Effects of Yiweining Recipe on expressions of metalloproteinase-2 and cyclooxygenase-2 mRNAs in ectopic endometrium of rats with endometriosis

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ABSTRACT  Objective: To explore the effects of Yiweining Recipe (YWNR), a compound Chinese herbal medicine, on expressions of metalloproteinase-2 (MMP-2) and cyclooxygenase-2 (COX-2) mRNAs in rats with endometriosis (EM). Methods: Operational self-transplantation was applied in establishing the rat models. Detection of MMP-2 and COX-2 mRNAs was conducted with hybridization \textit{in situ}. Results: There were significant differences in the expressions of MMP-2 and COX-2 mRNAs between the untreated group and the high-dose YWNR-treated group. YWNR could reduce the expressions of MMP-2 and COX-2 mRNAs. Conclusion: YWNR can treat EM through reducing the positive expressions of MMP-2 and COX-2 mRNAs.

KEY WORDS endometriosis; matrix metalloproteinase-2; cyclooxygenase-2; rats


Endometriosis (EM) is a common female disease associated with infertility, and the pathogenesis of the disease is unclear. According to Sampson’s theory, retrograde menstruation, peritoneal adhesion of shed endometrial tissue, and outgrowth of these endometrial cells are essential steps. Cyclooxygenase-2 (COX-2) mRNA expression was only recently described in eutopic endometrium.
lesions of endometriosis and adenomyosis. A correlation between the metalloproteinase-2 (MMP-2) concentrations in serum and peritoneal fluid of the patients and the endometriosis has also been demonstrated in recent years. This research mainly explored the effects of Yiweining Recipe (YWNR), a compound Chinese herbal medicine, on the expressions of MMP-2 and COX-2 mRNAs in rats with endometriosis.

1 MATERIALS AND METHODS

1.1 Materials

Female adult Wistar rats weighing (200 ± 20) g were supplied by the Animal Laboratory Center of Heilongjiang University of Chinese Medicine, Harbin, China. Hybridization in situ (HIS) reagents of MMP-2 and COX-2 were supplied by Boshide Biological Technology Institute, Wuhan, China. YWNR composed of Radix Paeoniae Rubra, Rhizoma Curcumae, Rhizoma Gastrodiae, Radix Scutellariae, Semen Coicis, Radix Bupleuri, Flos Lonicerae and Scolopendra) was supplied by the First Affiliated Hospital of Heilongjiang University of Chinese Medicine. Danazol was supplied by Lianhuan Medicine Company, China. The guidelines for animal care and use were approved by the committee on animal research.

1.2 Animal model

Nine rats were randomly picked out, which were then assigned to the normal control group without model made procedure. Operational self-transplantation was applied to establish the animal model. After each rat was injected with 0.2 mg of diethylstilbestrol to stimulate estrus, 20% urethane (1.5 g/kg) was then injected intra-abdominally. The animal models were then made as follows: (1) The abdominal fur was shaved and the skin was disinfected; (2) The abdominal cavity was opened; (3) Procedures were done to separate the uterus away from the right ovary by 0.5 cm, and a 2-centimeter long section of the uterus was removed; (4) The endometrium was separated and divided into three parts; (5) The uterine branch, left ovary, and parietal peritoneum were respectively sutured; (6) The abdominal cavity was then closed, and gentamicin sulfate (0.1 ml) was injected into each rat for three days after the above operations were completed.

1.3 Group and administration

Four weeks after the model was made, the rats were divided randomly into five groups with 9 rats in each group: (1) Untreated group; (2) High-dose YWNR-treated group (54 g/kg); (3) Low-dose YWNR-treated group (18 g/kg); (4) Danazol-treated group (36 mg/kg); (5) Normal control group. Rats in the untreated and normal control groups were administered with normal saline. The isotonic NaCl and drugs were administered by gavage. The drug dosage was calculated according to the proportion of the weights between the human and the rat. Four weeks later, the tissues of the ectopic and normal endometrium of the rats were taken out under aseptic conditions, which were then put into 4% formaldehyde. Regular methods of paraffin section at a thickness of 6 μm were taken.

1.4 Detection of MMP-2 and COX-2 mRNAs

All the procedures were rigorously conducted based on the manuals of the reagents. Diethylpyrocarbonate (DEPC) of 1 ml/L was added into all the solutions in the experiment to inactivate the RNA enzymes. Buffer solution of nitrate was used as the negative control in staining, and the positive slices above were used as the positive controls.

1.5 Standard of result evaluation

Double-blinded methods and high power microscope were used in the experiment. For each section preparation, 5 visual fields were randomly picked out for observation. Positive cells representing the positive expressions of MMP-2 mRNA and COX-2 mRNA turned yellow under staining. The evaluation standard is as follows: no color was expressed as (−), pale brown yellow (+), medium brown yellow (++), heavy brown yellow (+++). Scores of 0, 1, 2, and 3 respectively correspond to (−), (+), (++), (+++).

1.6 Statistical analysis

Results were analyzed by an independent university-based statistician. Data were analyzed by using the computer software, Statistical Package for Social Sciences (SPSS 10.0 for Windows). To compare the score of different groups with the normal control group, Mann-Whitney Test (a kind of two-independent samples tests of nonparametric test) was used. For all hypothesis tests a 5% significance level ($P < 0.05$) and two-tailed tests were used. Ninety-five percent Mann-Whitney confidence intervals for the median difference among four groups were determined.

2 RESULTS

2.1 Expression of MMP-2 mRNA
There was significant difference between the normal control group and the untreated group on the expression of MMP-2 mRNA \((P<0.01)\). YWNR could reduce the expression of MMP-2 mRNA. There was significant difference between the high-dose YWNR-treated group and the untreated group \((P<0.05)\). Results were shown in Table 1.

2.2 Expression of COX-2 mRNA

There was significant difference between the untreated group and the normal control group \((P<0.01)\). YWNR could reduce the expression of COX-2 mRNA. There were significant differences between the high-, low-dose YWNR-treated groups and the normal control group \((P<0.05, P<0.01)\). Results were shown in Table 2.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Number of cases that exhibits various staining conditions of the positive cells representing the positive expression of MMP-2 mRNA</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>9</td>
<td>-1 + 2 ++ 3</td>
<td>9* **</td>
</tr>
<tr>
<td>Untreated</td>
<td>9</td>
<td>-1 + 2 ++ 3</td>
<td>21</td>
</tr>
<tr>
<td>High-dose YWNR-treated</td>
<td>9</td>
<td>-1 + 2 ++ 3</td>
<td>10*</td>
</tr>
<tr>
<td>Low-dose YWNR-treated</td>
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<td>-1 + 2 ++ 3</td>
<td>12</td>
</tr>
<tr>
<td>Danazol-treated</td>
<td>9</td>
<td>-1 + 2 ++ 3</td>
<td>12</td>
</tr>
</tbody>
</table>

* \(P<0.05\), ** \(P<0.01\), vs untreated group.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Number of cases that exhibits various staining conditions of the positive cells representing the positive expression of COX-2 mRNA</th>
<th>Score</th>
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</thead>
<tbody>
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<td>-1 + 2 ++ 3</td>
<td>4* **</td>
</tr>
<tr>
<td>Untreated</td>
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<td>-1 + 2 ++ 3</td>
<td>20</td>
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<td>-1 + 2 ++ 3</td>
<td>7*</td>
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<td>-1 + 2 ++ 3</td>
<td>11*</td>
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<tr>
<td>Danazol-treated</td>
<td>9</td>
<td>-1 + 2 ++ 3</td>
<td>12*</td>
</tr>
</tbody>
</table>

* \(P<0.05\), ** \(P<0.01\), vs untreated group.

3 DISCUSSION

MMP-2 plays a major role in tumor angiogenesis and implantation of endometrial tissue. MMP-2 contributes to extracellular matrix proteolysis and increases cell invasiveness in many cell types including endothelial cells in basement membrane and interstitial matrix degradation\(^6,7\). After being transplanted in peritoneal cavities, endometrial grafts from the patients with endometriosis could express more MMP-2 than those from the women without endometriosis, suggesting that MMP-2 might play an important role in early-stage evolution of endometriosis\(^8,9\). Strong MMP-2 immunoreactivities have been found out both in glandular epithelial and interstitial cells of ectopic endometrium\(^8,9,10\). The endometrial fragments dispersed throughout the peritoneal cavity remain responsive to ovarian hormone regulation. The activity of MMPs is controlled by growth factors, hormones, cytokines, immune cells and tissue inhibitors of metalloproteinase. Present study found that MMP-2 mRNA expression levels in pigmented lesions of endometriosis were significantly higher than those in normal ectopic endometrium. Moreover, the expression in pigmented lesions was higher than that in non-pigmented lesions.

Cyclooxygenase (COX) is the enzymatic protein, involved in the synthesis of prostaglandin E\(_2\) (PGE\(_2\)) from prostaglandin G\(_2\) (PGG\(_2\)) and exists in two subtypes, COX-1 and COX-2. The related genes have been identified upon different chromosomes, i.e., COX-1 gene is located on chromosome 9, whereas COX-2 genes is on chromosome 1\(^{[11,12]}\). COX-2 is expressed in normal endometrial glandular epithelia as well as in vascular endothelia \(^{[12]}\), showing some changes through the menstrual cycle, being lower during proliferate phase and reaching the peak in the secretive phase of endometrium. The release of prostaglandin synthetase (PGs) in ectopic endometrial cells is involved in the pathogenesis of endometriosis and higher concentrations of prostaglandins have been found in
the peritoneal fluid of those patients.[16] COX-2 overexpression was detected by the method of immunohistochemistry in the endometrium of patients with endometriosis and in lesions of endometriosis and high expression of COX-2 could facilitate the formation of new vessels which then make the ectopic endometrium reproduce, implant and grow thus worsening EM[1, 2, 16]. Moreover, it was found that all examined extragenital lesions of endometriosis (rectovaginal endometriosis, cicatrix endometriosis or omentum endometriosis) expressed COX-2, although differential COX-2 expression was detected in the glands and stoma cells[19]. According to other data, COX-2 expression was found in 78.5% of ovarian foci of endometriosis, 11.1% of peritoneal lesions, 13.3% of rectovaginal lesions, although no correlation could be confirmed with clinical parameters or symptoms[17]. It is an established fact that large amounts of prostaglandins have been found in lesions of endometriosis and Douglas fluid[18]. These data are in good agreement with the described apoptosis resistance of ectopic endometrium lesion[19] as well as in the endopic and ectopic endometrium of endometriosis patients compared to those without endometriosis[20, 21].

Some researches[22, 23] have found that there may be some links between MMP-2 mRNA and COX-2 mRNA in cancer patients. As some of the biological behaviors of endometriosis are very close to cancer, MMP-2 mRNA and COX-2 mRNA were then chosen as the indexes in the research. Sivula et al[20] analyzed protein expressions of COX-2 and MMP-2 in tissue array specimens of 278 invasive breast cancers by immunohistochemical methods and found that high COX-2 expression in 30% and high MMP-2 expression in 83% of the breast cancer specimens, and there was a positive association between the expressions of these two factors (P = 0.003). Clinical research found[11] that the success rate for culture of endometriotic cells can be elevated through improving the method of primary culture and the ultrastructures of endometriotic glandular and interstitial cells are obviously different from those of entopic endometrial glandular and interstitial cells from endometriosis-free women and patients with endometriosis.

According to the theory of traditional Chinese medicine, EM is blood stasis, because the blood stasis causes fire syndrome and the accumulation of damp-heat in lower jiao, based on which the therapeutic principle of "reducing fire, resolving dampness and smoothing channels by removing blood stasis" was established. In the prescription, Radix Paeoniae Rubra is the main herb to resolve dampness, remove blood stasis, and reduce fire, which adapts to the pathogenesis of EM. Rhizoma Zedoariae could remove blood stasis, promote flow of qi, soften solid masses and relieve pain, promote the circulation of the efficient components of the drugs in blood. All these herbs, used together, play a role of promoting the circulation of blood and removing blood stasis and act as monarch herbs. Rhizoma Corydalís can promote the flowing of qi and relieve pain in order to increase the function of promoting the circulation of blood and removing blood stasis. Radix Scutellariae and Semen Coicis in the prescription function to reduce fire and resolve dampness. The gross observation shows that after treatment, the size and the weight of EM focus in the high-dose YWNR-treated group were significantly decreased. The experiment shows that YWNR could treat EM through reducing the expressions of MMP-2 and COX-2 mRNAs in the ectopic endometrium. There were significant differences between the high-, low-dose YWNR-treated groups and the untreated group in the expressions of MMP-2 and COX-2 mRNAs. The study fully demonstrates the advantages of traditional Chinese medicine in preventing and treating EM.

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