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ACUPUNCTURE IMPROVES THE EFFECTS OF CHINESE HERBAL MEDICINE IN TREATING ENDOMETRIOSIS MODEL RATS

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ABSTRACT

The present study was aimed to investigate whether acupuncture could significantly enhance the efficacy of Chinese herbal medicine (CHM) in treating endometriosis model rats. A total of 40 female Sprague-Dawley (SD) rats with body weight of 200 ±20 g were included. Operational transplantation was used with animal models. The rats were randomly divided into 5 groups: sham-operation control group (Group A), model group (Group B), CHM combined with acupuncture group (Group C), CHM group (Group D), Danazol group (Group E) with 8 rats in each group. During the treatment, two rats in Group B and one rat in Group E passed away. When the treatment ended, all the left rats were sacrificed. The samples of peritoneal fluids, serum and the ectopic endometrium were taken. The serum levels of cancer antigen 125 (CA-125) and interleukin 18(IL-18) in the peritoneal fluids were detected using enzyme-linked immune-sorbent assay. The cyclooxygenase-2 (COX-2) mRNA expression levels in the ectopic endometrium were measured by Real-time PCR. The results showed that in the rats from Groups A, C, D and E, the serum CA-125 levels, COX-2 mRNA expression in the ectopic endometrium and the IL-18 levels in the peritoneal fluids were significantly lower than those of Group B ($P<0.05$). The serum CA-125 levels and COX-2 mRNA expression in the ectopic endometrium of the rats in Group C were significantly lower than those of Group D ($P<0.05$), while there was no significant difference between Group C and E ($P>0.05$). The levels of IL-18 in the peritoneal fluids of the rats in Group C were markedly lower than those of Group D and E ($P<0.05$). It is then concluded that acupuncture treatment can improve the effects of CHM in treating endometriosis model rats.

Key words: Chinese herbal medicine acupuncture; endometriosis.

INTRODUCTION

Endometriosis in humans is characterized by the presence of endometrial tissue outside the uterus (Giudice and Kao, 2004). It can cause both pain symptoms (dysmenorrhoea, deep dyspareunia, and chronic pelvic pain) as well as infertility (Ferrero *et al.*, 2010). The disease affects from 10% of all reproductive-aged women to as many as 70% of the women suffering from infertility or chronic pelvic pain worldwide (Ferrero *et al.*, 2010; Guo and Wang, 2006a; Guo and Wang, 2006b; Simoens *et al.*, 2007; Sinaii *et al.*, 2007; Vigano *et al.*, 2004). However, the pathogenesis of endometriosis remains incomplete (Sharpe-Timms and Young, 2004). Although medical treatments for endometriosis have benefits (Brown *et al.*, 2010; Davis *et al.*, 2007; Prentice *et al.*, 2000; Selak *et al.*, 2001), they are often characterized by the possibility of unpleasant short- and long-term side-effects (Sagsveen *et al.*, 2003). The treatments may usually lead to high rates of relapse once medication is stopped and the treatment of endometriosis is far from satisfactory (Flower *et al.*, 2011).

Chinese herbal medicine (CHM) and acupuncture have advantages in treating endometriosis (Chen *et al.*, 2010; Flower *et al.*, 2009; Lundeberg and Lund, 2008; Meissner *et al.*, 2010; Schnyer *et al.*, 2008; Wang, 1994; Xiang *et al.*, 2011; Zhu *et al.*, 2011). CHM is useful in relieving endometriosis-related pain with fewer side effects than experienced with conventional treatment (Flower *et al.*, 2009). A commonly used anti-endometriosis herb preparation (Channel Flow) consisting of nine individual Chinese medical herbs was found to have direct effects on cell proliferation, apoptosis, and CCL5 production in endometriotic stromal cells (Wieser *et al.*, 2009). The findings support the potential of novel, potentially safe and well-tolerated botanical products as the future endometriosis treatments (Wieser *et al.*, 2009). Although a recent systematic review recommended well-designed, double-blinded, randomized controlled trials that assess various types of acupuncture in comparison to conventional therapies need to be conducted, the limited effectiveness of acupuncture for pain in endometriosis is demonstrated (Zhu *et al.*, 2011).

However, few studies have been focused on the effects of CHM combined with acupuncture on the endometriosis model rats. The study was aimed to investigate whether acupuncture could significantly

enhance the efficacy of CHM in treating endometriosis model rats. The serum levels of cancer antigen 125 (CA-125), interleukin 18(IL-18) in the peritoneal fluids and cyclooxygenase-2 (COX-2) mRNA expression levels in the ectopic endometrium were detected in this study.

MATERIALS AND METHODS

Preparation of the **Extracts of the Chinese Formula:** The composition of CHM formula used to treat endometriosis was shown as Table 1. The crude drugs of this formula were extracted by 10x boiling water for 60min. After filtrate, the extraction procedure was repeated once with 8x boiling water. All the filtrates were combined and condensed under reduced pressure and freeze dried.

Animals, groups and **administration:** A total of 40 female Sprague-Dawley (SD) rats with body weight of 200 ±20 g were purchased from the Laboratory Animal Center of Zhejiang University (Hangzhou, China). The whole protocol of the study was conducted based on the National Research Council's protocol for the care and use of laboratory animals and was approved by the Institutional Review Board. Among the 40 rats, 8 rats were randomly taken as the sham-operation control group (Group A). For the rats in Group A, after the abdominal fur was shaved and the skin disinfected, the abdominal cavity was opened and then closed, which was used to insure aseptic conditions (references). The other 32 rats were used to establish the model rats with endometriosis. Operational transplantation was used with animal models. Each rat was injected with 0.2mg Diethylstilbestrol to stimulate estrus. Then 20% Urethane (1.5g/kg) was intra-abdominally injected. The animal models were made: 1)the abdominal fur was shaved and the skin disinfected, 2)the abdominal cavity was opened, 3) procedures were done to separate the uterus away from the right ovary by 0.5cm, and 2cm long section of the uterus removed. 4) the endometrium was separated and divided into three parts, which were respectively sutured in the respective uterine branch, left ovary and parietal peritoneum. 6) the abdominal cavity was closed. Gentamycin Sulfate (0.1ml) was injected into each rat for 3 days after finishing the above operation. Danazol was provided by Lianhua Medicine Company (Jiangsu, China).

Four weeks after the 32 model rats were established, after confirming the ectopic endometrium were survived with histopathology examination, the model rats were randomly divided into 4 groups. In addition to Group A, all the rats were then divided into 5 groups as shown in Table 2. In the acupuncture treatment, Guanyuan (RN4), Sanyinjiao (SP 6), Zusanli (ST 36), Shensu (BL23) and Dazhui (Du14) were selected as the acupoints. All the acupoints and the stainless steel needles were sterilized with 75% alcohol. The rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) before acupuncture treatment. After the needles were inserted into the acupoints, they were connected to an electro-therapeutic apparatus (Model G6805-2, Shanghai, China) for 25 min through clip electrodes.

Detection index and method: During the treatment, two rats in Group B and one rat in Group E passed away. When the treatment ended, the remaining rats were sacrificed. The samples of peritoneal fluids, serum and the ectopic endometrium were taken. The peritoneal fluid samples of the rats were centrifuged at 12,000 rpm for 10 min at 4 □. Then, the supernatants were collected, aliquoted, and stored frozen at −80 °C until used for further evaluation. The serum levels of CA-125 and the levels of IL-18 in the peritoneal fluids were detected using enzyme-linked immune-sorbent assay (ELISA, RUIQI Bio Co. Ltd, Shanghai, China). All the measurements were carried out in duplicate and were conducted according to the manufacturer's instruction. Both intra- and inter-assay coefficients of variation were less than 10%. The COX-2 mRNA expression levels in the ectopic endometrium were measured by Real-time PCR.

Detection of COX-2 mRNA expression by Real-time PCR: Isolation of the total RNA was performed by using the RNAiso^{TM} Reagent (TAKARA, Dalian, China), according to the manufacturer's instructions. The purity and concentration of RNA were determined by NanoDrop$^{®}$ND-100 Spectrophotometer (Thermo Fisher Scientific Inc, USA).The cDNA was prepared from 500ng of total RNA by reverse transcription, using the PrimeScript^{TM} RT reagent Kit (Perfect Real Time, TAKARA, Dalian, China). The cDNA samples were diluted in DNase- and RNase-free water at a proportion of 1:3 before further analysis. Quantitative real-time PCR was performed by using the iCycler iQ Real-Time Detection System (Bio-Rad). The PPARG and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene specific primers for rats were provided by Sangon, Shanghai, China. PCR reactions were carried out using 2μL of cDNA, 1 μL of 10 μM of each primer, 8.5 μL of dH_2O and 12.5μL of 2×SYBR$^{®}$ Premix Ex Taq^{TM} (TAKARA) in 25-μL reactions. Thermal cycling conditions were 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds and 60.0°C for 30 seconds. A final melting curve verified formation of single-product. Gene starting quantity was based on the cycle threshold (Ct) method. A control cDNA dilution series of known concentration was constructed for each gene to establish a standard curve, plotting the logarithm of the standard concentration against the Ct values. The samples were quantified from the measured Ct values by interpolation, using the regression equation.

Statistical analysis: Data were analyzed using the Statistical Package for Social Sciences (SPSS 15.0 for Windows). The comparisons among different groups were performed with one-way analysis of variance (ANOVA) and multiple comparison tests were conducted with Bonferroni correction procedure. For all the hypothesis tests, significance level was set at P=0.05 and two-tailed tests were used.

RESULTS

Serum CA-125 levels: The results for the serum CA-125 are shown in Figure 1.The rats from Groups A, C, D and E had significantly lower CA-125 levels than those of Group B (P<0.05). The serum CA-125 levels of the rats in Group C were significantly lower than that of Group D (P<0.05), while there was no significant difference between Group C and E (P>0.05).

Table 1. The composition of CHM formula

Chinese medicinal herb	Dosage (g)
Concha Ostreae	25
Radix Scutellariae	20
Radix Paeoniae Rubra	20
Rhizoma Curcumae	15
Radix Bupleuri	15
Rhizoma Atractylodis Macrocephalae	10
Radix Salviae Miltiorrhizae	10
Semen Coicis	10
Spica Prunellae	10

Table 2. Treatment of the rats in each group.

Groups	N (original)	N (analyzed)	Treatment
Sham-operation control group (Group A)	8	8	The rats were orally administrated saline at 8ml/kg once daily for 56 consecutive days.
Model group (Group B)	8	6	The rats were orally administrated saline at 8ml/kg once daily for 56 consecutive days.
CHM combined with acupuncture group (Group C)	8	8	The rats were orally administrated the extracts of CHM at 380 mg/kg once daily and acupuncture treatment was taken every two days for 56 consecutive days.
CHM group (Group D)	8	8	The rats were orally administrated the extracts of CHM at 380 mg/kg once daily for 56 consecutive days.
Danazol group (Group E)	8	7	The rats were orally administrated danazol at 36 mg/kg once daily for 56 consecutive days.

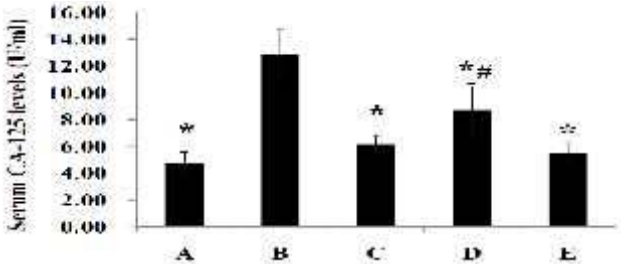

Figure 1. The serum CA-125 levels. Data were shown as mean ± SD. (n=8 in Group A, C and D; n=6 in Group B; n=7 in Group E). The significant difference was set at * P<0.05, compared with Group B; # P<0.05, Group D and E compared with Group C.

COX-2 mRNA expression in the ectopic endometrium: The results for the COX-2 mRNA expression in the ectopic endometrium are shown in Figure 2. The rats from Groups A, C, D and E had significantly lower COX-2 mRNA expression in the ectopic endometrium than those of Group B (P<0.05). COX-2 mRNA expression in the ectopic endometrium of the rats in Group C were significantly lower than that of Group D (P<0.05), while no significant difference existed between Group C and E (P>0.05).

IL-18 levels in the peritoneal fluids: The results for the IL-18 levels in the peritoneal fluids are shown in Figure 3. The rats from Groups A, C, D and E had significantly lower IL-18 levels in the peritoneal fluids than those of Group B (P<0.05). The Compared with Group D and E, the levels of IL-18 in the peritoneal fluids of the rats in Group C were markedly lower (P<0.05). The levels of IL-18 in the peritoneal fluids of the rats in Group E were markedly lower than that of Group D (P<0.05).

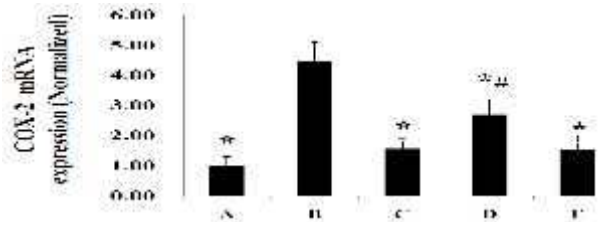

Figure 2. The COX-2 mRNA expression in the ectopic endometrium. Data were shown as mean ± SD. (n=8 in Group A, C and D; n=6 in Group B; n=7 in Group E). The significant difference was set at * P<0.05, compared with Group B; # P<0.05, Group D and E compared with Group C.

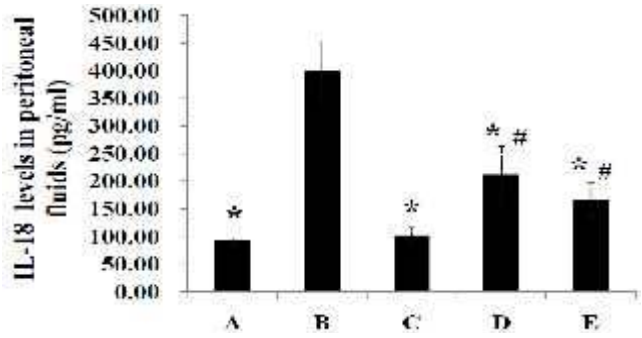

Figure 3. The IL-18 levels in the peritoneal fluids. Data were shown as mean ± SD. (n=8 in Group A, C and D; n=6 in Group B; n=7 in Group E). The significant difference was set at * P<0.05, compared with Group B; # P<0.05, Group D and E compared with Group C.

DISCUSSION

Endometriosis is a common, benign, oestrogendependent, chronic gynaecological disorder associated with pelvic pain and infertility (Giudice and Kao, 2004; Guillebaud, 1993; Parazzini and Ferraroni, 1993; Prentice, 1993; Vessey *et al.*, 1993). However, the aetiology and pathogenesis remain uncertain (Giudice and Kao, 2004), which may lead to no satisfactory treatment for endometriosis. In the present study, we successfully established the endometriosis model rats and we found acupuncture treatment can improve the effects of CHM in treating endometriosis model rats.

CA-125 is the most widely used marker for endometriosis (Barbieri, 1987; Ozaksit *et al.*, 1995; Patton *et al.*, 1986; Pittaway *et al.*, 1995; Ramos *et al.*, 2012). Some studies have assessed the value of CA-125 measurements during treatment (Chen *et al.*, 1998; Matalliotakis *et al.*, 2004). IL-18 levels in the peritoneal fluid was found to elevate in women with endometriosis(Arici *et al.*, 2003; Zhang *et al.*, 2005). IL-18-607 A homozygote and A allele were found to be positively correlated with the risk of developing endometriosis or the stage of endometriosis(Ayaz *et al.*, 2011). COX-2 expression increased in the eutopic endometrium and ovarian endometriotic tissue of the patients with endometriosis, indicating that COX-2 may be involved in the pathogenesis and progression of endometriosis(Cho *et al.*, 2010). The increased COX-2 expression in eutopic and ectopic endometria was believed to be strongly correlated with pathological abnormalities in endometriosis (Ota *et al.*, 2001). Hyper activation of COX-2 with abnormal prostaglandin generation is considered to contribute to the patho-physiology of endometriosis and the progression (Chishima *et al.*, 2002). The elevated COX-2 expression in stromal cells in eutopic endometrium from patients with deep endometriosis may play a role in severe, endometriosis-related dysmenorrheal (Matsuzaki *et al.*, 2004) and COX-2 selective inhibitors could be effective to suppress the establishment and growth of endometriosis, partially through their antiangiogenic activity(Machado *et al.*, 2010). Although we have demonstrated the effects of acupuncture in improving CHM in treating endometriosis rats, a more systematic study is needed and more studies designated to explore the underlying mechanism underlying acupuncture improving the effects of CHM in treating endometriosis should be done in the future.

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