Preventive Effect of Juzen-taiho-to on Endometrial Carcinogenesis in Mice Is Based on Shimotsu-to Constituent

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Juzen-taiho-to, a Kampo formula, originally consists of a mixture of Shimotsu-to and Shikunshi-to formulas together with two other crude ingredients. Juzen-taiho-to is reported to have a preventive effect on endometrial carcinogenesis in mice. Shimotsu-to exerts an inhibitory effect on estrogen-induced expression of c-fos, interleukin (IL)-1α and tumor necrosis factor (TNF)-α in uteri of ovariectomized mice. In the present study, short- and long-term experiments were designed to determine the effects of Juzen-taiho-to and Shimotsu-to on the estrogen-related endometrial carcinogenesis in mouse uteri, associated with the expression of cyclooxygenase (COX)-1 and -2. In the short-term experiment, exposure to Juzen-taiho-to or Shimotsu-to significantly reduced estradiol-17β (E2)-stimulated expressions of COX-2 mRNA (p<0.05) as well as the protein. However, no effects on the expression of COX-1 were observed. Shikunshi-to did not affect COX expression. In the long-term experiment, 90 female ICR mice were given N-methyl-N-nitrosourea (MNU) into their uterine corpora. The animals were divided into four groups as follows: group 1, a diet containing 0.07% Shimotsu-to and 5 ppm E2; group 2, a diet containing 5 ppm E2; group 3, a diet containing 0.07% Shimotsu-to; group 4 served as a control. Exposure of Shimotsu-to reduced the incidence of MNU- and E2-induced endometrial adenocarcinoma and atypical hyperplasia at the termination of the experiment (30 weeks). The above findings and our previous reports suggest that Shimotsu-to is responsible for the preventive effects of Juzen-taiho-to on estrogen-related endometrial carcinogenesis in mice, through the inhibition of estrogen-related COX-2 as well as c-fos, IL-1α and TNF-α expressions.

Key words endometrial carcinogenesis; prevention; Shimotsu-to; Juzen-taiho-to; cyclooxygenase-2; mouse

Juzen-taiho-to is a tonic formula in Kampo Medicine, mainly consisting of Shimotsu-to and Shikunshi-to (Table 1). Juzen-taiho-to is reported to reduce metastatic potential and to enhance cytokine induction, production of antibody or anti-tumor activity. Shimotsu-to is composed of four crude herbs (Rehmanniae radix, Paeoniae radix, Cnidii rhizoma, and Angelicae radix in Table 1), possessing the ability to improve deficiencies of ‘Ketsu’ (a concept referring to blood), hormones, the autonomic nervous system and other regulatory functions of the body’s internal environment. In Japan, Shimotsu-to is indicated to be used for poor blood circulation and irregular menstruation, etc. Meanwhile, Shikunshi-to is also composed of four crude herbs (Ginseng radix, Atractylodis radix, Astilbe radix, and Glycyrrhizae radix in Table 1), and used for improving a depression of ‘KI’ (a concept that encompasses mental nervous function), especially the appetite for food and the actual process of digesting and absorbing nutrients. In Japan, Shikunshi-to is indicated to be used for gastro-intestinal weakness and chronic gastritis, etc.

The transient expression of the immediate early gene, c-fos/jun, is considered to be necessary for cellular proliferation and differentiation. c-Fos/jun mRNA in the uterine corpora of the ovarectomized mice is overexpressed by estrogen. In addition, internal cytokines, such as interleukin (IL)-1α or tumor necrosis factor (TNF)-α contribute to tumor promotion or progression in chemical carcinogenesis. Previously, we reported that Juzen-taiho-to has an inhibitory effect on estradiol-17β (E2)-related endometrial carcinogenesis in mice. More recently, we have also reported that Shimotsu-to is the main part of Juzen-taiho-to for the inhibitory effects in E2-induced expression of c-fos, IL-1α and TNF-α mRNA and their proteins in the uterine corpora of the ovarectomized mice. Meanwhile, it is known that cyclooxygenase (COX), an enzyme activating production of prostaglandins from arachidonic acid, has two isoforms (1 and 2). The function of inducible COX-2 is associated with carcinogenesis in the large bowel, prostatic gland, stomach, breast and other organs. In addition, the expression of COX-2 is noted to increase in human endometrial carcinoma. These circumstances prompted us to determine if Shimotsu-to exerts inhibitory effects on mouse endometrial carcinogenesis induced by MNU and E2. Furthermore, the effects of Juzen-taiho-to and Shimotsu-to on the expression of COX-1 and -2 mRNAs and their proteins were investigated.

MATERIALS AND METHODS

Animals and Chemicals Female ICR mice, 10 weeks of age, were purchased from Japan SLC Co. (Shizuoka). The base diet (Oriental MF, Oriental Yeast Co., Tokyo) and filtered tap water were available ad libitum throughout the experiment. E2 and MNU were purchased from Sigma Chem Co. (St. Louis, MO, U.S.A.). Juzen-taiho-to, Shimotsu-to and Shikunshi-to were purchased from Tsumura Co. (Tokyo). The ingredients of Juzen-taiho-to, Shimotsu-to and Shikunshi-to are shown in Table 1. Juzen-taiho-to was prepared as follows. A mixture of Angelicae radix (3.0 g), Cnidii rhizoma (3.0 g), Paeoniae radix (3.0 g), Rehmanniae radix (3.0 g), Ginseng radix (3.0 g), Atractylodis radix (3.0 g), Astilbe radix (3.0 g), and Glycyrrhizae radix (1.5 g), Astragalus radix (3.0 g), and Cinnamomi cortex (3.0 g) was added to 285 ml of water and a solution extracted at 100°C for 1 h. The extracted solution was filtered, and the filtrate was spray-dried to obtain the
Dry extract powder (5.0 g). Shimotsu-to and Shikunshi-to were prepared in the same way as Juzen-taiho-to, and 2.5 g dry powder was obtained from crude ingredients, respectively (Table 1).

**Experimental Protocol for Short-Term Effects of Kampo Formula** Female ICR mice, 12 weeks-of-age, were ovariectomized under general anesthesia with diethylether. Two weeks later, the ovariectomized mice were divided into five groups. Group 1 was given a diet containing 0.2% Juzen-taiho-to and 5 ppm E2 (n = 5). The dose of Juzen-taiho-to in the diet (0.2%) was equivalent to the clinical dose (7.5 g/50 kg daily); group 2 was given 0.07% Shimotsu-to and 5 ppm E2 (n = 5), and 0.07% Shimotsu-to was equal to the weight ratio of Juzen-taiho-to; group 3 was fed on a diet containing 0.08% Shikunshi-to and 5 ppm E2 (n = 5), and 0.08% Shikunshi-to was equal to the weight ratio of Juzen-taiho-to; group 4 was fed on a diet containing 5 ppm E2 alone (n = 5), and 0.07% Shimotsu-to was equal to the weight ratio of Juzen-taiho-to; group 5 served as a non-treatment control (n = 5). Five weeks after the start of the experiment, all mice were subjected to pathological examinations. The weight of each uterine corpus was cut in half longitudinally. One half was quickly frozen in liquid nitrogen for the following experiments, and the other was subjected to pathological examinations.

**Reverse Transcriptase-PCR (RT-PCR)** Total RNA was isolated from frozen tissues by a guanidium thiocyanate–phenol–chloroform extraction method. Total RNA (3 μg) was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (MMLV–RTase, 200 units, Gibco BRL, Gaithersburg, MD, U.S.A.) in 20 μl Tris–HCl buffer (PH 8.4) with 50 μM KCl, 2.5 μM MgCl2, 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 carried out at 94 °C for 5 min to inactivate MMLV–RTase. For COX-2 (583 bp), treatment included 35 cycles of PCR consisting of 15 s at 94 °C for denaturation, 1 min at 55 °C for annealing, and 1 min at 72 °C for extension. For COX-1, treatment included 35 cycles of 1 min at 94 °C for denaturation, 1 min at 57 °C for annealing, and 1 min at 72 °C for extension. These processes were carried out in reverse transcribed cDNAs with the 0.1 μM specific primers described below, using an IWIKA thermal sequencer TSR-300 (IWIKA Glass, Tokyo) with Vent DNA polymerase (New England Biolabs, Beverly, MA, U.S.A.) in 20 μl Tris–HCl buffer (PH 8.8) with 10 μM KCl, 10 μM (NH4)2SO4, 2 μM MgSO4, 0.1% Triton X-100, and 0.15 μM deoxynucleotide phosphates. Twenty cycles of PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, a house-keeping gene) mRNA (252 bp) as an internal standard were performed at the same time.

The following oligodeoxynucleotides were synthesized as specific primers in PCR according to the published information [cDNA for COX-1, 28) and GAPDH29] as shown in Table 2.

**Semi-quantitative Analysis of COX-1 and COX-2 mRNA Expression in the Mice Uterine Corpus 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 with Bio image (Nihon Millipore Corp., Tokyo). The intensity of specific bands was standardized with that of GAPDH mRNA.

**Experimental Protocol for Long-Term Effects of Kampo Formulas** A total of 90 female ICR mice, 10 weeks of age, underwent 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 the exposure to MNU, the animals were divided into the following four experimental groups. Group 1 (20 mice) was given a diet containing 0.07% Shimotsu-to and 5 ppm E2. Group 2 (30 mice) was treated with 5 ppm E2 alone. Group 3 (10 mice) were fed with a diet containing 0.07% Shimotsu-to. The doses of E2 and Kampo formulas were the same as in the short-term assay, and the above diets were given throughout the experiment. Group 4 (30 mice) served as a control group. Thirty weeks after the start of the experiment, all major organs, especially the reproductive organs, were grossly inspected. The uterus, ovaries, vagina, and other lesions suspected of being hyperplastic or neoplastic were cut in half. One half was quickly frozen in liquid nitrogen for the following experiments, and the other was subjected to pathological examinations. The weight of each uterine corpus was

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**Table 1. The Ingredients and Botanical Origins of Juzen-taiho-to Formulas**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Botanical origin</th>
<th>Representative compounds</th>
<th>Weight ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shimotsu-to</td>
<td>Angelicae acutiloba</td>
<td>Ligustilide</td>
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<tr>
<td>Cnidii rhizoma</td>
<td>Cnidium officinale</td>
<td>Cnidilide, ligustilide</td>
<td>3.0</td>
</tr>
<tr>
<td>Paoniae radix</td>
<td>Paonia lactiflora</td>
<td>Paconflorin, paconol</td>
<td>3.0</td>
</tr>
<tr>
<td>Rehmanniae radix</td>
<td>Rehmannia glutinosa</td>
<td>Acteoside</td>
<td>3.0</td>
</tr>
<tr>
<td>Shikunshi-to</td>
<td>Panax gingseng</td>
<td>Ginsenoside Ro</td>
<td>3.0</td>
</tr>
<tr>
<td>Atractylodes japonica</td>
<td>Sclerotium of Poria cocos</td>
<td>Erurico acid</td>
<td>3.0</td>
</tr>
<tr>
<td>Holen</td>
<td>Wolf (Poylarpaceae)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycyrrhiza uralesis</td>
<td>Glycyrrhizin, formononetin</td>
<td></td>
<td>3.0</td>
</tr>
<tr>
<td>Others</td>
<td>Astragalus membraneceus</td>
<td>Formononetin</td>
<td>3.0</td>
</tr>
<tr>
<td>Cinnamomum cassia</td>
<td>Cinnamic acid</td>
<td>Cinnamic acid</td>
<td>3.0</td>
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</table>

This table is cited from refs. 1, 7 and is modified. a) Weight ratio was mentioned in Materials and Methods.

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**Table 2. DNA Sequences for Specific Primers**

<table>
<thead>
<tr>
<th>Primers</th>
<th>DNA sequences</th>
<th>Citation</th>
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</thead>
<tbody>
<tr>
<td>COX-1</td>
<td>Sense 5'-TGCATGTGCGTGGATGTACATCAA-3'</td>
<td>28)</td>
</tr>
<tr>
<td></td>
<td>Anti-sense 5'-CATAAGACAGACCCCTTCACTCCA-3'</td>
<td></td>
</tr>
<tr>
<td>COX-2</td>
<td>Sense 5'-ACTCCTCAGTTGTTAGTTCATTC-3'</td>
<td>28)</td>
</tr>
<tr>
<td></td>
<td>Anti-sense 5'-TTTGATAGCTACAGTAGGTTAATG-3'</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense 5'-CAAGGTGCATCCCAAGAGCTGAAA-3'</td>
<td>29)</td>
</tr>
<tr>
<td></td>
<td>Anti-sense 5'-GAATGCGACCCGCCGCACTCG-3'</td>
<td></td>
</tr>
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</table>
determined. After being fixed in 10% formalin, tissues were sectioned at 3 \( \mu \text{m} \) and stained with hematoxyline and eosin.

**Immunohistochemical Expression of COX-1 and COX-2 Proteins** After being fixed in 10% formalin, half the uterine corpus was processed for the conventional staining. Briefly, the avidin–biotin–peroxidase complex was applied to the sections using a Vestain kit (Vector, Burlingame, CA, U.S.A.). The primary antibodies used were against the proteins of COX-1 (1:250, anti-mouse monoclonal, Cayman Chemial, Ann Arbor, MI, U.S.A.) and COX-2 (1:200, anti-mouse monoclonal, Alexis Biochem., Carlsbad, CA, U.S.A.).

Immunohistochemical COX-1 and -2 expressions in glandular and stromal cells were basically scored separately according to the criteria of Krajewska et al. \(^{30}\) The scoring methods were modified by Fujiwaki et al. \(^{31}\) 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, 51—75%; 3, 50—75%; and 4, 76—100%. Intensity of immunostaining was rated as follows: 0, none; 1, weak; 2, moderate; 3, intense. Thus, the immunohistochemical COX scores were ranged from 0 to 12. \(^{31}\)

**Histology of the Uterine Lesions** Uterine endometrial lesions were divided into 4 lesions according to the WHO criteria. \(^{32}\) a) endometrial hyperplasia, simple; b) endometrial hyperplasia, complex; c) atypical endometrial hyperplasia; d) adenocarcinoma.

**Semi-quantitative Analysis of COX-1 and COX-2 mRNA Expression of the Hyperplastic and Neoplastic Endometrial Lesions** The frozen tissues of group 2 (treated with MNU and E\(_2\)) in the long-experiment were served for evaluations for COX-1, -2 mRNA expressions. The examination methods were same as the short-term experiments. Adenocarcinoma (\(n=5\)), atypical hyperplasia (\(n=4\)), endometrial hyperplasia, complex (\(n=5\)), simple (\(n=5\)) and the hitopathologically normal uterine corpus were examined.

**Statistical Analysis** Statistical analysis was done according to the \( \chi^2 \) test or Student’s \( t \) test.

**RESULTS**

**Short-Term Experiment** The levels of COX-1 and -2 mRNA expression are shown in Fig. 1. Juzen-taiho-to or Shimotsu-to treatment significantly reduced the \( E_2 \)-induced expression of COX-2 (\( p<0.05 \)) alone, whereas no change was found for COX-1 in each group. Shikunshi-to did not affect COX expression.

Representative immunohistochemical expression of COX-2 in the uteri treated with \( E_2 \) with or without Shimotsu-to is shown in Fig. 2. Shimotsu-to treatment reduced the COX-2 expression induced by \( E_2 \). The immunohistochemical score COX-1 and -2 is summarized in Table 3. Juzen-taiho-to treatment significantly reduced the COX-2 immunohistochemical scores of the glandular (\( p<0.01 \)) and stromal cells (\( p<0.05 \)) induced by \( E_2 \) treatment. Shimotsu-to treatment also significantly reduced the COX-2 immunohistochemical scores of the glandular cells compared with \( E_2 \)-treated group (\( p<0.01 \)), whereas it showed a decreased tendency in the stromal cells. Shikunshi-to appeared to have no effect on COX-expression.

**Long-Term Experiment** One mouse in group 1, four in group 2, three in group 3, and four in group 4 died within 15 weeks, yet no pathological abnormalities other than pneumonia were found. The remaining animals survived until the termination of the experiment and were enrolled as effective animals (Table 4). No significant difference in mean body weights was found between the corresponding groups with or without \( E_2 \)-treatment. Under \( E_2 \)-treatment, the mean wet
Significantly less than that of group 2 (weight of left and right uterine corpora of group 1 was significantly lower than that of group 2 (treated with E2 alone). The incidence of adenocarcinoma, atypical hyperplasia and endometrial hyperplasia, complex on the treated side of the uterine corpus of group 1 showed a decreased tendency compared with that of group 2 (treated with Shimotsu-to) was significantly lower than that of group 2 (MNU/saline alone). The incidence of adenocarcinoma in the present study were as the same as in our previous report.8,12,14) All adenocarcinomas seen in the endometria were well- or moderately-differentiated. The incidence of adenocarcinoma and atypical hyperplasia on the right side of group 1 (MNU/saline alone) was 17/19 (96%) (92%) (32%) (8%) (15%) (31%) (4%) (4%).

Although the enzyme activity was not evaluated in the pre-neoplastic and neoplastic endometrial lesions of the endometria were well- or moderately-differentiated. The incidence of adenocarcinoma and atypical hyperplasia on the right side of group 1 showed a decreased tendency compared with that of group 2. The levels of COX-1, -2 mRNA expression of adenocarcinoma was significantly higher than that of the normal control (p<0.05). The level of COX-2 mRNA showed an increased tendency according to the hyperplastic changes (Atypical hyperplasia> endometrial hyperplasia, complex>endometrial hyperplasia, simple), although significant differences could not be found. Meanwhile, the COX-1 mRNA expression was almost same in each group.

**DISCUSSION**

In our previous experiment, Juzen-taiho-to showed a suppressive effect on E2-related endometrial carcinogenesis in mice, possibly through suppression of estrogen-induced c-fos/jun expression.13) More recent report noted that Juzen-taiho-to and Shimotsu-to suppress the estrogen-mediated expression of c-fos, IL-1α, and TNF-α in the mouse uterine corpora treated with E2.20) There is more evidence showing that TNF-α and IL-1α play a significant role in chemical carcinogenesis.15,17,33) TNF-α is thus considered to stimulate not only tumor promotion but also progression in carcinogenesis.18)

Since COX-2 expression is known to be associated with estrous cycle in the uterus,14) ovariecetomized mice were used for the evaluation of COX-2 expression in the present study. Although the enzyme activity was not evaluated in the present study, COX-2 mRNA and protein expressions in the...
metastatic effects in different animal models.\textsuperscript{1,6)} The major mechanism for the effects are speculated to be the activation of macrophages and/or T cells in the host immune system.\textsuperscript{1)} Juzen-taiho-to as well as Shimotsu-to is reported to inhibit the production of cytokines, such as IL-1\(\alpha\) and TNF-\(\alpha\).\textsuperscript{20)} Such inhibitory effects may be related to the suppression of endometrial carcinogenesis in mice.

In summary, Shimotsu-to exerted inhibitory effects on the expression of COX-2 as well as c-fos, IL-1\(\alpha\) or TNF-\(\alpha\) in the endometrium on MNU- and E2-induced endometrial carcinogenesis in mice, suggesting that Shimotsu-to in Juzen-taiho-to is a key formula for the prevention of the endometrial carcinogenesis.

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