect the protein expression of RANTES in the SCID mice and U937 cells. Compared with the protein expression levels of RANTES in the sham and control groups, the protein expression of RANTES in the endometriosis model group was higher (Fig. 5A). Treatment with marrubiin significantly inhibited the protein expression of RANTES in the mice with endometriosis and U937 cells (Fig. 5A and B).

**Protective effect of marrubiin inhibits the protein expression of TNF-α.** To probe the protective effects of marrubiin on the protein expression of TNF-α, western blot analysis was used to detect the protein expression of TNF-α. The results from the western blot analysis showed that the protein expression of TNF-α in the mouse model of endometriosis was higher, compared with that in the sham group (Fig. 6). Treatment with 25 or 50 mg/kg marrubiin significantly inhibited the protein expression of TNF-α in the mice with endometriosis (Fig. 6).

**Protective effect of marrubiin inhibits the expression levels of IL-6 and IL-1β.** To examine the protective effects of marrubiin on the expression levels of IL-6 and IL-1β, the activities of IL-6 and IL-1β were measured using ELISA kits. As shown in Fig. 7, the activities of IL-6 and IL-1β were higher, compared with those in the sham group. Treatment with 25 or 50 mg/kg marrubiin significantly inhibited the activities of IL-6 and IL-1β in the mice with endometriosis (Fig. 7).

**Protective effect of marrubiin induces calcium levels.** To examine the protective effects of marrubiin on calcium levels, the levels of calcium were determined in the sham and endometriosis groups of mice. Compared with level of calcium migration in the sham group, the level in the endometriosis mice was lower (Fig. 8). Treatment with 25 or 50 mg/kg marrubiin significantly increased calcium levels in the mice with endometriosis (Fig. 8).

**Protective effect of marrubiin inhibits the protein expression of PGE2.** To investigate the protective effects of marrubiin on the protein expression of PGE2, western blot analysis was used to measure the protein expression of PGE2. The results from the western blot analysis showed that the protein expression of PGE2 in the mouse model of endometriosis was significantly higher, compared with that in the sham group (Fig. 9). Treatment with 25 or 50 mg/kg marrubiin significantly reduced the protein expression of PGE2 in the mice with endometriosis (Fig. 9).

**Protective effect of marrubiin inhibits the formation of TXB2.** The present study also investigated the protective effects of marrubiin on TXB2 formation in mice with endometriosis.
The formation of TXB2 in the endometriosis model was higher, compared with that in the sham group (Fig. 10). Treatment with 25 or 50 mg/kg marrubiin significantly reduced the formation of TXB2 in the endometriosis model (Fig. 10).

Discussion

Endometriosis is one of the common diseases frequently occurring in women of childbearing age, and has a significant impact on quality of life (4). The morbidity rate has increased significantly, however, the etiology and pathogenesis remain to be fully elucidated. The classic Sampson implantation theory has been widely accepted, however, blood reflux is a common phenomenon, whereas only a small proportion of individuals have disease (14). The implantation theory cannot explain this phenomenon, and investigations of endometriosis have shown that the ectopic adhesion, invasion and angiogenesis of endometrial tissue are closely associated with the successful ectopic plantation of the endometrium (6). Therefore, a novel theory of etiology, namely, the angiogenesis theory, has been suggested and has become a focus among obstetricians and gynecologists. The study of marrubiin on the angiogenesis and proliferation of endometriosis has become a potential new drug discovery direction.
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Gynecologists. In the present study, it was found that marrubiin significantly inhibited endometrial lesions in a mouse model of endometriosis.

The current understanding of the function of ion channel proteins has surpassed the traditional concept that the ion channel is a channel only for the movement of ions into and out of the cell (15). Studies have indicated that potassium channels are widely distributed in various tissues and have different functions, predominantly including effects on cell growth, differentiation and apoptosis, effects on cell volume and shape, the regulation of myocyte contraction and relaxation, regulation of the release of neurotransmitters by neurons, and regulation of hormone secretion by secretory cells (15-17). Based on the physiological and pathological effects of the Ca+-activated K⁺ pathway, particularly its effects on the growth, differentiation and apoptosis of cells, it is possible to develop Ca²⁺-activated K⁺ channels with a high level of specificity (17). Channel inhibitors and agonists may offer novel clinical therapeutic options for several diseases (15). In the present study, it was found that marrubiin significantly inhibited monocyte chemotaxis in mice with endometriosis and inhibited U937 cell migration.

A previous study revealed that there is a significant process of angiogenesis during endometriosis plantation, and that the angiogenesis regulatory factors, TNF-α and vascular endothelial growth factor, are important in the formation of blood vessels, however, the mechanism of action in angiogenesis in endometriosis remains to be elucidated (18). TNF-α is a type of cell factor with vascular activity, which may be involved in the angiogenesis of ectopic endometrium plantation (19). Investigations of TNF-α in endometriosis have predominantly focussed on the blood and peritoneal fluid, rather than the systematic investigation of expression in normal and ectopic endometrial tissue (20). It is well known that marrubiin significantly inhibited monocyte chemotaxis in mice with endometriosis and inhibited U937 cell migration.

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RANTES is an activation regulatory factor from the expression and secretion of normal T cells (21). As an all-round type of chemokine, RANTES belongs to the family of rapid growth chemokines, which are primarily produced by memory...
T cells, epithelial cells and mesothelial cells (9). RANTES can exert chemotactic effects specifically on memory T cells, monocytes and other immune cells, is involved in the regulation of the immune response, and interacts with other cytokines and ovarian hormones (22). Through its chemotactic effects, RANTES can amass inflammatory cells into a local lesion for involvement in the inflammatory reaction, which can cause the release of a variety of proinflammatory cytokines and angiogenic factors, leading to a positive feedback effect and peritoneal microenvironment change; this creates favorable conditions for the incidence and development of endometriosis (23). It has been demonstrated that the level of RANTES in the peritoneal fluid of patients with endometriosis is significantly higher, compared with that in patients without endometriosis, and this is positively correlated with disease severity (24). The present study showed that marrubiin significantly inhibited the protein expression of RANTES in the endometriosis model and U937 cells, and induced calcium levels, protein expression levels of PGE2 and the formation of TXB2 in the endometriosis model. Stulzer et al (11) demonstrated that marrubiin exerts pro-inflammatory agent-induced microvascular extravasation of Evans blue in the mouse ear through PGE2.

In conclusion, the present study showed that marrubiin significantly inhibited endometrial lesions and monocyte chemotaxis in mice with endometriosis, and reduced the migration of U937 cells. The mechanism underlying the protective effects of marrubiin may involve the inhibition of inflammation and downregulating the expression of RANTES in the mice with endometriosis, followed by mediating the levels of calcium, PGE2 and TXB2. Further investigations of marrubiin may provide a basis for the development of novel drugs for use in the treatment of endometriosis.

References


