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Combination use of ferulic acid, ligustrazine and tetrahydropalmatine inhibits the growth of ectopic endometrial tissue: A multi-target therapy for endometriosis rats

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ABSTRACT

Ethnopharmacological relevance: Ferulic acid (FA), ligustrazine (LZ) and tetrahydropalmatine (THP) are separately isolated from Chinese Angelica, Szechwan Lovage Rhizome and Rhizoma in the Jiawei-Foshou-San formula, a popular traditional Chinese medicine for irregular menses. It has been reported that the combination use of FA+LZ+THP has similar effect on endometriosis, but the underlying mechanisms are unclear. This study was to investigate the combination effects and mechanisms of FA+LZ+THP on endometriosis rats.

Materials and methods: Fifty endometriosis rats were intragastricly treated with FA+LZ+THP for 4 wk. The volume of ectopic endometrial tissue was measured to evaluate the effects. Then the changes in hypothalamic–pituitary–ovarian axis and ERE pathway were indicated by the levels of E_2, GnRH, FSH and LH, and the expressions of ER, HSP90 and COX-2, respectively. In addition, peritoneal macrophages of each rat were cultured *in vitro* and treated with (FA+LZ+THP)-medicated serum for 24 h. The proliferation and phagocytosis abilities, the levels of IL-1β and TNF-α, and the expression of IκBα were then measured for the changes of peritoneal macrophage activities.

Results: Combination use of FA+LZ+THP diminished the volume of the ectopic endometrial tissues ($P<0.05$ or $P<0.01$). It also decreased the E_2 level, suppressed the expression of GnRH, FSH and LH, and decreased the protein expression of ER, HSP90 and COX-2 (all $P<0.05$ or $P<0.01$). The phagocytosis ability of peritoneal macrophage was enhanced by (FA+LZ+THP)-medicated serum ($P<0.05$) with no change of proliferation ($P>0.05$). Moreover, IL-1β and TNF-α were downregulated (both $P<0.05$ or $P<0.01$) and IκBα was upregulated by the (FA+LZ+THP)-medicated serum ($P<0.01$).

Conclusions: The combination use of FA, LZ and THP could inhibit the growth of ectopic endometrial tissue in endometriosis rats. It might be related to the down-regulation of hypothalamic–pituitary–ovarian axis, the amelioration in ERE pathway and the improvement of peritoneal macrophage activities.

Abbreviations: FA, Ferulic acid; LZ, Ligustrazine; THP, Tetrahydropalmatine; GnRH, Gonadotropin-releasing hormone; FSH, Follicle-stimulating hormone; LH, Luteinizing hormone; E_2, Estradiol; ER, Estrogen receptor; HSP90, Heat-shock protein 90; COX-2, Cycloxygenase-2; ERE, Estrogen response element; TNF-α, Tumor necrosis factor; IL-1β, Interleukin-1β; ELISA, Enzyme linked immunosorbent assay; NF-κB, Nuclear transcription factor-κB; IκBα, Inhibitor of nuclear factor-κB; β-actin, An internal control used in Western Blot; IgG, Immunoglobulin G; RPMI, Roswell Park memorial institute; PBS, Phosphate buffered saline; RIPA, Radio immunoprecipitation assay; NP-40, Nonyl phenoxypolyethoxylethanol; SDS, Sodium dodecyl sulfate; PMSF, Phenylmethanesulfonyl fluoride; PAGE, Polyacrylamide gel electrophoresis; TBST, Tris-buffered saline and Tween 20; GTN, Gestrinone; SP, Streptavidin–peroxidase; DAB, Diaminobenzidine; HRP, Horseradish peroxidase; LPS, Lipopolysaccharide; FBS, Fetal bovine serum; MTT , 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PVDF, Polyvinylidene difluoride; ECL, Enhanced chemiluminescence; CMC-Na, Sodium Carboxymethycellulose; CLIA, Chemiluminescent Immunoassay

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1. Introduction

Endometriosis is a disease characterized by the presence of endometrium-like glandular tissue and stroma outside the uterus (Vinatier et al., 2001). It is thought to be a common disease with an estimated morbidity as 10–15% in women of reproductive age and most with pelvic pain and/or infertility (Eskenazi and Warner, 1997). The pathogenesis of endometriosis is still controversial. Three theories have been proposed to explain it, and of which the menstrual reflux implantation theory is mostly accepted, *i.e.* endometrial cells reflux to abdominal cavity following retrograde menstrual blood during menstruation (Kodati et al., 2008). Recent studies suggest that endometriosis sufferers have higher content of estrogen than normal ones (Rižner, 2009). Peritoneal macrophages of endometriosis sufferers not only grow in number, but also secrete lots of proinflammatory cytokines such as TNF-α and IL-1β. However, their phagocytic capacities were obviously weakened (Tariverdian et al., 2007). All of these abnormalities promote the development of endometriosis. Otherwise, the growth of ectopic endometrium is regulated by hormone secretion of ovarian (Chen and Li, 2005). The hypothalamus, pituitary and ovarian interacts with each other, which is essential to form a complete and coordinated neuroendocrine system, namely hypothalamic–pituitary–ovarian axis (Zhou and Xue, 2005). At present, the treatment of endometriosis is primarily by surgical eradication and/or hormone drugs, which may bring about severe adverse reaction. For example, hormone treatment can induce mood changes, body configuration changes and so on. Surgical treatment can bring about great insults to the endometriosis sufferers, and it often does not provide a definitive treatment (Evers et al., 1991). Therefore, searching for new drugs with less adverse reactions to treat endometriosis is significant.

Ferulic acid (FA), ligustrazine (LZ) and tetrahydropalmatine (THP) (the structures can be seen in Fig. 1) are three compounds isolated from Chinese Angelica, Szechwan Lovage Rhizome and Rhizoma in the Jiawei-Foshou-San formula, a popular traditional Chinese medicine for treating irregular menses. It has been reported that the combination use of FA+LZ+THP has similar effect on treating endometriosis with the Jiawei-Foshou-San formula (Wang et al., 2011; Yang et al., 2011), but the underlying mechanisms are not totally understood. Therefore, the purpose of this study is to investigate the combination effects of FA, LZ and THP on endometriosis rats′ ectopic endometrial tissues and explore the relationship of this effect with the hypothalamic–pituitary–ovarian axis, the ERE pathway and the peritoneal macrophage.

2. Materials and methods

2.1. Animals and reagents

Female Sprague–Dawley rats of 180–220 g were purchased from the Experimental Animal Center, Chongqing Medical University (Chongqing, China) and housed under controlled environment (22 ± 2 °C, 12 h light/dark cycle, free access to food and water) in the Experimental Animal Center, College of Pharmaceutical Sciences, Southwest University (Chongqing, China). The animal approval number was SYXK 2009-0002. All the experiments were performed in accordance with China′s Guidelines for Care and Use of Laboratory Animals.

FA, LZ and THP were provided by Nanjing Zelang Medical Technology Co., Ltd. (Nanjing, China). They were physically mixed by a ratio of 10:5:3 in this study (*i.e.* FA+LZ+THP=10+5+3). Gestrinone was from Zizhu Pharmaceutical Co., Ltd. (Beijing, China). Antibodies against GnRH, FSH, LH, ERα, HSP90, COX-2, IκBα and β-actin were from Santa Cruz (Santa Cruz, CA, USA). Streptavidin–Peroxidase (SP) kit, Diaminobenzidine (DAB) kit, Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were from ZSGB-BIO (Beijing, China). E_2 kit was from Abbott Laboratories (Abbott Park, IL, USA). TNF-α ELISA kit and IL-1β ELISA kit were from Boster Biological Engineering Co., Ltd. (Wuhan, China). Lipopolysaccharide (LPS) was from Sigma (St Louis, MO, USA). RPMI 1640 medium was from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was from Hyclone (Thermo scientific, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), polyvinylidene difluoride (PVDF) membranes and enhanced chemiluminescence (ECL) kit were from Millipore (Bedford, MA, USA).

2.2. Experimental design and treatment

Fifty rats were used for the experimental preparation of endometriosis. The induction procedures were conducted as described by Vernon and Wilson (Vernon and Wilson, 1985). Briefly, fresh endometrial tissues were collected and cut into fragments ($5 \times 5\ mm^2$). Under sterile environment, one piece of endometrial fragment was transplanted into the peritoneum and secured by suturing with nonabsorbable sutures. After 4 wk of development of endometriosis, the spherical volume of each ectopic uterine tissue was calculated by the formula: $V(mm^3) = 0.52 \times length \times width \times height$ (all in millimeters) (Gazi et al., 2010). Only rats whose spherical volume of ectopic uterine tissue is between 20 and 60 mm^3 were remained in the study (Fig. 2).

The surgically induced-endometriosis rats were randomly allocated into five groups with 10 rats for each group. Group 1 (EMS group) was treated with 0.5% sodium carboxymethycellulose (CMC-Na) as a negative control. Group 2 (GTN group) was treated with Gestrinone 0.5 mg kg^{-1} d^{-1} as a positive control. Group 3–5 (FA+LZ+THP groups) were experimental groups treated with FA+LZ+THP 0.045, 0.09 and 0.18 g kg^{-1} d^{-1}, respectively. Another 10 rats with Sham operations were treated with same volume of 0.5% CMC-Na as a normal control (Sham group).

2.3. Preparation of experimental samples from rats

2.3.1. Preparation of serum

Four weeks after treating, rats were taken blood from femoral artery when they were at estrus. After set for 4 h at 4 °C, the blood was centrifuged at 800 (× *g*) for 20 min to obtain serum. Finally, the serum was divided into two parts: One (serum I) was stored at −20 °C in freezer for subsequent determination of E_2, and the

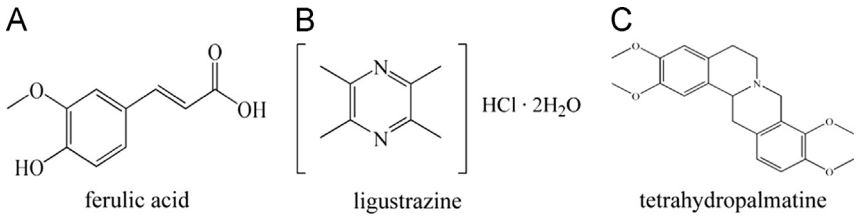

Fig. 1. Structures of the 3 experimental compounds. (A) Ferulic acid; (B) ligustrazine; (C) and tetrahydropalmatine.