Efficacy of Lubeikangru formulation in mice with hyperplasia of the mammary glands induced by estrogen and progesterone

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Abstract

OBJECTIVE: To evaluate the protective effects of Lubeikangru formula (LF) on hyperplasia of the mammary glands (HMG) induced by estrogen and progesterone in mice.

METHODS: Female mice were divided randomly into five groups: normal, model, tamoxifen (3 mg/kg), Rupixiao (900 mg/kg) and LF (900 mg/kg). All mice except those in the normal group were treated sequentially with estradiol and progesterone to induce HMG. From the tenth day of induction, mice in normal and model groups received distilled water and mice in the other groups were given the corresponding drugs by gavage, once a day, for 30 d. At the end of treatment, the mammary glands, ovaries, hypothalamus, and serum was collected for whole-mount and hematoxylin and eosin (HE) staining, enzyme-linked immunosorbent assays (ELISAs), or western blotting.

RESULTS: Whole-mount and HE staining of mammary glands showed that LF rescued (at least in part) the hyperplasic morphology of the mammary glands, and the number of branch points decreased after LF treatment ($P < 0.05$). ELISAs revealed that levels of estrogen and progesterone were decreased following LF treatment, whereas levels of gonadotropin-releasing hormone, follicle-stimulating hormone, and luteinizing hormone were increased in serum and tissues. Western blotting confirmed that LF treatment led to a reduction in expression of phosphorylated (p)-Erk, p-p38 and p-c-Jun N-terminal kinase. LF was also confirmed to be safe by acute-toxicity tests.

CONCLUSION: LF can protect the mammary glands of mice from estrogen- and progesterone-induced hyperplasia by adjusting hormone levels and regulating the mitogen-activated protein kinase pathway.

INTRODUCTION

Hyperplasia of the mammary glands (HMG) is one of the most common breast diseases in women of childbearing age. Usually, HMG is induced by disorders of estrogen and progesterone. It has been reported that HMG (especially atypical HMG) leads to a much higher risk of carcinoma of the mammary glands.

Keywords: Hyperplasia; Mammary glands, animal; Hormones; Tamoxifen; Mitogen-activated protein kinases; Lubeikangru formulation
ceptable in most cases due to the high prevalence of relapse, adverse reactions and even carcinogenesis. Clinical and experimental studies have demonstrated that several Traditional Chinese Medicine (TCM) prescriptions are efficacious against HMG. Lubeikangru formulation (LF) is a simple formulation guided by TCM principles. It consists of Lujiao Tuopan (the base of Cervi Cornu), Zhebeimu (Bulbus Fritillariae Thunbergii), Loulu (Radix Stemmacanthae Uniflori), Xiakucao (Spica Prunellae Vulgaris), Xiangfu (Rhizoma Coryperi). These ingredients are used frequently in TCM prescriptions for HMG treatment. According to TCM theory, LF can attenuate HMG by tonifying the kidneys, relieving the depressed liver, promoting the blood circulation to remove stasis, and alleviating pain. However, the effect of their combination on HMG has not been shown.

We addressed the potential of LF to protect the mammary glands of mice from the hyperplasia induced by estrogen and progesterone. Also, we investigated the possible mechanism of action of LF.

MATERIALS AND METHODS

Reagents
Rupixiao tablets (Liaoning Haohushi Pharmaceutical Group, Liaoning, China), tamoxifen citrate tablets (Yangtze River Pharmaceutical, Taizhou, China), estradiol valerate tablets (Progynova®, Bayer, Leverkusen, Germany), progesterone soft capsules (Urtoestan®, Laboratoires Besins International, Bangkok, Thailand), carmine (Sigma-Aldrich, Saint Louis, MO, USA) and Permount (Thermo Scientific, Waltham, MA, USA) were purchased. Enzyme-linked immunosorbent assay (ELISA) kits for anti-mouse estradiol, progesterone, follicle-stimulating hormone (FSH), luteinizing hormone (LH) and gonadotropin-releasing hormone (GnRH) were obtained from Shanghai Lianshuo Biological Technology (Shanghai, China). Antibodies against Erk1/2 (catalog number, YT1623), c-Jun N-terminal kinase (JNK; YT2440), p38 (YT3513), phospho-Erk1/2 (YP1197), phospho-JNK (YP0156), and phospho-p38 (YP0203) were purchased from ImmunoWay Biotechnology (Plano, TX, USA).

LF preparation
Lujiao Tuopan powder was kindly provided by Shenyang Sanshan Deer Development (Shenyang, China). All other herbs were purchased from the Affiliated Hospital of Liaoning University of Traditional Chinese Medicine (LUTCM, Shenyang, China). The five ingredients of LF were mixed at a ratio of 15:8:5:4:4 by weight, rinsed in water and boiled for 30 min; this process was repeated thrice. The three decoctions were combined, filtered by gauze, concentrated under reduced pressure, and left to dry in air. The w/w yield of LF was 13.3%. The powdered LF was stored at 4°C and dissolved in distilled water before use.

Animals
Procedures involving animals and their care were conducted in accordance with the guidelines set by the Chinese government on the ethical use and care of laboratory animals. The study protocol was approved by the Animal Care and Use Committee of LUTCM (20160307). Female Kunming mice [(20 ± 2) g] were provided by the Animal Center of LUTCM. Female C57BL/c mice (6-7 weeks) were purchased from Vital River Laboratory (Beijing, China) and housed in a specific pathogen-free room with a 12-h light-dark cycle at 22-25°C and relative humidity of 40%-60%. Mice had access to food and water ad libitum. Experiments were conducted after mice became acclimatized to their surroundings.

Acute toxicity of LF
Twenty female Kunming mice were divided randomly into two groups of 10. They were starved overnight but had free access to water before experimentation. Mice in the control group were given distilled water. Mice in the LF group were administered LF (20 g/kg body weight, i.g., t.d.s.) on the first day and then observed twice-daily for 14 d. The body weight of each mouse was measured before and after experiments. Blood was collected from the eyeballs of mice. One part was transferred into 1.5-mL Eppendorf tubes containing 2% ethylene diamine tetraacetic acid (EDTA) for hematology. The remainder was collected into 1.5-mL Eppendorf tubes, allowed to clot for 30 min, and centrifuged at 3000 g for 15 min at 4°C for biochemical analyses. Mice were sacrificed by cervical dislocation and vital organs observed macroscopically.

Induction of HMG and drug administration
Suspensions of estradiol and progesterone were prepared, and HMG induced as described previously. Seventy-five female C57BL/c mice were allocated random into five groups of 15. Mice in the normal control group were administered distilled water or sesame oil by gavage. Mice in the other groups were treated with estradiol (2.5 mg/kg body weight) once every 2 d for 12 times, followed by progesterone (20 mg/kg body weight) once a day for 5 consecutive days to induce HMG. From the 10th day of induction, mice in the normal control group and model group received distilled water by gavage. Mice in the other groups were treated daily with drugs for 30 d. The drug doses were: LF group (900 mg/kg, LF), rupixiao group (900 mg/kg, rupixiao), tamoxifen group (3 mg/kg, tamoxifen). At the end of treatments, blood and the fourth pair of mammary glands were collected for analyses.

Hormone analyses
The concentrations of estradiol, progesterone, FSH, LH and GnRH in serum and tissues were measured by ELISA kits according to manufacturer instructions.

Whole-mount staining of mammary glands
The fourth pair of mammary glands (left inguinal) was...
spread on glass slides. They were fixed in Carnoy’s fixative overnight, washed in 70% ethanol for 30 min, which was changed gradually to distilled water, and stained overnight in carmine alun. Then, tissues were dehydrated in a graded series of ethanol solutions, immersed in xylene until the fat was cleared, and mounted with Permount™. Images of whole mounts of mammary glands were taken with a digital camera for morphology. Secondary and tertiary branch points within five fields per mammary gland were counted manually under the 4× objective of a microscope.

**Hematoxylin and eosin (HE) staining of mammary glands**

The fourth pair of mammary glands (left inguinal) was fixed in Carnoy’s fixative, embedded in paraffin, and sectioned at 6 microns. Tissue sections were processed for standard HE staining. Signals were visualized with Permount™.

**Protein extraction and western blotting**

Mammary glands (100 mg) were ground into powder using liquid nitrogen and homogenized in RIPA Buffer supplemented with a proteinase inhibitor and phosphatase inhibitor. After centrifugation, the supernatant was collected and the protein concentration determined using a bicinchoninic acid protein assay kit. Protein (60 μg) was loaded on a 12% sodium dodecyl sulfate-polyacrylamide gel and then transferred to a polyvinylidene difluoride (PVDF) membrane. The latter was incubated with primary antibody overnight at 4°C. Immunoblots were created with a chemiluminescence imaging system (5200; Tanon Science & Technology, Beijing, China). Experiments were repeated at least thrice. Images were analyzed by ImageJ (San Diego, CA, USA).

**Real-time polymerase chain reaction (PCR)**

Total RNA was extracted from mammary glands with an EZ-10 DNA Away RNA Mini-prep kit (Sangon Biotech, Shanghai, China). First-strand cDNA was synthesized using a ProtoScript® II First Strand cDNA Synthesis kit (New England Biolabs, Ipswich, MA, USA). Real-time PCR was done with Fast SYBR® Green Master Mix in a Mx3000P instrument (Agilent Technologies, Santa Clara, CA, USA). The primer sequences (forward and reverse, respectively) for cyclin D1 were 5′-CCT GAC TGC CGA GAA GTT GT-3′ and 5′-TCA GCC TCT GGC ATT TT-3′ and for β-actin (housekeeping gene) were 5′-CCA CCA TGT ACC CAG CCA TT-3′ and 5′-ACG CAG CTC AGT AAC AGT CC-3′, respectively. Conditions were: 95°C for 10 min and 40 × (95°C for 15 s, 59°C for 15 s and 72°C for 30 s). All runs were done in triplicate and specific amplification was checked with melting-curve analyses. The relative expression of the target gene was calculated using the 2^−ΔΔCt method.

**Statistical analyses**

Data are the mean ± standard deviation (̅ x ± s). Differences between groups were tested by one-way analysis of variance followed by Tukey’s multiple comparison. P < 0.05 was considered significant. Data were analyzed by SPSS 17.0 (IBM, Armonk, NY, USA).

**RESULTS**

**Acute toxicity of LF**

To measure the acute toxicity of LF, mice received LF at a maximal dose of 60 g·kg⁻¹·d⁻¹. During 14-day observation, all mice survived and looked healthy with active behaviors and shining fur. The body weights of LF-treated mice increased from (19.8 ± 1.8) to (25.8 ± 2.1) g, as much as that of control mice from (19.3 ± 1.5) to (24.5 ± 2.6) g (n = 10, P > 0.05). There were no significant differences between the control group and LF group in terms of counts of red blood cells, white blood cells, platelets or hemoglobin level (P > 0.05) (Table 1). LF-treated mice, compared with control mice, had similar levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) as well as similar concentrations of urea and creatinine in serum, indicating that liver and kidney functions were normal (P > 0.05) (Table 2).

### Table 1: Effect of LF on the hematology parameters of female mice (̅ x ± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Hb (g/L)</th>
<th>WBC (×10⁹/L)</th>
<th>RBC (×10¹²/L)</th>
<th>PLT (×10⁹/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>155.7±13.0</td>
<td>9.5±1.1</td>
<td>10.5±0.6</td>
<td>828.1±68.4</td>
</tr>
<tr>
<td>LF</td>
<td>10</td>
<td>159.3±10.1</td>
<td>10.0±1.2</td>
<td>11.3±1.3</td>
<td>869.3±74.7</td>
</tr>
</tbody>
</table>

**Notes:** control: mice were given distilled water. LF: mice were administered LF (20 g/kg body weight, i.g., t.d.s.) on the first day and then observed for 14 d. LF: lubeikangru formula; Hb: hemoglobin; WBC: white blood cells; RBC: red blood cells; PLT: platelets. P > 0.05, compared with control group.

### Table 2: Effect of LF on the kidney and liver function of female mice (̅ x ± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Urea (mM)</th>
<th>Creatinine (μM)</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>6.72±0.18</td>
<td>51.47±8.54</td>
<td>36.73±9.76</td>
<td>50.69±11.25</td>
</tr>
<tr>
<td>LF</td>
<td>10</td>
<td>6.46±0.45</td>
<td>53.28±9.03</td>
<td>37.32±8.31</td>
<td>50.75±14.94</td>
</tr>
</tbody>
</table>

**Notes:** control: mice were given distilled water. LF: mice were administered LF (20 g/kg body weight, i.g., t.d.s.) on the first day and then observed for 14 d. LF: lubeikangru formula; ALT: alanine aminotransferase; AST: aspartate aminotransferase. P > 0.05, compared with control group.
Inhibition by LF of estrogen- and progesterone-induced HMG

Whole-mount staining was conducted to assess the morphology of mammary glands (Figure 1A-E). The mammary glands of mice in the model group displayed hyperplasia as characterized by an increased number of branches, side-branches and terminal end buds (Figure 1A-B, 1K). HE staining revealed that the intensity of the mammary epithelium increased and lumina filled with fluid in the model group (Figure 1G). LF treatments as well as treatments with tamoxifen and rupixiao, which have been used to treat HMG in China, could notably decrease the number of branch points (Figure 1K) and rescue (at least in part) the hyperplastic morphology of mammary glands induced by estrogen and progesterone. Counting of branch points indicated that the anti-hyperplasia effect of LF was comparable with those of rupixiao and tamoxifen ($P > 0.05$) (Figure 1K).

LF regulation of sex hormones

Hypothesizing that LF may regulate hormone levels to inhibit HMG, we used ELISAs to measure hormone levels in mice. Administration of estradiol and progesterone resulted in an obvious increase in serum levels of estradiol and progesterone, and a marked decrease in serum levels of GnRH, FSH, and LH, when comparing the model group with the control group ($P < 0.05$) (Figure 2A-E). After treatments, serum levels of estradiol and progesterone declined considerably in the LF group compared with those in the model group, whereas serum levels of GnRH, FSH and LH increased notably ($P < 0.05$). Meanwhile, comparisons were made between LF and reference drugs. Serum levels of FSH, LH and GnRH in the LF group were much higher than those in rupixiao and tamoxifen groups ($P < 0.05$). However, serum levels of estradiol and progesterone in the LF group were slightly higher than those in the tamoxifen group but lower than those in the rupixiao group ($P < 0.05$). These results suggested that LF had a stronger effect on serum hormone levels than rupixiao, and that its effects were also stronger than those of tamoxifen. The trends of estradiol and progesterone in the mammary glands, FSH and LH in ovaries, and GnRH in the hypothalamus were similar to those in the serum among treatment groups (Figure 2F-J).

Inhibition by LF of mitogen-activated protein kinase (MAPK) activity

MAPK signaling is required for branching morphogenesis in the mammary glands of mice. Therefore, expression of three major MAPKs (p38, Erk1/2 and JNK) was measured by western blotting to ascertain if MAPK signaling was involved in the mammary gland-protective effect of LF. The phosphorylation levels of these three MAPKs were upregulated in the model group, suggesting that
pared with those in the model group (Figure 3A-D). Besides, the mRNA level of cyclin D1 (a target gene of MAPK signaling) also declined after LF treatment (Figure 3E).

**DISCUSSION**

Development of the mammary glands in adults is controlled mainly by the hypothalamus-pituitary-ovary (HPO) axis. The hypothalamus secretes GnRH to regulate production of LH and FSH in the pituitary gland. In response to LH and FSH, the ovaries produce the sex hormones estrogen and progesterone, which are pivotal for elongation and branching of the mammary ducts during puberty. Disorders of the HPO axis contribute to breast diseases. Patients with benign breast disease often show increased levels of estrogen and progesterone in blood. Our results demonstrated that excessive intake of estrogen and progesterone can induce HMG in mice, along with a decrease in levels of GnRH, FSH and LH in serum and nearby organs. LF could recover (at least in part) the morphology of mammary glands, and relieve the disorder in the HPO axis. Compared with the well-known anti-estrogen drug tamoxifen, LF had a slightly weaker effect in reducing estrogen levels in mouse mammary glands. However, there was no obvious difference in the morphology or branch number of mammary glands between LF-treated and tamoxifen-treated mice, suggesting that the effect of LF may not have been limited to regulating estrogen levels. Besides, the effects of LF on levels of GnRH, FSH and LH were much stronger than those of tamoxifen, which may explain the similar morphology observed in LF and tamoxifen groups.

Estrogen and progesterone interact with local signaling networks to regulate the growth of the mammary glands. We found that MAPK signaling was involved in the local effect of LF upon HMG treatment. MAPK signaling, which is required for the proliferation and migration of cells and branch elongation in the development of the mammary glands, is one of the downstream signals of estrogen and progesterone. This information is consistent with our
results showing that MAPK signaling was activated in the mammary glands treated with estradiol and progesterone. LF can modulate the activity of MAPK signaling by suppressing the phosphorylation of p38, Erk1/2 and JNK. Furthermore, we found that mRNA levels of cyclin D1, a target of MAPK signaling, was increased in the mammary glands affected by hyperplasia, and that this increase could be attenuated by LF. Cyclin D1 is known to control cell proliferation.17 Downregulation of expression of cyclin D1 in LF-treated mammary glands could suggest lower proliferation of epithelial cells in the mammary glands. However, further studies are needed to ascertain if MAPK and cyclin D1 are required for the effect of LF on HMG.

In conclusion, LF can protect the mammary glands of mice from estrogen- and progesterone-induced hyperplasia by adjusting hormone levels and regulating the MAPK pathway.

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