Effect of warming Yang and removing blood stasis method on matrix metalloproteinases / tissue inhibitor metalloproteinases levels secreted by cultured endometrial cells from patients with endometriosis

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Abstract

OBJECTIVE: To investigate the effect of Chinese medicines using the warming Yang and removing blood stasis method on the levels of matrix metalloproteinases (MMPs)/tissue inhibitor metalloproteinases (TIMPs) secreted by cultured endometrial cells from patients with endometriosis.

METHODS: Ectopic and eutopic endometrial cells obtained from 15 endometriosis patients were cultured in vitro, and divided randomly into five groups: high dose; moderate dose; low dose; nemestran; blank control. The three dose groups were treated with a decoction prepared according to the principle of warming Yang and removing blood stasis; nemestran and 0.9% NaCl were administered to the nemestran group and blank control group, respectively. Eutopic endometrial cells obtained from 10 hysteromyoma patients were cultured in vitro, as the normal control group, 0.9% NaCl were administered to the normal control group. Cell culture supernatants were collected and levels of matrix metalloproteinase-1 (MMP-1), matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9), tissue inhibitor metalloproteinase-1 (TIMP-1) and tissue inhibitor metalloproteinase-2 (TIMP-2) detected by enzyme-linked immuno sorbent assay (ELISA).

RESULTS: Compared with the normal control group, levels of MMP-1, MMP-2, and MMP-9 in eutopic and ectopic endometrium cell supernatants in the blank control group were increased, whereas levels of TIMP-1 and TIMP-2 were decreased (P < 0.05). Compared with the blank control group, levels of MMP-1 and MMP-2 in ectopic and eutopic endometrium cell supernatants cultured in low-dose, middle-dose, and high-dose groups were decreased, whereas levels of TIMP-1 and TIMP-2 were increased significantly (P < 0.05).
CONCLUSION: The warming Yang and removing blood stasis method affects expression of MMPs and TIMPs.

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Key words: Endometriosis; Matrix metalloproteinases; Tissue inhibitor of metalloproteinases; Warming Yang; Removing blood stasis

INTRODUCTION
The incidence of endometriosis (EMS) in women of childbearing age is about 5%-10%. EMS is a relatively common gynecologic disease characterized by the proliferation, invasion and metastasis of cells, as well as recurrence, and malignancy.1,2 Studies have shown that matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) are associated with EMS onset.3-5
MMPs are a group of proteases that can degrade almost all components of the extracellular matrix (ECM; e.g., collagen, protein-fiber connections, proteins, polysaccharides). MMPs are a class of highly conserved enzymes in nature. TIMPs are a family of natural, specific inhibitory factors of MMPs that can combine with the corresponding MMP preferment or active enzyme to inhibit MMP activity. These two factors have important roles in maintenance of the stability and structural integrity of the internal environment of the ECM.4-6 Studies have shown that disorder of MMPs/TIMPs can promote destruction and degradation of the peritoneal ECM and prompt the development of EMS.4-7 However, the occurrence, development, and prognosis of EMS warrants further study because these factors are incompletely understood.

EMS treatment with Traditional Chinese Medicine (TCM) has gained increasing attention in recent years. Main pathologic change of EMS is periodic bleeding of the ectopic endometrium (known as “blood circulating out of vessels” in TCM). Numerous scholars believe that the basic pathogenesis of EMS (in terms of TCM theory) is blood stasis; treatment is based on promotion of blood circulation and removal of blood stasis. However, the clinical outcome for some EMS patients is not significant, which suggests that the pathogenesis and therapeutic principle of TCM in EMS merits further investigation.

Clinical observations as well as ancient and modern literature suggest that the pathogenesis of EMS in terms of TCM is Yang deficiency and blood stasis. Thus, EMS treatment should consider both of these aspects. In clinics, warming Yang and removing stasis (by consumption of Dangguisini soup) has been applied, replacing the convention of activating blood and removing stasis for EMS treatment. Results have shown that the curative effect has been obviously improved (especially in terms of relieving dysmenorrhea and chronic pelvic pain) for EMS patients.

In the present study, the effect of warming Yang and removing blood stasis on cultured endometrial cells in EMS patients was observed. Pathogenesis of EMS, as well as the therapeutic effect of warming Yang and removing blood stasis on EMS, was investigated further.

METHODS

Subjects
Twenty-one cases of EMS patients and 12 cases of Hysteromyoma patients were recruited from the Department of Gynecology in Guangdong Provincial Traditional Chinese Medicine Hospital (Guangdong, China) from October 2010 to August 2012. ALL patients were underwent surgery, the ectopic endometrium and eutopic endometrium in patient were gain and cultured in vitro. The cultivation of 15 cases of EMS and 10 cases of Hysteromyoma patients were successful. The average age of the recruited patients was (32 ± 4) years. Hormonal drugs had not been used within 3 months in all cases.

Preparation of Chinese medicines for warming Yang and removing blood stasis
Chinese medicines used for warming Yang and removing blood stasis were purchased from Kangmei Pharmaceutical Company (Puning, China) and were: Danggui (Radix Angelicae Sinensis), 1.5 × 10^7 mg; Guizhi (Ramuclus Cinnamomi), 1.0 × 10^7 mg; Baishao (Radix Paeoniae Alba), 1.5 × 10^7 mg; Xixin (Herba Asari Mandshurica), 3.0 × 10^7 mg; honey-fried Gancao (Radix Glycyrrhizae), 1.0 × 10^7 mg; Tongcao (Medulla Tetrapanaceis), 1.0 × 10^-7 mg; Dazao (Fructus Jujubae), 1.0 × 10^-7 mg; Wuyao (Radix Linderae Aggregatae), 1.0 × 10^-7 mg; Fuzi (Radix Aconiti Lateralis Preparata), 6.0 × 10^-7 mg; Ganjiang (Rhizoma Zingiberis), 9.0 × 10^-7 mg. Total weight of these ingredients was 9.6 × 10^-7 mg. Ingredients were boiled in a ceramic frying pan and centrifuged (1699 × g for 10 min, Again, 3999 × g for 10 min) to obtain a crude drug concentration of 700 mg/mL in liquid; and then stored in a sterile vessel. Phosphate-buffered saline was used to dilute nemestrain to 8 mmol/L.

Culture and identification of cultured endometrial cells
Culture and identification of cultured endometrial cells were undertaken according to a method reported previously,8 with slight modification. Obtained tissue was shredded and washed with Dulbecco’s modified Eagle’s media: Nutrient Mixture F-12 ("FD medium"; Sigma-Aldrich, Saint Louis, MO, USA). After centrifugation, a double-enzyme digestion liquid was added in an incubator. After washing, centrifugation and discarding supernatants, the mixture was filtered. The filtrate contained mainly interstitial cells, with clusters of glandular cells on the filter. Cells in the filtrate were in-
tocated in six-well plates with coverslips in a culture bottle (concentration, $5\times6\times10^4$ cell/mL), followed by culture in an incubator (at 37 °C in an atmosphere of 5% CO$_2$). After cells had covered 90% of the wall of the culture bottle, passaging was undertaken. The medium was discarded, and then 5 mL of 0.25% trypsin (Cusabio Biotech Co., Ltd., Wuhan, China) was added, followed by incubation at 37 °C. Cells on the wall of the culture bottle were "blown down" using a suction tube. After centrifugation, the supernatant was discarded. FD medium containing 10% fetal bovine serum (Cusabio Biotech Co., Ltd., Wuhan, China) and penicillin (Cusabio Biotech Co., Ltd., Wuhan, China) was added to precipitated cells. After "blowing", the cell suspension was obtained, and the concentration adjusted to 1-2.5×10$^4$ cell/mL. Cells were inoculated into a 96-well culture plate (Cusabio Biotech Co., Ltd., Wuhan, China), followed by culture in an incubator (at 37 °C in an atmosphere of 5% CO$_2$). The medium was renewed once for every 3-5 days until cell aggregation. Vimentin, keratin, and prolactin were used to identify cultured endometrial cells. Changes in cell morphology were documented.

**Cell grouping and administration**

Third-generation cultured endometrial cells were obtained and seeded in 96-hole plates ($1\times10^5$/mL). When about 90% of cultured cells had aggregated, those from the EMS patients were divided randomly into five groups using the random number table method: high-dose, medium-dose, low-dose, nemestran, blank control (3 cells per hole). According to preliminary test results, the high-dose, medium-dose and low-dose warming Yang and removing blood stasis groups were treated with 14.0, 1.4 and 0.7 mg/mL TCM, respectively. The nemestran group were treated with 8 mmol/L nemestran (Sanofi-Aventis, Paris, France). The blank control group were treated with physiologic (0.9%) NaCl. Those from the Hysteromyoma patients were treated with physiologic (0.9%) NaCl, as the normal control group. After 24 h of incubation, cell culture supernatants were collected and stored at −70 °C. All samples were evaluated by an enzyme-linked immunosorbent assay (ELISA; Bender MedSystems, Vienna, Austria).

**Detection indicators and methods**

Contents of MMP-1, MMP-2, MMP-9, TIMP-1, and TIMP-2 in cell culture supernatants were detected by ELISA in accordance with kit instructions. All laboratory reagents were purchased from Cusabio Biotech Co., Ltd. (Wuhan, China).

**Statistical analyses**

Statistical analyses were carried out using SPSS v17.0 (IBM, Armonk, NY, USA). Measurement data with a normal distribution were expressed as the mean ± standard deviation ($\bar{x} \pm s$). Differences between multiple groups were compared with analysis of variance using SPSS v17.0. Differences between two groups were compared using the Mann-Whitney U-test. $P < 0.05$ was considered significant.

**RESULTS**

**Changes in cell morphology**

Cultured endometrial cells in the ectopic endometrium and eutopic endometrium grew well, and most of them had normal morphology (Figure 1A, 1B). In warming Yang and removing blood stasis groups,
with increasing dose, the number of normal cells decreased, and the number of cells that exhibited shrinkage, vacuolization, fat bodies, mitochondrial swelling and even disintegration increased (Figure 1C-1E). The number of normal cells in the nemestran group also decreased, and had a similar morphology (Figure 1F).

Effect of warming Yang and removing blood stasis on levels of MMP-1 and MMP-2
Levels of MMP-1 and MMP-2 in the ectopic endometrium and eutopic endometrium of the blank control group were higher than those of the normal control group ($P < 0.05$) (Table 1). Levels of MMP-1 in the ectopic endometrium and eutopic endometrium, as well as MMP-2 levels in the eutopic endometrium, in medium-dose, high-dose, and nemestran groups were lower than those in the blank control group ($P < 0.05$). MMP-2 levels in the eutopic endometrium in the high-dose group and nemestran group were lower than those in the control group ($P < 0.05$). Levels of MMP-1 and MMP-2 in the eutopic endometrium in the high-dose group were lower than those in the control group, but the difference was not significant ($P > 0.05$). Levels of MMP-1 and MMP-2 in the ectopic endometrium in high-dose and low-dose groups were less than those in the control group, but this difference was not significant ($P > 0.05$). MMP-9 levels in the eutopic endometrium in high-dose and low-dose groups were less than those in the control group, but this difference was not significant ($P > 0.05$). MMP-9 levels in the eutopic endometrium were lower than those in the normal control group ($P < 0.05$). These results suggested that EMS patients may have increased expression of MMP-1 and MMP-2 in the ectopic endometrium and eutopic endometrium. The warming Yang and removing blood stasis method may decrease MMP-1 and MMP-2 levels in the ectopic endometrium and eutopic endometrium. With the increase of drug concentration, the inhibition effect was enhanced, which was dose dependent.

Effect of eutopic and ectopic endometrial in vitro culture on MMP-9 levels in cell supernatants by the warming Yang and removing blood stasis method
MMP-9 levels in the eutopic endometrium and ectopic endometrium of the blank control group were higher than those in the normal control group ($P < 0.05$) (Table 2). MMP-9 levels in the eutopic endometrium in the medium-dose group and nemestran group were lower than those in the control group ($P < 0.05$). However, MMP-9 levels in the eutopic endometrium in high-dose and low-dose groups were less than those in the control group, but this difference was not significant ($P > 0.05$). MMP-9 levels in the eutopic endometrium in high-dose, medium-dose, and low-dose groups were similar with those in the control group. Compared with the control group, the difference was not significant ($P > 0.05$) (Table 2). However, MMP-9 levels in the eutopic endometrium and ectopic endometrium of the blank control group were higher than those in the normal control group ($P < 0.05$) (Table 1). These results suggested that EMS patients may have increased expression of MMP-1 and MMP-2 in the ectopic endometrium and eutopic endometrium. The warming Yang and removing blood stasis method may decrease MMP-1 and MMP-2 levels in the ectopic endometrium and eutopic endometrium. With the increase of drug concentration, the inhibition effect was enhanced, which was dose dependent.

Table 1 Effect of the warming Yang and removing blood stasis method on levels of MMP-1 and MMP-2 from stromal cells (ng/mL ± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>MMP-1 level in eutopic endometrium (ng/mL)</th>
<th>MMP-1 level in ectopic endometrium (ng/mL)</th>
<th>MMP-2 level in eutopic endometrium (ng/mL)</th>
<th>MMP-2 level in ectopic endometrium (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low dose</td>
<td>15</td>
<td>137 ± 40</td>
<td>136 ± 20</td>
<td>50 ± 11</td>
<td>59 ± 19</td>
</tr>
<tr>
<td>Medium dose</td>
<td>15</td>
<td>70 ± 17</td>
<td>88 ± 12</td>
<td>43 ± 8</td>
<td>55 ± 17</td>
</tr>
<tr>
<td>High dose</td>
<td>15</td>
<td>43 ± 24</td>
<td>45 ± 16</td>
<td>40 ± 8</td>
<td>40 ± 10</td>
</tr>
<tr>
<td>Nemestran</td>
<td>15</td>
<td>45 ± 17</td>
<td>45 ± 16</td>
<td>40 ± 9</td>
<td>42 ± 9</td>
</tr>
<tr>
<td>Blank control (0.9% NaCl, 24 h)</td>
<td>15</td>
<td>150 ± 45</td>
<td>133 ± 18</td>
<td>55 ± 6</td>
<td>60 ± 18</td>
</tr>
<tr>
<td>Normal control</td>
<td>10</td>
<td>40 ± 13</td>
<td>-</td>
<td>39 ± 8</td>
<td>-</td>
</tr>
</tbody>
</table>

Notes: the high-dose, medium-dose and low-dose warming Yang and removing blood stasis groups were treated with 14.0, 1.4 and 0.7 mg/mL TCM for 24 h, respectively. The nemestran group was treated with 8 mmol/L nemestran for 24 h. The blank control group and the normal control group were treated with physiologic (0.9%) NaCl for 24 h. Compared with the blank control group, $P < 0.05$, following are the same. TCM: Traditional Chinese Medicine; MMP: metalloproteinase.

Table 2 Effect of eutopic and ectopic endometrial in vitro culture on MMP-9 levels in the warming Yang and removing blood stasis method (ng/mL ± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>MMP-9 level in the eutopic endometrium (ng/mL)</th>
<th>MMP-9 level in the ectopic endometrium (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low dose</td>
<td>15</td>
<td>88 ± 21</td>
<td>99 ± 16</td>
</tr>
<tr>
<td>Medium dose</td>
<td>15</td>
<td>61 ± 7</td>
<td>100 ± 18</td>
</tr>
<tr>
<td>High dose</td>
<td>15</td>
<td>89 ± 21</td>
<td>99 ± 12</td>
</tr>
<tr>
<td>Nemestran</td>
<td>15</td>
<td>44 ± 7</td>
<td>51 ± 7</td>
</tr>
<tr>
<td>Blank control</td>
<td>15</td>
<td>99 ± 15</td>
<td>99 ± 18</td>
</tr>
<tr>
<td>Normal control</td>
<td>10</td>
<td>43 ± 22</td>
<td>-</td>
</tr>
</tbody>
</table>

Notes: the high-dose, medium-dose and low-dose warming Yang and removing blood stasis groups were treated with 14.0, 1.4 and 0.7 mg/mL TCM for 24 h, respectively. The nemes tran group was treated with 8 mmol/L nemestran for 24 h. The blank control group and the normal control group were treated with physiologic (0.9%) NaCl for 24 h. Compared with the blank control group, $P < 0.05$. TCM: Traditional Chinese Medicine; MMP: metalloproteinase.
levels in the ectopic endometrium in the nemestran group were lower than those in the blank control group ($P < 0.05$). These data showed that EMS patients may have increased expression of MMP-9 in the ectopic endometrium and eutopic endometrium. Nemestran may decrease MMP-9 levels in the ectopic endometrium and eutopic endometrium, but the warming Yang and removing blood stasis method had little effect on endometrial levels of MMP-9.

**DISCUSSION**

MMPs are a class of endopeptidases dependent on Zn$^{2+}$, Ca$^{2+}$, and Mg$^{2+}$. MMPs have 30 members that can participate in the cyclical growth, differentiation, and collapse of endometrial cells in childbearing-age women.$^{10,11}$ The ectopic endometrium of patients with EMS can secrete MMPs. "Planting" of the ectopic endometrium involves invasion of surrounding tissues to cause the collapse and reconstruction of the ECM.$^{12,13}$

Disorders in the relationship between MMPs and TIMPs may promote the degradation and destruction of the peritoneal ECM and, consequently, prompt EMS development.$^{14-17}$ Stilly and colleagues found that MMP-1/TIMP-1 levels could induce endometrial tissue to increase the chance of cell invasion, and to have important roles in EMS development.$^{18}$ We showed that expression of MMP-1, MMP-2, and MMP-9 in the ectopic endometrium and eutopic endometrium in the control group was significantly higher than that in the control group. Levels of TIMP-1 and TIMP-2 in the control group were significantly lower than those in the control group. These results are consistent with previous studies.$^{19,20}$

Regulation of MMPs and their inhibitors is a complex and multiple-stage process affected by many factors that can control gene transcription and enzyme activation. Steroid hormones, cytokines, and growth factors can regulate the secretion and expression of MMPs and TIMPs.$^{21,22}$

Bruner et al.$^{23}$ found that inhibition of MMP secretion prevented the formation of ectopic lesions in nude mice. These enzymes were chosen because of the biological properties of endometrial cells: they are good markers in clinical observations regarding therapeutic effects. The present study showed that warming Yang and removing blood stasis could reduce the number of EMS patients with ectopic disorders, and involved expression of MMP-1, MMP-2, TIMP-1 and TIMP-2. Thus, the mechanism of action of the warming Yang and removing blood stasis method may have involved restoration of the balance of MMPs/TIMPs levels to suppress cellular invasion in the ectopic endometrium and eutopic endometrium. This method could also change the endometrial biologic characteristics of EMS patients, thereby reversing pathologic processes.

### Table 3 Effect of eutopic and ectopic endometrial in vitro culture on levels of TIMP-1 and TIMP-2 by the warming Yang and removing blood stasis method (ng/mL, x ± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>TIMP-1 level in the eutopic endometrium</th>
<th>TIMP-1 level in the ectopic endometrium</th>
<th>TIMP-2 level in the eutopic endometrium</th>
<th>TIMP-2 level in the ectopic endometrium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low dose</td>
<td>15</td>
<td>10.2±1.5</td>
<td>12.5±2.7</td>
<td>11.4±3.4</td>
<td>6.2±1.6</td>
</tr>
<tr>
<td>Medium dose</td>
<td>15</td>
<td>21.7±3.4</td>
<td>13.2±2.4</td>
<td>13.3±6.8</td>
<td>11.1±3.3</td>
</tr>
<tr>
<td>High dose</td>
<td>15</td>
<td>24.8±5.0</td>
<td>26.1±4.8</td>
<td>22.6±8.8</td>
<td>16.7±2.4</td>
</tr>
<tr>
<td>Nemestran</td>
<td>15</td>
<td>25.0±4.2</td>
<td>26.3±5.1</td>
<td>22.8±9.3</td>
<td>16.6±3.1</td>
</tr>
<tr>
<td>Blank control</td>
<td>15</td>
<td>10.4±1.1</td>
<td>12.7±2.6</td>
<td>8.6±3.4</td>
<td>6.1±1.9</td>
</tr>
<tr>
<td>Normal control</td>
<td>10</td>
<td>25.6±2.7</td>
<td>-</td>
<td>15.5±5.9</td>
<td>-</td>
</tr>
</tbody>
</table>

Notes: the high-dose, medium-dose and low-dose warming Yang and removing blood stasis groups were treated with 14.0, 1.4 and 0.7 mg/mL TCM for 24 h, respectively. The nemestran group was treated with 8 mmol/L nemestran for 24 h. The blank control group and the normal control group were treated with physiologic (0.9%) NaCl for 24 h. Compared with the blank control group, *$P < 0.05$, TCM: Traditional Chinese Medicine; TIMP: tissue inhibitor of metalloproteinase.
concepts merit verification in rigorous, prospective, double-blind randomized controlled clinical trials.

REFERENCES


