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• BASIC RESEARCH •

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

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Abstract

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, T_{84}.

METHODS: Colonic transepithelial electrophysiological characteristics and colonic anion secretion were studied using the short circuit current (I_{SC}) 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 T_{84} cells with MP (15 μg/mL) for 72 h significantly increased basal potential difference, transepithelial resistance and basal I_{SC}. 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 μg/mL, EC_{50} = 293.9 μg/mL) increase in I_{SC}. MP-induced I_{SC} 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′-diisothiocyanostilbene-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 I_{SC} 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 *lligustrazine* 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.

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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 Baifeng 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[1], 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 HCO_3^- secretion[11,15,16] 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 T_{84} 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'-diisothiocyanostilbene-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-Henseit (K-H) solution had the following composition (mmol/L): NaCl, 117; KCl, 4.5; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 24.8; KH_2PO_4, 1.2; glucose, 11.1. The solution was gassed with 950 mL/L O_2 and 50 mL/L CO_2, 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 T_{84} 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 I_{SC} recording the cells ($2\text{-}3\times10^5$/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 cm^2). For the RT-PCR analysis, cells were seeded on the Millipore filter with a confined culture area of 4.5 cm^2. Cultures were incubated at 37 °C in 950 mL/L O_2 and 50 mL/L CO_2 for 6 d before experiments. For the experiments of MP and *ligustrazine* pretreatments, MP (15 μg/mL) or *ligustrazine* (100 μmol/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 I_{SC} 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 enclosing the reservoir. The Krebs-Henseit solution was bubbled with 950 mL/L O_2 and 50 mL/L CO_2 to maintain the pH of the solution at 7.4. Drugs could be added directly to apical or basal side of the epithelium. Usually, the epithelia exhibited a basal transepithelial potential difference for every monolayer examined, which was measured by the Ag/AgCl reference electrodes (World Precision Instruction) connected to a preamplifier which was in turn connected to a voltage-clamp amplifier (World Precision Instruction, DVC-1000). In most of the experiments, the change in I_{SC} was defined as the maximal rise in I_{SC} following agonist stimulation and it was normalized to current change per unit area of the epithelial monolayer (μA/cm^2). The total charges transported for 15 min (the area under the curve of the agonist-induced I_{SC} responses) were also used to describe the agonist-induced responses (μC/cm^2). In each experiment, a transepithelial potential difference was 0.1 mV. The change in current in response to the applied potential was used to calculate the transepithelial resistance (TER) of the monolayer using Ohm's Law. Experiments were normally repeated in different batches of culture to ensure that the data were reproducible.

Reverse transcription PCR (RT-PCR) analysis

Total RNA (15 μg) was extracted from the T_{84} (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 CGGTCCTCTGAGCCTGTAAG for sense and GGA TCTACATGCGACGACAA for antisense corresponding to nucleotides 3 100-3 470 with an expected cDNA of 371 bp[19], and for ZO-2 was GCCAAAACCCAGAACAAAGA for sense and ACTGCTCTCTCCCACCTCCT 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 T_{84} cells. The specific oligonucleotide primers for GAPDH were TCC CAT CAC CAT CTT CCA G for sense and TCC ACC ACT GAC ACG TTG for antisense corresponding to nucleotides 249-764 bp with an expected cDNA of 515 bp[20].

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. EC_{50} values were determined by nonlinear regression using GraphPad Prism software.

RESULTS

Effect of pretreatment with MP on electrophysiological characteristics

Pretreatment of T_{84} 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 I_{SC} from 3.05±0.44 to 7.14±1.80 μA/cm^2 (Figure 1B, P<0.05) and transepithelial resistance (TER) from 0.14±0.01 to 0.37±0.04 μC/cm^2 (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 T_{84} cells (Figure 2A). Semi-quantitative analyses showed that the expression levels of both *ZO-1* and *ZO-2* after MP pretreatment were significantly elevated, the ratio of *ZO-1* to GAPDH was from 0.46±0.08 to 0.81±0.10 (n = 6, P<0.05, Figure 2B), and the ratio of *ZO-2* to GAPDH was from 0.76±0.12 to 1. 27±0.12 (n = 4, P<0.001, Figure 2C), indicating the enhancement of epithelial barrier function.

MP-induced I_{SC} response

As shown in Figure 3, apical addition of MP (10-5000 μg/mL) produced an I_{SC} increase which was concentration-dependent (Figure 3A) with an apparent EC_{50} of about 293.9 μg/mL (Figure 3B). MP-induced changes in I_{SC} were calculated as total charges transported for 15 min (μC/cm^2, the area under the curve of the MP-induced I_{SC} 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 I_{SC} increases of 306.7±25.5 (n = 4), 673.3±91.3 (n = 4), 1380.0±119.4 (n = 4), 7624.0±309.7 (n = 5), 9580.0±734.9 (n = 6) and 10053.3±979.1 μC/cm^2 (n = 4), respectively.

Anion dependence of MP-induced I_{SC}

In order to study the ion species involved in mediating MP-induced I_{SC}, a Na^+ channel blocker, amiloride and a couple of Cl^- channel blockers, DPC, glibenclamide and DIDS were examined (Figure 4). The change in I_{SC} was defined as the maximal rise in I_{SC} following MP stimulation and it was normalized to current change per unit area of the epithelial monolayer (μA/cm^2). DPC (1 mmol/L, n = 4, Figure 4A) or glibenclamide (1 mmol/L, n = 5) added to the apical side reduced MP (500 μg/mL)-induced responses from 10.0±0.97 μA/cm^2 to 1.78±0.18 μA/cm^2 (P<0.01) or from 9.44±0.49 μA/cm 2 to 1.39±0.5 μA/cm^2 (P<0.001) respectively, but apical addition of amiloride (10 μmol/L, n = 4) or DIDS (100 μmol/L, n = 4) had no significant effects (Figure 4B). Basolateral addition of bumetanide (100 μmol/L, n = 6), a strong inhibitor of the Na^+-K^+-$2Cl^-$ cotransporter reduced the MP-induced I_{SC} from 9.33±0.64 to 2.31±0.74 μA/cm^2 (Figure 4B, P<0.01).

Mimicking effects of MP by ligustrazine

Similar to the effects of pretreatment with MP, treating T_{84} 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)

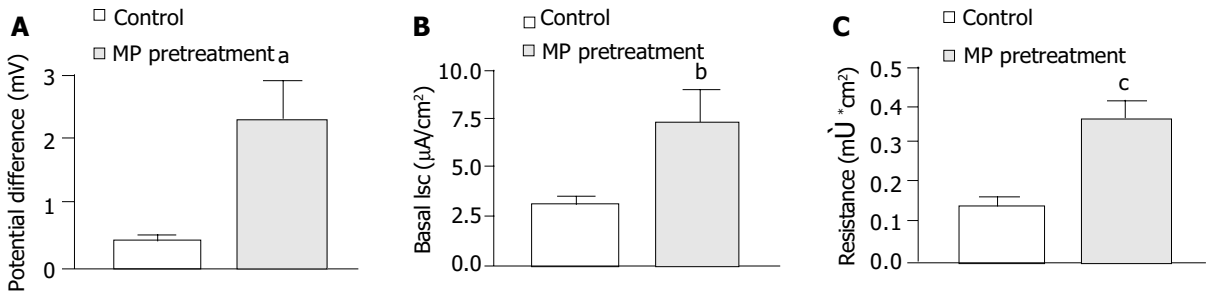

Figure 1 Effects of *MP* pretreatment on transepithelial electrophysiological characteristics. Comparison of potential difference (A) transepithelial I_{SC} (B) and transepithelial resistance (C) in T_{84} cells with and without MP (15 μg / mL, 72 h) pretreatment. Values are mean±SE; $^{a(b)}P<0.01$; $^{b(a)}P<0.05$; $^{c(d)}P<0.001$.

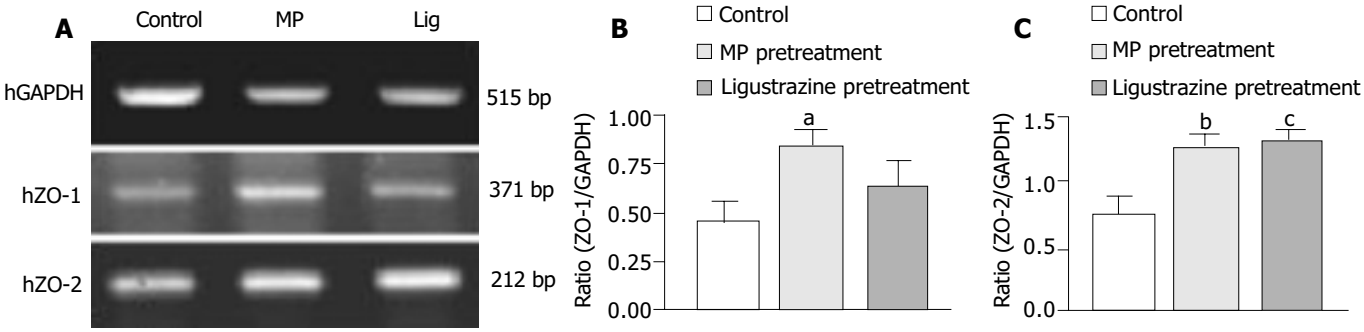

Figure 2 RT-PCR analyses of mRNA expressions of *ZO-1* and *ZO-2* in T_{84} cells. (A) RT-PCR results with products as expected of *ZO-1* and *ZO-2* found in control, MP pretreatment and *ligustrazine* pretreatment. Semi-quantitative analyses of *ZO-1* (B) and ZO-2 (C) expressions in T_{84} cells without 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; ^{a}P and $^{b(c)}P<0.05$; $^{c(d)}P<0.001$.

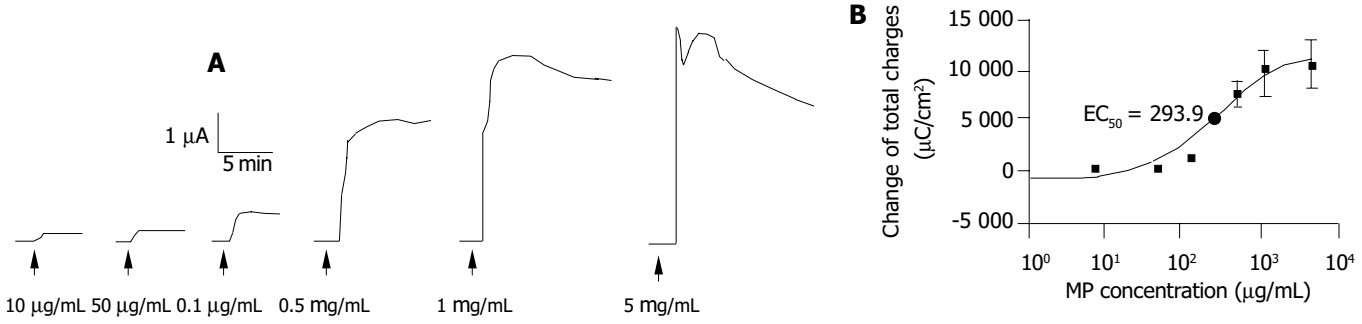

Figure 3 MP-induced I_{SC} in T_{84} cell lines. A: Representative I_{SC} recordings in response to MP (10, 50, 100, 500, 1 000 and 5 000 μg / mL) added to the apical side. Arrowheads indicate the time of MP addition. B: The concentration-response curve for MP-induced responses. Different concentrations 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 I_{SC} increase.

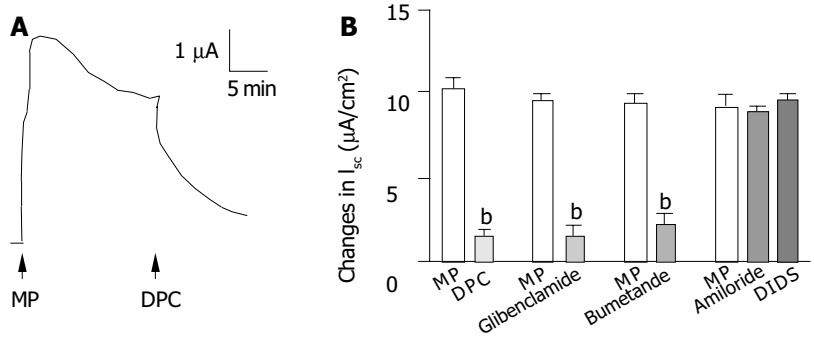

Figure 4 Anion dependence of MP-induced I_{SC}. A: Representative I_{SC} recording with arrows indicating the time for apical addition of MP (500 μg / mL) and DPC (1 mmol / L). B: Summary of the effects of DPC (1 mmol / L, apical), glibenclamide (1 mmol / L, apical), bumetanide (100 μmol / L, basolateral), amiloride (10 μmol / L, apical) and DIDS (100 μmol / L, apical) on MP-induced I_{SC}. Values are mean±SE; $^{b}P<0.01$.

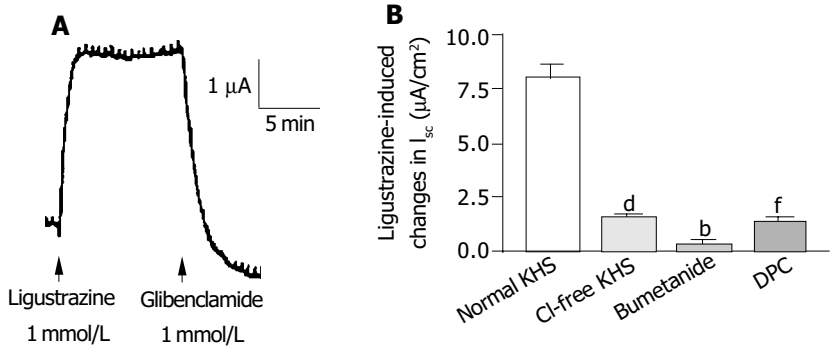

Figure 5 Anion dependence of *ligustrazine*-induced I_{SC}. A: Representative I_{SC} recording with arrows indicating the time for apical addition of *ligustrazine* (1 mmol/L) and glibemclamide (1 mmol/L). B: Summary of the effects of removal of extracellular Cl^-, basolateral addition of bumetanide (100 μmol/L) and apical addition of DPC (1 mmol/L) on *ligustrazine*-induced I_{SC}. Values are mean±SE; ^dP, ^bP and ^fP<0.001.

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 T_{84}. 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 epithelia[11,15,16], 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 T_{84} 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 T_{84} 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 T_{84} monolayers[21]. Since *Ligustrazine* has been shown to decrease intracellular Ca^{2+} by inhibiting Ca^{2+} entry and/or Ca^{2+} 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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