Effect of Lichong Decoction on expression of IGF-I and proliferating cell nuclear antigen mRNA in rat model of uterine leiomyoma

Donghua Li, Yalan Zhang, Hongjuan Han, Jianguo Geng, Xiaolei Xie, Jiubo Zheng, Yasong Wang, Xiaoli Zou

OBJECTIVE: To study the effect of Lichong Decoction (Lichong Decoction for strengthening anti-pathogenic Qi and eliminating blood stasis) on the expression of insulin-like growth factor-I (IGF-I) and proliferating cell nuclear antigen (PCNA) mRNA in a rat model of uterine leiomyoma.

METHODS: Fifty female Wistar rats were randomized into a normal control group, model group, Lichong Decoction group, Guizhifuling Capsule (Capsule containing Cassia Twig and Poria) group, and Mifepristone group. The uterine leiomyoma model was established by peritoneal injections of exogenous estrogen and progesterone hormone. The ultrastructural changes in cells of rat uterine tissues were observed with transmission electron microscopy, and the expression of IGF-I and PCNA mRNA was detected by real-time fluorescent quantitative PCR.

RESULTS: Following treatment, cells in the Lichong Decoction group appeared to be arranged normally, the cellular morphology were almost in a normal state, hyperplasia and hypertrophy of the chondroosome was reduced, collagen fibers were arranged in a regular manner, without obvious hyperplasia, and the expression of IGF-I and PCNA mRNA was significantly decreased compared with the model group (P<0.01).

CONCLUSIONS: The effect of Lichong Decoction on uterine leiomyoma is related to its function in reducing the expression of IGF-I and PCNA mRNA.

INTRODUCTION

Uterine leiomyoma, which is a common disease of the female reproductive system, has a great influence on women’s health and quality of life. Studies have shown an association between uterine leiomyoma and excessive proliferation of uterine smooth muscle cells. In addition, the expression of insulin-like growth factor-I (IGF-I) and proliferating cell nuclear antigen (PCNA) has been shown to affect cell proliferation, thus the molecular basis of uterine leiomyoma development is related to the overexpression of IGF-I and PCNA.

Through the establishment of a rat model of uterine fibroids, using the real-time quantitative PCR technique, this study observed the effect of Lichong Deco-
tion, which has the function of strengthening anti-pathogenic Qi and eliminating blood stasis, on the expression of IGF-I and proliferating cell nuclear antigen (PCNA) mRNA in a rat model of uterine leiomyoma. The study also explored the molecular mechanisms of this Chinese medicine in strengthening anti-pathogenic Qi and eliminating blood stasis to inhibit uterine fibroids for the purpose of providing a scientific experimental basis for clinical treatment.

MATERIALS AND METHODS

Reagents and instruments
Fermentas K1622 RT-PCR Kit (ABI, USA); SYBR Green PCR Master Mix (ABI, USA); TRIZOL reagent (Invitrogen life technologies, USA); PRISM 5700 fluorescent quantitative PCR (ABI, USA); DU-600 ultraviolet spectrophotometer (BECKMAN, USA); High speed refrigerated centrifuge (BECKMAN, USA), JEM-1230 transmission electron microscope (JEOL, JAPAN).

Animals
Fifty healthy, non-pregnant, adult female Wistar rats, weighing 220 ± 20 g, of SPF grade, were purchased from the Academy of Military Medical Sciences, certified SCXK (Army) 2007-0004. Rats were bred in SPF animal rooms of the Ministry of Experimental Animals, Capital Medical University, with normal light conditions and free access to food and water, and were housed at room temperature (18°C-22°C).

Drugs
Lichong Decoction from the Yixue Zhong Zhong Can Xi Lu (Records of Traditional Chinese and Western Medicine in Combination) as was written by the famous doctor Xichun Zhang of Qing Dynasty. The quantity of each medicine was converted from the original to the following prescription: raw Huangqi (Radix Astragali seu Hedysari) 9 g, Sangshen (Radix Codonopsis Pilosulae) 6 g, Baizhu (Rhizoma Atractylodis Macrocephalae) 6 g, raw Shanyao (Rhizoma Dioscoreae) 15 g, Tianhuafen (Radix Trichosanthis) 12 g, Zhiru (Rhizoma Anemarrhenae) 12 g, Sanling (Rhizoma Sparganii) 9 g, Ezhu (Rhizoma Zedoariae) 9 g, raw Jin-ejin (Endothelium Corneum Gigeriae Galli) 9 g. The medicines were first processed by water extraction twice, and then the preparation was concentrated to 3.30 g/mL. The daily dose for one rat was 22 g·kg⁻¹.

Modeling and drug administration
Experimental rats were fed normally for a week. After their adaptation, they were divided into groups according to the random number table for model building, the normal control group (n=10 rats), and the model group (n=40 rats). Rats from the two groups were caged separately and allowed to drink water and eat freely. The model group was injected intraperitoneally with estradiol benzoate(0.5 mg·kg⁻¹) once a day for 25 days in succession, and then injected intraperitoneally with progesterone (4 mg·kg⁻¹) once a day for 5 days in succession. After modeling, the rat models were subdivided according to the random number table into model control group, Lichong Decoction group, Guizhifuling Capsule group, and Mifepristone group. Gavage was given to the rats in each treatment group, once a day, and the dosage for each rat was equal to two times of that for an adult person. The normal and model control groups were given gavage with an equal amount of distilled water every day, for 4 weeks in succession. The rats were weighed once a week. Observations on hair color and spontaneous activities of rats was monitored. After drug administration, the rats were made to fast for 12 h overnight without any limitation on water intake, and then weighed. Intraprteroneal anesthesia was applied with 30% (v/v) chloral hydrate (3 mL·kg⁻¹) and the uterus of each rat was removed for testing.

Cellular ultrastructure
Uterine tissue 1 cm above the uterine cornu was put into glutaraldehyde liquid, immediately cut into 1 mm³ pieces, fixed, washed with the 2% (v/v) PBS, and embedded for sectioning. Changes in the cellular ultrastructure of tissue were observed using transmission electron microscopy.

Expression of IGF-I and PCNA mRNA
Using the real-time PCR, the influence of Lichong Decoction on the expression of IGF-I and PCNA mRNA was tested. TIRZOL (1 mL) was added to uterine samples (50 mg-100 mg) and RNA was extracted. Primers were designed using Primer Expression 3.0 (Table 1). Sample RNA was reversely transcribed to cDNA using the Fermentas K1622 RT-PCR Kit. The reaction con-
ditions for real-time PCR amplification and testing were: 95°C for 5 min; 95°C for 30 s; 53°C for 30 s; 72°C for 50 s, 40 cycles; 72°C for 8 min. After the reaction, sample CT values were determined.

**Statistical analysis**
Data are shown as the mean ± standard deviation. The data were analyzed with SPSS13.0. If data were normally distributed, a one-way ANOVA was used to analyze differences among groups. When there was homogeneity of variance, the LSD test was adopted. If not, a Tamhane’s T2 test was performed. If data were not normally distributed, the Kruskal-Wallis H test was performed. P<0.05 was regarded as statistically significant.

**RESULTS**

**Transmission electron microscopy**
Normal control group: Uterine smooth muscle cells were in an ordered spindle shape. Around the cells, collagen fibers arranged regularly. The cellular organelles in the cytoplasm were normal (Figure 1A).

Model control group: Fibroid cells increased in size, the nucleus increased in volume, and some were observed to be malformed. Cells were rich in actin microfilament cables. Mitochondria were hypertrophied and hyperplasia was evident. The rough endoplasmic reticulum expanded and the number of free ribosomes increased. Around the cells, collagen fibers were hyperplastic, disordered, and irregular. These results indicated that model establishment was successful (Figure 1B).

Lichong Decoction group: Cells appeared ordered, and organelles were almost normal. The presence of actin microfilament cables was reduced when compared to the normal control group. Mitochondria were less hypertrophied. Collagen fibers were arranged relatively regularly. Hyperplasia was not obvious (Figure 1C).

Guizhifuling Capsule group: Cells were observed to have slight hyperplasia. The number of cellular organelles decreased. Some mitochondria appeared empty with bubble-like changes. The rough endoplasmic reticulum appeared to expand, but not to the same extent as the model control group. Collagen fibers were irregular and slightly hyperplastic (Figure 1D).

Mifepristone group: Cellular hyperplasia was reduced and the cell was rich in cellular organelles. There were some hyperplastic and irregular collagen fibers surrounding cells (Figure 1E).

**Influence of Lichong Decoction on the expression of IGF-I and PCNA mRNA**
Amplified PCR products: results: β-actin (264bp), IGF-I (201bp), and PCNA (231bp) Bands were bright and visible (Figure 2).

![Figure 1 Transmission electron microscopy](image_url)
A: The normal control group (x8000)
B: The model control group (x8000)
C: Lichong decoction group (x8000)
D: Guizhifuling group (x8000)
E: Mifepristone group (x8000)
IGF-I and PCNA mRNA expression

Compared with the normal control group, the level of IGF-I and PCNA mRNA expression in the model group increased significantly ($P<0.01$). Compared with the model group, the level of IGF-I and PCNA mRNA expression in the Lichong Decoction group and Guizhi Fuling Capsule group decreased significantly ($P<0.01$); and the level of IGF-I mRNA expression in the Mifepristone group decreased significantly ($P<0.01$), while PCNA mRNA expression was without statistical significance (Table 2).

**DISCUSSION**

Uterine leiomyoma, the incidence of which is rising every year, is a commonly seen benign tumor of the female reproductive organs. It is one of the major reasons for hysterectomies and greatly influences women’s physical and psychological health. The pathogenesis of uterine leiomyomas is not clear. Modern medicine suggests it is related to environmental factors, gene mutations, progestational hormones, growth factors, and changes in the extracellular matrix. Modern medical treatments include surgery and hormone therapy. However, these treatments have a great influence on a woman’s quality of life and have side effects, so most sufferers refuse to use them. Traditional Chinese Medicine has its own unique advantages in treating early-stage uterine leiomyomas, including good outcomes and few side effects. Therefore, it is necessary to investigate the effect of Traditional Chinese Medicine on uterine leiomyomas.

Uterine leiomyoma falls into the category of an “abdominal mass” in TCM according to its clinical manifestations. *Jing-yue Quan Shu and Fu Ren Gui* (The Complete Works of Jingyue Zhang – Woman Rules) state that "Long-term stasis developing into lumps is only a symptom observed in women. The symptoms may appear during menstruation or postpartum because of raw or cold food, invasion of cold, or anger damaging the liver, thus, *Qi* stagnation results in blood stasis; or long-term worries damage the spleen, leading to *Qi* deficiency, thus, causing blood stasis; or extreme physical weakness from exhaustion causes *Qi* deficiency, thus, causing blood stasis. The tumor is the final presentation of blood stasis". This statement points out that "deficiency" and "blood stasis" are the basic pathogenesis of uterine leiomyoma.

Jiaozhu Furen Liangfang (Collated and Annotated Effective Prescriptions for Women) states: "In general, dyspepsia and lumps are usually caused by excess pathogens during duration of deficiency in anti-pathogenic *Qi*. Reinforcing the anti-pathogenic *Qi* and removing excess pathogens makes the lumps dissolve automatically.” Reinforcing the anti-pathogenic *Qi* to remove the excess pathogens, that is blood stasis, is the treating principle for uterine leiomyoma.

Lichong Decoction from the Records of Traditional Chinese and Western Medicine in Combination written by the famous doctor Xichun Zhang in the Qing Dynasty advocates reinforcing anti-pathogenic *Qi* to remove blood stasis, which both strengthens and elimi-
nates at the same time. In the prescription, raw Huangqi (Radix Astragali seu Hedyrsyi), Danshen (Radix Codonopsis Pilosulae), Baizhu (Rhizoma Atractylodis Macrocephalae), and raw Shanyao (Rhizoma Dioscoreae) function to reinforce Qi and strengthen the spleen; Sanleng (Rhizoma Spargani), Ezhu (Rhizoma Zedoariae), and raw Jineinin (Endothelium Cornu Gigeriae Galli) are effective in dissolving lumps; Tianhuaifen (Radix Trichosanthis) is used to replenish Yin and remove toxins; and Zhimu (Rhizoma Anemarrhenae) acts to cool the heat of Huangqi and Danshen and supplement the kidney with water. Collectively, this prescription functions to reinforce Qi without disturbing the removal of stasis, and eliminates pathogens without damaging anti-pathogenic Qi.

During our early studies, we examined the effect of Lichong Decoction on the expression of related genes that control the death of cells and found that it could induce the death of myoma cells. In order to further study the functions of Lichong Decoction, we monitored its effect on IGF-I and PCNA mRNA expression to understand its mechanism of action at the molecular level. The complex pathogenesis of uterine fibroids is related to the abnormal expression of proliferation-related genes of uterine smooth muscle cells. IGF-I, containing 70 amino acids, is a peptide that has cell differentiation and proliferation functions, and insulin-like metabolism. This peptide has intense mitotic activity in normal and proliferating functions, and insulin-like metabolizing 70 amino acids, is a peptide that has cell differentiation and proliferation functions, and insulin-like metabolism. This peptide has intense mitotic activity in normal and proliferating functions, and insulin-like metabolism. This peptide has intense mitotic activity in normal, actin microfilaments were reduced, mitochondria were less hypertrophied, and hyperplasia was not obvious. These results indicate that Lichong Decoction was effective on uterine leiomyoma, the mechanism of which is related to the reduction of IGF-I and PCNA mRNA expression. Further studies are needed to reveal the molecular mechanisms of Lichong Decoction in uterine leiomyomas.

**REFERENCES**