

## Original Research Article

# Anti-endometriotic effect of *Angelica sinensis* (Oliv.) Diels extract in human endometriotic cells and rats

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### Abstract

**Purpose:** To study the anti-endometriotic effect of *Angelica sinensis* (Oliv.) Diels extract (ASDE) in human endometriotic cells and rats.

**Method:** Forty female rats were randomly divided into four groups (10 rats/group): control, endometriosis+danazol, endometriosis+high dose of ASDE and low dose of ASDE. The rats were orally administered either vehicle (200  $\mu$ L of PBS) alone or ASDE (140, 280 and 560 mg/kg/day) for 5 weeks. Danazol was used as the control drug. After induction of endometriosis for 4 weeks, the rats were sacrificed by cervical dislocation and the peritoneum and visceral organs examined visually to measure the number of endometriotic lesions. Serum levels of cancer antigen 125 (CA-125) and interleukin 13 (IL-13), interleukin 18 (IL-18) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) of peritoneal fluids of rats were measured using ELISA kits. Western blot assay was performed to measure the levels of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) expressions after 24 h of treatment with ASDE (30, 60, and 120  $\mu$ g/mL).

**Results:** ASDE-treated rats displayed reduced numbers of total endometriotic lesions when compared with vehicle-treated controls ( $p < 0.01$ ). When the rats were treated with high dose of ASDE, serum CA-125 level, as well as IL-18 and TNF- $\alpha$  levels in peritoneal fluids were significantly lower than that of the control group ( $p < 0.01$ ); however, IL-13 level in peritoneal fluids was significantly higher than that of the control group ( $p < 0.01$ ). ASDE treatment significantly suppressed the levels of MMP-2 and MMP-9 protein in 11Z cell ( $p < 0.01$ ).

**Conclusion:** The results reveal that ASDE exhibits significant anti-endometriotic effect by inhibiting inflammatory factors in rats. Thus, the plant extract can potentially be developed for the clinical management of endometriosis.

**Keywords:** *Angelica sinensis*, Endometriosis, Cancer antigen, Endometriotic lesions, Matrix metalloproteinase

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## INTRODUCTION

Endometriosis is a common gynaecological disease that affects approximately 1 in 7 women and 30 - 50 % of infertile women [1]. The

symptoms of the disorder are dysmenorrhoea, chronic pelvic pain and infertility. Endometriosis is characterised by ectopic implantation and growth of endometrial tissue and local sterile inflammation of peritoneal cavity. Proliferation,

adhesion, and migration of ectopic endometrial tissue are required to establish endometriotic lesions in the peritoneal cavity [2]. In addition, the expression of matrix metalloproteinase (MMP)-2 and MMP-9 have been shown to be higher in women with endometriosis than in healthy controls [3].

Endometriosis-associated inflammatory response, tissue repair and neo-vascularization are dependent on the peritoneal fluids macrophages and their secretory products/cytokines [4]. These cytokines may play major roles in regulating cell proliferation, activation, motility, adhesion, chemotaxis and morphogenesis in the pathogenesis of endometriosis [5]. Increased peritoneal fluid concentrations of cytokines that lead to the migration, proliferation, and activation of macrophages have been reported in patients with endometriosis.

The biomarkers such as cancer antigen 125 (CA-125) and IL-13, IL-18 and TNF-alpha levels in the peritoneal fluids were related with endometriosis [6]. Although surgical and hormonal treatment are applied as the common interventions, the unpleasant side effects limited the use of these interventions [7]. Some traditional plant medicine were shown to be potential treatment for the disease [8].

*Angelica sinensis* (Oliv.) Diels has been used to treat endometriosis for many years and has achieved good curative action. In this study, the anti-endometriotic effect of ASDE was investigated in human endometriotic cells and rats.

## EXPERIMENTAL

### Preparation of ASDE

The medicinal herbs of *Angelica sinensis* (Oliv.) Diels weighing 5 kilogram were collected from Guilin City, Guangxi Province in China in October 2018. Taxonomic identification of the plants was performed by Prof Meng Wang of Wuhan University China. A voucher specimen (no. ASDE 20171008) was deposited in the herbarium of College of Pharmacy, Wuhan University, China for future reference.

One batch of *Angelica sinensis* (Oliv.) Diels was dried in an oven. ASDE was prepared by steeping the dried *Angelica sinensis* (Oliv.) Diels in hot water (60 °C) three times (1 h each time). The extract was dried first in an oven and then freeze-dried. The yield was 73.45 %.

### Animals and grouping

Female Wistar rats weighing 200 - 220 g were obtained from Experimental Animal Center of Hubei Province, Hubei, China. The animals had free access to feed and water, and were allowed to acclimatize for at least one week before use. All animal experiments were approved by the Animal Care and Use Committee of Wuhan Center hospital (approval ref no. 20110508) and were carried out in compliance with the Animal Welfare Act and the NIH guidelines (NIH publication no. 80-23, revised 1996) [9]. Endometriosis was induced in rats using a previously established method with modifications [10]. The uterine horns of the donor mice were removed and put into a dish containing PBS. The endometrium-rich fragments (1 cm) from the middle-third of the uterine horn were finely and uniformly chopped.

The fragments (~ 20 sections) suspended in PBS were injected into the peritoneal cavity of recipient mice with a micropipette to induce the formation of endometriosis-like lesions. Forty (40) rats with induced endometriosis were randomly divided into four groups (10 rats/group): control, Danazol®, high dose ASDE and low dose ASDE. Ten normal rats were used as sham group. Rats were orally administered either vehicle (200 µL of PBS) alone or ASDE (140, 280 and 560 mg/kg/day) for 5 weeks. Danazol was used as the control drug. Danazol produces high androgen and low estrogen levels, which leads to the atrophy of endometriotic implants [11]. After induction of endometriosis for 4 weeks, the rats were sacrificed by cervical dislocation, and the peritoneum and visceral organs were examined visually to measure the number of endometriotic lesions (1 > mm).

### Enzyme linked immunosorbent assay

The rats were sacrificed by carbon dioxide execution and the samples of peritoneal fluids and serum were taken. The peritoneal fluid samples were centrifuged at 12,000 rpm for 10 min at 4 °C. Then, the supernatants were collected, aliquoted, and stored frozen at -80 °C until used for further evaluation. Serum CA-125 levels, and the levels of IL-13, IL-18 and TNF-α of the peritoneal fluids were determined using ELISA as directed by the manufacturer (RUIQI Bio Co. Ltd, Shanghai, China).

### Cell culture and cell viability

Immortalised human endometriotic cells 11Z cell were kindly provided by Nanjing Ke Biological Technology Co., Ltd., Nanjing, China. The

endometriotic cells were cultured in DMEM/F12 medium supplemented with 100 U/mL penicillin G, 100 g/mL streptomycin, and 10 % FBS. Met5A cells (human mesothelial cells) is originally from the American Type Culture Collection (Manassas, VA, USA). Met5A cells were cultured in Medium 199 medium supplemented with 100 U/mL penicillin G, 100 g/mL streptomycin, 10 % FBS and 400 nM hydrocortisone.

The cells were maintained in a humidified atmosphere of 5 % CO<sub>2</sub> – 95 % air at 37 °C. Cell viability was estimated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma-Aldrich) assay. Cells were seeded in 96-well plates at a density of 5 × 10<sup>3</sup> cells per well and incubated for 24 h. To study the effect of ASDE, the cells were treated with ASDE (30, 60 and 120 ug/mL) for 48 h. MTT solution was added into the medium and the cells were incubated at 37 °C for 4 h. The MTT-containing medium was removed and the cells were solubilized in DMSO (100 µL) for 30 min.

The absorbance was determined at 490 nm using a microplate spectrophotometer (Fisher Scientific Ltd., Ottawa, ON, Canada).

### Statistical analysis

Data are expressed as mean ± SD. Statistical comparison between two groups was made using Student's *t*-test. For comparing more than two groups, one-way analysis of variance (ANOVA) was used followed by Tukey test with statistical significance set at *p* < 0.05.

## RESULTS

### ASDE inhibited the formation of endometriosis-like lesions in rats

As shown in Table 1, compared with vehicle-treated controls, ASDE-treated rats had reduced number of total endometriotic lesions (*p* < 0.01). Thus, ASDE inhibited the formation of endometriosis-like lesions in the rats.

**Table 1:** Effect of ASDE on the formation of endometriosis-like lesions in rats (n = 10)

Group	No. of lesions/mouse
Normal	9.1±1.2**
Control	24.3±1.1
Danazol	18.2±1.4*
ASDE-H	10.4±1.1**
ASDE-M	13.8±1.8*
ASDE-L	15.2±1.2*

\**P* < 0.05, \*\**p* < 0.01 compared with control group

### Effect of ASDE on serum biomarkers in rats

As shown in Table 2, the serum CA-125 level, and the levels IL-18 and TNF-α of peritoneal fluids in control group rats were higher than those of sham group (*p* < 0.01). The serum CA-125, IL-18 and TNF-α levels of peritoneal fluids of high dose of ASDE decreased significantly than that of the control group (*p* < 0.01). The IL-13 level of peritoneal fluids in control group was significantly lower than that of sham group (*p* < 0.01). After treatment with high dose of ASDE, the IL-13 level of peritoneal fluids was significantly higher than that of the control group (*p* < 0.01).

## DISCUSSION

Endometriosis is usually associated with inflammation of the pelvic area and peritoneum. This hallmark has led to searching for inflammatory markers in the circulation which could potentially predict the presence of endometriosis, and the possibility of a clinically silent systemic inflammatory state in women with endometriosis. These results are largely similar with minimal differences at a level which precludes their use as diagnostic biomarkers for endometriosis. This may explain why there has been no unequivocal consensus of the circulating cytokine levels in endometriosis [13].

In this study, CA125, IL-13, IL-18 and TNF-α levels were chosen to reflect the effect of ASDE on the model rats established with endometriosis.

**Table 2:** Effect of ASDE on biochemical profile of rats

Group	CA-125 (U/mL)	IL-13 (pg/mL)	IL-18 (pg/mL)	TNF-α (pg/mL)
Normal	4.6±0.5**	8.2±1.2**	124.3±9.4**	225.1±16.3**
Control	14.2±1.2	2.1±0.8	312.4±18.2	846.4±11.3
Danazol	6.4±1.5**	6.1±1.1**	120.1±8.4**	252.2±13.5**
ASDE-H	6.7±1.3**	7.8±0.7**	113.1±7.2**	234.1±11.4**
ASDE-M	8.56±2.4*	6.4±1.2*	138.27±9.5*	257.9±12.3*
ASDE-L	10.2±2.2*	5.2±0.8*	148.3±10.2	299.3±15.4*

\**P* < 0.05, \*\**p* < 0.01 compared with control group

Serum CA-125 measurement is now a consolidated method for diagnosing endometriosis, and the serum CA-125 values were found significantly elevated in patients with ovarian and mixed endometriosis lesions [14]. The level of IL-18 in peritoneal fluids was markedly higher in women with peritoneal, minimal-to mild-stage endometriosis than in controls [15]. The level of TNF- $\alpha$  in peritoneal fluids was demonstrated as a biomarker to discriminate between patients with and without endometriosis [16]. It was found that ASDE markedly decreased the serum levels of CA-125 as well as the levels of IL-18 and TNF- $\alpha$  in the peritoneal fluids, and significantly increased the levels of IL-13 in the peritoneal fluids.

Matrix metalloproteinase (MMPs), a family of zinc-dependent endopeptidases, are known to regulate the migration, invasion, and proliferation of various cell types [17]. In recent years, accumulating data suggest that MMPs are associated with the establishment and progression of endometriosis. For example, MMP levels were shown to be enhanced in ectopic endometriotic tissues [18]. It was reported that MMP levels in endometrial tissue are related to the ability of the tissue to progress into ectopic endometriotic lesions in a mouse endometriosis model [19]. Among the various MMPs, gelatinases MMP-2 and MMP-9, which degrade the principal component of basement membranes, (collagen IV), have been intensively investigated in the context of endometriosis.

MMP-2 and MMP-9 are elevated in the peritoneal fluid of patients with endometriosis compared to healthy people [20]. Similarly, induction of endometriosis enhanced the levels of MMP-2 and MMP-9 in peritoneal fluid and cells in an *in vivo* study using BALB/c mice [21]. In addition, human endometriotic tissues were shown to have higher levels and activities of MMP-2 and MMP-9 [22]. In this study, it was showed that ASDE significantly suppressed the mRNA and protein levels of MMP-2 and MMP-9 in 11Z cell, suggesting that ASDE modulated MMP-2/9 expression at the transcriptional level. Down-regulation of MMP-2 and MMP-9 by ASDE may involve the inhibition of ASDE-induced migration in human endometriotic cells, considering that MMPs have been implicated in the endometriotic cell migration.

## CONCLUSION

The findings of this study reveal that ASDE shows significant anti-endometriotic effect by inhibiting inflammatory factors. Thus, it can

potentially be developed as a medicinal agent for the management of endometriosis.

## DECLARATIONS

### Conflict of interest

No conflict of interest is associated with this work.

### Contribution of authors

We 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 the authors. Qi-Xiang Xiong and Xiao-Yun Ruan contributed to this work equally and are co-first authors of this paper.

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