

Effects of Inhalable Microparticles of Seonpyejeongcheon-Tang in an Asthma Mouse Model

- Effects of Microparticles of SJT -

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Key Words

asthma, cigarette smoking, inhalable microparticles, Seonpyejeongcheon-tang (SJT), spray drying

Abstract

Objectives: Allergic asthma generally presents with symptoms of wheezing, coughing, breathlessness, and airway inflammation. Seonpyejeongcheon-tang (SJT) consists of 12 herbs. It originated from Jeongcheon-tang (JT), also known as Ding-chuan-tang, composed of 7 herbs, in She-sheng-zhong-miao-fang. This study aimed to evaluate the effects of local delivery of SJT *via* inhalable microparticles in an asthma mouse model.

Methods: Microparticles containing SJT were produced by spray-drying with leucine as an excipient. SJT microparticles were evaluated with respect to their aerodynamic properties, *in vitro* cytotoxicity, *in vivo* toxicity, and therapeutic effects on ovalbumin (OVA)-induced asthma in comparison with orally-administered SJT.

Results: SJT microparticles provided desirable aerodynamic properties (fine particle fraction of $48.9\% \pm 6.4\%$ and mass median aerodynamic diameter of $3.7 \pm 0.3 \mu\text{m}$). SJT microparticles did not show any cytotoxicity against RAW 264.7 macrophages at concentrations of 0.01 - 3 mg/mL. Inhaled SJT microparticles decreased the levels of IL-4, IL-5, IL-13, IL-17A, eotaxin and OVA-IgE in bronchoalveolar lavage fluid (BALF) in mice with OVA-induced asthma. These effects were verified by histological evaluation of the levels of infiltration of inflammatory cells and collagen, destructions of alveoli and bronchioles, and hyperplasia of goblet cells in lung tissues. The effects of SJT microparticles in the asthma model were equivalent to those of orally-administered SJT extract.

Conclusion: This study suggests that SJT is a promising agent for inhalation therapy for patients with asthma.

1. Introduction

Allergic asthma is a chronic inflammatory disease of the airways, characterized by airway eosinophilia, goblet-cell hyperplasia, and mucus hypersecretion in response to inhaled allergens and nonspecific stimuli [1, 2]. Asthma patients generally present with symptoms such as wheezing, coughing, breathlessness, and

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airway inflammation. The features of asthma are airway inflammation, reversible airflow obstruction, and an increased sensitivity to bronchoconstricting agents, termed airway hyperresponsiveness (AHR) [3]. Immune cells involved in the regulation of allergic airway inflammatory responses include monocytes/macrophages, dendritic cells, neutrophils, basophils, mast cells, eosinophils, and T and B lymphocytes [4]. Asthma causes excess production of Th2 cytokines (IL-4, IL-5, IL-13) from inflammation cells in the airways and eosinophilic infiltration into the lungs. Increased eosinophils and T lymphocytes in the bronchial mucosa and bronchoalveolar lavage fluid (BALF) are distinctive features of the inflammatory responses in asthma patients, and the extent shows an apparent correlation with the severity of the disease [5-7].

Seonpyejeongcheon-tang (SJT) consists of 12 herbs. It originated from Jeongcheon-tang (JT), also known as Ding-chuan-tang, composed of 7 kinds of herbs (*Farfarae flos*, *Ephedrae herba*, *Armeniaca Amarum semen*, *Scutellariae radix*, *Pinelliae tuber*, *Mori cortex*, *Glycyrrhizae radix*), in She-sheng-zhong-miao-fang. JT has been used for antipyretics, as an expectorant, and for stopping asthma by aerating lung “Qi (氣); i.e., lung energy. JT has also

shown anti-asthmatic effects in pre-clinical [8] and clinical studies [9]. SJT contains five additional components: *Platycodi radix* and *Fritillariae Cirrhosae bulbus* for expectoration and cough remedy; *Lonicerae flos* for antipyretics and detoxification; *Liriopsis tuber* for supplying fluid and reinforcing lung Qi; and *Schizandrae fructus* for cough remedy. SJT showed protective effects for the lungs in an elastase-induced lung injury mouse model via reduction of caspase-3, tumor necrosis factor (TNF)- α and IL-1 β [10]. SJT also showed an antitussive effect in chronic cough patients [11]. These studies collectively support the effectiveness of SJT in treating patients with asthma and related respiratory symptoms and have prompted the Korean Ministry of Food and Drug Safety (MFDS) to approve SJT as an investigator-initiated investigational new drug (IND) for treating patients with cough variant asthma [12]. In this study, we aimed to develop an inhalable microparticle form of SJT and evaluate its delivery efficiency, biocompatibility in the lungs, and bioactivity in an animal model of asthma. We investigated the effects of SJT microparticles on the total pulmonary airflow in mice, the eosinophil influx, the total leukocyte number, cell surface markers, and cytokine production in BALF.

Table 1 Composition of SJT

Herbal name	Scientific name	Part used	Place of origin	Amount used (g)
Lonicerae Flos	<i>Lonicerae japonica</i> Thunberg	Flower	China (Henan)	6.0
Farfarae Flos	<i>Tussilago farfara</i> L.	Flower	China (Neimenggu)	6.0
Ephedrae Herba	<i>Ephedra sinica</i> Stapf	Whole plant	China (Neimenggu)	6.0
Armeniaca Amarum Semen	<i>Prunus armeniaca</i> L. var. <i>ansu</i> Maxim.	Seed	China (Shanxi)	4.0
Scutellariae Radix	<i>Scutellaria baicalensis</i> Georgi	Root	China (Heilongjiang)	3.0
Pinelliae Tuber	<i>Pinellia ternata</i> Breitenbach	Root tuber	China (Gansu)	3.0
Mori Cortex	<i>Morus alba</i> L.	Bark	China (Henan)	3.0
Platycodi Radix	<i>Platycodon grandiflorum</i> A. De Candolle	Root	China (Neimenggu)	3.0
Fritillariae Cirrhosae Bulbus	<i>Fritillaria thunbergii</i> Miquel	Bulb	China (Zhejiang)	3.0
Liriopsis Tuber	<i>Liriope platyphylla</i> Wang et Tang	Root tuber	South Korea (Kyeongnam)	3.0
Schizandrae Fructus	<i>Schisandra chinensis</i> Baillon	Fruit	South Korea (Gangweon)	2.0
Glycyrrhizae Radix	<i>Glycyrrhiza uralensis</i> Fischer	Root	China (Neimenggu)	2.0
Total	44.0			

SJT, Seonpyejeongcheon-tang.

2. Materials and Methods

The herbs of SJT were obtained from Human Herbs Co. Ltd. (Kyeongsan, Korea), a licensed herb company (Table 1). The herb samples were identified by Professor C. G. Son (College of Korean Medicine, Daejeon University, Daejeon, Korea). Voucher specimens (No. 2014-028) of the collected herb samples were deposited in the herbarium, according to the procedure described by Park [13]. SJT was suspended in 70% ethanol and extracted at 60 - 70°C for 3 hours by using reflux extraction. The ethanolic extract was evaporated at 45°C and subsequently lyophilized. The extraction yield was 26.1%.

Five-week-old male and female BALB/c mice were obtained from Daehan Biolink Co. LTD (Eumseong, Korea). The procedures used in this study were approved by the Committee for Animal Welfare at Daejeon University (Written approval number DJUAR2013-002), and all animal procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea).

Inhalable microparticle forms of SJT extract were prepared by spray-drying using the LabPlant SD-05 spray dryer (Lab-Plant Ltd., Huddersfield, UK). Specifically, lyophilized SJT extract (80 wt%) and an excipient mixture (20 wt%), consisting of leucine, dipalmitoylphosphatidylcholine (DPPC), or a 4:1 mixture of leucine and DPPC, were dissolved in 70% ethanol. Typically, the solution was introduced to the spray dryer at 17 - 20 mL/min and atomized through a 1-mm nozzle by using compressed air with an inlet temperature of 150°C. The formed microparticles were referred to as Seonpyejeongcheon-tang Microparticles (SJT-MPs).

The aerodynamic properties of the SJT-MPs were evaluated using the eight-stage Mark II Anderson Cascade Impactor (ACI), as we previously reported [14, 15]. Dry SJT-MPs (10 mg) were manually loaded into a gelatin capsule (size 3), put in a Rotahaler; the capsule was then split open to release the particles. Each set of SJT-MPs was drawn through the induction port into the ACI operating at a flow rate of 28.3 L/min for 8.5 seconds. The number of SJT-MPs deposited at each impaction stage was determined by measuring the difference in the weights of the collection plates (for the filter stage, a glass filter with pore size < 1 µm was used; Thermo Fisher). The effective cutoff aerodynamic diameters for stages 0 to 7 were 9 µm, 5.8 µm, 4.7 µm, 3.3 µm, 2.1 µm, 1.1 µm, 0.65 µm, and 0.43 µm, respectively. The fine particle fraction (FPF) was defined as the number of SJT-MPs with aerodynamic sizes < 4.7 µm (particles deposited at stage 3 and lower) divided by the initial total number of

particles loaded into the Rotahaler (10 mg, nominal dose). The cumulative mass of particles with sizes less than effective cutoff diameter as percent of the total mass recovered in the ACI was plotted against the effective cutoff diameter. The mass median aerodynamic diameter (MMAD) was defined on this graph as the particle size at which the line crossed the 50th percentile.

The morphologies of the SJT-MPs, critical information for explaining the aerodynamic properties of the particles, were examined using scanning electron microscopy. Dry SJT-MPs were attached to specimen stubs by using a double-sided tape and were sputter-coated with gold-palladium in the presence of argon gas by using a Hummer I sputter coater (Anatech Ltd.). THE SJT-MPS were imaged with a JEOL JSM-840 scanning electron microscope (JEOL USA, Inc.) at an accelerating voltage of 5 kV, a working distance of 10 mm, an objective aperture of 70 µm, and a probe current of 6×10^{11} A.

Cytotoxicity was tested by using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assay. The RAW 264.7 murine macrophage-like cell line was maintained in a Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were placed in a 96-well plate at a density of 2.5×10^4 per well. After incubation, the medium was replaced with one containing the original SJT extract or SJT-MPs at final concentrations equivalent to 0.01, 0.03, 0.1, 0.3, 1, and 3 mg/mL. The MTT assay was performed after a 24-hour incubation with SJT extract or SJT-MPs. The cytotoxicity was quantified by measuring the ultraviolet (UV) absorbance at 570 nm with a microplate reader. The measured absorbance was normalized to the absorbance of non-treated control cells.

A mouse model of ovalbumin (OVA)-induced asthma was created based on a previously-described protocol, but with modifications [16]. Briefly, OVA (500 µg/mL) (Sigma-Aldrich Korea, Korea) in phosphate buffer saline (PBS) was mixed with equal volumes of 10% (w/v) aluminum potassium sulfate (alum; Sigma-Aldrich Korea, Korea) in distilled water, incubated for 60 minutes at room temperature (RT) after pH adjustment to 6.5 by using 10-N NaOH, and centrifuged at $750 \times g$ for 5 minutes. The OVA/alum pellet was resuspended at the original volume in distilled water. As shown in Fig. 1, all mice were immunized on two different days (i.e., days 0 and 7) by using intraperitoneal injections of 0.2 mL of an OVA/alum solution equivalent to 100 µg of OVA.

Animals were initially challenged via intratracheal injection of 100 µL of OVA solution (equivalent to 50 µg OVA). Seven days after the initial challenge, the mice were exposed to aerosolized OVA for 30 minutes per day, 3 days

Table 2 Aerodynamic properties of SJT-MPs

ED (mg)	FPF of CD (%)	FPF of ED (%)	FPF of ND (%)	MMAD (µm)
6.8 ± 0.4	61.3 ± 4.7	48.9 ± 6.4	33.4 ± 6.6	3.7 ± 0.3

SJT-MP, Seonpyejeongcheon-tang microparticles; ED, emitted dose; FPF, fine particle fraction; CD, collection dose; ND, nominal dose (10.0 ± 0.3 mg), MMAD, mass median aerodynamic diameter.

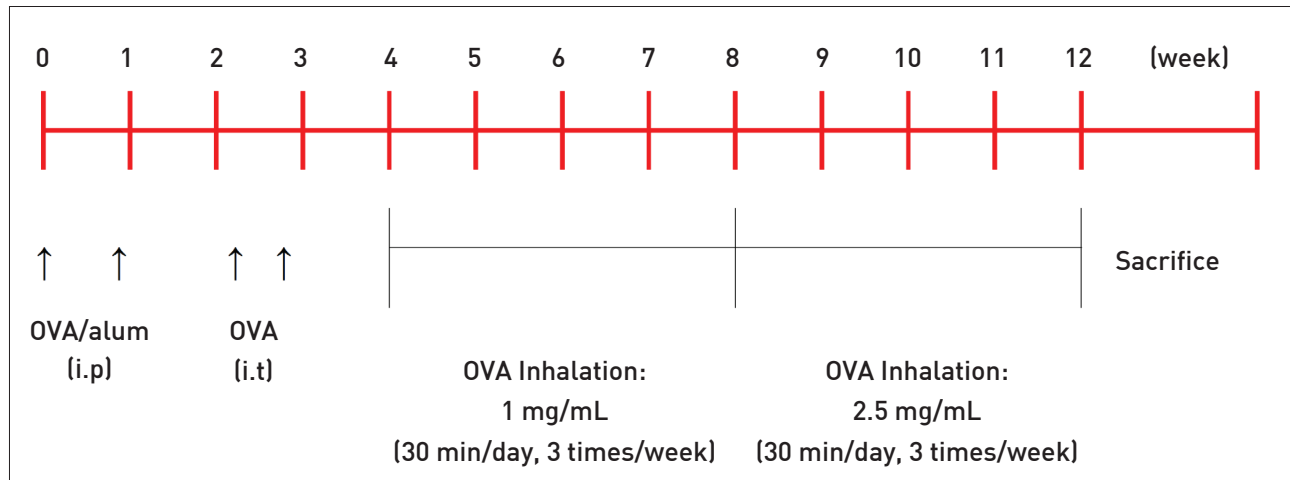
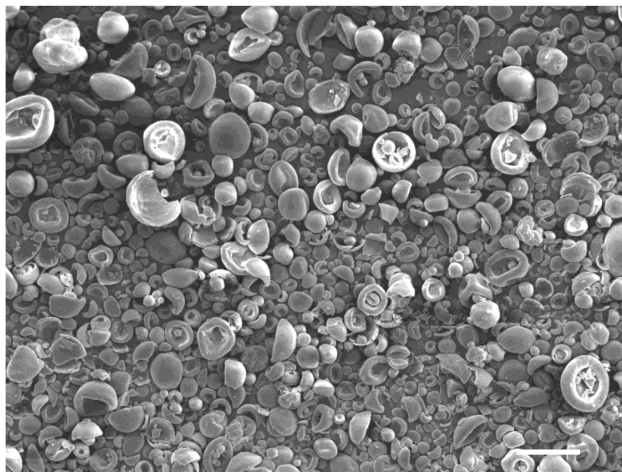


Figure 1 Experimental plan of repeated OVA exposure.

OVA, ovalbumin; i.p., intraperitoneal; i.t., intratracheal.



A



B

Figure 2 Scanning electron microscopic images of SJT-MPs.

Magnification: (A) 1000 ×, (B) 2500 ×. Scale bar: 10 μm. SJT-MPs, Seonpyejeongcheon-tang Microparticles.

per week, for 8 weeks (1 mg/mL OVA in normal saline for the first 4 weeks and 2.5 mg/mL OVA in normal saline for the last 4 weeks). Specifically, the particles were introduced into the lungs through a catheter by using a PennCentury dry-powder insufflator (Wyndmoor, PA, USA) [17]. SJT (200 and 400 mg/kg) was orally administered every day for a week during the last 2 weeks before sacrifice. SJT-MPs (50 and 100 mg/kg) were administered directly to the lungs via endotracheal intubation once per every three days during the last 2 weeks before sacrifice. One day after the last OVA exposure, animals were sacrificed using an i.p. injection of sodium pentobarbitone (100 mg/kg), and their bronchoalveolar lavage fluid and lung tissues were collected for further molecular analyses.

Following the sacrifice, the trachea was cannulated, and BALF was obtained by washing the airway lumen. Briefly, cells of the lungs were recovered by flushing 1 mL of fluid

(1 mM ethylenediaminetetraacetic acid (EDTA), 10% FBS, PBS) into the lungs via the trachea. The supernatant of the BALF was stored at -25°C and was later used to determine the cytokine levels. The levels of interleukins (IL-4, IL-5, IL-13, IL-17A, eotaxin) and anti-OVA IgE in the BALF of the indicated mice were measured by using enzyme-linked immunosorbent assay, enzyme-linked immunospecific assay (ELISA) monoclonal antibody-based mouse interleukin ELISA kits (eBioscience, San Diego, CA, USA) and mouse OVA IgE ELISA kits (Shibayagi, Shibukawa, Japan) according to the manufacturers' instructions.

The lungs were examined histologically using a previously-described protocol with some modifications [16]. The lung tissues were embedded in paraffin, cut into 3-μm-thick sections, and stained with hematoxylin and eosin (H&E) or M-T solution. The degree of inflammatory cell in-

filtration into the airways was scored in a double-blinded manner by two independent observers. The degree of peribronchiole and perivascular inflammation was evaluated on a scale of 0 - 2, where 2 indicates the highest severity [16]. Periodic acid-Schiff (PAS) staining was performed to identify mucus secretion in the lung tissue. Frozen sections (30 μ m in thickness) were prepared and mounted on gelatin-coated slides, stained with PAS reagents, dehydrated, and cover-slipped with permount. The PAS-positive goblet cells were counted manually, normalized to the length of the bronchial epithelial perimeter on the basal side, and expressed as the number of PAS-positive cells per millimeter of the basement membrane.

Data were analyzed by using the analysis of variance (ANOVA), followed by the Dunnett's multiple comparison test (SPSS version 12.0 statistic software). Results (presented as mean \pm standard error of mean) were considered statistically significant if the *P* values were < 0.05 (*), < 0.01 (**), or < 0.001 (***).

3. Results

The aerodynamic properties of the SJT-MPs were evaluated using an ACI. The fine particle fraction of the microparticles increased with the addition of an excipient (leucine, DPPC, or their mixture) added as a dispersion enhancer. Based on their aerodynamic properties, SJT-MPs contain-

ing 20% leucine were chosen for subsequent studies (Table 2). The SJT-MPs were deformed spheres with sizes ranging from 1 to 10 μ m (Fig. 2). Their hollow interiors suggest that the particles had low densities, consistent with the MMAD estimated by using the ACI evaluation.

The cytotoxicities of the SJT extract and the SJT-MPs for RAW 264.7 macrophages were evaluated by using the MTT assay. RAW 264.7 cells were used to evaluate the anti-inflammatory effects for asthma as a heterogeneous airway inflammatory disease [17]. The MTT assay was performed after the cells had been incubated with SJT extract or SJT-MPs for 24 hours at concentrations equivalent to 0.01 - 3 mg/mL of SJT. The results showed that the SJT itself and the SJT-MPs themselves had no cytotoxic effect on the RAW 264.7 cells (Fig. 3).

To analyze whether the SJT and the SJT-MPs induced Th2 cytokine secretion, we measured the levels of IL-4, IL-5, and IL-13 in the BALF after the final challenge. The IL-4, IL-5 and IL-13 levels were significantly reduced in the SJT-treated (200 and 400 mg/kg oral) and the SJT-MP-treated (50 and 100 mg/kg endotracheally) mice as compared to the non-treated control group. In addition, the IL-17 level was significantly reduced in both the SJT-treated and the SJT-MP-treated mice. The eotaxin level was also reduced in the SJT-MP-treated (100 mg/kg) mice. An important feature of the allergic asthma model is the production of OVA-specific IgE. The SJT-MP-treated (100 mg/kg) mice showed significantly reduced levels of OVA-specific IgE (Fig. 4).

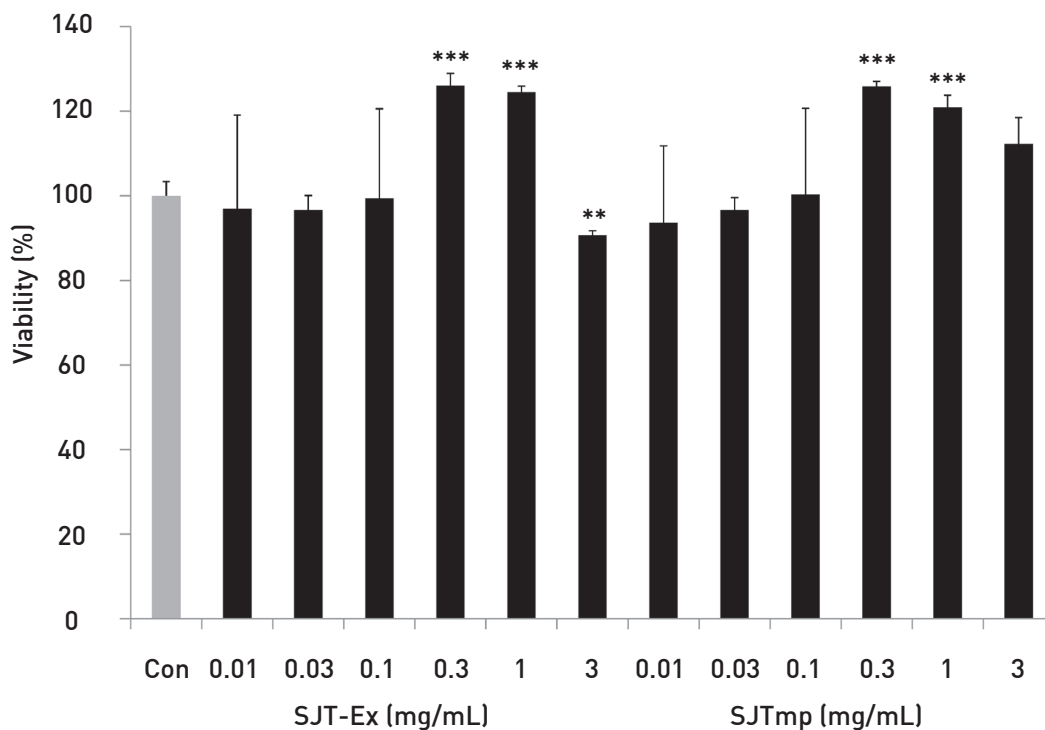


Figure 3 Cell viability of SJT-MPs on RAW 264.7 cells, where SJT-Ex and SJT-MPs. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 compared to control. ANOVA followed by Dunnett's multiple comparison.

SJT-MPs, Seonpyejeongcheon-tang Microparticles; SJT-Ex, Seonpyejeongcheon-tang extract; ANOVA, analysis of variance.

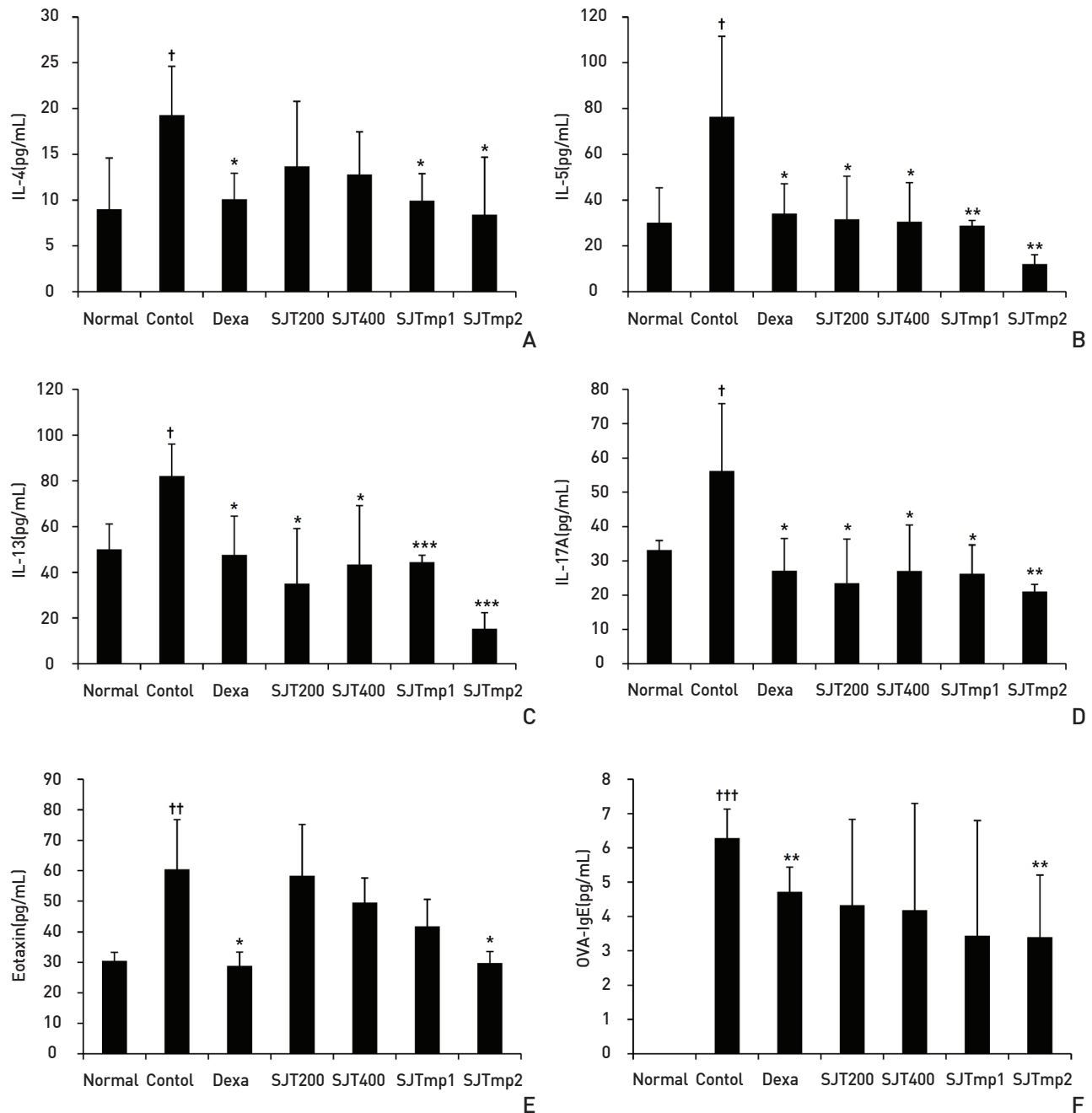


Figure 4 Effects of SJT-MPs on cytokine production in a mouse asthma model. Mice were challenged by OVA inhalation and then treated with dexamethasone *p.o.* (Dexa: 0.5 mg/kg), SJT extract *p.o.* (SJT200: 200 mg/kg, SJT400: 400 mg/kg), and SJT-microparticle inhalation (SJT-MP1: 50 mg/kg, SJT-MP2: 100 mg/kg). The (A) IL-4, (B) IL-5, (C) IL-13, (D) IL-17A, (E) eotaxin, and (F) OVA-IgE productions were measured by using ELISA. Data are represented as mean \pm SD (n = 6). [†]*P* < 0.05, ^{††}*P* < 0.01, ^{†††}*P* < 0.001 compared to normal. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared to control. ANOVA followed by Dunnett's multiple comparison.

SJT-MPs, Seonpyejeongcheon-tang Microparticles; OVA, ovalbumin; ANOVA, analysis of variance.

After OVA challenge, the signs of inflammation in the peribronchial and the perivascular regions of the mice receiving SJT and SJT-MPs were observed and compared with those of the non-treated group. The non-treated mice and the SJT-treated mice showed inflammatory histological changes as compared with normal mice. Infiltration of leukocytes was observed in sections of the lungs from the

non-treated mice. Inflammatory infiltration and erosion were apparent in the peribronchial and perivascular areas. The peribronchial and the perivascular inflammatory infiltrates consisted of eosinophils, mast cells, and lymphocytes. Eosinophil infiltration was mainly observed in the peribronchial parts of the lungs. In contrast, SJT- and SJT-MP-treated mice showed reduced airway inflammation in

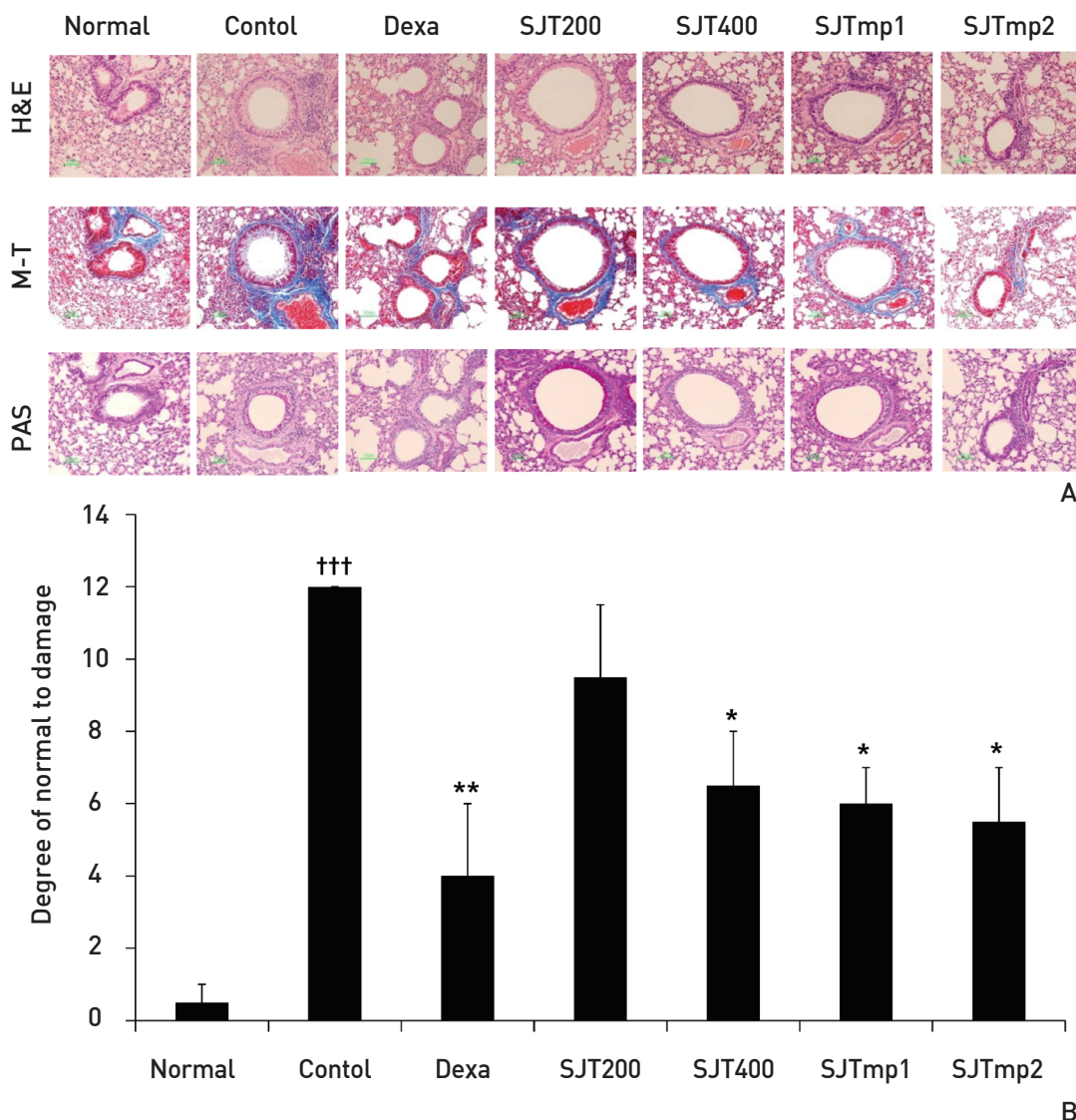


Figure 5 Effects of SJT-Ex and SJT-MPs on the histology of lung tissues in an asthma mouse model. Mice were challenged with OVA inhalation and then treated with dexamethasone *p.o.* (Dexa: 0.5 mg/kg), SJT-Ex *p.o.* (SJT200: 200 mg/kg, SJT400: 400 mg/kg), and SJT-MP inhalation (SJT-MP1: 50 mg/kg, SJT-MP2: 100 mg/kg). (A) Mouse lung sections were stained by using H&E, Masson's trichrome (M-T) and PAS methods. (B) The indices of lung damage were calculated by using a subjective scale. The degree of lung damage was evaluated on a scale of 0 - 2, with 2 indicating the highest severity. Data are represented as mean \pm SD ($n = 4$). ††† $P < 0.001$ compared to normal. * $P < 0.05$, † $P < 0.01$ compared to control. ANOVA followed by Dunnett's multiple comparison.

SJT-Ex, Seonpyejeongcheon-tang extract; SJT-MPs, Seonpyejeongcheon-tang Microparticles; OVA, ovalbumin; H&E, hematoxylin and eosin; PAS, periodic acid-Schiff; ANOVA, analysis of variance.

the lung tissue (Fig. 5).

4. Discussion

Both clinical research and experimental research have shown the potential effectiveness of many herbal medicines in the treatment of patients with pulmonary disease. However, herbal medicines have not been widely used for this purpose due to challenges in their administration [18]. Most herbal medicines are commonly taken as oral drugs,

either in the form of a decoction or a granular extract. For effective levels to be obtained *via* oral administration, large quantities of the herbal medicines are often required, which is not only inconvenient but also carries a potential risk of systemic side effects [19]. In this regard, inhalation might be a logical alternative route of administration; however, virtually no effort has been made to develop inhalable forms of herbal medicines.

Asthma is characterized by airway obstruction, which is variable and reversible. This is due to chronic inflammation of the respiratory tract, which is mediated by the increased

expression of multiple inflammatory proteins, including cytokines, chemokines, adhesion molecules, inflammatory enzymes, and receptors [20]. In allergic asthma, the activation of allergen-induced Th2 cells in the lungs leads to eosinophilic lung inflammation, increased secretion of mucus, and recurring bronchospasm. Eventually, with repeated allergen exposure, the bronchial smooth muscle and basement membrane thicken, lung tissue is destroyed, and lung function is impaired [21]. Th2 cells and their signature cytokines IL-4, IL-5, and IL-13 have key pathogenic roles in asthma [22]. Numerous therapeutic agents have been proposed for the treatment of patients with asthma. For example, anti-IL-5 inhibits eosinophil adhesion, infiltration, and mediator release [23] because IL-5 is the most critical cytokine for eosinophil differentiation, activation, and survival [24]. The recruitment and the activation of eosinophils appear to be controlled by the release of cytokines such as IL-5 and chemotactic agents such as eotaxin from Ag-stimulated T lymphocytes [25]. A suggestion has been made that IL-5 and eotaxin may collaborate in the regulation of blood and tissue eosinophilia in mice.

We tested the possibility of using SJT, a beneficial treatment for asthma based on traditional medicine clinical practice, in an inhalable dry-microparticle form. We produced SJT-MPs by using the spray-drying process. Leucine, DPPC, or a combination of the two was additionally included as an inactive ingredient due to their known abilities to enhance the aerodynamic properties of the resulting microparticles [14, 26]. The aerodynamic properties and the biological effects of SJT-MPs were measured using a set of standard *in vitro* techniques and an animal model of asthma. The goals were for the MMAD to be in the range of 0.5 - 5 μ m [27] and for the emitted dose (ED) and the FPF of the nominal dose to be comparable to those of the "large porous particles" introduced by Edwards *et al* [28]. The addition of leucine and/or DPPC helped achieve the target ED and FPF.

The secretions of the Th2 cytokines (IL-5, IL-13, IL-17A) and eotaxin and the level of OVA-IgE following OVA challenge were suppressed by the SJT-MPs to extents comparable or superior to those of dexamethasone at 0.5 mg/kg, a standard corticosteroid used in Western medicine as a treatment for patients with asthma. Suppression of these protein levels is important because the releases of IL-5 and eotaxin are involved in the inhibitions of eosinophil proliferation, respiratory influx, and the level of IL-17A in mucus secretion. Worth mentioning is the observation that SJT-MPs were comparable to orally-administered SJT extract in the inhibitions of IL-4, eotaxin, and IgE production at lower doses (50 and 100 mg/kg for SJT-MPs compared to 200 and 400 mg/kg for SJT). This suggests that the local administration of SJT-MPs can reduce the dose requirement of SJT. Following OVA challenge, inflammatory-cell infiltration, goblet-cell proliferation, collagen deposition, and damage in the bronchi and the alveoli were clearly observed in the lungs of the non-treated group; these were significantly reduced in the SJT-MP-treated animals.

5. Conclusion

SJT-MPs were more effective in successfully inhibiting the adverse changes associated with asthma than oral dexamethasone or SJT extract administered at a greater dose. This result suggests that SJT-MPs, by suppressing the production of pro-inflammatory cytokines, may have protective effects against the lung injury experienced by asthma patients.

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Conflicts of interests

The authors declare that there are no conflicts of interest.

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