Promoting Effects of Chinese Pangolin and Wild Pink Medicines on the Mammary Gland Development in Immature Mice

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ABSTRACT. The effects of the mixture of crude aqueous extracts from Chinese pangolin and wild pink (C+W), traditional Chinese medicine, on the proliferation and differentiation of mammary gland epithelium in intact and ovariectomized immature mice were investigated by light and electron microscopy and BrdU immunohistochemistry. Although there were no significant differences in mammary gland fat pad and parenchyma areas between the intact experimental groups, the numbers of duct branchings and buds were significantly larger in the C+W treated mice than in the control mice. The ratio of BrdU immunopositive cells to total epithelial cells was higher in C+W treated intact mice. Ultrastructurally, epithelial cells of the mammary buds and ducts possessed an oval and lucent nucleus, and ribosomes increased in number or developed to a greater degree in C+W treated intact mice than in the control mice. Conversely, there were no significant differences in any measurements of mammary gland between the experimental groups of ovariectomized mice. BrdU immunoreactive cells were never seen and the ultrastructure of mammary epithelial cells indicated the inactive cell phase in both ovariectomized mice. In comparison between the intact and ovariectomized mice, the mammary fat pad area was larger in the ovariectomized mice than in the intact mice, although another four measurements were larger in the intact groups. These observations suggest that administration with C+W could promote the development of mammary glands via ovary in immature mice.

KEY WORDS: Chinese pangolin and wild pink, development, mammary gland, mouse.

The mammary gland is one of the unique organ systems in mammals. The growth and differentiation of the mammary gland takes place in postnatal period and completes its morphology [5]. It is well known that various hormones such as estrogen, progesterone and prolactin regulate mammary gland development [9, 16, 23]. In a previous study, we observed mammary ducts with numerous branches and well-developed buds, and many epithelial cells possessed an oval and lucent nucleus, numerous mitochondria and ribosomes, well-developed rER and Golgi apparatus in ovariectomized mice administered 17-β estradiol (E) and progesterone (P) [12, 13].

Although infectious diseases and epidemics have been dealt with comparatively easily through the development of various new medicines, traditional Chinese medicines (TCM) have been utilized as therapeutic drugs for patients with diminished health and chronic conditions that have proven difficult to treat using Western medicine [22]. These include hypertension and diabetes as the lifestyle-related diseases, allergic diseases such as atopic dermatitis, and psychosomatic illnesses and stress diseases. Based on improving the self-power of healing to fix poor health conditions, TCM are used, not only in the treatment of the ailments, but also in balancing the entire human body. Therefore, TCM utilize combinations of plant-, animal-, and mineral-derived herbal medicines [22, 25]. TCM alleviate health condition symptoms through the interaction of many known and unknown elements.

Traditionally, TCM have also been employed in the field of veterinary medicine in China in the prevention and treatment of various diseases [25]. For example, the mixture of crude aqueous extracts from Chinese pangolin and wild pink is effective in promoting milk secretion in cattle after parturition. It has been known that such a prolactin-like lactogenic activity is included in the crude aqueous extracts from scales of Chinese pangolin although the constituents have been unclear until now. The extracts may primarily promote milk secretion in cattle after parturition. Prolactin also causes mammary lobuloalveolar development in combination with E and P [16]. Prolactin and other lactogenic hormones may affect mammary development directly via interaction with the prolactin receptor [1]. Prolactin may also act indirectly via function of other endocrine organs, especially ovary, because prolactin and related lactogenic hormones provide trophic support to the corpus luteum, allowing E and P production [8]. Mammary glands, even if in immature state, are already responsive to these hormonal stimuli from ovary and pituitary gland [18]. Therefore, the effects of the crude aqueous extract mixture from Chinese pangolin and wild pink on the morphogenesis can be also evaluated in immature mammary gland. However, there are no reports of morphological investigations on the effects of TCM on mouse mammary gland development.

The present study was designed to evaluate the effects of

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the mixture of crude aqueous extracts from Chinese pangolin and wild pink on the development of immature mouse mammary gland morphology. Furthermore to confirm whether prolactin-like mammogenic effect acts directly or indirectly via ovary to mammary gland, ovariectomy was carried out and the morphological differences between intact and ovariectomized mice were compared.

MATERIALS AND METHODS

**TCM:** Two TCM, Chinese pangolin (C) and wild pink (W), were used in this study (Table 1). These TCM were sold for the medicines under the permission of the government and purchased from Chinese Inner Mongolia Hohhot Huameng Medicine Limited Liability Company, Hohhot, China. The extraction from TCM was performed by a traditional method as following. Each TCM (50 g) was boiled in 500 ml distilled water (DW) for 30 min, and then the supernatant fluid was collected by filtration. Remained TCM was boiled again with 500 ml DW for the same time before filtration. This procedure was repeated once again. After centrifugation at 3,000 rpm for 10 min, the supernatants were condensed by additional boiling, and obtained at a final total volume of 100 ml. The extract was prepared just one time for the whole experiment and kept in a refrigerator until use. Each solution was mixed with another solution by the same volume of 100 ml. The extract was prepared just one time for the whole experiment and kept in a refrigerator until use. Each solution was mixed with another solution by the same volume prior to administration to mice.

**Animals:** A total of 24 Jcl-ICR female mice, bred and maintained as a closed colony in our laboratory, were used in this study. The animals were either kept intact or ovariectomized at 21 days of age under sodium pentobarbital anesthesia. All mice (28 days old) were orally administered with TCM extracts once a day for 14 days. Each group consisted of 7 (intact) and 5 (ovariectomy) mice. The animals were housed in an environmentally controlled air-conditioned room (temperature, 23 ± 3°C; humidity, 60 ± 10%; light-dark cycle, 12–12 hr) and fed a commercial diet (MF, Oriental Yeast Co., Tokyo, Japan) and tap water ad libitum.

At 43 days of age, mice were euthanized by exsanguination under sodium pentobarbital anesthesia. The left first abdomino-inguinal mammary gland was excised for whole mount samples and the right one for light and electron microscopy.

**Whole mount samples:** The left abdomino-inguinal mammary gland was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, containing 0.85% NaCl, the sections were blocked with proteinase K (DakoCytomation, CA) for 6 min at room temperature to remove nonspecific staining. The sections were washed extensively with DW and then incubated with 4N HCl for 30 min at room temperature. After washing with 0.1 M borate buffer (pH 8.5) the sections were incubated with a monoclonal mouse anti-BrdU antibody (NeoMarkers, CA) diluted to 1 µg/ml with 0.1% BSA/PBS and 4 µg/ml horseradish peroxidase-conjugated goat anti-mouse IgG (Pierce, IL) suspended in 0.1% BSA/PBS. Peroxidase activity was visualized with 0.05% diaminobenzidine tetrahydrochloride (Sigma, MO) and 0.01% H₂O₂ in PBS. After washing with PBS, the sections were counterstained with hematoxylin for 20 sec, dehydrated, and mounted. The number of BrdU immunopositive epithelial cells was counted, and the ratio of these cells to total mammary epithelial cell number in each section was calculated.

**Transmission electron microscopy:** Small pieces were dissected out from the central part between superficial lymph node and nipple of the right first abdomino-inguinal mammary gland. They were immediately fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer for 2 hr at 4°C, post-fixed in 1% osmic acid in the same buffer for 2 hr at 4°C, and routinely embedded in Epon812. Ultrathin sections were stained with uranyl acetate and lead citrate and observed with H-7000 KU transmission electron microscope at 75 kV.

**Statistical analysis:** The data were statistically analyzed using ANOVA to compare between the control and treated group, and between the intact and ovariectomized mice.

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### Table 1. List of traditional Chinese medicines used in this study

<table>
<thead>
<tr>
<th>Item</th>
<th>Part</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chinese pangolin</td>
<td>Manis</td>
<td>C</td>
</tr>
<tr>
<td>wild pink</td>
<td>pentadactyla</td>
<td>scale</td>
</tr>
<tr>
<td>Vaccaria segetalis</td>
<td>Cuan</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>shan</td>
<td>W</td>
</tr>
<tr>
<td></td>
<td>jia</td>
<td></td>
</tr>
<tr>
<td></td>
<td>seed</td>
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</tbody>
</table>

Bromodeoxyuridine (BrdU) immunohistochemistry: In order to detect S-phase cells in the mammary gland, BrdU (50 mg/kg BW, Sigma, MO) was injected intraperitoneally into mice. One hour after injection, the right first abdomino-inguinal mammary gland was excised under pentobarbital anesthesia and fixed with 10% neutral buffered formalin for 12 hr at 4°C. The specimens were routinely embedded in paraffin wax and sectioned at 3 µm thick.

BrdU immunohistochemical staining was employed for S-phase cell detection. Six different sections at 12-µm intervals were immunostained as previously described [24]. Briefly, the sections were deparaffinized and soaked in 0.3% H₂O₂ in 100% methanol for 30 min at room temperature. After hydration and rinsing in PBS (10 mM sodium phosphate buffer, pH 7.2, containing 0.85% NaCl), the sections were blocked with proteinase K (DakoCytomation, CA) for 6 min at room temperature to remove nonspecific staining. The sections were washed extensively with DW and then incubated with 4N HCl for 30 min at room temperature. After washing with 0.1 M borate buffer (pH 8.5) the sections were incubated with a monoclonal mouse anti-BrdU antibody (NeoMarkers, CA) diluted to 1 µg/ml with 0.1% BSA/PBS and 4 µg/ml horseradish peroxidase-conjugated goat anti-mouse IgG (Pierce, IL) suspended in 0.1% BSA/PBS. Peroxidase activity was visualized with 0.05% diaminobenzidine tetrahydrochloride (Sigma, MO) and 0.01% H₂O₂ in PBS. After washing with PBS, the sections were counterstained with hematoxylin for 20 sec, dehydrated, and mounted. The number of BrdU immunopositive epithelial cells was counted, and the ratio of these cells to total mammary epithelial cell number in each section was calculated.

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EFFECTS OF TCM ON MAMMARY GLAND DEVELOPMENT

RESULTS

Whole mount morphometry: Table 2 shows fat pad and parenchyma areas, the ratio of parenchyma to fat pad, and the numbers of duct bifurcations and buds in whole mount samples of the first abdomino-inguinal mammary gland in intact mice. There were no significant differences in fat pad and parenchyma areas, or in parenchyma to fat pad ratios between the 2 groups. However, the numbers of duct bifurcations and buds were significantly greater in C+W treated intact mice than in the control group (Fig. 1a, b).

BrdU immunohistochemistry: BrdU immunoreactivity was observed in both duct and bud epithelial cells, with especially high immunoreactivity in the bud cells. In intact group, C+W treated mice possessed numerous immunoreactive cells (Fig. 2). The higher ratio of BrdU immunopositive cells to total cells of the mammary parenchyma was seen in C+W treated intact mice (Table 2).

Electron microscopy: The epithelial cells of the mammary ducts and buds in control intact mice were character-

Table 2. Areas of mammary fat pad and parenchyma, and numbers of duct branchings and buds and ratio of BrdU immunopositive cells to total epithelial cells in intact and ovariectomized mice treated with C+W. Each value represents mean ± SE

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Intact control</th>
<th>C+W</th>
<th>Ovariectomy control</th>
<th>C+W</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>7</td>
<td>7</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Fat pad (mm²)</td>
<td>113.0 ± 7.3</td>
<td>116.8 ± 13.8</td>
<td>194.8 ± 14.1ᵇ)</td>
<td>199.7 ± 5.6ᵃ)</td>
</tr>
<tr>
<td>Parenchyma (mm²)</td>
<td>79.1 ± 3.5</td>
<td>77.9 ± 7.1</td>
<td>22.9 ± 5.1ᵇ)</td>
<td>37.3 ± 7.3ᵃ)</td>
</tr>
<tr>
<td>Ratio (%)</td>
<td>66.4 ± 3.2</td>
<td>68.2 ± 4.2</td>
<td>11.9 ± 2.8ᵇ)</td>
<td>18.5 ± 3.4ᵃ)</td>
</tr>
<tr>
<td>Duct branchings</td>
<td>468.1 ± 26.1</td>
<td>604.1 ± 38.4ᵇ)</td>
<td>57.6 ± 8.2ᵇ)</td>
<td>87.8 ± 10.7ᵃ)</td>
</tr>
<tr>
<td>Buds</td>
<td>506.2 ± 24.5</td>
<td>662.0 ± 36.8ᵇ)</td>
<td>73.4 ± 8.6ᵇ)</td>
<td>95.0 ± 13.4ᵃ)</td>
</tr>
<tr>
<td>BrdU(%)</td>
<td>5.3 ± 1.2</td>
<td>8.6 ± 1.1</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Superscripts a) and b) indicate significant difference (P<0.05) versus intact control, and c) versus intact C+W, respectively. ND: not detected.

Fig. 1. Whole mount samples of the first abdomino-inguinal mammary gland in intact (a, b) and ovariectomized (c, d) mice treated with distilled water (a, c) and C+W (b, d). Scale bar, 5 mm.
ized by a nucleus with condensed chromatin and an irregular contour, round mitochondria, numerous ribosomes, underdeveloped rough-surfaced endoplasmic reticulum, and Golgi apparatus (Fig. 3a). These cells also occasionally contained a few lipid droplets. Conversely, most cells in C+W treated intact mice were characterized by an oval and lucent nucleus, numerous ribosomes, and well-developed Golgi apparatus (Fig. 3b).

**Effects of ovariectomy**: The whole mount morphometry in ovariectomized mice is shown in Table 2. No significant differences were seen in mammary gland fat pad and parenchyma areas, parenchyma to fat pad ratios, or the numbers of duct branchings and buds between the control and C+W treated ovariectomized mice, although these measurements indicated a tendency to be larger in C+W treated mice (Fig. 1c, d). BrdU immunohistochemistry revealed no immunoreactivity in both groups of ovariectomized mice (data not shown). Ultrastructurally, the mammary duct and bud epithelial cells possessed a nucleus with deep invagination, a small number of mitochondria and ribosomes, and underdeveloped rough-surfaced endoplasmic reticulum in all ovariectomized experimental groups (Fig. 4).

In comparison with intact groups, the ratios of parenchyma to fat pad and the numbers of duct branchings and buds among the experimental groups in ovariectomized mice were significantly lower (Table 2, Fig. 1). BrdU immunoreactivity was observed in all intact groups but never seen in any group of ovariectomized mice. Electron microscopically, mammary epithelium with active cell phase was frequently revealed in intact mice but not in ovariectomized mice.
DISCUSSION

The development of mammary glands is regulated by various hormones; estrogen elongates the ducts and progesterone promotes alveolar formation [9, 16, 22], and prolactin causes alveolar development resembling that of pregnancy [2, 16]. We reported that in immature mice, the administration of a combination of estrogen and progesterone affected the maximal changes observed in duct elongation and branching and in the ultrastructure of the mammary epithelium in an ovariectomized experiment [13]. The present study showed a significant increase in buds and duct bifurcations and the S-phase of mammary epithelial cells in C+W treated intact mice. Moreover, the ultrastructure of the mammary epithelium changed to an active cell phase in C+W treated intact mice. However, no significant differences in parenchyma area were seen between the 2 groups. Thus, we confirmed that the increase in numbers of buds and duct branching was induced by mammary epithelial cell proliferation in intact immature mice treated with C+W and that mammary epithelial cells could be differentiated by C+W administration.

However, these findings were not established in ovariectomized mice, indicating that C+W may not directly stimulate the development of mammary glands. Instead, C+W may promote structural progesterone-like changes by activating the ovary directly and/or via the pituitary gland indirectly. As TCM are also used for improving the blood circulation of affected areas, they assist in the recovery of target organs [22, 25]. Actually it is known that both Chinese pangolin and wild pink possess a function that improves amenorrhea caused by low blood circulation to ovary [25]. The present findings suggest that C+W in intact mice might increase the blood supply to the ovary and activate ovarian function.

Crude aqueous extracts from scales of Chinese pangolin have been known to demonstrate prolactin-like lactogenic activity [25]. However, to our knowledge, there are no reports on the constituents extracted from scales of Chinese pangolin. Bazan suggested that prolactin and other lactogenic hormones might affect mammary lactogenesis directly via interaction with the prolactin receptor [1]. It is speculated that this prolactin-like lactogenic activity from Chinese pangolin may promote milk secretion directly in C+W treated cattle after parturition. Conversely, although any alveolar buds were not observed, the significant increases in numbers of duct bifurcations and buds were indicated in C+W treated immature intact mice. However, these morphogenic changes were not confirmed in ovariectomized mice. Galosy and Talamantes [8] suggested that prolactin may also act indirectly via function of other endocrine organs, especially ovary, because prolactin and related lactogenic hormones provide trophic support to the corpus luteum, allowing E and P production. Brisken et al. [2] speculated that the absence of side branching in the prolactin receptor knockout mice is due to reduce ovarian progesterone production in those mice. The present study speculates that prolactin-like activity of crude aqueous extracts from Chinese pangolin may promote morphogenesis indirectly via ovary.

Wild pink have been known to include saponin which induced a prolactin-like stimulation of ornithin decarboxylase activity in mouse mammary gland explants [10]. Vaccarosides, cytotoxic triterpenoid saponins, are also isolated from wild pink [11], but those effects on the mammary glands are unclear. Morita et al. [15] isolated segetalins, cyclic peptides with estrogen-like activity extracted with hot methanol from seeds of wild pink, and reported segetalins increase in uterine weight against ovariectomized rats [15]. Recently Shoemaker et al. [20] reported that crude aqueous extract from wild pink seeds demonstrated growth inhibit activity against 2 breast cancer cell lines, MCF-7 and MCNeuA, and normal human mammary epithelial cells in vitro. However, it is uncertain which constituents from wild pink are related to the morphogenesis in the present experiment.

BrdU, an analog of thymidine, has been useful as an alternative to autoradiographic techniques for detecting S-phase cells in various organs, including the mammary gland [19]. BrdU immunoreactivity was observed in the epithelial cells of ducts and buds, with an especially large number of immunopositive bud cells. In intact group, C+W treated mice exhibited the higher ratio of BrdU immunopositive cells to total cells of the mammary parenchyma. Thus, C+W appears to promote the proliferation of mammary gland epithelial cells in intact immature mice.

No significant differences were observed in fat pad area in the both groups of intact and ovariectomized mice. We confirmed that the adipose tissue was histologically composed of unilocular fat cells and showed no marked differences in any experimental group. It was reported that mammary adipose tissues were in a mature state by 50 days after birth [17]. We reported in the previous study [13] that vast majority of fat cells were unilocular type in ovariectomized immature mice treated with E and E+P, and that estrogen may promote fat cells from multilocular type to...
unilocular one. This indicates that mammary adipose tissue may support the proliferation of mammary parenchyma since the formation of blood vascular beds is induced by the development of fat cells in mammary fat pad [5, 12, 13, 17]. However, the present findings suggest that C+W doesn’t promote the change of adipocyte morphology.

The elements of some TCM have been isolated from natural sources, such as glycyrrhizin from liquorice [4], crocin from crocus [3], phytoestrogens from Bupleurum and Peony Formula [14]. Glycyrrhizin has been reported to have antiviral activity against SARS-associated coronavirus [4]. Crocin inhibits the cell growth of cancer cells [3, 6, 21]. Phytoestrogens are steroid-like plant compounds which may act as precursors to sex hormones [14]. In the present study, we indicated that the crude aqueous extracts from Chinese pangolin and wild pink are capable to promote the mammary gland development in immature mice although the constituents of both TCM effective in morphogenesis have remained undefined. Further investigations are necessary to clarify the mechanism of morphogenesis by TCM.

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