Inhibitory Effects of Several Korean Traditional Herbs on Anaphylactic Reaction and Mast Cell Activation

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아나필락시스 반응과 비만세포 활성화에 대한 한국전통약제의 억제효과

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ABSTRACT
Korean traditional herbs, especially Anemarrhena asphodeloides (A. asphodeloides), Salvia miltiorrhiza (S. miltiorrhiza), and Terminalia chebula (T. chebula) have been known to have the immunomodulatory, anti-tumor, and anti-inflammatory effects. However, direct cellular mechanism underlying the mast cell-mediated anaphylaxis-like reaction has poorly been understood.

In the present study, the effects of the methanol extracts of A. asphodeloides (MEAA), S. miltiorrhiza (MESM), and T. chebula (METC) on anaphylactic reaction were investigated. Using in vitro and in vivo experiments, the influences of MEAA, MESM, and METC on the compound 48/80-induced anaphylaxis-like reaction and mast cell activation, and IgE-mediated PCA were examined.

Results are below; 1) MEAA, MESM, and METC significantly inhibited systemic anaphylaxis-like reaction, ear swelling response, and IgE-mediated PCA. 2) the compound 48/80-induced mast cell degranulation, histamine release of rat peritoneal mast cells (RPMC) were significantly inhibited by the pretreatment with MEAA, MESM, and METC, and 3) the compound 48/80-induced calcium influx in RPMC was significantly inhibited by the pretreatment with MEAA, MESM, and METC.

These results suggest that MEAA, MESM, and METC may be an activity to inhibit the compound 48/80-induced or anti-DNP IgE-mediated anaphylactic reactions by blocking histamine release and calcium uptake into RPMC. MEAA, MESM, and METC potentially may serve as an effective therapeutic tool for allergic diseases.

Keywords: Anaphylactic reaction, Anemarrhena asphodeloides, Mast cell, Salvia miltiorrhiza, Terminalia chebula

INTRODUCTION
Mast cells are recognized as the principal contributors to inflammatory responses such as the allergy and hypersensitivity. Immediate-type hypersensitivity, anaphylaxis, is mediated by histamine release in response to antigen cross-linking of IgE
bound to FcεRI receptor on mast cells. Mast cell degranulation can be elicited by the basic secretagogues. The most potent secretagogues include compound 48/80, which is a mixed polymer of phenethylamine cross-linked by formaldehyde. Compound 48/80 can increase the intracellular calcium content, which leads to histamine release from mast cells. Thus, it has been used as a direct and convenient reagent to study the mechanism of allergy and anaphylaxis. All the mast cell-mediated inflammatory reactions are characterized by an accumulation of mast cells in the inflammatory sites (Mekori & Metcalfe, 2000; Bradding, 2008). For example, the selective microlocalization of mast cells within specific airway structures, such as the airway smooth muscle and submucosal glands, is important in the pathophysiology of inflammatory lung disease. Apart from the release of inflammatory mediators, mast cells also play their multiple immunopathological roles in many inflammatory disorders by releasing multiple chemokines to recruit different subtypes of leukocytes. Activated mast cells can produce histamine, as well as a wide variety of other inflammatory mediators such as proteoglycans, proteases and proinflammatory and chemotactic cytokines (Puxeddu et al., 2005; Klemm & Ruland, 2006).

*Anemarrhena asphodeloides* (A. asphodeloides) is widely used in Chinese and Korean traditional medicines. The rhizoma of *A. asphodeloides* are known to have anti-diabetic, anti-platelet aggregation, and diuretic activities (Li et al., 2001; Chiang et al., 2003). Cis-hinokiresinol isolated from *A. asphodeloides* has an inhibitory effect on endothelial cell proliferation, vascular endothelial growth factor and basic fibroblast growth factor stimulate endothelial cell proliferation and invasion (Chang et al., 2001). Yeum et al. (2007) reported that *Fritillaria cirrhosa* A. asphodeloides and Lee-Mo-Tang inhibit OVA-induced eosinophils accumulation and Th2-mediated bronchial hyperresponsiveness in a murine model of asthma. *Salvia miltiorrhiza* (S. miltiorrhiza) has been reported the radical scavenging and antioxidant activities, and protection effect on the compound 48/80-induced systemic anaphylaxis and anti-dinitrophenyl (DNP) IgE-mediated passive cutaneous anaphylaxis (PCA), and the inhibitory mechanisms of *A. asphodeloides*, *S. miltiorrhiza*, and *T. chebula* methanol extracts on mast cell activation were investigated.

### MATERIALS AND METHODS

1. **Materials**

*A. asphodeloides*, *S. miltiorrhiza*, and *T. chebula* were extracted. Compound 48/80, anti-DNP IgE, DNP-human serum albumin (HSA), bovine serum albumin (BSA) and HEPES were purchased from Sigma Chemical Co. (MO, USA). Percoll solution was purchased from Pharmacia (Uppsala, Sweden).

2. **Animals**

Male ICR mice and Sprague-Dawley rats were purchased from Damool Science (Daejeon, Korea). They were housed 3–5 per cages in laminar air-flow cabinet maintained at 23 ± 2°C and relative humidity of 55 ± 10% throughout the study. Animal Research Committee of Chonbuk National University approved the animal study in accordance with the guidelines of the National Institutes of Health (NIH publication #85-23, 1985).

3. **Preparation of methanol extracts of**

*A. asphodeloides* (MEAA), *S. miltiorrhiza* (MESM), and *T. chebula* (METC)

*A. asphodeloides*, *S. miltiorrhiza*, and *T. chebula* used in this study were purchased from Jangso Oriental Pharmacy (Jeonbuk, Korea). A voucher specimens (number 2005-AAMw705031, SMMw705032, TCMw705032) were deposited at the Herbarium of the Research Center for Allergic Immune Diseases, Chonbuk National University Medical School. Each 100 g of these air-dried herbs were immersed in 400 mL of 70% methanol, kept overnight in a refrigerator, and boiled under reflux for 2 h. These methanol extractions were conducted twice. The resulting extracts were filtered through a 0.45 μm filter, and concentrated to approximately 100 mL under reducing pressure. The concentrated extracts were finally lyophilized, yielded 46.0 g (MEAA), 49.0 g (MESM) or 52.0 g (METC), dried powder and kept at 4°C until use. The dried extracts were dissolved in saline or HEPES-Tyrode buffer (136 mM NaCl, 5 mM KCl,
2 mM CaCl₂, 11 mM NaHCO₃, 0.6 mM NaH₂PO₄, 2.75 mM MgCl₂, 5.4 mM HEPES, 1.0 mg/mL bovine serum albumin, 1.0 mg/mL glucose, 0.1 mg/mL heparin) before use.

4. Compound 48/80-induced systemic anaphylaxis

Mice were given an intraperitoneal injection of 8 mg/kg body weight (BW) of the mast cell degranulator compound 48/80. MEAA, MESM, or METC (1, 10 or 100 mg/kg BW) was dissolved in saline and administered orally at 24, 12 and 1 h before the injection of compound 48/80 (n=10/group). Mortality was monitored for 1 h after induction of anaphylactic shock (Chai et al., 2005).

5. Compound 48/80-induced ear swelling response

Ear swelling response was investigated by the method described previously (Choi et al., 2006a, b). Compound 48/80 was freshly dissolved in saline (5 mg/mL) and injected intradermally in the ventral aspect of left side of mouse ear (100 μg/site, 20 μL) using a 30-gauge hypodermic needle. Sham saline was injected intradermally in the ventral aspect of right side of mouse ear. Ear thickness was measured with a digital micrometer (Mitutoyo, No. 7326, Japan) under mild anesthesia induced by intraperitoneal injection of 1:1 mixture (50 μL) of ketamin (1 mg/mL) and xylazine hydrochloride (23.32 mg/mL). Mice were kept in immobility state during the measurement. Ear swelling response represented an increment in thickness above baseline control values. Ear swelling response was determined at 1 h after injection of compound 48/80 or vehicle. MEAA, MESM, or METC (1 to 100 g/kg BW) was administered orally at 24, 12 and 1 h before injection of compound 48/80.

6. Anti-DNP IgE-mediated passive cutaneous anaphylaxis (PCA)

An IgE-dependent cutaneous reaction was generated by sensitizing the skin with an intradermal injection of anti-DNP IgE followed 48 h later with an injection of DNP-HSA into the rat’s penis vein. The DNP-HSA was diluted in phosphate-buffered saline (PBS). The rats were injected intradermally with 500 ng of anti-DNP IgE into each of 4 dorsal skin sites that had been shaved 24 h earlier. The sites were outlined with a water-insoluble red marker. After 48 h, each rat received an injection of 20 μg DNP-HSA in PBS containing 0.5% Evans blue via the penis vein. MEAA, MESM, or METC was orally administered 24, 12 and 1 h before the challenge. Thirty minutes after the challenge, the rats were anesthetized with ether and the dorsal skin was removed for measurement of the pigment area. The amount of dye was then determined colorimetrically after extraction with 1 mL of formamide based on the method of Li et al. (2005a). The absorbent intensity of the extraction was measured at 620 nm in a spectrophotometer (Spectra MAX PLUS, Molecular Devices, CA, USA), and the amount of dye was calculated with the Evans blue measuring line.

7. Preparation of RPMC suspension and microscopic observation

RPMC were isolated as previously described (Choi et al., 2006a). In brief, rats were anesthetized with ether and injected with 10 mL of calcium-free HEPES-Tyrode buffer into the peritoneal cavity, and the abdomen was gently massaged for about 90 s. The peritoneal cavity was opened, and the fluid was aspirated by a Pasteur pipette, and RPMC were purified by using a percoll density gradient as described in detail elsewhere (Hachisuka et al., 1988). RPMC preparations were at least 95% pure and at least 98% of these cells were viable as assessed by trypan blue exclusion (Yoshimura et al., 2004). Purified mast cells (1 × 10⁶ cells/mL) were resuspended in HEPES-Tyrode buffer. Mast cells were observed under phase contrast microscope and photographed as described (Na et al., 2009).

8. Mast cell viability assay

To test the viability of RPMC at time zero and subsequent time-points as indicated, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay was performed as described previously (Mascotti et al., 2000).

9. Assay of histamine release

Mast cell suspensions (2 × 10⁵ cells in 200 μL) were preincubated with MEAA, MESM, or METC (1, 10 or 100 μg/mL) for 10 min at 37°C and then incubated with compound 48/80 (10 μg/mL). The cells were separated from the released histamine by centrifugation at 150 × g for 10 min at 4°C. Residual histamine in the cells was released by disrupting the cells with boiling. After centrifugation, histamine content was measured by the radioenzymatic method described by Choi et al. (2006b). The inhibition percentage histamine release was calculated using the following formula: % Inhibition═{(Histamine release without MEAA, MESM, or METC)/Histamine release with MEAA, MESM, or METC} × 100.
10. Measurement of $^{45}$Ca uptake

The calcium uptake of mast cells was measured according to the method described by Li et al. (2005b). Purified mast cells were resuspended in HEPES-Tyrode buffer containing $^{45}$Ca (1.5 mCi/mL; 1 Ci = $3.7 \times 10^{10}$ becquerels; PerkinElmer Life Sciences, MA, USA), and incubated for 10 min at 4°C. Mast cell suspensions were preincubated with MEAA, MESM, or METC (1, 10 or 100 μg/mL) for 10 min at 37°C and then incubated with compound 48/80 (10 μg/mL). The reaction was stopped by the addition of 1 mM lanthanum chloride and centrifuged 3 times at 4°C for 10 min, then the cells in the pellet were disrupted with 10% Triton X-100 by vigorous shaking. Radioactivity of the solution was measured in a scintillation β-counter (Liquid scintillation Analyzer, A canberra company, Australia).

11. Statistical analysis

The results obtained were expressed as mean ± SEM for the number of experiments. Student’s t-test and ANOVA with Dunnett’s test were used to make a statistical comparison among the groups. Results with $P < 0.05$ were considered statistically significant.

RESULTS

1. Effect of MEAA, MESM, and METC on compound 48/80-induced systemic anaphylaxis

To investigate the effects of MEAA, MESM, and METC in anaphylactic reactions, the in vivo model of systemic anaphylaxis was used. After the injection of compound 48/80 (concentration ranging from 5, 10, 15 mg/kg BW), the mice were monitored for 1 h to determine their mortality. As shown in Table 1, compound 48/80 induced fatal shocks in ranging from 80 to 100% of some groups. Oral administration of MEAA, MESM, and METC reduced compound 48/80-induced mortality in a dose-dependent manner. At the dose of 1 g/kg BW of MEAA, MESM, and METC, the compound 48/80-induced death of mice was completely inhibited (Table 1).

2. Effect of MEAA, MESM, and METC on compound 48/80-induced ear swelling response

Ear swelling was induced by the injection of compound 48/80 (100 μg/site) as described. Oral administration of MEAA, MESM, and METC reduced the ear swelling response induced by compound 48/80 in a dose-dependent way (Fig. 1).

3. Effect of MEAA, MESM, and METC on anti-DNP IgE-mediated PCA

Another way to test the anaphylactic reaction is to induce PCA. As described in Materials and methods, local extravasation was induced by a local injection of anti-DNP IgE followed by an intravenous antigenic challenge. Oral administration of MEAA, MESM, and METC (10 or 100 mg/kg BW) inhibited
4. Effect of MEAA, MESM, and METC on mast cell viability

Fig. 3 shows the results of exposing the RPMC to MEAA, MESM, and METC on the viabilities of the cells determined by the MTT assay. Viability was not affected by concentration of 1~100 μg/mL MEAA, MESM, and METC for 2 h. Treatment of MEAA, MESM, and METC had no cytotoxicity on RPMC (Fig. 3).

5. Effect of MEAA, MESM, and METC on mast cell degranulation

To investigate the inhibitory mechanism of MEAA, MESM,
and METC on the anaphylactic reactions, we observed the compound 48/80-induced mast cell activation (Fig. 4). The normal rat peritoneal mast cells (RPMC) were generally spherical, or oval in shape, and had many fine granules surrounding a prominent nucleus (Fig. 4A). After stimulation with compound 48/80 for 5 min, RPMC were degranulated (Fig. 4B). The characteristics of mast cell degranulation were the cell swelling, cytoplasmic vacuoles, and extruded granules near the cell surface and in the surrounding medium. When RPMC were incubated with MEAA (Fig. 4C), MESM (Fig. 4E), or METC (Fig. 4G) alone, RPMC were similar to ones as seen in Fig. 1A. After the compound 48/80 stimulation of RPMC pretreated by MEAA (Fig. 4D), MESM (Fig. 4F), or METC (Fig. 4H), RPMC showed cell swelling without degranulation.

6. Effect of MEAA, MESM, and METC on histamine release from RPMC

The inhibitory effect of MEAA, MESM, or METC on the compound 48/80-induced histamine release from RPMC was shown (Fig. 5). MEAA, MESM, and METC dose-dependently inhibited the compound 48/80-induced histamine release at concentrations of 1 ~ 100 μg/mL. These results indicate that MEAA, MESM, and METC may be activity to inhibit the compound 48/80-induced or anti-DNP IgE-mediated anaphylactic reactions by blocking histamine release from mast cells.

7. Effect of MEAA, MESM, and METC on calcium uptake into RPMC

Because elevation of intracellular calcium level occurs during mast cell degranulation and calcium ions are also involved in the histamine release from RPMC, we wanted to find out whether MEAA, MESM, and METC also suppress calcium uptake into the RPMC. MEAA, MESM, and METC alone did not affect the calcium uptake into RPMC, while compound 48/80 increased the calcium uptake into RPMC. However, MEAA, MESM, and METC inhibited the compound 48/80-induced calcium uptake increment into RPMC in a concentration-dependent manner (Fig. 6). These results suggest that MEAA, MESM, and METC may inhibit histamine release by blocking of calcium uptake into mast cells.

DISCUSSION

The antiallergic effects of A. asphodeloides, S. miltiorrhiza, and T. chebula were analyzed, as part of continuing search for biologically active antiallergic agents from the medicinal sources. In this study, MEAA, MESM, and METC inhibited the compound 48/80-induced systemic anaphylaxis and anti-DNP IgE-mediated PCA. These results indicated that nonspecific and specific mast cell-dependent allergic reactions were signi-
Significantly inhibited by MEAA, MESM, and METC. It is believed that stimulation of mast cells with compound 48/80 or anti-DNP IgE initiates the activation of a signal-transduction pathway, which leads to histamine release. The release of histamine and other pharmacological mediator from mast cells is a prominent feature of acute inflammatory processes including the immediate type anaphylactic reactions (Lantz et al., 1998). Histamine released from mast cells is stored in secretory granules. There are a various agents to induce the mast cell degranulation, which are commonly associated with the anaphylactic shocks in human and other mammals. Compound 48/80 increases the permeability of the lipid bilayer membrane by causing a perturbation of the cell membrane, and elicits mast cell activation that is mediated by the mast cell degranulation, and by the change of free cytoplasmic calcium concentration (Lorenz et al., 1998). MEAA, MESM, and METC might act on the lipid bilayer membrane affecting the prevention of the perturbation being induced by compound 48/80. The MEAA, MESM, and METC-administered mice were protected from IgE-mediated local allergic reaction.

MEAA, MESM, and METC inhibited the compound 48/80-induced mast cell degranulation and histamine release from RPMC in concentration-dependent manner. The close correlation among the concentration of the histamine-releasing stimulus, the calcium ion influx, and the amount of released histamine suggest a cause- and effect-relationship between the influx of calcium and the release of histamine (Hachisuka et al., 1988). Compound 48/80 caused the release of histamine from RPMC following calcium uptake, but the pretreatment with MEAA, MESM, and METC inhibited the compound 48/80-induced calcium uptake into RPMC. It is conceivable that MEAA, MESM, and METC inhibited the initial phase of immediate-type allergic reactions, probably through interference with the mast cell-histamine-calcium system.

The early phase PCA response is mediated by the fast degranulation of mast cells and the rapid release of histamine and serotonin resulting in locally increased blood vessel permeability (Inagaki et al., 1986a, b). Several reports show the relation of intracellular calcium and inflammatory cytokine production from mast cells (Jeong et al., 2002; Tanaka et al., 2005). Consistent with the in vivo PCA experiments, MEAA, MESM, and METC decreased the elevated calcium uptake into mast cells in our present study. These data suggest that inhibition of calcium uptake into the mast cell by MEAA, MESM, and METC modulates inflammatory cytokine production.

The active components in MEAA, MESM, and METC have not been purified phenolic compounds. However, steroidal saponins isolated from A. asphodeloides (Lee et al., 2010), tanshinone derivatives from the S. miltiorrhiza (Jang et al., 2006; Fang et al., 2008), and Aller-7 from T. chebula (D’Souza et al., 2004; Pratibha et al., 2004) shown to have anti-inflammatory, anti-histaminic, and anti-oxidant activities. Tanshinone isolated from MESM (Trinh et al., 2010) and mangiferin from A. asphodeloides (Lee et al., 2009) were reported to possess inhibitory effects passive cutaneous anaphylaxis reaction and pruritis in mice. These reports and my studies suggest the possibility that phenolic compounds such as tanshinones, saponins, and mangiferin may inhibit the compound 48/80-induced or anti-DNP IgE-induced anaphylactic reactions by mast cell degranulation, histamine release, and calcium uptake into RPMC. However, further studies are needed to elucidate the purification and characterization of the bioactive ingredients.

In conclusion, I have show that MEAA, MESM, and METC can inhibit the compound 48/80-induced or anti-DNP IgE-induced anaphylactic reactions by mast cell degranulation, histamine release, and calcium uptake into RPMC. These MEAA, MESM, and METC inhibition of mediator release appear to be involved in the suppression of calcium uptake in mast cells. Given these findings, MEAA, MESM, and METC could contribute to the prevention or treatment of mast cell-mediated allergic diseases.

REFERENCES

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</국문초록>

한방에서 사용되는 약재 중 가자와 지모

이 페로미네와 약초에 능효과가 있다고 알려졌다

그러나 비만세포와 관련된

항염증효과가 있다고 알려졌다

단삼은 면역조절 및 항

암효과, 항염증효과가 있다고 알려졌다. 그러나 비만세포와 관련된
아나필락시스 이해할 수 있는 세포학적 기전에 대한 가자와 지모, 단삼의 효과에 대해서는 알려진 바 없다. 본 연구에서는 가자와 지모, 단삼의 메탄올 추출물이 아나필락시스 반응에 대해 어떠한 영향을 가져오지 않는지 조사하였다. 시험관내 실험과 생체 실험을 통하여, 가자와 지모, 단삼의 메탄올 추출물이 compound 48/80에 의한 아나필락시스와 비만세포 활성화 및 IgE에 의한 피부반응을 관찰하였다.

실험결과는 다음과 같다. 1) 가자와 지모, 단삼의 메탄올 추출물은 compound 48/80에 의한 전신성 아나필락시스와 귀부종 반응, IgE에 의한 피부반응을 억제하였다. 2) Compound 48/80에 의한 비만세포 탈과립과 흰쥐 복강 비만세포로부터 히스타민 유리가 가자와 지모, 단삼의 메탄올 추출물 전처리에 의해 억제되었다. 3) Compound 48/80에 의한 귀부종 비만세포내로의 칼슘 유입이 가자와 지모, 단삼의 메탄올 추출물 전처리에 의해 현저하게 억제되었다.

이상의 결과로 가자와 지모, 단삼의 메탄올 추출물은 비만세포 탈과립과 비만세포로부터 히스타민 유리, 비만세포내로 칼슘유입을 억제함으로써 compound 48/80에 의한 아나필락시스와 IgE에 의한 피부반응을 억제한다는 것을 알 수 있었다. 이로써 가자와 지모, 단삼은 알레르기 질환에 대한 효과적인 치료제로 이용될 수 있을 것으로 생각된다.