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Original Research Article

Jing Tong Yu Shu, a traditional Chinese medicine, suppresses IL-1β and IL-6 gene expressions in macrophages, and alleviates endometriosis

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Abstract

Purpose: *To evaluate the effect of a traditional Chinese medicine, Jing Tong Yu Shu (JTYS) on endometriosis in a rat surgical model.*
Methods: *Endometriosis was induced in 40 female rats. The rats were randomly divided into 4 groups: three JTYS groups given different doses of the drug, and a saline group. After four weeks of treatment with JTYS, the volume of the endometriotic explants was measured, and the levels of IL-1β and IL-6 in peritoneal fluid and serum were determined by enzyme-linked immunosorbent assay (ELISA). The production of cytokine IL-1β and IL-6 by peritoneal macrophages was also measured for each group.*
Results: *JTYS treatment brought about regression of implants and inhibition of IL-1β and IL-6 production in a dose-dependent manner, with high-dose JTYS eliciting 66.76 % reduction in mean endometriotic explant volume. Plasma and peritoneal fluid levels of IL-1β and IL-6 were significantly lower in the high-dose JTYS group than in the saline group ($p < 0.05$). However, JTYS treatment significantly inhibited IL-1β and IL-6 production in peritoneal macrophages ($p < 0.05$).*
Conclusion: *These results suggest that JTYS treatment leads to regression of endometriotic lesions in rat. Thus, JTYS has the potential to be developed into a new drug for the treatment of endometriosis.*

Keywords: *Endometriosis, Interleukins, Traditional Chinese medicine, Jing Tong Yu Shu Macrophages*

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INTRODUCTION

Endometriosis (EMs), defined by the presence of proliferative endometrial tissue outside the uterine cavity, is a common and serious illness affecting women in their reproductive years [1]. The current treatment approaches for endometriosis, which are dependent on the fact that it is an estrogen-associated disease, may cause unwanted side effects. Thus, there is need to develop new strategies for endometriosis treatment. Endometriosis is a disease associated

with inflammation and activation of immune cells in the peritoneal environment [2,3]. Inflammatory responses during endometriosis depend on activated peritoneal fluid macrophages (PMs) [4]. Activated PMs can release a variety of cytokines, such as interleukin (IL)-1β and IL-6, into the peritoneal fluid and serum. These cytokines are involved in the pathophysiology of endometriosis [5-7].

A previous meta-analysis [8] has revealed that Chinese herbal medicine may be more effective, and may have fewer side effects than conventional strategies, in endometriosis treatment. The traditional Chinese medicine *Jing Tong Yu Shu* (JTYS) consists of eleven herbs: *Dioscorea polystachya*, *Corydalis yanhusuo*, *Faeces trogopterori*, *Paeonia lactiflora* Pallas, *Radix cyathulae*, *Angelica sinensis*, *Whitmania pigra* Whitman, *Cornus officinalis*, *Foeniculi Fructus*, *Cortex cinnamom* and *Asarum sieboldii*. In the present study, the effect of JTYS on endometriosis was investigated in a rat model.

EXPERIMENTAL

Animals and reagents

Adult female Sprague-Dawley rats (mean body weight 200 ± 10 g) were used. All rats were housed under a standard 12 h light: 12 h dark cycle, and maintained under standard conditions for one week to observe their health condition before the experiment. The study was approved by the Animal Ethic Committee of Harbin University of Commerce (Approval ref no. 2016185). The animal experiments were in full compliance with the Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985). The dosage of JTYS for an adult human is comprised of the following: 20 g *Dioscorea polystachya*, 20 g *Corydalis yanhusuo*, 12 g *Faeces trogopterori*, 12 g *Paeonia lactiflora* Pallas, 15 g *Radix cyathulae*, 15 g *Angelica sinensis*, 3 g *Whitmania pigra* Whitman, 15 g *Cornus officinalis*, 10 g *Foeniculi fructus*, 10 g *Cortex cinnamomi*, and 5 g *Asarum sieboldii*. These eleven herbs were purchased from LBX Drugs (Harbin, Heilongjiang). The equivalent dose (D) for the rat was calculated as in Eq 1.

$$D = (A/S)6.3R \quad \text{..... (1)}$$

where A, S and R are adult human dose, standard adult human weight and rat weight, respectively. This was taken as medium dose for rats. The low and high doses of JTYS were half and twice the medium dose, respectively. The herbs were mixed and kept in water for 1 h, after which they were boiled continuously for 30 min. The resultant residue was decocted for another 30 min, and the two decoctions were pooled and concentrated to 2 mL.

Animal model

A rat model of endometriosis was surgically produced as described previously, with slight modifications [10]. The rats were anesthetized with chloral hydrate (350 mg/kg). After routine disinfection of each rat skin, the abdominal cavity was opened and the uterus was exposed under sterile conditions. One centimeter-long distal segment was resected from the right uterus and was split longitudinally, revealing the endometrium. A 5 × 5 mm piece was sectioned and implanted onto the peritoneal surface of the right abdominal wall. Sham operations were performed in another 10 rats. During the operation, saline was used to keep the peritoneal cavity moist. The abdominal incision was closed at the end of the surgery.

Three weeks after the surgery, a second operation was performed to observe the ectopic endometrium and check whether the endometriosis model was successfully established. The volume of implant (V) was calculated as in Eq 2.

$$V\ (mm^3) = (\pi/6)LWH \quad \text{..... (2)}$$

where L, W and H are the length, width and height of the implant.

The rats with endometriosis were randomly divided into four groups (n = 10): Group 1 was the EMs group without medication (EMs group); Groups 2, 3 and 4 rats were treated with JTYS at low, medium and high doses, respectively. The extract was given once a day via intra-gastric administration for four weeks. After 4 weeks of treatment of JTYS, the implant volumes were measured.

Specimen collection

Rats were sacrificed after 4-week treatment. After routine disinfection of each rat's skin, peritoneal fluid was collected under sterile conditions through rinsing of the peritoneal cavity with 2 ml sterile saline. The peritoneal fluid was centrifuged at 500 g for 10 min, and the supernatant was stored at −80 °C until analysis. The peritoneal fluid macrophages were enriched by centrifugation using Ficoll-Hypaque (Sigma-Aldrich, St. Louis, USA) as reported previously [11,12], and cultured in 48-well plates for 24 h. After 24 h of *in vitro* culture, the conditioned media and macrophages were obtained and

stored at −80 °C until analysis. Blood samples were centrifuged after collection, and the serum samples were frozen at −80 °C until analysis. The volume of the implants from EMs rats was computed as in Eq 2 above.

In vitro culture of peritoneal fluid macrophages

The peritoneal fluid macrophages were cultured in RPMI1640 complete medium containing 15 % FCS. After attachment for 24h, the cells were washed with complete medium and stimulated by serum from JTYS- or saline-treated EMs rats for another 24 h. Cells in the control group were cultured in complete medium. Peritoneal fluid macrophage activation was achieved through the addition of 10 ng/mL lipopolysaccaride (LPS) (Sigma-Aldrich, St. Louis, USA) in the cell culture system. The conditioned media and macrophages were collected and frozen at −80 °C until analysis.

Assay of IL-1β and IL-6 using ELISA

The levels of IL-1β and IL-6 were assayed using a commercial ELISA kit (Abcam, Cambridge, UK) according to the manufacturer's instructions.

Western blotting for IL-1β and IL-6

Western blot analysis of IL-1β and IL-6 was performed using mouse anti-rat IL-6 monoclonal antibody (Abcam, Cambridge, UK) and rabbit anti-rat IL-1β polyclonal antibody (Abcam, Cambridge, UK). β-Actin was used as loading control. Immunocomplexes were visualized using enhanced chemiluminescence (Cell Signaling Technology, Beverly, MA).

Statistical analysis

All data are presented as mean ± standard deviation (SD). Mann-Whitney U test was used for statistical analysis with the aid of SPSS software. $P < 0.05$ were taken as indicative of statistical significance.

RESULTS

JTYS treatment induced regression of ectopic endometrial explants in a rat model of endometriosis

Viable implants were observed in 36 out of the 40 rats. As shown in Table 1, after 4-week treatment with saline, mean explant volumes in pre-treatment and post-treatment were similar (45.73 ± 17.77 mm^3 and 47.78 ± 16.51 mm^3, respectively, $p > 0.05$). However, JTYS treatment significantly decreased the mean explant volume, relative to pre-treatment: low dose (49.26 ± 18.45 mm^3 versus 35.46 ± 12.57 mm^3, $p < 0.05$); medium dose (43.07 ± 15.53 mm^3 versus 20.13 ± 9.77 mm^3, $p < 0.01$) and high dose (46.99 ± 16.24 mm^3 versus 15.55 ± 8.75 mm^3, $p < 0.01$). The implant volume was inhibited by 20.56 %, 53.39% and 66.76% in the low dose, medium dose and high dose groups, respectively.

JTYS treatment inhibited the production of inflammation-associated cytokines in endometriosis

The serum and peritoneal fluid levels of inflammatory cytokines IL-1β and IL-6 were tested first. Using ELISA, it was found that IL-1β and IL-6 levels increased significantly ($p < 0.01$) in EMs rats, when compared with the Sham group. However, the production of IL-1β and IL-6 was down-regulated in the JTYS group (especially the high dose group). These results are shown in Figure 1.

JTYS treatment inhibited IL-1β and IL-6 production by peritoneal fluid macrophages

The production of IL-1β and IL-6 by peritoneal fluid macrophages was significantly increased in the macrophage lysates and their conditioned media, as revealed by western blotting and ELISA, respectively. More importantly, JTYS treatment depressed the production of IL-1β and IL-6 by peritoneal fluid macrophages in a dose-dependent manner (Figure 2).

Table 1: Comparison of the implant volumes between pre-treatment and post-treatment in both EM group and JTYS groups

Group	Graft volume (pre-treatment) (mm^3)	Graft volume (post-treatment) (mm^3)	Volume change (mm^3)	% Inhibition
EMs	45.73 ± 17.77	47.78 ± 16.51	-2.11 ± 3.22	-
Low dose	49.26 ± 18.45	35.46 ± 12.57*	10.13 ± 6.99	20.56
Medium dose	43.07 ± 15.53	20.13 ± 9.77**	23.08 ± 11.67	53.59
High dose	46.99 ± 16.24	15.55 ± 8.75**	31.37 ± 15.49	66.76

Values are expressed as mean ± SD; *$p < 0.05$, compared with pre-treatment; **$p < 0.01$, compared with pre-treatment

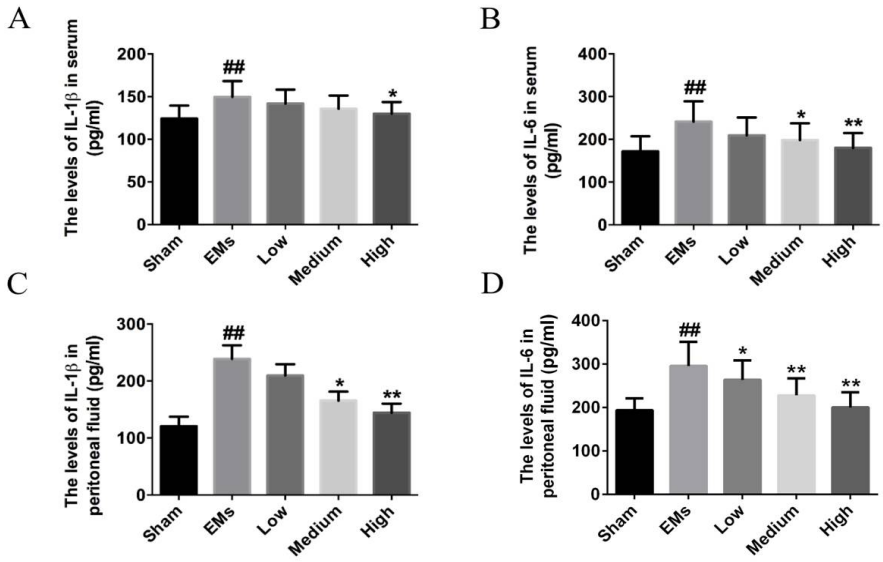

Figure 1: Serum and peritoneal fluid levels of inflammatory cytokines in sham and EMs rats. Serum levels of IL-1β (A) and IL-6 (B), together with peritoneal fluid levels of IL-1β (C) and IL-6 (D) were quantified by ELISA; ##p < 0.01, compared with sham; *p < 0.05, compared with EMs; **p < 0.01, compared with EMs

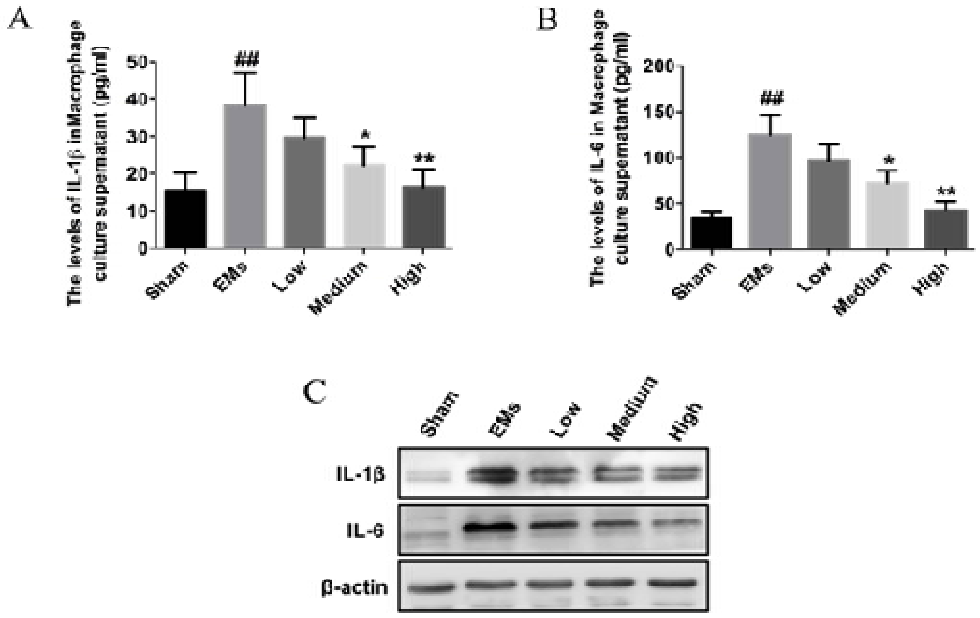

Figure 2: IL-1β and IL-6 production by peritoneal fluid macrophages. IL-1β (A) and IL-6 (B) levels in macrophage conditioned media were determined by ELISA. Western blot analysis was also carried out to determine the IL-1β and IL-6 production in PBS-washed macrophage lysates (C); ##p < 0.01, compared with sham; *p < 0.05, compared with EMs; **p < 0.01, when compared with Ems

JTYS suppressed IL-1β and IL-6 production by activated peritoneal fluid macrophages

Peritoneal fluid macrophages were isolated from untreated rats, and their levels of IL-1β and IL-6 levels were measured after *in vitro* culture, with or without activation. To rule out any influences due to differences in initial status of peritoneal fluid macrophages, these cells were obtained from untreated rats, but not from rats with endometriosis. Irrespective of activation status, IL-1β and IL-6 secretions were detected. However, LPS activation significantly (p < 0.01) elevated the secretions of IL-1β and IL-6 by these cells, relative to control (Figure 3). Direct *in vitro* stimulation of LPS-activated peritoneal fluid macrophages by serum from JTYS-treated EMs rats resulted in approximately 1.5- to 2-fold decreases in IL-1β and IL-6 secretions. Thus, JTYS treatment directly suppressed the production of IL-1β and IL-6 in activated peritoneal fluid macrophages. These results are depicted in Figure 3.

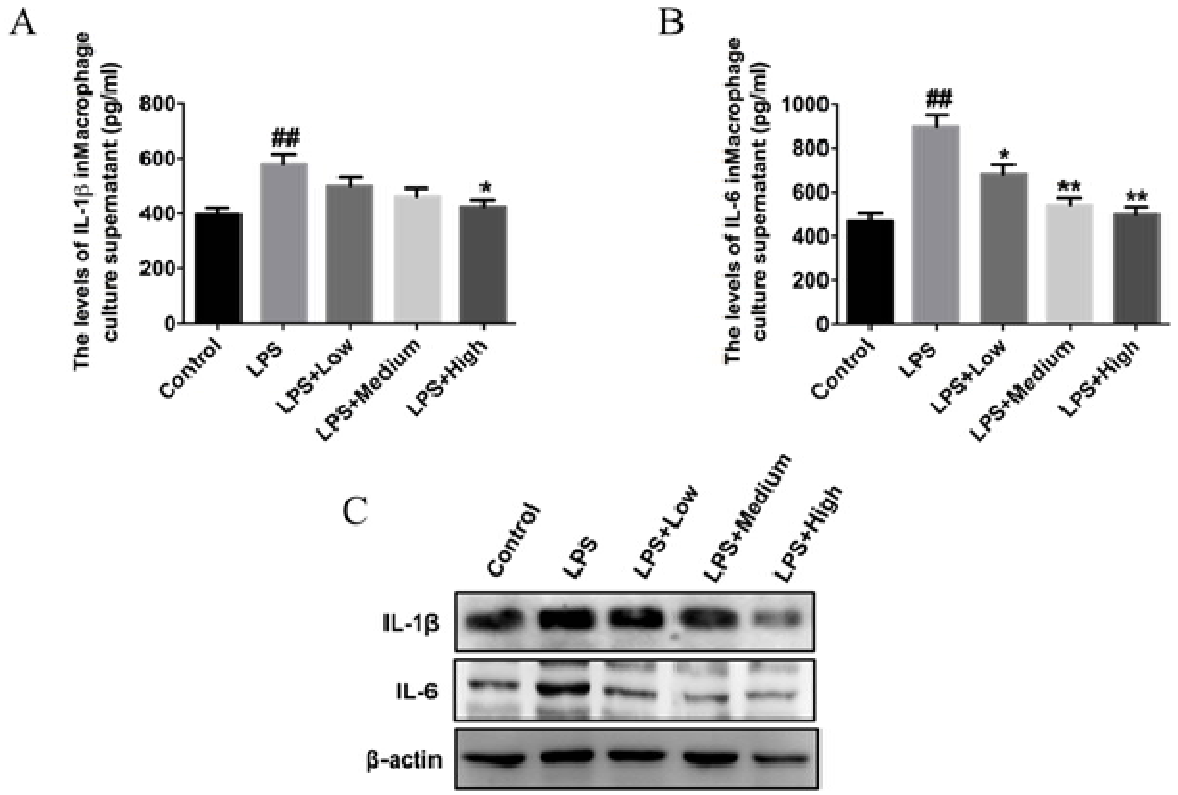

Figure 3: IL-1β and IL-6 production from LPS-activated and/or JTYS-stimulated peritoneal fluid macrophages. The peritoneal fluid macrophages were isolated from rats without endometriosis. These macrophages were cultured without special treatment, or activated by LPS (10 ng/ml) together with serum from saline- or JTYS-treated rats for 24 h. The levels of IL-1β (A) and IL-6 (B) in the conditioned media were determined by ELISA. Western blot analysis was carried out to determine the production of IL-1β and IL-6 in PBS-washed macrophages (C); ##$p < 0.01$, compared with control; *$p < 0.05$, compared with LPS; **$p < 0.01$, compared with LPS

DISCUSSION

Previous clinical data show that JTYS has significant protective effects against endometriosis. Rat models of endometriosis are regarded as useful tools for testing potential therapeutic agents, and for understanding the pathophysiology of endometriosis [13,14]. In the present study, it was shown that the volume of ectopic endometriotic implants was decreased by JTYS treatment in a surgically induced endometriosis rat model.

Inflammatory mediators secreted by peritoneal macrophages play important roles in the pathophysiology of endometriosis [15,16]. Inflammatory cytokine IL-1β is significantly involved in the regulation of inflammation and immune responses. It has been revealed that IL-1β can function as angiogenic factor [17] and increase sICAM-1 shedding from endometrial cells [18], thereby contributing to the pathophysiology of endometriosis. IL-6 is a multifunction cytokine which is known to induce VEGF expression, and to exhibit angiogenic effects [19]. This implies the involvement of IL-6-secreting macrophages in promotion of vascularity during endometriosis. The results of the present study revealed that JTYS treatment significantly decreased the levels of IL-1β and IL-6 in peritoneal fluid and serum. Thus, the therapeutic effect of JTYS on endometriosis might be supported by the suppressed production of IL-1β and IL-6 by activated peritoneal fluid macrophages.

However, it should be noted that IL-1β and IL-6 can also be produced by other cell types apart from peritoneal fluid macrophages. In addition, other important inflammatory factors and immune cells are involved in the pathophysiology of endometriosis [20,21]. Therefore, it will be necessary to investigate other possible mechanisms underlying the therapeutic effects of JTYS on endometriosis in further studies.

CONCLUSION

The results obtained in this investigation suggest that JTYS is a potential treatment strategy for endometriosis. The underlying mechanisms of action of JTYS most likely involve its anti-

inflammatory action and its ability to decrease the size of ectopic endometriotic implants. However, further studies are required to determine if these findings can be replicated in humans.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. The Tianchan Zhang and Qiao Zhang conceived and designed the study, Huifang Cong, Shan Zhao and Xumeng Gu collected and analysed the data, Tianchan Zhang wrote the manuscript. All authors read and approved the manuscript for publication.

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