Improvement of barrier function and stimulation of colonic epithelial anion secretion by *Menoease Pills*

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AIM: *Menoease Pills* (MP), a Chinese medicine-based new formula for postmenopausal women, has been shown to modulate the endocrine and immune systems[1]. The present study investigated the effects of MP and one of its active ingredients, *ligustrazine*, on epithelial barrier and ion transport function in a human colonic cell line, T84.

METHODS: Colonic transepithelial electrophysiological characteristics and colonic anion secretion were studied using the short circuit current (Isc) technique. RT-PCR was used to examine the expression of cytoplasmic proteins associated with the tight junctions, ZO-1 (zonula occludens-1) and ZO-2 (zonula occludens-2).

RESULTS: Pretreatment of T84 cells with MP (15 μg/mL) for 72 h significantly increased basal potential difference, transepithelial resistance and basal Isc. RT-PCR results showed that the expressions of ZO-1 and ZO-2 were significantly increased after MP treatment, consistent with improved epithelial barrier function. Results of acute stimulation showed that apical addition of MP produced a concentration-dependent (10-5 000 μmol/L) increase in Isc. MP-induced Isc was inhibited by basolateral treatment with bumetanide (100 μmol/L), an inhibitor of the Na+-K+-2Cl- cotransporter, apical addition of Cl channel blockers, diphenylamine-2, 2'-dicarboxylic acid (1 mmol/L) or glibenclamide (1 mmol/L), but not 4, 4'-disothiocyanostilbene-2, 2'-disulfonic acid or epithelial Na+ channel blocker, amiloride. The effect of MP on ZO-1 and ZO-2 was mimicked by *ligustrazine* and the *ligustrazine*-induced Isc was also blocked by basolateral application of bumetanide and apical addition of diphenylamine-2, 2'-dicarboxylic acid or glibenclamide, and reduced by a removal of extracellular Cl-.

CONCLUSION: The results of the present study suggest that MP and *ligustrazine* may improve epithelial barrier function and exert a stimulatory effect on colonic anion secretion, indicating the potential use of MP and its active ingredients for improvement of GI tract host defense and alleviation of constipation often seen in the elderly.

INTRODUCTION

It is well known that the gastrointestinal (GI) epithelium of the host, as the first defense line, plays an important role in protecting enteric epithelia from invasion of most pathogens. Intestinal epithelial barrier function regulates epithelial ions and nutrient transport as well as host defense mechanisms. Epithelial membrane pumps, ion channels and tight junctions tightly control epithelial transcellular and paracellular fluxes[2,3]. Cl- secretion also provides an essential driving force for lubrication of intestinal contents during regular bowel movements or flushing of microbial organisms or artificial irritants in host defense responses[4,5]. Epithelial Cl- channels play an important role in regulation and maintenance of normal GI physiological functions. Abnormal regulation of Cl- channels may result in diarrhea[6-8] or constipation[9,10]. While the later represents one of the frequently encountered conditions in aged people, few remedies are available for alleviation of the condition in the elderly.

*Menoease Pills* (Modified Bak Foong Pills, MP), a newly developed formula based on traditional Chinese medicine Bak Foong Pills (BFP, also known as Baifen Wan)[11-17], has been designed for the use of postmenopausal women. It has been demonstrated that MP can regulate hormonal profiles (Gou *et al*., unpublished data) and immune system in the elderly[11], indicating its beneficial effects for postmenopausal or elderly women. Since our previous studies have demonstrated that BFP could increase colonic epithelial Cl- and pancreatic duct epithelial HCO3- secretion[13,15,16] and both BFP and MP have a common active ingredient, *ligustrazine*, we undertook the present study to examine whether MP and *ligustrazine* exerted any effect on Cl- secretion and epithelial electrophysiological characteristics using human colonic T84 cells in conjunction with the short-circuit current technique and RT-PCR.

MATERIALS AND METHODS

Chemicals and solutions

Dulbecco’s Modified Eagle’s medium (DMEM)/F12, Hank’s balanced salt solution (HBSS), and fetal bovine serum were from Gibco Laboratories (New York, NY). 4, 4'-disothiocyanostilbene-2, 2'-disulfonic acid (DIDS) and glibenclamide were from Sigma (St. Louis, MO). MP was obtained from Eu Yan Sang Ltd (Hong Kong). Diphenylamine-2, 2'-dicarboxylic acid (DPC) was purchased from Riedel-de Haen Chemicals (Hannover, Germany). Calbiochem (San Diego, CA) was the source for amiloride hydrochloride and bumetanide. Krebs-Henseleit (K-H) solution had the following composition (mmol/L): NaCl, 117; KCl, 4.5; CaCl2, 2.5; MgCl2, 1.2; NaHCO3, 24.8; KH2PO4, 1.2; glucose, 11.1. The solution was gassed with 950 mL/L O2 and 50 mL/L CO2, at pH 7.4.
**MP extraction**

Five hundred gram of MP powder in 700 mL/L ethanol at a ratio of 1 to 10 (g/mL) was put in round-bottomed flask and boiled under reflux for 2 h. The mixture was filtered and the residues of MP were subject to the same treatment for a second time. The filtrates from the two treatment procedures were collected and put in the vacuum rotary evaporator for concentration. The extracts were collected and lyophilized by a freeze dryer.

**Cell culture**

Human colonic T84 cells were purchased from American Type Culture Collection (Rockville, MD). The cells were grown in DMEM/F12 with 100 mL/L fetal bovine serum. For Isc recording the cells (2-3x10^5/mL) were plated onto each floating permeable support, which was made of a Millipore filter with a silicone rubber ring attached on top of it for confining the cells (culture area 0.45 cm²). For the RT-PCR analysis, cells were seeded on the Millipore filter with a confined culture area of 4.5 cm². Cultures were incubated at 37°C in 950 mL/L O₂ and 50 mL/L CO₂ for 6 d before experiments. For the experiments of MP and ligustrazine pretreatments, MP (15 µg/mL) or ligustrazine (100 µm/L) was added into the culture medium at 72 h before experiments, when the cells became semi-confluent.

**Short-circuit current measurement**

The measurement of Isc has been described previously[18]. Monolayers grown on permeable supports were clamped vertically between two halves of the Ussing chamber. The monolayers were bathed in both sides with Krebs-Henseit solution, which was maintained at 37°C by a water jacket wall. For experiments, when the cells became semi-confluent. (100 mL/L CO₂ for 6 d before experiments. For the experiments of MP and ligustrazine pretreatments, MP (15 µg/mL) or ligustrazine (100 µm/L) was added into the culture medium at 72 h before experiments, when the cells became semi-confluent.

**Reverse transcription PCR (RT-PCR) analysis**

Total RNA (15 µg) was extracted from the T84 (control, MP and ligustrazine pretreated). Expressions of ZO-1 and ZO-2 were analyzed by competitive RT-PCR. The specific oligo nucleotide primers for ZO-1 was CGGTCTTCTGGACCTTGAAG for sense and GGA TCTACATGCAGCAACA for antisense corresponding to nucleotides 3 100-3 470 with an expected cDNA of 371 bp[19], and for ZO-2 was GCAAAAACCCAGAAACAAAGA for sense and ACTGCTCTTCCCCACCTCTCT for antisense corresponding to nucleotides 3 018-3 283 with an expected cDNA of 212 bp[19]. GAPDH was used as an internal marker for semi-quantitative analysis of expressions of ZO-1 and ZO-2 of T84 cells. The specific oligonucleotide primers for GAPDH were TCC CAT CAC CAT TCTTCCAG for sense and TCC ACC ACT GAC ACG TTG for antisense corresponding to nucleotides 249-764 bp with an expected cDNA of 515 bp[220].

**Data analysis**

Results were expressed as mean±SD. The number of experiments represents independent measurements on separate monolayers. Comparisons between groups of data were made by Student’s t-test. A P value less than 0.05 was considered statistically significant. EC₅₀ values were determined by nonlinear regression using GraphPad Prism software.

**RESULTS**

**Effect of pretreatment with MP on electrophysiological characteristics**

Pretreatment of T₈₄ cells with MP 15 µg/mL (n = 15) for 72 h significantly increased the basal transepithelial potential difference from 0.39±0.07 to 2.27±0.59 mV (Figure 1A, P<0.01), basal Isc from 3.05±0.44 to 7.14±1.80 µA/cm² (Figure 1B, P<0.05) and transepithelial resistance (TER) from 0.14±0.01 to 0.37±0.04 µΩ/cm² (Figure 1C, P<0.001).

**Effect of pretreatment with MP on expressions of ZO-1 and ZO-2**

In order to see weather MP-induced TER increase was related to the cytoplasmic proteins associated with tight junctions, ZO-1 (zonula occludens-1) and ZO-2 (zonula occludens-2), we used RT-PCR analysis to examine the expression levels of ZO-1 and ZO-2 in T₈₄ cells (Figure 2A). For ISC recording experiments, the changes in current kinetics did not sustain. MP at 10, 50, 100, 500, 1 000 µm/L produced ISC increases of 306.7±25.5 (n = 4), 673.3±93.3 (n = 4), 1 380.0±119.4 (n = 4), 7 624.0±309.7 (n = 5), 9 580.0±734.9 (n = 6) and 10 053.3±979.1 µA/cm² (n = 4), respectively.

**Anion dependence of MP-induced Isc**

As shown in Figure 3, apical addition of MP (10-5000 µg/mL) produced an Isc increase which was concentration-dependent (Figure 3A) with an apparent EC₅₀ of about 293.9 µg/mL (Figure 3B). MP-induced changes in Isc were calculated as total charges transported for 15 min (µC/cm²), the area under the curve of the MP-induced Isc responses for the given time period) since the current kinetics did not sustain. MP at 10, 50, 100, 500, 1 000 and 5 000 µg/mL produced Isc increases of 306.7±25.5 (n = 4), 673.3±93.3 (n = 4), 1 380.0±119.4 (n = 4), 7 624.0±309.7 (n = 5), 9 580.0±734.9 (n = 6) and 10 053.3±979.1 µA/cm² (n = 4), respectively.

**Mimicking effects of MP by ligustrazine**

Similar to the effects of pretreatment with MP, treating T₈₄ cells with ligustrazine, one of the active ingredients of MP, for 72 h also increased the levels of ZO-1 and ZO-2, the ratio of ZO-1 to GAPDH was raised from 0.46±0.08 to 0.65±0.11 (n = 6, Figure 2B).
Figure 1 Effects of MP pretreatment on transepithelial electrophysiological characteristics. Comparison of potential difference (A) transepithelial ISC (B) and transepithelial resistance (C) in T84 cells with and without MP (15 µg/mL, 72 h) pretreatment. Values are mean±SE; abP<0.01; a(b)P<0.05; c(d)P<0.001.

Figure 2 RT-PCR analyses of mRNA expressions of ZO-1 and ZO-2 in T84 cells. (A) RT-PCR results with products as expected of ZO-1 and ZO-2 found in control, MP pretreatment and ligustrazine pretreatment. Semi-quantitative analyses of ZO-1 (B) and ZO-2 (C) expressions in T84 cells without and with MP or ligustrazine pretreatment, which were shown in ratio of ZO-1 or ZO-2 to GAPDH (internal marker). Values are mean±SE; aP and b(c)P<0.05; c(d)P<0.001.

Figure 3 MP-induced ISC in T84 cell lines. A: Representative ISC recordings in response to MP (10, 50, 100, 500, 1 000 and 5 000 µg/mL) added to the apical side. Arrowheads indicate the time of MP addition. B: The concentration-response curve for MP-induced responses. Different concentrations of MP were added to the apical side and each data was obtained from at least 3 individual experiments. Values are mean±SE of maximal ISC increase.

Figure 4 Anion dependence of MP-induced ISC. A: Representative ISC recording with arrows indicating the time for apical addition of MP (500 µg/mL) and DPC (1 mmol/L). B: Summary of the effects of DPC (1 mmol/L, apical), glibenclamide (1 mmol/L, apical), bumetanide (100 µmol/L, basolateral), amiloride (10 µmol/L, apical) and DIDS (100 µmol/L, apical) on MP-induced ISC. Values are mean±SE; bP<0.01.
and the ratio of ZO-2 to GAPDH was from 0.76±0.12 to 1.33±0.07 (n=4, P<0.001) (Figure 2C).

Acute stimulation with ligustrazine (1 mmol/L, apical side) produced a current increase which was similar to that induced by acute addition of MP (0.5 mg/mL, apical) (n=6, Figure 5A). Removal of Cl− from KHS (n=4), apical addition of DPC or glibenclamide (1 mmol/L) (n=3) and basolateral administration of bumetanide (100 mmol/L) (n=3) reduced ligustrazine-induced current increases by 79.9% (P<0.001), 82.4% (P<0.001) and 96.2% (P<0.001), respectively (Figure 5B).

**DISCUSSION**

The present study has provided scientific evidence for the pharmacological action of MP, a Chinese medicine-based formula for postmenopausal women, on the GI tract. The results demonstrated that MP could stimulate Cl− secretion in human colonic epithelial cell line T84. The supporting evidence includes: MP-induced responses were insensitive to Na+ channel blockers; the response was inhibited by Cl− channel blockers; and substantially inhibited by the Na+–K+–2Cl− cotransporter inhibitors. The stimulatory effects of MP on colonic anion secretion were mimicked by its active ingredient, ligustrazine. Since ligustrazine is an active ingredient common in both MP and BFP, a traditional formula previously shown to stimulate anion secretion by GI tract epithelial cells, the present results suggest that Ligustrazine may be one of the responsible ingredients involved in mediating the secretory effects of both MP and BFP.

Apart from its acute stimulatory effects on colonic anion secretion, MP, by treating T84 cells for 72 h, was also demonstrated to significantly alter the electrophysiological characteristics of the colonic epithelia. Increases in transepithelial potential and basal I_{sc} may represent an increased driving force for anion secretion and basal secretion, respectively. These results indicate long-term treatment of MP can promote colonic anion secretion, consistent with its acute effects. On the other hand, pretreatment of T84 cells with MP also increased the transepithelial resistance, indicating its effect on improving epithelial barrier function. This was confirmed by RT-PCR results, which showed that pretreatment with MP significantly up-regulated gene expressions of tight junction related proteins, ZO-1 and ZO-2. Similar results were obtained using Ligustrazine, suggesting that ligustrazine was able to improve barrier function in addition to colonic secretion. It has been reported that an elevation of intracellular calcium could decrease the tight junction resistance in T84 monolayers[21]. Since Ligustrazine has been shown to decrease intracellular Ca2+ by inhibiting Ca2+ entry and/or Ca2+ release[22,23], ligustrazine as well as ligustrazine-containing MP may strengthen tight junctions, thereby enhancing transepithelial resistance. In fact, we have found that intracellular calcium could also be reduced by an apical addition of MP (data not shown), indicating a possible mechanism for improving barrier function. Further studies may be required to understand the detailed mechanisms.

Taken together, the present results have demonstrated that MP and Ligustrazine exert a stimulatory effect on gastrointestinal Cl− secretion and improvement of epithelial barrier function. Since MP is designed for postmenopausal or elderly women, its demonstrated effects on the colonic epithelia, in addition to its beneficial effect on endocrine (Gou et al., unpublished data) and immune systems previously shown[1], suggest that MP and its active ingredient, ligustrazine, may be used to alleviate some of the GI tract disorders, such as infection and constipation, often seen in the elderly.

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