Effect of Lichong decoction on expression of Bcl-2 and Bcl-2-associated X protein mRNAs in hysteromyoma model rat

Donghua Li, Xin Xu, Ruiya Qian, Jianguo Geng, Yan Zhang, Xiaolei Xie, Yasong Wang, Xiaoli Zou

Abstract

OBJECTIVE: To study on effects of Lichong decoction on expression of apoptosis-controlling genes, Bcl-2 and Bcl-2-associated X protein (Bax) mRNAs in hysteromyoma tissue of the hysteromyoma model rat.

METHODS: Fifty Wistar female rats were randomly divided into a normal group, a model group, a Lichong decoction group, a Guizifuling capsule group and a Mifepristone group. The hysteromyoma rat model was established by intraperitoneal injection of exogenous estrin and progestogens. Pathological examination of uterine tissue, uterine coefficient and uterine transverse diameter were made under optic microscope and expressions of Bcl-2 and Bax mRNAs in uterine tissue in the groups were detected with real-time fluorescent quantitative polymerase chain reaction (PCR) technique.

RESULTS: After treatment, under microscope it was found that in the Lichong decoction group myometrium thinned, muscle fiber slightly overgrowth or long and thin, regular arrangement, inserting phenomenon of inner circular muscle and external longitudinal muscle was occasionally or not seen in the Lichong decoction group. The uterine coefficient and the uterine transverse diameter significantly decreased ($P<0.01$), and Bcl-2 mRNA expression significantly decreased ($P<0.01$) and Bax mRNA expression significantly increased in hysteromyoma tissue ($P<0.01$) in the Lichong decoction group as compared with the model group.

CONCLUSION: Therapeutic effects of Lichong decoction on hysteromyoma is related with decrease of Bcl-2 mRNA expression and increase of Bax mRNA expression.

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Key words: Myoma; Apoptosis; Genes, bcl-2; Bcl-2-associated X protein; Lichong decoction

INTRODUCTION

Hysteromyoma is a commonly and frequently encountered disease of female reproductive system, severely influencing female health and quality of life. Lichong decoction originates from Interpretation of Medical Problems by Combined Chinese and Western Medicine written by Mr. Xichun Zhang, a famous physician in the Qing Dynasty, which has functions of strengthen-
ing genuine Qi and removing blood stasis and is used for treatment of hysteromyoma and other gynecopaties with better clinical therapeutic effects, but the pharmacologic effect is not very clear. A study indicates that production and development hysteromyoma is closely related with abnormal expression of apoptosis-regulating genes, Bcl-2 and Bcl-2-associated X protein (Bax) form positive and negative regulation of apoptosis, over high Bcl-2 expression level and over lower Bax expression level are molecular basis of pro-

MATERIALS AND METHODS

Experimental animals

Fifty Wistar rats, female, health, adult, un-pregnancy, weighing (220±20) g, SPF grade, were purchased from Academy of Military Medical Sciences, Certificate of quality No. SCXK (Army) 2007-0004, and they were fed in the animal room of SPF grade, the Experimental Animal Department, Capital Medical University, with normal illumination, free access to food and water, at room temperature of 18°C-22°C.

Medicines for modeling Estradiol benzoate Injection: Tianjin Jinyao Amino Acid Co. Ltd., Batch No. 0703121, 1 mg/mL; Progesterone Injection: Shanghai Tongyong Pharmaceutical Co. Ltd., Batch No. 070902, 20 mg/mL.

Drugs for investigation Lichong decoction was composed of crude Huangqi (Radix Astragali) 9 g, Dangshen (Radix Codonopsis) 6 g, Baizhu (Rhizome Atractylodis Macrocephalae) 6 g, fresh Shan Yao (Rhizome Dioscoreae) 15 g, Tianhuafen (Radix Trichosanthis) 12 g, Zhuimu (Rhizome Anemarrhenae) 12 g, Sanleng (Rhizome Sparganii) 9 g, Ezhu (Rhizoma Curcumae) 9 g, fresh Jineijin (Endothelium Comune Gigeriae Gallii) 9 g, which were purchased from Beijing Tong Ren Tang Prepared Herbal Pieces Co. Ltd., and were extracted twice with water and prepared a concentrated solution containing crude drugs 3.3 g/mL, kept at 0°C-4°C, and the dosage was 22 g/kg per day for the animal with mixed before use; Gui Zhi Fu Ling capsules, Jiangsu Kangyuan Pharmaceutical Co. Ltd. Batch No.: 080305, was dissolved in distilled water and prepared a solution of 0.10 g/mL, and the dosage was 0.70 mg/kg per day for the animal; Mifepristone, Zhejiang Xianju Pharmaceutical Co. Ltd. batch No.: 080501, was dissolved in distilled water and prepared a solution of 0.94 mg/mL, and the dosage of was 6.25 mg/kg per day.

Main reagents and equipments: fermentas K1622 RT reverse transcription kit (Mbi, California, USA); SYBR Green PCR Master Mix (Abi, North Carolina, USA); TRIZOL reagent (Invitrogen life technologies, California, USA); PRISM 5700 fluorescent quantitative PCR meter (Abi, North Carolina, USA); DU-600 spectrophotometer (Beckman, California, USA); high speed refrigerated centrifuge (Beckman, California, USA).

Modeling and administration

The animals were conveniently fed for one week and were randomly divided into two groups by random number table, a normal control group (n=10) and a modeling control group (n=40). They were fed in separated cages with free access to water and food. The rats in the modeling group were intraperitoneally injected with estradiol benzoate injection, 0.5 mg/kg, once a day for 25 consecutive days and then with progesterone injection, 4 mg/kg, once each day for 5 consecutive days. After end of modeling, the model rats were randomly divided into a model control group, a Lichong decoction group, a Guizifuling capsule group and a Mifepristone group by random number table. The rats in the medical groups were intragastrically administrated once each day in 2-fold adult dosage. The rats in the normal control and the model control group were administrated intrastragically with equal volume of distilled water for 4 consecutive weeks.

Experimental methods

The rats were weighed and hair color and automatic activity were observed each week. After administration for 4 weeks, the rats were fasted for 12 h over night, with free access to water and the body weight was weighed, and then anesthetized with 30% chloral hydrate (3 mL/kg) ip, and the uterus was weighed. The indexes and methods investigated are showed as follows.

Pathological examination

The uterine segment of same part 0.5 cm above the uterine dividing horn was taken and fixed with 10% neutral formalin, and embedded with paraffin and sectioned, HE staining and observed under a microscope.

Uterine coefficient and uterine transverse diameter detection

Uterine coefficient (mg/g): uterine weight/body weight of rat.

Uterine transverse diameter: the transverse diameter bellow the uterine dividing horn (above the uterine neck) and the root of the dividing horn (the uterine root of dividing horn on one side) was measured.

Real-time fluorescent quantitative PCR

Amount of 50-100 mg uterine tissue of same part above the uterine dividing horn was added with 1 mL TRIZOL reagent, and Real-time PCR primer was de-
RNA in the detected sample was extracted and qualitatively detected, and was reversely transcribed as cDNA by using Fermentas K1622 RT reverse transcription kit; Standard curve samples were prepared, and amplified and detected by Real-Time PCR at 95°C 5 min; 95°C 30 s, 53°C 30 s, 72°C 50 s, 40 cycles; 72°C 8 min. After end of reaction, CT values of the samples were detected. The mixed cDNA solution after the reverse transcription was used as standard sample, which was diluted in 10-fold gradient dilution. The linear relative quantitative standard curve based on 5 points was constructed by drawing a lattice figure of each standard CT value (Y axis) and corresponding Log value (X axis) of total RNA amount, and then RNA levels of Bcl-2, Bax and β-actin of each sample were calculated on the basis of the standard curve, and RNA levels of Bcl-2 and Bax genes were corrected with β-actin RNA level, their ratio was relative expression amount of Bcl-2 and BaxmRNAs in each sample.

Statistical methods
Quantitative data were expressed as mean±standard deviation. SPSS 13.0 software (SPSS, Chicago, IL, USA) was used for detection of normal distribution of the data. For the data conforming to normal distribution, One-way ANOVA was used for comparison between groups, LSD method was used for equal variance assumed and Tamhane’s T2 method was used for equal variance not assumed; For the data in-conforming to normal distribution, Kruskal-Wallis H test was adopted. P<0.05 was used as the limitation of statistical significance.

RESULTS

Pathological detection
Under microscope (HE × 200): in the normal control group uterine muscular layer was thin, muscle fiber had no proliferation and hypertrophy, inner circular muscle and external longitudinal muscle regularly arranged (Figure 1A); In the model control group the uterine muscle fiber had obvious proliferation and hypertrophy, the muscular layer obviously thickened and the muscle fiber arranged irregularly showing a slight network form or a whirlpool form, approaching to lio-myoma-like proliferation, indicating the modeling is successful (Figure 1B); In the Lichong decoction group the uterine muscular layer obviously thinned, the muscle fiber had slight hypertrophy or long and thin, and arranged regularly, inserting phenomenon of inner circular muscle and external longitudinal muscle occasionally was seen or was not seen (Figure 1C). In the Guizifuling capsule group the muscular layer obviously thinned, the muscle fiber had slight hypertrophy or long and thin, and arranged regularly, inserting phenomenon of inner circular muscle and external longitudinal muscle occasionally was seen or was not seen (Figure 1D). In the Mifepristone group uterine muscle fiber had proliferation and hypertrophy, the muscular fiber arranged slightly-irregularly, a part of them showed whirlpool form (Figure 1E).

Table 1 Gene orders of the primer

<table>
<thead>
<tr>
<th>Gene</th>
<th>Orders of primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>5’ gagaccttcaacacccccagcc 3’</td>
</tr>
<tr>
<td></td>
<td>5’ aatgtcacgcacgatttccc 3’</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>5’ gggatgcctttggaacta 3’</td>
</tr>
<tr>
<td></td>
<td>5’ atttgtttggggcaggtct 3’</td>
</tr>
<tr>
<td>Bax</td>
<td>5’ agacacctgagctgaccttg3’</td>
</tr>
<tr>
<td></td>
<td>5’ aagttgccatcagcaaaat 3’</td>
</tr>
</tbody>
</table>

Note: Bax: Bcl-2-associated X protein.
Comparison of uterine coefficient and transverse diameter among the groups (n=10, ±±s)

<table>
<thead>
<tr>
<th>Group</th>
<th>Uterine coefficient</th>
<th>Uterine transverse diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Diameter below the dividing horn</td>
</tr>
<tr>
<td>Normal control</td>
<td>2.5±0.6</td>
<td>4.8±1.0</td>
</tr>
<tr>
<td>Model control</td>
<td>6.9±1.2</td>
<td>7.8±1.1</td>
</tr>
<tr>
<td>Lichong</td>
<td>2.6±0.4</td>
<td>5.0±0.7</td>
</tr>
<tr>
<td>Guizhifuling capsule</td>
<td>2.5±0.6</td>
<td>6.1±0.9</td>
</tr>
</tbody>
</table>

Notes: Compared with the model control group, *P<0.01; †P<0.05; .

**Uterine coefficient and transverse diameter**

The uterine coefficient and transverse diameter in the model control group were significantly increased as compared with the normal control group (P<0.01), and in the Lichong decoction group and the Guizhifuling capsule group significantly decreased as compared with the model control group (P<0.01), and in the uterine coefficient there was significant difference between the Mifepristone group and the model control group (P<0.01) with no significant difference in the uterine transverse diameter (Table 2).

**Lichong decoction effect on expressions of Bcl-2 and Bax mRNAs**

PCR amplified products: the results of 1.2% agarose gel electrophoresis indicated that the specific strip-zones for β–actin (264 bp), Bcl-2 (163 bp ) and Bax (193 bp) were bright and visible, suggesting that the amplified fragments are accurate (Figure 2).

**DISCUSSION**

Hysteromyoma belongs to the category of “stony uterine mass”, “female abdominal mass”, etc. in TCM. TCM holds that “blood stasis” is the main pathogenesis of this disease, with pathological basis of insufficient genuine Qi. For its treatment, supplementing Qi, reinforcing kidney, activating blood circulation and removing blood stasis are commonly used methods. Strengthening genuine Qi to remove blood stasis has good clinical effects on early Hysteromyoma. Lichong decoction originates from Yi Xue Zhong Zhong Can Xi Lu written by Mr. Xichuan Zhang, a famous physician in the Qing Dynasty, which is a represent formula treating hysteromyoma with the therapeutic principle of strengthening genuine Qi to remove blood stasis. In the formula, Shenghuangqi (Radix Astragalii), Danshen (Radix Codonopsis), Baizhu (Rhizome Atractylodis Macrocephalae), and Shengshanyao (Rhizome Cornus Officinalis), and Shengjineijin (Endothelium Corneum Gigeriae Galli) have functions of supplementing Qi and strengthening spleen, and Sanleng (Rhizome Sparganii), Ezu (Rhizoma Curcumae), Shengjineijin (Endothelium Corneum Gigeriae Galli) have functions of disintegrating masses, dispersing accumulation of pathogen, and Tianhuafen (Radix Trichosanthis) functions nourishing Yin and removing toxic substances, Zhimu (Rhizoma Anemarrhenae) has function of cooling-moistening. The whole
Bcl-2/Bax is particularly important. Bcl-2 protein is course regulating apoptosis, among them, action of They interact and are commonly involved in the members such as Bcl-2/Bax, Bcl-XL/Bcl-Xs, Bak, etc. one of oncogenes of the most attaching attention in with occurrence of human many tumors. Bcl-2 gene is same family of Bcl-2, and they are genes closely related to tumor. Bcl-2 and Bax are a pair of cytokines of regulat-

crease of cell death. When apoptosis is inhibited, cells strengthen of cellular proliferation, but also de-

crease of cell death. When apoptosis is inhibited, cells abnormally proliferate, finally, forming focal mass or tumor. Bcl-2 and Bax are a pair of cytokines of regulat-

The results in the study indicate that compared with the normal control group, Bax mRNA expression signifi-

cently decreases (P<0.01), Bcl-2 mRNA expression significantly increases (P<0.01) in the model control group, suggesting that occurrence of hysteromyoma has the molecular basis of over high Bcl-2 expression and over low Bax expression. Compared with the model control group, Bcl-2 mRNA expression significantly decreases (P<0.01) and Bax mRNA expression significantly increases (P<0.01) in the Lichong decoction group. Pathological results showed that after treatment of Lichong decoction, the uterine muscular layer obviously thinned, the muscle fiber had slight hypertrophy or long and thin, and arranged regularly, the inserting phenomenon of inner circular muscle and external lon-
gitudinal muscle occasionally was seen or was not seen, and the uterine coefficient and the uterine transverse diameter significantly decreased as compared with the model control group (P<0.01). The above results indicate that therapeutic action of Lichong decoction on hysteromyoma is related with decrease of Bcl-2 mRNA expression and increase of Bax mRNA expression, so as to promote apoptosis of hysteromyoma.

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