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The New Phytoformula Containing *Morus alba*, *Schizandra sinensis* and *Asparagus cochinchinensis* Inhibits Lung Inflammation in vitro and in vivo

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Abstract – A phytoformula containing the root barks of *Morus alba*, the fructus of *Schizandra sinensis* and the roots of *Asparagus cochinchinensis* (MSA) was prepared as a potential new herbal remedy, and its therapeutic potential for alleviating inflammatory lung conditions was examined. For in vivo evaluation, an animal model of lipopolysaccharide (LPS)-induced acute lung injury (ALI) in mice was used. With oral administration of 6 - 60 mg/kg, MSA potently and dose-dependently inhibited bronchitis-like symptoms in acute lung injury induced by intranasal treatment of LPS as judged by the number of cells in the bronchoalveolar lavage fluid (BALF) and histological observation. The inhibitory potency was comparable with that of dexamethasone. For in vitro assay, the effects on the production of proinflammatory molecules in lung epithelial cells and alveolar macrophages were examined. Although MSA inhibited IL-6 production in IL-1β-treated lung epithelial cells (A549) only at a high concentration (300 μg/ml), the formula strongly and concentration-dependently inhibited NO production in LPS-treated alveolar macrophages (MH-S) at 20 - 300 μg/ml. Based on all of these findings, the new phytoformula MSA is suggested to have the potential to control inflammatory lung diseases including bronchitis, at least in part, by inhibiting inducible nitric oxide synthase-catalyzed NO production.
Keywords – *Morus alba*, *Schizandra chinensis*, *Asparagus cochinchinensis*, Lung Inflammation, Bronchitis

Introduction

Lung inflammation may be provoked by exposure to bacteria, viruses, tobacco smoke, etc. Although acute inflammatory responses such as acute bronchitis are controlled using currently developed drugs including antibiotics, chronic inflammation in the lung may not be easily treated even with several classes of drugs such as anti-inflammatory steroids, antitussives, mucolytics and/or bronchodilators.[1] In particular, it is difficult to cure chronic obstructive pulmonary diseases (COPD), which consist of chronic asthma, chronic bronchitis and emphysema, all characterized by chronic recurrent inflammation in the lung. Thus, there is a need for new therapeutic agents to treat these disorders. In the disease progress of COPD, many important molecules such as cyclic nucleotides, proinflammatory cytokines, chemokines and matrix metalloproteinases, and oxidative stress are involved.[2] Because these diseases have complex etiological and pathological processes, new therapeutic agents that have multiple cellular action mechanisms to target multiple points of disease process are preferable in some respects. For this purpose, plant-based herbal drugs that have many different classes of constituents possessing complex action mechanisms could be the choice. Anti-inflammatory herbal preparations are particularly desirable in that COPDs are essentially inflammatory disorders.

The root barks of *Morus alba* L. have been widely used in traditional medicine for treating various inflammatory conditions, including bronchitis, in northeast Asia.[3] Previously, the methanol and ethylacetate extracts of the root barks of *M. alba* were reported to demonstrate anti-inflammatory activity.[4,5] As major constituents, the prenylated flavonoids such as morusin, kuwanon C and kuwanon G have been shown to possess various anti-inflammatory properties.[6,7] Recently, we demonstrated that the 70% ethanol extract of the root barks of *M. alba* possesses strong inhibitory action against lung inflammation in a lipopolysaccharide (LPS)-treated acute lung injury (ALI) model in mice.[8] The fructus of *Schizandra chinensis* Baillon have also been frequently used in traditional medicine to treat lung inflammation, especially asthma.[9] Some inhibitory actions of 5-lipoxygenase (5-

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LOX) by major lignan derivatives isolated from the fructus of *S. chinensis* have been reported,[10] and these pharmacological properties may explain its pharmacological use for treating asthma. Indeed, the anti-asthmatic activity of this plant material has been described,[11] and the water extract of the Schizandra fructus was revealed to possess inhibitory action against airway inflammation in mice.[12] In addition, the roots of *Asparagus cochinchinensis* are also prescribed in traditional medicine for treating lung inflammatory disorders. Dioscin and methyl protodioscin as major ingredients have been reported to inhibit mucin production.[13] Recently, we established the inhibitory action of the extract and its major constituent, methyl protodioscin, against lung inflammatory response by oral administration.[14] All of these previous findings suggest that these plant materials may be useful for preparing phyto-combinations to alleviate lung inflammatory disorders.

Here in the present study, a new phytoformula containing the above three herbal materials was prepared, and its pharmacological action on lung inflammation was evaluated to identify a potential new therapeutic agent.

Experimental

Chemicals – 2-Amino-5,6-dihydro-6-methyl-4H-1,3-thiazine hydrochloride (AMT) was purchased from Tocris Cookson Ltd. (UK). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dexamethasone, IL-1β and LPS (*Escherichia coli* 0127:B8) were purchased from Sigma Chem. (St. Louis, MO). RPMI and other cell culture reagents including FBS were products of HyClone Lab. (South Logan, UT). Protein assay kit was purchased from Bio-Rad Lab. (Hercules, CA).

Animals – Male ICR mice (18 - 22 g, specific pathogen-free) were obtained from KOATECH (Korea). Animals were fed with standard lab. chow and water *ad libitum*. The animals were maintained in animal facility (KNU) at 20 - 22 ºC under 40 - 60% relative humidity and a 12 h/12 h (light/dark) cycle for at least 7 days prior to the experiment. The experimental design using the animals was approved by the local committee for animal experimentation, KNU (KW-140929-1). In addition, the ethical guideline described in the Korean Food and Drug Administration guide for the care and use of laboratory animals was followed throughout the experiments.

Plant materials – The root barks of *Morus alba* L. (Moraceae), the fructus of *Schizandra chinensis* (Magnoliaceae) and the roots of *Asparagus cochinchinensis* (Liliaceae) were obtained in local herbal store (Chunchon, Korea), and authenticated by one of the authors (Prof. Y. S. Kwon). The voucher specimens were deposited in the College of Pharmacy, KNU.

Preparation of the plant extracts – The dried root barks of *M. alba* (1 kg) were extracted with 70% ethanol (10 L) for 7 days. Evaporation under reduced pressure gave the dried extract (ME, 70.6 g). The dried fructus of *S. chinensis* (1 kg) was extracted with 70% ethanol (10 L) for 7 days and the extract was dried to give the residue (SE, 203.1 g). The dried roots of *A cochinchinensis* (1 kg) were extracted with methanol (10 L) for 7 days and evaporation under reduced pressure gave the dried residue (AM, 32.7 g) AM (30.0 g) was further suspended in water and fractionated with *n*-butanol. Evaporation under reduced pressure provided dried *n*-butanol fraction (AB, 14.3 g). All these extraction and fractionation procedures were implemented at room temperature. MSA is the mixture of ME:SE:AB (1:1:0.2, w/w/w), and used throughout this study.

LPS-induced airway inflammation in mice (ALI) – Mice were divided into the control, LPS-treated, LPS/MSA-treated and LPS/dexamethasone-treated groups (n = 9). MSA and the reference drug were dissolved in 0.3% carboxymethylcellulose (CMC) and were orally administered. The control and LPS treatment groups also received the CMC solution. One hour later, for inducing bronchitis, LPS (*E. coli* 0127:B8, 800 μg/ml, saline) was administered intranasally to mice (10 μl/mouse, 5 times). At 16 h after LPS treatment, mice were sacrificed (n = 7), and bronchoalveolar lavage fluid (BALF) was collected via intratracheal cannulation after 700 μl of saline was administered 3 times. BALFs collected were approximately 2,000 μl/mouse. From BALF, the total cell number was counted with a haemocytometer, and the cells were differentially counted with fluorescence-activated cell sorter (FACS, BD Bioscience). The remaining mice (n = 2) were sacrificed and lung tissues were excised. Histology was carried out using conventional methods of H&E staining.

A549 cell culture and measurement of IL-6 concentration – A549 cells, a human lung epithelial cell line, obtained from American Type Culture Collection (ATCC, Rockville, VA) were cultured with RPMI supplemented with 10% FBS, 1% L-glutamine and 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) in a 5% CO_2 atmosphere at 37 ºC. After pre-incubation for 24 h, IL-1β (10 ng/ml) was added simultaneously with/without test compounds. Four hours later, media was collected and the concentration of IL-6 was determined from the media with ELISA kit (eBioscience) according to the manufacturer's recommendation. The cell viability was checked using an MTT bioassay as

previously described.[15]

MH-S cell culture and measurement of nitric oxide (NO) concentration – MH-S cells, a mouse alveolar macrophage cell line, obtained from ATCC were cultured with RPMI supplemented with 10% FBS, 1% L-glutamine and 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) in a 5% CO_2 atmosphere at 37 °C. As previously described,[16] cells were plated in 96-well plates (2×10^5 cells/well). After pre-incubation for 24 h, test compounds and LPS (0.1 μg/ml) were added and the cells were incubated for 24 h. Media was collected and NO concentration was determined. To assess NO production, the stable conversion product of NO, nitrite (NO_2^-), was measured using Griess reagent and the optical density was determined at 550 nm.

The test compounds including reference drug were dissolved in DMSO and properly diluted with complete RPMI media. The final concentration of DMSO in the cell culture including A549 and MH-S cells was adjusted to 0.1% (v/v), and this concentration of DMSO did not affect the cell viability and the levels of IL-6 and NO production (data not shown).

Statistical analysis – Experimental values were represented as arithmetic mean ± SD. One way ANOVA followed by Dunnett's test was used to determine the statistical significance.

Result and Discussion

Initially, the synergistic effects of the three components of MSA on lung inflammation were verified using in vivo experiments. In LPS-treated ALI in mice, MSA (44 mg/kg, 20 + 20 + 4 mg/kg of ME, AE and AB) clearly showed significant inhibitory action on cell infiltration in the BALF (Fig. 1). In contrast, however, at 20 mg/kg of ME and SE each, they did not show significant reduction of cell infiltration. AB did not show the inhibitory action at 4 mg/kg in mice under the same experimental conditions. With respect to the total number of infiltrated cells, the % inhibition of MSA (65.3% reduction) was much higher than the % inhibition of the sum of the three components (42.6% reduction).

Next, the dose-dependent pharmacological activity of MSA was investigated. When orally administered at doses of 6 - 60 mg/kg, MSA clearly inhibited cell infiltration in the BALF dose-dependently (Fig. 2A). FACS analysis indicated that MSA strongly reduced the number of infiltrated neutrophils, an important acute inflammatory biomarker, and the numbers of other cells were also reduced. As a reference compound, dexamethasone, a

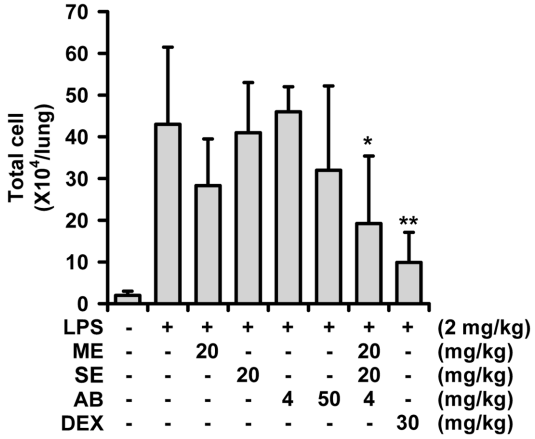

Fig. 1. Potentiation of MSA in inhibiting LPS-induced acute lung injury in mice.
The number of total cells in the BALF is presented here, and BALF was obtained 16 h after LPS-treatment in mice. ME: 70% ethanol extract of *M. alba*, SE, 70% ethanol extract of *S. chinensis*, AB: n-butanol fraction of *A. cochinchinensis*, DEX: dexamethasone, $*P < 0.05$, $**P < 0.01$, Significantly different from the LPS-treated control group ($n = 7$).

potent steroidal anti-inflammatory drug, strongly inhibited cell infiltration in LPS-induced ALI in mice at 30 mg/kg as expected. The inhibitory potency of MSA (60 mg/kg) is comparable with that of dexamethasone (30 mg/kg). MSA possesses approximately 1/2 - 1/3 the potency of dexamethasone in this parameter. These pharmacological actions of MSA and dexamethasone were also verified by histological observation. Fig. 2B shows that the lung sections of the MSA-treated group (especially at 60 m/kg) reduced cell infiltration and the thickness of alveolar wall. The dexamethasone-treated group showed similar responses.

In order to evaluate the cellular action on lung-related cells, A549 (lung epithelial cells) and MH-S (alveolar macrophages) were used. Lung epithelial cells and alveolar macrophages are major cell types in lung tissue and importantly participated in provoking inflammatory responses. In IL-1β-treated A549 cells, MSA inhibited the production of IL-6, a proinflammatory cytokine, at the high concentration of 300 μg/ml. When MSA was incubated in LPS-treated MH-S cells, it inhibited NO production strongly and concentration-dependently (Fig. 3 and 4). The reference agents, dexamethasone and AMT [an inducible nitric oxide synthase (iNOS) inhibitor] strongly inhibited IL-6 and NO production, respectively, at the indicated concentrations. No cytotoxic effect of the test compounds at the concentrations examined was found in these in vitro studies. All of these findings strongly demonstrated that MSA possesses strong inhibitory action against lung inflammation in vitro and in vivo.

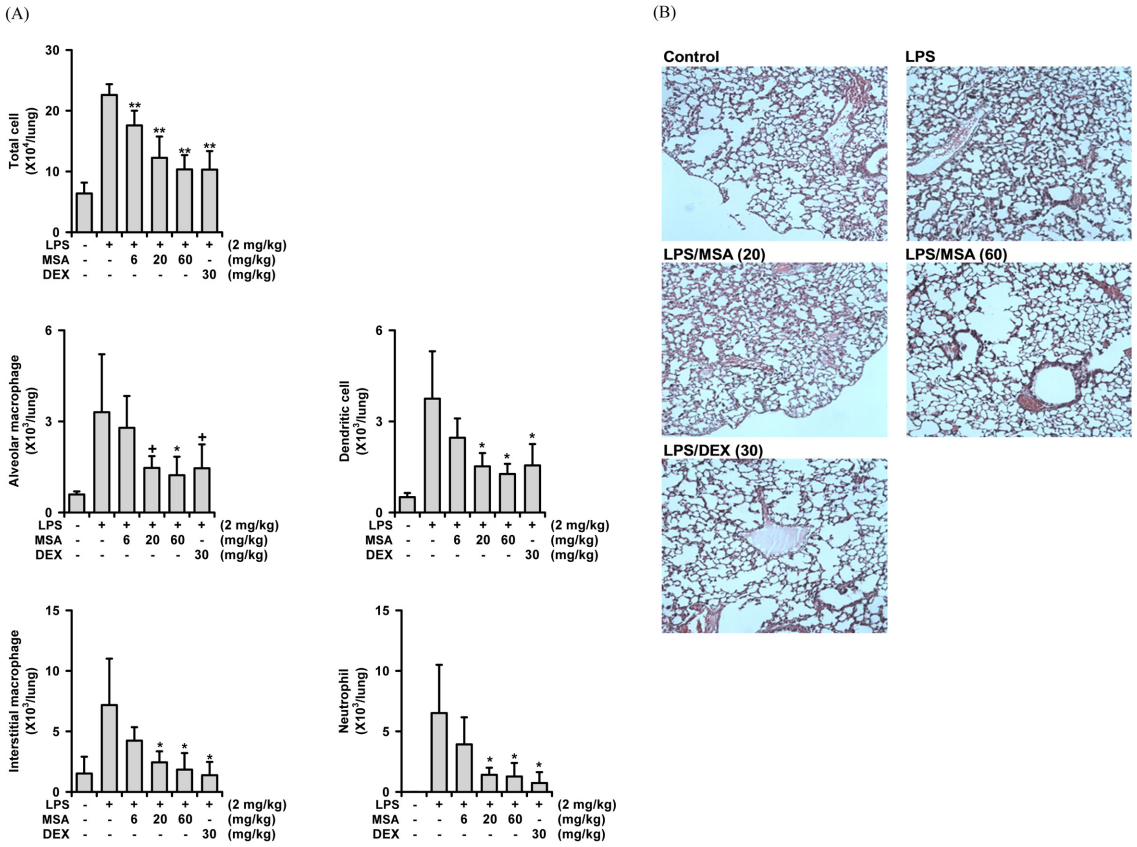

Fig. 2. Dose-dependent inhibition of MSA against LPS-induced acute lung injury in mice.
Mice were sacrificed 16 h after the LPS challenge. From the BALF, the numbers of cells were counted. (A) Effects on the cell numbers in the BALF. Total cell numbers were counted using a haemocytometer. FACS was used to differentiate between each type of inflammation-related cell. DEX: dexamethasone, $^{+}P < 0.1$, $*P < 0.05$, $**P < 0.01$, Significantly different from the LPS-treated group ($n = 7$). (B) Histological observation of lung tissues (H&E staining). Presented here is the result of one sample from two sets stained (× 100).

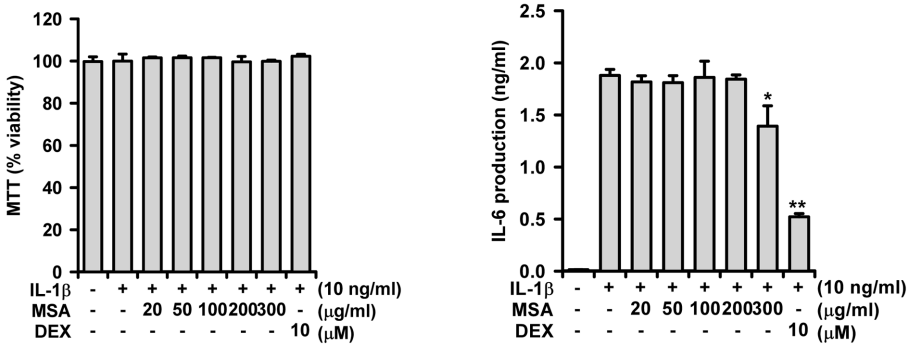

Fig. 3. Inhibition of MSA on IL-6 production in IL-1β-treated A549 cells.
$*P < 0.05$, $**P < 0.01$, Significantly different from the IL-1β-treated control group.

MSA is a mixture of extracts of *M. alba*, *S. sinensis*, and *A. cochinchinensis*. These three plant materials have been widely used in traditional medicine for treating lung inflammatory disorders. In the present study, MSA was demonstrated to possess strong pharmacological activity against in vitro and in vivo models of airway inflammation. Moreover, MSA exerted a meaningful synergistic action against airway inflammation compared with that of each of the herbal materials individually, providing a scientific rationale for this new formulation.

Previously, we found that the ethanol extract of *M. alba* possesses significant inhibitory action against an ALI

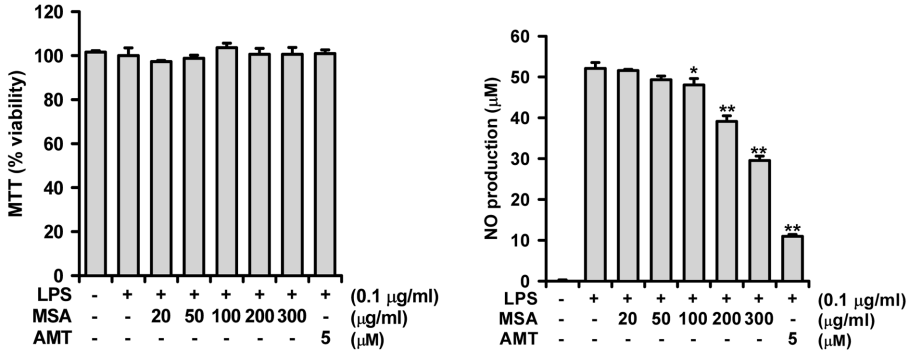

Fig. 4. Inhibition of MSA on LPS-induced NO production in MH-S cells.
$*P < 0.05$, $**P < 0.01$, Significantly different from the LPS-treated control group.

model with oral administration at 100 - 400 mg/kg.[8] *S. chinensis* was also reported to show inhibitory action against an ALI model at 50 - 100 mg/kg.[12] Recently, we found that *A. cochinchinensis* shows significant inhibition in the same animal model, ALI, at 100 - 400 mg/kg.[14] However, MSA showed strong inhibitory action on the ALI model at 6 - 60 mg/kg, suggesting its potent action and therapeutic potential for inflammatory lung disorders. It has also been revealed that the inhibitory action against lung inflammation by MSA could be at least in part attributable to NO and proinflammatory cytokine inhibition.

Phytochemical studies on these plant materials have previously demonstrated that the major active constituents of *M. alba* are prenylated flavonoids and that various lignan derivatives are the major constituents of *S. chinensis*.[17,18] *A. cochinchinensis* has varieties of steroidal saponins as its major constituents. Prenylated flavonoids were reported to contribute anti-inflammatory activity. The multiple prenylated flavonoids in *M. alba* showed strong anti-inflammatory action,[6] and, in particular, kuwanon E and norartocarpanone from *M. alba* were found to inhibit IL-6 production in lung epithelial cells.[8] These findings may again confirm an important role of the flavonoids in MSA for exerting its anti-inflammatory action against lung inflammation. Certain lignan derivatives also possess anti-inflammatory properties. Particularly, lignans such as schizandrin and gomisins from *S. chinensis* demonstrated an inhibitory action on 5-LOX.[10] Given that 5-LOX products including leukotrienes are importantly involved in the pathology of asthma, lignan derivatives from *S. chinensis* may also participate in the pharmacological action of MSA. In addition, steroidal saponins were revealed to possess anti-inflammatory characteristics.[19] In particular, dioscin and methyl protodioscin inhibited mucin production in vitro.[13] Methyl protodioscin from *A. cochinchinensis* also showed strong inhibitory action on the ALI model by oral administration.[14] Thus, all of these constituents in MSA may contribute to its pharmacological action. These multiple constituents that are presumed to have multiple cellular action mechanisms could provide beneficial effects for controlling the symptoms of COPD having complex pathological and etiological parameters.

Against airway inflammatory disorders including bronchitis, a number of plant-based herbal medicines are currently prescribed in European and Asian countries. They include the extracts of *Hedera helix*,[20] *Echinacea purpurea*,[21,22] and *Pelargonium sidoides*.[23] In line with these products, MSA may have therapeutic potential for lung inflammation-related disorders.

In conclusion, the present investigation was carried out to find plant-based products with the potential to serve as new and alternative medicines for treating inflammatory lung disorders. MSA, composed of *Morus alba*, *Schizandra chinensis* and *Asparagus cochinchinensis*, showed potent and synergistic action against lung inflammation in vitro and in vivo. Thus, it is suggested that MSA may be a new candidate for treating lung inflammation-related disorders including COPD.

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