Guizhi Fuling capsule, an ancient Chinese formula, attenuates endometriosis in rats via induction of apoptosis


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ABSTRACT

Objective The Guizhi Fuling (GZFL) capsule has been a traditional Chinese medicine for the treatment of gynecological inflammation for the past thousands of years. However, as a formula, its therapeutic mechanism has not been clearly elucidated. The aim of this study is to investigate the role of apoptosis during GZFL capsule therapy for the treatment of endometrial hyperplasia.

Methods The rat model of endometriosis was established, and the rats were given different doses of GZFL capsule. Uterine histomorphometric analysis, real-time quantitative PCR (qPCR) and Western blotting were performed. The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP biotin nick end labeling (TUNEL) method was performed to analyze the apoptosis induced by the GZFL capsule.

Results The TUNEL assay showed that different doses of GZFL capsule were able to induce apoptosis in rat endometriotic cells. qPCR and Western blot analysis showed that the GZFL capsule can inhibit the mRNA levels of the survivin gene. In addition, the GZFL capsule can inhibit the mRNA level of the mitochondrial apoptotic pathway-related apoptosis-inhibiting factor Bcl-2 but increases the mRNA level of apoptosis-promoting factor Bax.

Conclusions These results indicate that the GZFL capsule can induce apoptosis of endometriotic cells and inhibit cell proliferation and metastasis of endometriotic cells through the mitochondrial apoptotic pathway.

INTRODUCTION

Endometriosis is a common hormone-dependent benign lesion; its incidence rate is 10–15% in women of child-bearing age. This ratio accounts for more than 30% of the cases of common gynecological surgery and the trend is growing1. Endometriosis is the main reason for dysmenorrhea, infertility, and chronic pelvic pain in women. This disease has a higher relapse rate after drug treatment2.

Apoptosis is a physiologic process that eradicates undesired cells without inducing an inflammatory reaction3. It is an important regulator of eutopic endometrial function and evidence suggests that apoptosis aids in maintaining cellular homeostasis during the menstrual cycle by eliminating aging cells from the functional layer of the uterine endometrium4. Endometriosis, which is characterized by the growth of endometrial tissue outside the uterus, could result from increasing cellular proliferation or decreasing apoptosis in response to appropriate stimuli. The oncoprotein Bcl-2 is a well-known regulator of cellular apoptosis, inhibiting the occurrence of this process5. Apoptotic activity coincides with reduced Bcl-2 activity in the endometrium during the late luteal phase, and persistent Bcl-2 expression is found in endometrial hyperplasia and endometrial cancers, thus indicating that Bcl-2 plays an important role in endometrial hyperplasia.

In addition, survivin is a key inhibitor of apoptosis. Konno and Masatsugu found that the expression level of survivin was higher in endometriotic compared with normal endometrial tissue6. The changes in expression of survivin have no co-relation with the stage in the menstrual cycle or the stage of endometriosis. Survivin is mainly expressed in glandular epithelial cells; it can permit ectopic cells to escape environmental stimuli-induced apoptosis and allow cell proliferation to remain in the mitotic state, which may partly explain the malignancy-like invasion of tumor in endometriosis7. The drugs goserelin and celecoxib could induce apoptosis of
ectopic endometrial cells in vitro; this may indicate the inhibition of survivin expression in ectopic endometrial cells.

Currently, the therapies for endometriosis in Western countries are surgery and hormone treatment. However, the 5-year relapse rate is more than 40%. There is no breakthrough research for treatment in this area. Also, because of the direct treatment of ectopic lesions, it is difficult to improve patients’ fertility. Studies have attempted to reduce macrophage activity and cytokine production in order to eliminate the influence of macrophages on fertility. Studies have attempted to reduce macrophage activity and cytokine production in order to eliminate the influence of macrophages on fertility.

The remaining 70 rats were used for the rat model of endometriosis that was established according to a new protocol by Ping Yang and improved by Ji X. After routine disinfection of each rat’s skin, an approximately 3-cm midline incision was made in the lower abdomen; the two sides of the left uterine horn were ligated, one side at about 1 cm from the proximal part of the left uterine horn and the other side at about 1 cm from the ovary. The excised uterine tissue was placed in sterile saline. The uterus was incised longitudinally and a 3 × 5 mm section of endometrial tissue was clipped. The tunnel between the abdominal muscle and subcutaneous fascia was extruded in order to just fit the uterus piece. Endometrial tissue was neatly placed in the bottom of the right side of the tunnel, the intimal surface was made tightly close to the abdominal muscles, and finally the opening was sutured. After surgery, penicillin was injected for 3 days with 400 000 Units per rat. The rats were fed normally, and 2 days after surgery they were injected with estradiol 0.1 mg/kg/day every 4 days, for a total of three times. This can promote the growth of ectopic endometrium. The rats were checked twice a week after surgery to determine the success of modeling.

After 15 rats were discarded because their size was inconsistent with the other rats, the remaining 55 rats were divided into five groups randomly as follows: model group, Sanjie analgesic group, GZFL capsule low-, middle- and high-dose groups, each group consisting of 11 rats. The rats were given drugs the day following surgery. The GZFL capsule low-, middle- and high-dose groups were given 4.13, 8.26, and 16.52 g/kg GZFL, respectively, and the Sanjie analgesia group was given 0.44 g/kg of Sanjie analgesic capsule (Zhunzi Z20030127) dissolved in 10 ml of water. The sham-operation control group and the model group were given the same volume of distilled water. The drugs were continuously administered for 30 days; the rats were then killed and the ectopic endometrium was rapidly removed for the next stage of analysis.

H&E staining

The rat ectopic endometrium was rapidly fixed in 10% formaldehyde and embedded in paraffin. Sections of 4 μm were mounted for H&E. Characteristics and differences of eutopic and ectopic endometrium and normal rat endometrial tissue morphology were observed under the microscope.

TUNEL assay

Paraffin sections were washed in two changes of xylene for 5 min each, and hydrated with two changes of 100% ethanol for 3 min each, and 95% ethanol for 1 min. They were rinsed in distilled water, incubated with 0.2% Triton X-100 in PBS-Tween for 30 min, rinsed in two changes of PBS-Tween.
20 min at room temperature, rinsed in PBS-Tween 20 for 3 × 2 min; incubated in TdT Reaction Buffer for 10 min, incubated in TdT reaction mixture for 1–2 h at 37–40°C in a humidified chamber, rinsed in stop wash buffer for 10 min, and rinsed in PBS-Tween 20 for 3 × 2 min. Then the sections were incubated with streptavidin-HRP in PBS for 20 min at room temperature, rinsed in PBS-Tween 20 for 3 × 2 min, incubated with DAB for 1–2 min, rinsed in tap water, counterstained with Gill’s hematoxylin for 30 s, rinsed in running tap water for 5 min, dehydrated through 95% ethanol for 5 min, 100% ethanol for 2 × 3 min, cleared in xylene for 2 × 5 min, and placed on a coverslip with xylene-based mounting medium.

Quantitative PCR analysis

Total RNA from the rat ectopic endometrial tissue was prepared using TRizol reagent (Invitrogen, Carlsbad, CA, USA). 1 μg RNA was reversely transcribed into cDNA using the AMV method (Maxima First Strand cDNA Synthesis Kit, Fermentas). qPCR was performed using 2X Maxima SYBR Green/ROX qPCR Master Mix kit with ABI7500. The program was initially 2 min at 95°C, followed by 40 cycles of 30 s at 95°C, and 60 s at 60°C. Data were analyzed by ΔΔCt relative quantification analysis. The primer sequences are given in Table 1.

Western blot analysis

Total protein was extracted and processed for the analysis. After boiling for 5 min in loading buffer, proteins were separated by 8% Tris–glycine gels for Bcl2, survivin, caspase-3 and caspase-9. Western blotting was performed using mouse anti-rat antibody (Abcam Biotech, USA) and goat anti-mouse secondary antibody. Immunocomplexes were visualized by enhanced chemiluminescence (Cell Signaling Technology) according to the manufacturer’s protocol.

Table 1  Sequences of primers

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<tr>
<th>Primer name</th>
<th>Sequences (5′→3′)</th>
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<tr>
<td>Rat Bax-F</td>
<td>AAACCTGGTGCTCAAGGCCCT</td>
</tr>
<tr>
<td>Rat Bax-R</td>
<td>AGCAGCCGCTCAACGGAG</td>
</tr>
<tr>
<td>Rat survivin-F</td>
<td>AAGCACGTCGCGGCGAGTCATT</td>
</tr>
<tr>
<td>Rat survivin-R</td>
<td>CAGGCAGGCCTGGAAAGCCTGG</td>
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<tr>
<td>Rat Bcl-2-F</td>
<td>TCCTTCAAGCCTGAGGAGCAACC</td>
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<tr>
<td>Rat Bcl-2-R</td>
<td>CGACGGTAGCGGACGAGAAGG</td>
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<tr>
<td>Rat β-actin-F</td>
<td>CACCCCGGAGTACAACCTTC</td>
</tr>
<tr>
<td>Rat β-actin-R</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td>Rat caspase-9-R</td>
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Statistical analysis

For statistical analysis, one-way analysis of variance (ANOVA) was used. The significance level was set at p < 0.05, and p-values < 0.01 indicated a strongly significant difference.

RESULTS

Growth of ectopic endometrium

As shown in Figure 1, the volumes of ectopic endometrial tissue in the groups receiving different doses of GZFL were significantly smaller than in the model group. The inhibitory effect of GZFL in these groups was better than in the group receiving the Sanjie analgesic capsule (SJZY) and showed characteristics of dose-dependence. This showed that the GZFL capsules could inhibit the proliferation of the rat endometriotic tissue.

H&E staining of ectopic endometrial tissue

As shown in Figure 2, H&E staining in the normal, control group showed endometrial tissue lined by simple columnar epithelium, rich in glands; the uterine stromal and smooth muscle cells had no obvious abnormalities. In the model group, the graft formed a gland cavity-like structure, and the epithelial cells on the cavity surface began to proliferate; their arrangement was crowded and presented a pseudostratified appearance, rich in interstitial cells. After positive drug (SJZT) processing, the gland cavity shrank inwards and epithelial cells in the cavity surface were significantly reduced compared with the model group. Also, the number of interstitial cells decreased compared with the model group. GZFL intervention had a similar therapeutic effect. After different doses of GZFL, the gland cavity shrank inwards. Epithelial cells on the cavity surface were significantly reduced compared with the model group. Also, the number of interstitial cells decreased compared with the model group and were arranged loosely.

TUNEL assay of ectopic endometrial tissue

TUNEL labeling showed that all endometriotic tissues were scattered with the Tunel-labeled cells (Figure 3), and positive signals were localized in the nucleus, with green fluorescence. Compared with the model group, endometrial tissue with different doses of GZFL and the Sanjie analgesic all showed fluorescence, and the positive cells increased with the increasing dose. This shows that GZFL can achieve an antiproliferative effect, possibly by inducing apoptosis of endometriotic tissue.
Western blot analysis on the translation levels of Bcl2, survivin, caspase-3 and caspase-9 revealed that GZFL could up-regulate caspase-3 and caspase-9 translation levels and showed a dose-dependence (Figure 6). At the same time, Bcl2 and survivin translation levels were significantly down-regulated. These results confirm that GZFL can promote apoptosis of endometriosis tissue cells.

DISCUSSION

Apoptosis is a physiologic process involved in the growth and regression of normal tissues\textsuperscript{17}. It can be induced by extrinsic factors such as chemotherapeutic drugs and radiation. It has been shown previously that the process of apoptosis is involved in the cyclic growth of normal endometrium\textsuperscript{18}. Endometrial hyperplasia is an overgrowth of normal endometrium.
or thickening of the endometrium, which may involve part or all of the endometrium.\(^{19}\)

The Chinese herb, the GZFL capsule, has been used as a traditional Chinese medicine for the treatment of gynecological inflammation over the past thousands of years. However, as a Chinese herbal compound, the composition is extremely complex, and its therapeutic mechanism has not been elucidated.\(^{20}\) In recent years, with modern biotechnology being used in the field of traditional Chinese medicine research, its molecular mechanisms have been gradually revealed. In past research, a

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**Figure 3** (a) TUNEL assay of ectopic endometrial tissue in the different groups (200× magnification); (b) quantitative fluorescence intensity of different groups (\(* *, p < 0.01\)). Model, group receiving distilled water only; SJZT, Sanjie analgesic group; GZFL(L), GZFL low-dose group; GZFL(M), GZFL medium-dose group; GZFL(H), GZFL high-dose group

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**Figure 4** Quantitative PCR detection of mRNA level changes in apoptosis-related genes Bcl-2, Bax and survivin after different doses of administration (\(p < 0.05\)). Model, group receiving distilled water only; SJZT, Sanjie analgesic group; GZFL(L), GZFL low-dose group; GZFL(M), GZFL medium-dose group; GZFL(H), GZFL high-dose group
Study confirmed that the GZFL capsule significantly lowered monocyte chemoattractant protein (MCP-1) and cell adhesion molecules (ICAM-1), and at the same time raised the activity of CD4+ cells and NK cells, thus inhibiting the proliferation and invasion of the endometrium14. However, its role in apoptosis has not yet been studied.

Our studies in rat uteri demonstrated that a physiological dose of GZFL capsule given for 1 month caused serious apoptosis of the uterine endothelial tissue cells, whereas it prevented the anti-apoptotic effect of uterine endothelial cells. In the model group, uterine H&E staining clearly showed the histological changes in comparison with the control group. Interestingly, all these changes were reversed when the GZFL capsule was administered. In addition, the expression of apoptotic genes such as caspase-3 and caspase-9 were up-regulated under the influence of the GZFL capsule both at gene transcription level and at expression level (Figures 4–6), thereby inducing the apoptosis of uterine endothelial cells.

In this study, we also found that different doses of the GZFL capsule were able to induce apoptosis of rat endometrial cells using the TUNEL assay. In addition, qPCR experiments show that the GZFL capsule can inhibit the mRNA levels of the survivin gene. The survivin gene is an inhibitor of apoptosis which is highly expressed in the endometrium21. Down-regulation of the expression of anti-apoptotic Bcl-2 and the up-regulation of pro-apoptotic Bax indicate that the Bax, Bcl-2, and caspase proteases are involved in regulating GZFL capsule-induced apoptosis.

In summary, this study proves that the GZFL capsule could induce apoptosis of endometrial cells through the inhibition of survivin and Bcl-2 and could promote the expression of Bax. Our results suggest that the GZFL capsule is a potent apoptosis inducer in the uterus and can be used as a therapeutic agent for endometrial hyperplasia.

Conflict of interest The authors report no conflict of interest. The authors alone are responsible for the content and writing of this paper.

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References
