Preventive effects of glycyrrhizin on estrogen-related endometrial carcinogenesis in mice

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Abstract. We have previously reported on the inhibitory effect of *Glycyrrhizae radix (Gl radix)* on mouse endometrial carcinogenesis. The present study was performed to clarify the effects of *Gl radix* and glycyrrhizin (GL), the main part of *Gl radix*, on estradiol (E_2)-related endometrial carcinogenesis. Both *Gl radix* and GL exerted a significant decrease in the COX-2, IL-1α and TNF-α mRNA expressions. GL generated a significant decrease in the incidence of endometrial adenocarcinoma. Accordingly, the preventive effects of *Gl radix* may be attributable to GL, thus being related with the suppression of COX-2, IL-1α and TNF-α. *Gl radix* and GL could therefore be a promising formula for the chemoprevention of human endometrial cancer.

Introduction

Glycyrrhizin (GL), a major constituent of *Glycyrrhizae radix (Gl radix)* which is ~10% of the dry weight of *Gl radix* extract, is the calcium and potassium salt of glycyrrhizinic acid. GL glycoside loses its sweet taste and upon hydrolysis is then converted to aglycone glycyrrhetinic acid plus two molecules of glucuronic acid. GL has an anti-estrogenic as well as an estrogenic effect (1). There is some evidence that GL has a protective effect on the skin (2,3) or liver carcinogenesis (4,5).

It is known that *Gl radix* is widely used as a Kampo medicine in Asian countries and ~75% of traditional Japanese and Chinese medicines contain this agent. We have previously reported that *Gl radix* has a preventive effect on endometrial carcinogenesis in mice, and this effect is related to the inhibition of the expression of estrogen-stimulated genes *c-fos/jun* mRNA and proteins (6). We also proved that the herbal complex containing *Gl radix* suppresses cell proliferation in a chemo-endocrine resistant cancer cell line (7). In general, the anti-estrogenic effects are considered to have a protective effect against the occurrence of estrogen-dependent cancers. Such effects are confirmed in animal models of uterine endometrial cancer (8).

Cyclooxygenase (COX), an enzyme that activates the production of prostaglandins from arachidonic acid, has two isoforms. COX-2 has attracted considerable attention in connextion with carcinogenesis in organs such as the large bowel, mammary glands and breast carcinogenesis (9-11). There is evidence that the COX activity is related to the promotion and progression in the tumorigenesis of the prostatic gland, stomach and lung (12-14). It remains unclear whether COX-2 is involved in E_2 related mouse endometrial carcinogenesis, although a high expression level of COX-2 has been confirmed in human endometrial carcinomas (15). In this study, we examined the possible association of the preventive effects of GL on E_2-induced mouse endometrial carcinogenesis with COX-2 activity.

We have reported that the expression of internal cytokines, interleukin (IL)-1α and tumor necrosis factor (TNF)-α, increase after E_2 treatment (16). Such an increase of the internal cytokines is suggested to contribute to both tumor promotion and progression (17-19). Such circumstances prompted us to clarify the effects of GL on endometrial carcinogenesis in mice. Therefore, the expression of IL-1α and TNF-α were examined to indentify any further mechanism(s) of the effects of *Gl radix* and GL in the uteri of ovarectomized mice.

Materials and methods

Animals and chemicals. Female ICR mice were purchased from Japan SLC Co. (Shizuoka, Japan). As a basal diet, Oriental MF (Oriental Yeast Co., Tokyo, Japan) was used. Both the basal diet and filtered tap water were available *ad libitum* throughout the experiment. E_2 and GL were purchased from Sigma Chemical Co. (St. Louis, MO), and

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Abbreviations: GL, glycyrrhizin; E_2, estradiol-17ß; COX, cyclooxygenase, IL-1α, interleukin-1α; TNF, tumor necrosis factor

Key words: glycyrrhizin, *Glycyrrhizae radix*, chemoprevention, endometrial carcinogenesis, COX-2

Table I. Sequences of primers.

Primers	Nucleotide sequences	Citation
COX-1 sense	5'-TGCATGTGGCTGTGGATGTCATCAA-3'	21
COX-1 antisense	5'-CACTAAGACAGACCCGTCATCTCCA-3'	
COX-2 sense	5'-ACTCACTCAGTTTGTTGAGTCATTC-3'	21
COX-2 antisense	5'-TTTGATTAGTACTGTAGGGTTAATG-3'	
IL-lα sense	5'-GATGGCCAAAGTTCCTGACTTG-3'	22
IL-lα antisense	5'-GCCTGACGAGCTTCATCA-3'	
TNF-α sense	5'-AGGCAGGTTCTGTCCCTTTCA-3'	23
TNF-α antisense	5'-TCCACTTGGTGGTTTGCTACG-3'	
GAPDH sense	5'-CAAGGTCATCCCAGAGCTGAA-3'	24
GAPDH antisense	5'-GCAATGCCAGCCCCGGCATCG-3'	

Gl radix (powder of the crude extract) was purchased from Tsumura Co. (Tokyo, Japan), respectively.

Experimental protocol for short-term experiment. Female (12 weeks old) ICR mice were ovariectomized at laparotomy under general anesthesia with diethylether. Two weeks later, the ovariectomized mice were divided into 6 experimental groups (6 mice in each). Group 1 was given daily 0.625% *Gl radix* and 5 ppm E_2-containing diet; Group 2 was fed with 0.0625% of GL and 5 ppm E_2; Group 3 was exposed to 5 ppm E_2-containing diet alone; Group 4 was given daily 0.625% *Gl radix* alone; Group 5 was fed 0.0625% GL alone. Group 6 was given the basal diet only as a control. The dose of 0.625% *Gl radix* in the diet has been proven to be sufficient to inhibit the estrogenic action of 5 ppm E_2 (1,2), and the concentration, at the GL diet was calculated to be 10% of the 0.625% *Gl radix*. After two weeks feeding with the diet containing the above agents, the mice uteri were resected and cut in half longitudinally. One half was quickly frozen in the liquid nitrogen for the following experiments, while the other specimen was submitted to a pathological examination.

Reverse transcription-polymerase chain reaction (RT-PCR). Total-RNA was isolated from frozen tissue specimens by the guanidium thiocyanate-phenol-chloroform extraction method (20). Total RNA (3 μg) was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (MMLV-RTase, 200 units, Gibco BRL, Gaithersburg, MO) in 20 μM Tris-HCl buffer (pH 8.4) with 50 μM KCl, 2.5 μM $MgCl_2$, 0.1 μg/ml bovine serum albumin, 10 μM dithiothreitol, and 0.5 μM deoxynucleotides to generate cDNAs, using random hexamers (50 ng, Gibco BRL) at 37˚C for 60 min. RT reaction was heated at 94˚C for 5 min to inactivate MMLV-RTase. For COX-1 (450 bp), extension of 35 cycles was performed consisting of 1 min at 94˚C for denaturation, 1 min at 57˚C for annealing, and for COX-2 (583 bp) of PCR consisting of 1 min at 72˚C 15 sec at 94˚C for denaturation, 1 min at 55˚C for annealing, and 1 min at 72˚C for extension. PCR (30 or 25 cycles), consisting of 1 min at 94˚C for denaturation, 1 min at 55˚C for annealing, and 1.5 min at 72˚C for extension, for TNF-α (369 bp) and IL-1α (401 bp). They were carried out in reverse transcribed cDNAs with 0.1 mM specific primers described below, using the IWAKI thermal sequencer TSR-300 (IWAKI Glass, Tokyo) with Vent DNA polymerase (New England Biolabs, Beverley, MA) in 20 μM Tris-HCl buffer (pH 8.8) with 10 μM KCl, 10 μM $(NH_4)_2SO_4$, 2 μM $MgSO_4$, 0.1% Triton X-100, and 0.15 μM deoxynucleotide phosphates. PCR (20 cycles) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, a house-keeping gene) mRNA (252 bp) as an internal standard was performed at the same time.

The following oligodeoxynucleotides were synthesized as specific primers in PCR according to the published information [cDNA for COX-1/2 (21), IL-1α (22) TNF-α (23) and GAPDH (24)] as shown in Table I.

Semi-quantitative analysis of the COX-1, COX-2, IL-1α and TNF-α mRNA expressions by PCR products. PCR products were applied on 1.5% agarose gel electrophoresis at 50-100 V. The quantification of the products was carried out using Bio image (Millipore Corp.). The intensity of specific bands was standardized with that of GAPDH mRNA.

Immunohistochemical expression of COX-1/2, IL-1α and TNF-α protein. After being fixed in 10% formalin, half the uterine corpus was processed by conventional staining methods. Briefly, the avidin-biotin-peroxidase complex was applied on the sections using a Vestain kit (Vector, Burlingame, CA). The primary antibodies used were against the proteins of COX-1 (1:250, anti-mouse monoclonal, Cayman Chemial, Ann Arbor, MI) and COX-2 (1:200, anti-mouse monoclonal, Alexis Biochem., Carlsbad, CA). IL-1α (1:200, anti-rabbit polyclonal, Santa Cruz Biotech Inc., Santa Cruz, CA) and TNF-α (1:100, anti-goat polyclonal, Santa Cruz Biotech Inc.).

Immunohistochemical COX-1/2 expressions in glandular and stromal cells were scored separately according to the criteria of Krajewska *et al* (25). The scoring methods were modified by Fujiwaki *et al* (26). Namely, the percentage of COX-1 and -2 immunostaining in the glandular and stromal cells were graded as follows: 0, no staining; 1, 1-25%; 2, 26-50%; 3, 51-75%; and 4, 76-100%. The intensity of immunostaining was rated as: 0, none; 1, weak; 2, moderate; 3, intense. As a result, the immunohistochemical COX scores

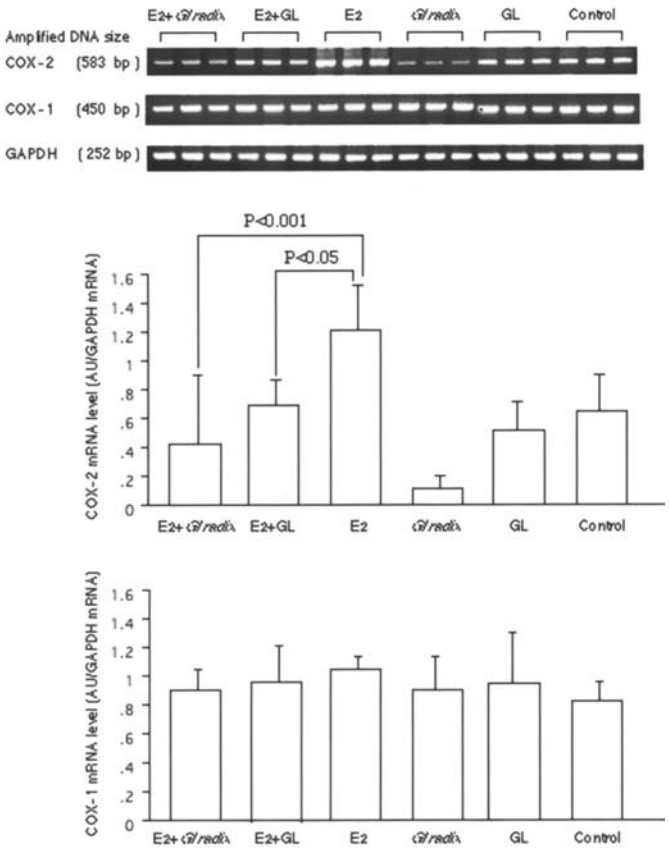

Figure 1. Expression of COX-1 and COX-2 mRNA in the uterus of the ovariectomized mice, treated continuously for two weeks with E_2 plus *Gl radix* or GL, E_2, and *Gl radix* or GL alone. *Gl radix*, *Glycyrrhizae radix*; GL, Glycyrrhizin. The COX-2 mRNA in the group treated with E_2 plus *Gl radix* or GL was significantly lower than that in the group treated with E_2 alone ($P<0.05$).

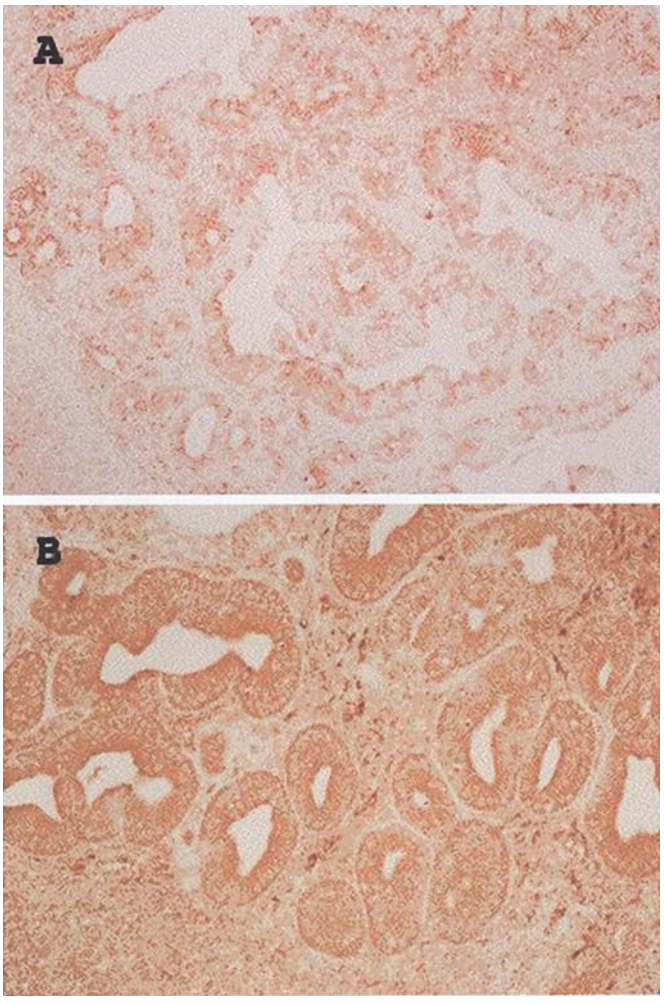

Figure 2. The expression of COX-2 in the uteri of ovariectomized mice treated orally for 2 weeks with E_2 and *Gl radix* to resection of the uteri (A) (sABC stain, x350), and with E_2 alone (B) (sABC stain, x350). The expression with E_2 and *Gl radix* was also prominent in the glandular cells, and it was weaker than that in the case of E_2 alone.

ranged from 0 to 12 (26). The immunohistochemical findings were analyzed by two independent investigators counting >200 cells and discordant results were reviewed jointly.

The staining intensity for IL-1α and TNF-α protein was assigned as: (+), positive; (+/-), minimally or randomly positive; (-), negative (27).

Experimental protocol for long-term effects of GL. The protocol was: A total number of 125 female ICR mice, 12 weeks of age, underwent a laparotomy under general anesthesia with diethylether. MNU solution (total volume: 0.1 ml) at a dose of 1 mg/100 g body weight was injected into the left uterine tube and normal saline into the right. One week after MNU exposure, the animals were divided into 6 groups. Group 1 (35 mice) was given the diet with 0.0625% GL and 5 ppm E_2. Group 2 (30 mice) was given the 5 ppm E_2-containing diet alone. Group 3 (30 mice) was given the diet with 0.0625% GL. Group 4 (30 mice) was treated with the basal diet only as a control. At 30 weeks after the MNU exposure, all animals were sacrificed and autopsied. All major organs, especially the reproductive organs, were grossly inspected.

Any lesions in the uterus, ovaries, vagina suspected of being neoplastic and hyperplastic were cut in half. The tissue specimens were submitted to a histopathological examination. The tissue specimens were then cut in 3-μm slices and then were stained with hematoxylin and eosin.

Histology of the uterine lesion. According to the WHO criteria (28), uterine endometrial lesions were divided into 4 types of lesions: a) endometrial hyperplasia, simple; b) endometrial hyperplasia, complex; c) atypical endometrial hyperplasia; d) adenocarcinoma.

Statistical analysis. A statistical analysis was done according to either the χ^2 test or Student's t-test.

Results

Short-term experiment. The expression of COX-1/2 mRNAs is shown in Fig. 1. The level of COX-1 mRNA expression did not change after any treatment, however, the COX-2 mRNA expression which had been overexpressed in the E_2 group significantly decreased after either the *Gl radix* or GL treatment ($P<0.001$, $P<0.05$, respectively). The results of the protein expression are summarized in Table II. The immunohistochemical score significantly decreased after treatment with either *Gl radix* or GL ($P<0.05$). The representative expression pattern of COX-2 protein is indicated in Fig. 2A and B. The

Table II. Immunohistochemical expression of COX-1/2 in the ovarectomized mouse uteri.

Group	Treatment	COX-1		COX-2	
		Glandular cells	Stromal cell	Glandular cells	Stromal cells
1	E_2 + Gl radix	2.5±0.8[a]	2.0±0.7	3.0±1.7[b]	4.6±1.2
2	E_2 + GL	3.0±0.4	1.8±0.5	3.7±1.5[b]	5.0±1.0
3	E_2 alone	3.5±0.6	2.0±0.2	7.6±2.1	6.0±2.0
4	Gl radix alone	2.1±0.7	1.1±0.3	4.6±1.2	0.8±0.2
5	GL alone	1.7±0.2	1.0±0.1	4.3±1.5	1.3±0.6
6	Control	1.5±0.2	1.2±0.2	5.0±1.7	3.3±1.5

[a]Mean ± SD. [b]Significantly different from E_2 group (P<0.05).

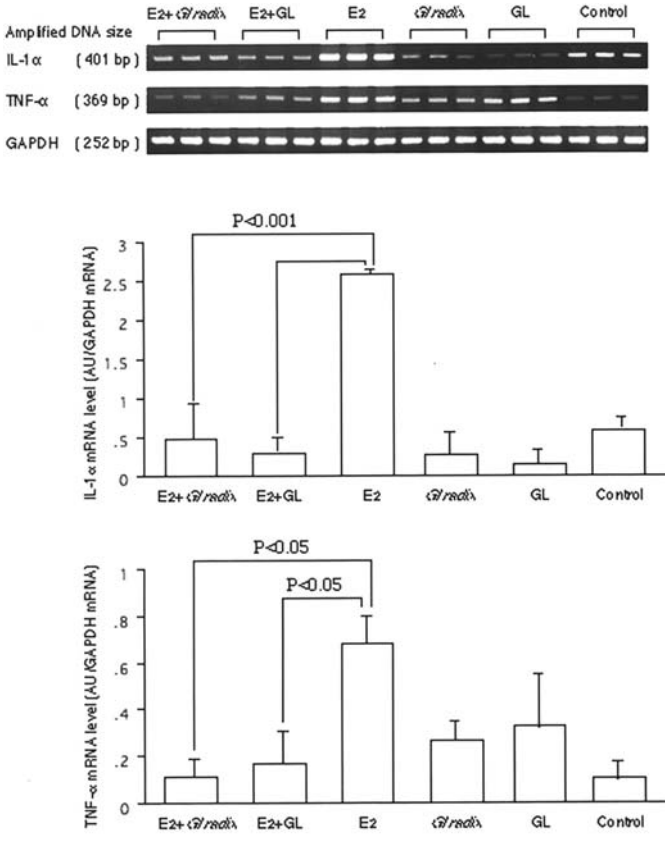

Figure 3. Expression of IL-1α and TNF-α mRNA in the uterus of ovarectomized mice, treated continuously for two weeks with E_2 plus *Gl radix* or GL, E_2, and or GL alone. *Gl radix*, *Glycyrrhizae radix*; GL, Glycyrrhizin.

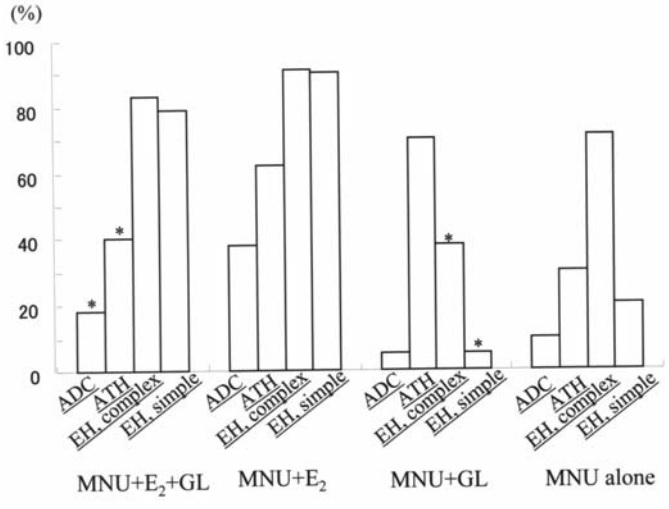

Figure 4. The incidence of neoplastic and preneoplastic endometrial lesions in each group. *P<0.05 compared with the control group.

protein expression decreased after the E_2 plus *Gl radix* treatment (Fig. 2A) in comparison to that of the E_2 group (Fig. 2B).

The expression of IL-1α and TNF-α mRNAs is shown in Fig. 3. *Gl radix* or GL exposure significantly decreased the level of IL-1α mRNA induced by the E_2 treatment (P<0.001). *Gl radix* or GL also decreased the expression of TNF-α mRNA generated by E_2 treatment (P<0.05). The results of the immunohistochemical analysis are summarized in Table III.

The protein expressions of IL-1α and TNF-α demonstrated a similar pattern to the mRNAs in the same groups.

Long-term experiment for GL. The mean body weight and weight of the uterine corpora are summarized in Table IV. The mean wet weights of the uterine corpus in the groups treated with either *Gl radix* or GL were significantly less than in the group not treated by either *Gl radix* or GL. The incidence of neoplastic and preneoplastic endometrial lesions is summarized in Fig. 4. The incidence of endometrial adenocarcinoma and atypical endometrial hyperplasia in the GL plus E_2-treated group was significantly lower than that of the E_2-treated group.

Discussion

We previously demonstrated that *Gl radix* has an inhibitory effect on E_2-related endometrial carcinogenesis in mice (6). In the present study, the exposure to GL decreased the uterine weight in the long-term experiment, thus suggesting that GL has some anti-estrogenic effects at the dose used in this experiment. In the long-term experiment, the incidences of

Table III. Immunohistochemical expression of IL-1α and TNF-α in the ovarectomized mouse uteri.

Group	Treatment	IL-lα		TNF-α	
		Glandular cells	Stromal cells	Glandular cells	Stromal cells
1	E_2 + Gl radix	+	±	+	±
2	E_2 + GL	+	±	±	±
3	E_2 alone	++	+	++	+
4	Gl radix alone	±	±	±	±
5	GL alone	±	±	±	±
6	Control	±	±	±	±

(++), strongly positive; (+), positive; (±), minimally or randomly positive.

Table IV. Mean body weight and mean left uterine corpora in each group.

Group	Treatment	Initial number of animals	Effective number of animals	Body weight (g)	Wet weight of uterine corpora (g)
1	MNU + E_2 + GL	35	32	44.3±6.2[a]	0.21±0.19
2	MNU + E_2 alone	30	25	43.1±3.0	0.65±0.20[b]
3	MNU + GL alone	30	27	36.9±4.1	0.17±0.10
4	MNU alone	30	27	48.0±6.0	0.46±0.31[b]

[a]Mean ± SD. [b]Significantly different from the corresponding group ($P<0.05$).

adenocarcinoma and atypical hyperplasia of the group treated with E_2 and *Gl radix* were significantly lower than those of the group treated with E_2 alone (6). A similar tendency was confirmed by GL treatment. Since atypical hyperplasia is considered to be a direct precursor of endometrial adenocarcinoma (29), the decreased expression of atypical hyperplasia was suggested to support the chemopreventive effects of these agents in the endometrial carcinogenesis.

It has been reported that *Gl radix* contains not only GL but also isoflavones such as liquiritin, licoricone, licoflavone and formononetin (30). Isoflavones are known to act as chemopreventive agents for carcinomas including endometrial carcinoma (16). As summarized in Fig. 4, the incidence of adenocarcinoma in the *Gl radix* plus E_2 group was lower than that in the GL plus E_2 group (11.8 and 19.4%). The effectiveness for the preventive effect of *Gl radix* may therefore be related to the presence of other isoflavones in *Gl radix*.

Clinical studies have shown that the COX-2 expression is associated with the estrous cycle in the uteri (31). In the present study, the COX-2 mRNA expression significantly increased after E_2 stimulation, and COX-2 mRNA was overexpressed in adenocarcinoma and atypical endometrial hyperplasia in the mouse uterus, thus suggesting the overexpression of COX-2 to be related to E_2-induced endometrial carcinogenesis in mice. It is therefore suggested that the suppression of the overexpression of COX-2 is an effective strategy for endometrital cancer prevention.

In this study, GL and *Gl radix* suppressed expression of COX-2 as well as the internal cytokines, IL-1α and TNF-α expression in the mouse uterine corpora. Meanwhile, it is known that TNF-α and IL-1α also play a significant role in both human and rodent carcinogenesis (16,32). TNF-α has also been reported to stimulate tumor promotion and the progression of initiated cells and premalignant cells in mice (33). Therefore, the inhibition of TNF-α and IL-1α mRNA expression is considered to contribute to cancer prevention (16,32,33).

In summary, we report herein that *Gl radix* is an efficient preventive agent for endometrial carcinogenesis, and that GL acts as an important constituent in *Gl radix*, for the suppression of the carcinogenesis related to the suppression of COX-2, TNF-α and IL-1α expression.

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