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Therapeutic effects of traditional Korean medicine, Jeechool-Whan in allergic rhinitis model

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ABSTRACT

Jeechool-Whan (JW) is a prescription of Ponciri Fructus Immaturus and Atractylodis Rhizoma Alba and improves the functions of the stomach and the spleen. Although it is said in Korean Medicine that the spleen and the stomach are the roots of the body's resistance, the meaning of 'improving the spleen and the stomach' is very comprehensive. Moreover, there are lots of drugs that are said to improve the spleen and the stomach, and the number of prescriptions using these drugs is huge. In this study, we focused on the new effect and mechanism of the JW on the ovalbumin (OVA)-induced allergic rhinitis (AR) model. The increased number of rubs and the increased levels of IgE and histamine in the OVA-sensitized mice were inhibited by JW administration. The balance of Th1/Th2 cytokine level was regulated by JW administration. The levels inflammatory proteins were decreased by JW administration in the nasal mucosa of the OVA-sensitized mice. Eosinophils and mast cells infiltration increased by OVA-sensitization was also decreased in the JW-administered mice. In addition, JW inhibited caspase-1 activity in the same nasal mucosa tissue. In activated human mast cells, JW inhibited the receptor interacting protein-2, IκB kinase-β, nuclear factor-κB/Rel A, and caspase-1 activation. In conclusion, this study will be support the clear understanding of the concept of the spleen and the stomach in traditional Korean medicine as well as for a possibility of finding a cure for this AR in traditional medical treatments.

Keywords Jeechool-Whan, allergic rhinitis, macrophage inflammatory protein-2, intercellular adhesion molecule-1, receptor interacting protein, caspase-1

INTRODUCTION

Allergic rhinitis (AR) is a global health problem that causes major illness and disability and common manifestation of allergic diseases, affecting approximately 500 million people worldwide (Bousquet et al., 2008). Although not life threatening, AR can deteriorate the quality of life and can be a major risk. Despite its clinical and socioeconomic impact, advances in its treatment still have a long way to go (Lee et al., 2007). Many of the symptoms of patients with AR-sneezing, itching, and respiratory obstruction- cause a lot of pain. However, the symptoms of AR do not end here. If prolonged, AR, can cause problems in the nasal voice box and can cause very severe eye and ear symptoms (Helling and Fokkens, 2006). These symptoms are due to the release of histamine and other active substances by mast cells, which stimulate the dilation of blood vessels, irritate nerve endings and increase the secretion of tears (Whitcup, 2006).

Since the discovery by Coffman and colleagues of two distinct types of Th in mice (Mosmann et al., 1986), mutual regulation between Th1 cells and Th2 cells has been considered important for homeostatic maintenance of the immune system in the whole body. Dysregulated Th1 and Th2 responses lead to excessive Th1 cell or Th2 cell activation, resulting in the development of autoimmune diseases associated with the accumulation of Th1 cells or in an induction of allergic diseases due to the accumulation of Th2 cells, respectively (Bach, 2002). In response to exposure to allergens, patients with AR present an inflammatory Immunoglobulin (Ig)E-mediated response characterized by a Th2 immunologic pattern with mast cells and eosinophils activation and the release of inflammatory mediators, interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α (Howarth, 2003; Johansson et al., 2001). Leukotrienes and prostanoids produced by the 5-lipoxygenase and cyclooxygenase (COX)-2 pathways have potent pro-inflammatory and vascular actions that implicate them in allergic and inflammatory reactions (Montuschi et al., 2007). Eosinophils are innate effector cells that are important in immune responses against helminthes parasitic infections and contribute to the pathology associated with allergic inflammatory conditions. Mast cells contribute to the induction and/or maintenance of eosinophilic inflammation by a variety of mechanisms, including IgE-dependent and IgE-independent processes (Pawankar et al., 2007). The recruitment of these mast cells to inflammatory sites occurs in response to chemotactic and activation signals (Bournazou et al., 2010). The intercellular adhesion molecule (ICAM)-1, a member of the immunoglobulin gene superfamily of adhesion molecules, is expressed on the surface of a variety of cells, including vascular endothelial cells, epithelial cells, alveolar macrophages, and fibroblasts (Calderon and Lockey, 1992; Guzman et al., 1994; Montefort et al., 1994). ICAM-1 was

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considered as a marker of allergic inflammation (Albelda et al., 1991; Marlin and Springer, 1987). Macrophage-inflammatory protein 2 (MIP-2) is a potent chemoattractant for immune cells (Tosi et al., 1992; Gupta et al., 1996).

Caspase-1 is a member of the cystein-aspartic acid protease (caspase) family (Stutz et al., 2009). Caspase-1 is characterized by its ability to activate the inactive precursors of IL-1β and IL-18 that are involved in inflammation. Caspase-1 contains an N-terminal caspase recruitment domain (CARD). This CARD promotes the proteolytic activation of the recruited caspase-1 in inflammation (Lamkanfi et al., 2003). Caspase-1 is activated within the inflammasome, a large cytosolic protein complex that is induced by a growing number of endogenous, microbial, chemical or environmental stimuli (Yazdi et al., 2010). Specific adaptor molecules of the receptor interacting protein-2 (RIP2, CARD containing kinase) regulate the activation of caspase-1 through CARD-CARD interaction (Yoo et al., 2002; Chin et al., 2002) RIP2 then recruits the IκB kinase (IKK) complex through direct interaction of its intermediate domain with IKK-β, leading to the activation of nuclear factor (NF)-κB (Kobayashi et al., 2002; Inohara et al., 2000; Shin et al., 2000).

In eastern Asia traditional medicine, it is hard to specifically explain the medical theory. The major reason is that most core medical terms used to explain medical theory come from philosophy. In addition, many of meanings are compressed in a word and a character (Kim et al., 2011). The fact that the Jeechool-Whan (Bitter Orange and Atractylodes Pill, JW) can have an effective result on AR reflects the general and basic theory of East Asian Medicine that the spleen and the stomach are the center of the body. In ‘Shanghanlun’, where the JW first appears, it is said that the JW was first created in order to solve problems in the spleen and the stomach that occur in the process of various changes in symptoms of contagious diseases. Records on AR, which this paper aspires to study, cannot be found in documents of ancient East Asian medicine. Certainly, there are such expressions of symptoms such as 'sneezes', 'runny nose', and 'tiredness', but there is no clear description on the consecutive process (Skoner, 2001) that starts from sneezing, itching, and clear rhinorrhea and continues on to congestion, fatigue, malaise, irritability, and possibly neurocognitive deficits. Several possibilities can be brought up on this matter. First, there is a possibility that, in the course of developing remedies for diseases with high mortality rates, such as contagious diseases, infections, and verminous diseases, a rather trivial rhinitis that emerges during the periods of seasonal changes got less attention. Next, it is possible that AR is a newly emerged syndrome in modern times because of drastically changed life environment. It is certain that there is no experience of treating this syndrome in the ancient East Asian medicine. Nevertheless, the reason it is said that AR can be cured with a traditional formula of Oriental Medicine is because of an extensive range of the application of treatment concept of Oriental Medicine.

Numerous prescriptions exist in East Asian medical records. In ‘Pujifang’, published in 1406 in China, there are about 61,000 prescriptions; ‘Euibang-yuchui’, published in 1445 in Korea, also contains about 50,000 prescriptions. ‘Dongeuibogam’, published in 1610, has 3917 prescriptions which are said to have been selected from those prescriptions. The reason these kinds of remedies could be accumulated several hundreds of years ago is that in Oriental Medicine, treatment is considered as balancing the disharmony between the human body and nature, and not as discovering an effective medicine that treats a certain disease. Nature here could mean germs or harmful substances as described in modern medicine. The human body has a self-healing power that forces/enables the body to overcome harmful factors and to return to healthy state in certain types and degrees of unhealthy environments. Symptoms are byproducts of discharging harmful factors or overcoming the harmful environment. In Oriental Medicine, it is thought that the right combination of crude drugs could maximize the self-healing power of the human body, and thus those kinds of skills have been developed over a long period of time. Numerous prescriptions recorded in Oriental Medical documents are the results of this kind of combination of simple drugs.

As shown in the history of development of Oriental Medical treatment techniques, it can be said that new prescriptions are continuously being created not only so that diseases that cannot be cured with existing prescriptions can be treated, but also because types of diseases have been continuously changing. In the 2nd century, prescriptions containing Ephedrae Herba and Cinnamomi Ramulus were mainly used for symptoms similar to epidemics or the common cold, but prescriptions in which Osterici Radix, Saposhnikoviae Radix, and Schizonepetae Spica are used as main drugs have been replacing the Cinnamon Twig Decoction and the Ephedra Decoction of ‘Shanghanlun’ since the 12th century. From the 17th to the 19th century, techniques and methods for treating febrile diseases were intensively recorded in Chinese medical society; numerous prescriptions that contain Gypsum Fibrosum are contained in these records. Examples of the use of Gypsum Fibrosum can be found in records from the 2nd century, but it seems that the time Gypsum Fibrosum-containing prescriptions were intensively developed and came to be widely known is after the 17th century.

Changes in remedies like this are often found in modern days. The YULDA-HANSO-TANG [Cool the Diaphragm and Dispel Fire Decoction] is applied to treat cerebral infection (Jeong et al., 2007); Prescriptions that contain Cinnamomi Ramulus or Ephedrae Herba that were used to treat contagious diseases or influenza in the ancient times are applied to atopic diseases and arthritis (Jeong et al., 2008; Sato et al., 1989; Chang et al., 1993; Kubo et al., 1996; Eum et al., 2005). Moreover, the fact that Oriental Medicine is being used in fields that did not get much attention in the ancient times, such as skin care and obesity treatment, tells us that Oriental Medicine does not depend on a certain ingredient in a drug that has a unique therapeutic effect toward a certain disease. That is, it shows an old Oriental Medicinal idea that applying drugs helps the change of energy flow in the human body, which in turn helps the body to overcome diseases and to have a self-healing effect.

In this study, the anti-allergic effects of JW on AR were observed. Generally, the fact that a drug that strengthens the functions of the spleen and the stomach can be used to improve troubles that occur in the nasal cavity, which pertains to the respiratory system, reflects an old idea of Oriental Medicine that the spleen and stomach functions play an important role in the activities of the whole body. To put it more concretely, the fact that prescriptions for fatigue induced by the common cold or respiratory syndromes, such as coughs, dyspnea, and sneezing, contain many herbs that treat the spleen and the stomach, such as Pinelliae Tuber, Citrus Unshius Pericarpium, and Atractylodis Rhizoma Alba, suggests that respiratory syndromes caused by AR can be connected to troubles of the gastrointestinal tract.

That this paper has shown that JW has an anti-allergic effect on AR does not mean that a new remedy that can cure AR has been found. Rather, it is supporting evidence for the concept of treatment, not the development of a new drug. The human body's allergic response is the result of resistance of the body to an external allergen. It is the result of the body's response to perceiving a stimulus that would otherwise be

harmless to healthy people as a harmful one. In Oriental Medicine, this process is interpreted as weakened resistance or immunity around the nasal cavity caused by the deteriorated functions of the spleen and the stomach. The reason that JW, an improvement measure for the spleen and the stomach, has an anti-allergic effect is explained like this: JW does not temporarily remove external symptoms of AR. Rather, it helps the body to create a resisting power toward external allergens.

Therefore, it can be said that this research is meaningful in the fields of both Western and East Asian Medicines. From the aspect of Western Medicine, this research is meaningful in that it shows a direct connection between allergic response and the functions of the spleen and the stomach, which has not been previously known; from the aspect of East Asian Medicine, this research shows that the traditionally described 'spleen and stomach' cannot simply be matched with the 'spleen and stomach' or the 'pancreas and stomach' of modern anatomy, and that they have a more comprehensive meaning. That is, this research suggests realistic evidence that could help reinterpret ancient theories of Oriental Medicine in a more modern way.

Concomitantly, a hint was found in the ancient ideas of East Asian Medicine that time and energy can be saved only by starting the recruiting process of drugs for allergic responses from searching for simple drugs or prescriptions in Oriental Medicine that are classified as improvement measures for the spleen and the stomach. However, more is to be found out about which combination of drugs can have the greatest effects, how to combine drugs in order to treat different kinds of symptoms that are classified as allergic, and how allergic responses and the spleen and stomach functions are related.

In contrast, the present study was designed to investigate the possibility of applying this JW for the regulation of AR. Furthermore, we aimed to validate a possible mechanism in the ovalbumin (OVA)-induced AR models and activated mast cells.

MATERALS AND METHODS

Materials

Dexamethasone (DEX), OVA, Compound 48/80 (Com 48/80), phorbol 12-myristate 13-acetate (PMA), A23187, O-phthaldialdehyde (OPA), avidin peroxidase (AP), 2'-azino-bis (3-ethylbenzithiazoline-6-sulphonic acid) tablets substrate (ABTS), bicinchoninic acid (BCA), and other reagents were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS), iscove's modified dulbecco's medium (IMDM), and streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). Anti-mouse IgE/IL-1β/L-4/IFN-γ antibody (Ab), biotinylated anti-mouse IgE/IL-1β/IL-4/IFN-γ Ab, recombinant mouse (rm) IgE/IL-1β/IL-4/IFN-γ, anti-human IL-1β Ab, biotinylated anti-human IL-1β Ab, and recombinant human (rh) IL-1β were purchased from Pharmingen (Sandiego, CA, USA). Ab for IKK-β, RIP2, caspase-1, COX-2, NF-κB/Rel A, IκB-α, histon, and actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The caspase-1 assay kit was supplied by R&D Systems Inc. (Minneapolis, MN, USA).

Preparation of JW

A sample of JW was obtained from an oriental drug store, Noa Pharmacy (Seoul, Republic of Korea), and then authenticated by Kim HM, College of Pharmacy, Kyung Hee University. A voucher specimen was deposited at the Department of Pharmacology of the College of Oriental medicine, Kyung Hee University. JW was extracted by decocting the dried herbs (total 48 g, Table 1) with boiling distilled water (DW, 1 L) for approximately 2 h 30 min. The crude extracts were filtered and concentrated in vacuo at 60℃. It was lyophilized and reduced to powder. The yield of dried extract from starting materials was about 20% (w/w). The JW was dissolved in DW and filtered with 0.22 μm syringe filter.

Table 1. The Amount and Composition of Jeechool-Whan

Herbal name	Scientific name	Dose (g)
Baekchool	*Atractylodis Rhizoma Alba*	32
Jisil	*Ponciri Fructus Immaturus*	16

OVA-induced AR animal model

We maintained 5-week-old female BALB/c (Charles River Technology) mice under pathogen-free conditions. Mouse care and experimental procedures were performed under approval from the animal care committee of Kyung Hee University [KHUASP (SE)-10-016]. The mice were sensitized on days 1, 5, and 14 by intraperitoneal (i.p) injection of 100 μg OVA emulsified in 20 mg aluminum hydroxide (Sigma) in 100 μl phosphate-buffered saline (PBS) and challenged the mice with 1.5 mg OVA in 2 μl PBS or PBS. JW (0.1 and 1 g/kg) and DEX (5 mg/ml), or a control vehicle (DW) administrated orally before the intranasal (i.n.) OVA challenge for 10 days. Nasal symptoms were evaluated by counting the number of nasal rubs that occurred in the 10 min after OVA i.n. provocation at the 10 day mark after the challenge. The numbers of mice in each group are 5.

Culture of HMC-1 cells

A human mast cell line (HMC-1) was grown in IMDM supplemented with 100 unit/ml penicillin, 100 mg/ml streptomycin and 10% heat-inactivated FBS at 37℃ 5% CO_2 and 95% humidity. HMC-1 cells (3×10^5 cells/ml) were treated with JW (0.01, 0.1, and 1 mg/ml) for 1 h prior to stimulation with PMA and calcium ionophore A23187 (PMACI) which had been incubated for 2 h or 8 h.

Preparation of rat peritoneal mast cells (RPMCs)

We maintained male SD (Charles River Technology) rats under pathogen-free conditions. In brief, rats were anesthetized by ether and injected with 30 ml of Tyrode buffer B (137 mM NaCl, 5.6 mM glucose, 12 mM NaHCO3, 2.7 mM KCl, and 0.3 mM Na2HPO4) containing 0.1% gelatin into the peritoneal cavity, and the abdomen was gently massaged for about 2 min. The peritoneal cavity was carefully opened, and the fluid containing peritoneal cells were aspirated with a Pasteur pipette. Thereafter, peritoneal cells were sedimented at 150 × g for 10 min at room temperature and resuspended in Tyrode buffer B. To separate mast cells from the major components of rat peritoneal cells, i.e., macrophages and small lymphocytes, suspended peritoneal cells were layered on 2 ml of 22.5% metrizamide (density, 1.12 g/ml) and centrifuged at 400 × g for 10 min at room temperature. The cells remaining at the buffer–metrizamide interface were aspirated and discarded; the cells in the pellet were washed and resuspended in 1 ml of Tyrode buffer A (10 mM HEPES, 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl2, 1 mM MgCl2, 5.6 mM glucose, 0.1% bovine serum albumin) containing calcium. Mast cell preparations were about 95% pure as assessed by toluidine blue staining. More than 97% of the cells were viable as judged by the trypan blue uptake.

Histamine assay

RPMCs (2×10^5) were treated with JW (0.01, 0.1, and 1 mg/ml) for 40 min prior to stimulation with Com 48/80 incuvated for 25 min. The histamine contents from serum and supernatant were measured by an OPA spectrofluorometric procedure. The fluorescent intensity was measured at 440 nm

(excitation at 360 nm) in a spectrofluorometer.

Isolation of tissue protein

100 mg tissue homogenized using 1 ml in the homogenization buffer (20 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1 M NaCl, 0.2 mM DTT, and 0.5 mM Na_3VO_4). After centrifugation 12,000 × g at 30 min, transfer the supernatant and mix 5% glycerol. Protein level of the sample was measured using a BCA.

Enzyme-linked immunosorbent assay (ELISA)

HMC-1 cells (3×10^5) were treated with JW (0.01, 0.1 and 1 mg/ml) for 1 h prior to stimulation with PMACI incubated for 8 h. Cytokines of serum, mucosa, and spleen tissue, and the supernatant were measured by ELISA. The ELISA was performed by coating 96-well plates with 1 μg/well of capture Ab. Before the subsequent steps in the assay, the coated plates were washed twice with 1 × PBS containing 0.05% tween-20 (PBST). All reagents and coated wells used in this assay were incubated for 2 h at room temperature. The standard curve was generated from known concentrations of cytokine, as provided by the manufacturer. After exposure to the medium, the assay plates were exposed sequentially to each of the biotin-conjugated secondary antibodies, AP, and an ABTS substrate solution containing 30% H_2O_2. The plates were read at 405 nm. Appropriate specificity controls were included, and all samples were run in duplicate. Cytokine levels in the spleen and nasal mucosa were divided according to the total protein. Protein level was determined using a BCA.

Reverse transcription-polymerase chain reaction (RT-PCR)

HMC-1 cells (3×10^6) were treated with JW (0.01, 0.1 and 1 mg/ml) for 1 h prior to stimulation with PMACI incubated for 6 h. Total RNA was isolated from cells and nasal mucosa according to the manufacturer's specification using an easy-BLUETM RNA extraction kit (iNtRON Biotech, Korea). The concentrations of total RNA in the final elutes were determined by spectrophotometry. Total RNA (2.5 μg) was heated at 65°C for 10 min and then chilled on ice. Each sample was reverse-transcribed to cDNA for 90 min at 37°C using a cDNA synthesis kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). PCR was performed with the following primers for mouse IL-1β (sense 5' AGG CCA CAG GTA TTT TGT CG 3'; antisense 5' GCC CAT CCT CTG TGA CTC AT 3'), mouse GAPDH (sense 5' TTC ACC ACC ATG GAG AAG GC 3'; antisense 5' GGC ATG GAC TGT GGT CAT GA 3'), human IL-1β (sense 5' GGG GTA CCT TAG GAA GAC ACA AAT TG 3'; antisense 5' CCG GAT CCA TGG CAC CTG TAC GAT CA 3'), and human GAPDH (sense 5' CCT GCT TCA CCA CCT TCT TG 3'; antisense 5' CAA AAG GGT CAT CAT CTC TG 3') was used to verify whether equal amounts of the RNA were used for reverse transcription and PCR amplification from different experimental conditions. The annealing temperature was 50°C for mouse and human IL-1β and 60°C for mouse and human GAPDH respectively. Products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide.

Western blot analysis

HMC-1 cells (3×10^6) were treated with JW (0.01, 0.1 and 1 mg/ml) for 1 h prior to stimulation with PMACI incubated for 2 h. Western blot analysis was used for nasal mucosa tissue extracts and cell extracts were prepared by a detergent lysis procedure. Samples with loading buffer were heated at 95°C for 5 min, and briefly cooled on ice. Following the centrifugation at 15,000 × g for 5 min, 50 μg aliquots were resolved by 10% SDS-PAGE. Resolved proteins were electrotransferred overnight to nitrocellulose membranes in 25 mM Tris, pH 8.5, 200 mM glycine, 20% methanol at 25 V. Blots were blocked for at least 2 h with PBST containing 5% nonfat dry milk and then incubated with primary antibodies for 1 h at room temperature. Blots were developed by peroxidase-conjugated secondary antibodies, and proteins were visualized by enhanced chemiluminescence procedures (Amersham Bioseciences, Piscataway, NJ, USA) according to the manufacturer's instructions.

Histological examination

Tissue samples were immediately fixed with 4% formaldehyde and embedded in paraffin. Sections of the nasal mucosa samples were 4 μm thick. Each section was stained with hematoxylin and eosin (H&E, for eosinophils), alcian blue and safranine O (A&S, for mast cells) or immunohistochemical stain (for IL-1β) before dewaxing and dehydration. The numbers of eosinophils, mast cells, and IL-1β on both sides of the septal mucosa were counted. Sections were coded and randomly analyzed by two blinded observers.

Caspase-1 assay

HMC-1 cells (3×10^6) were treated with JW (0.01, 0.1, and 1 mg/ml) for 1 h prior to stimulation with PMACI incubated for 2 h. Caspase-1 assay was used for nasal mucosa tissue and cell extracts. Caspase-1 activity was measured according to the manufacturer's specification using a caspase assay kit (R & D system). Equal amounts of total protein were quantified by a BCA protein quantification kit (Sigma) in each lysate. Catalytic activity of caspase-1 from the cell lysate was measured by proteolytic cleavage of WEHD-pNA for 4 h at 37°C. The plates were read at 405 nm.

Transient transfection and luciferase assay

For the transfection, we seeded the HMC-1 cells (1×10^7) in a 100 mm culture dish. We then used Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) to transiently transfect reporter gene constructs into HMC-1 cells. HMC-1 cells (3×10^6) were treated with JW (0.01, 0.1 and 1 mg/ml) for 1 h prior to stimulation with PMACI incubated for 24 h. We mixed 20 μl of cell extract and 100 μl of the luciferase assay reagent at room temperature. To measure the luciferase activity, we used a luminometer (1420 luminescence counter, Perkin Elmer) in accordance with the manufacturer's protocol. All the transfection experiments were performed in at least three different experiments, with similar results. The relative luciferase activity was defined as the ratio of firefly luciferase activity to renilla luciferase activity.

Statistical analysis

The experiments shown are a summary of the data from at least three experiments and statistical analyses were performed using SPSS statistical software (SPSS 11.5, USA). Treatment effects were analyzed by one-way ANOVA, offered by Tukey's multiple range tests, and $p < 0.05$ was used to indicate significance.

RESULTS

Effect of JW on clinical symptoms and histamine, IgE, IL-4, IFN-γ and IL-1β levels in the AR model

To investigate the inhibitory effect of JW in the AR model, we sensitized mice on days 1, 5, and 14 by i.p. injections of 100 μg OVA emulsified in 20 mg aluminum hydroxide and challenged mice with 1.5 mg OVA. Dexamethasone (5 mg/kg) was used as a positive control. The number of nasal and ear rubs after the

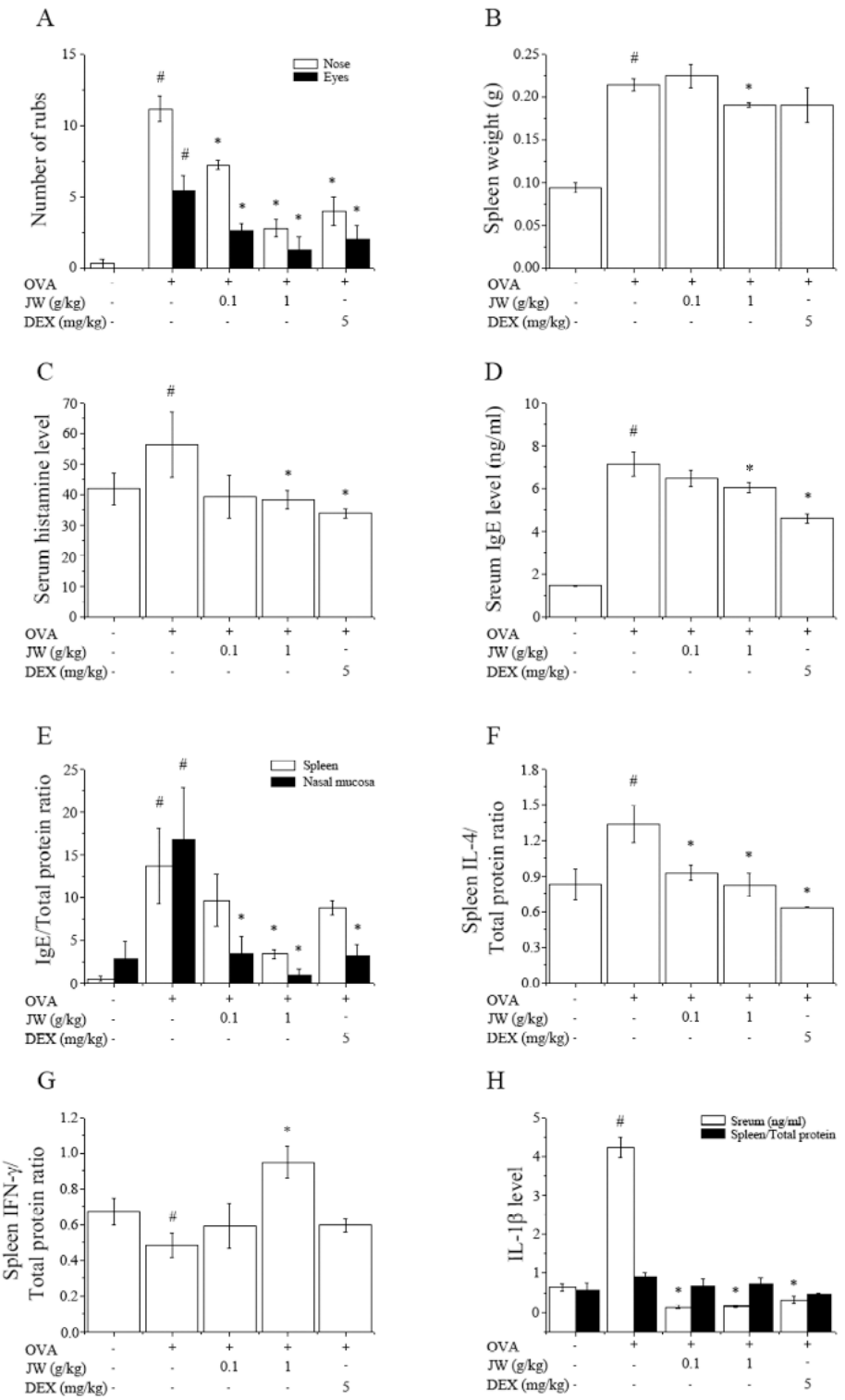

Fig. 1. Effects of JW on Clinical Symptoms, Spleen weight, and Histamine, IgE, IL-4, IFN-γ, and IL-1β level in The AR Model. We sensitized mice on days 1, 5, and 14 by intraperitoneal injections of 100 μg OVA emulsified in 20 mg of aluminum hydroxide and we challenged mice with 1.5 mg OVA. Mice received JW before the intranasal OVA challenge for 10 days. (A) The number of the nasal and ear rubs that occurred in the 10 min after the OVA intranasal provocation. (B) Spleen weight, (C) Serum were isolated from blood and then assayed about histamine. (D and E) IgE, (F) IL-4, (G) IFN-γ, and (H) IL-1β were measured by the ELISA method. All parameters measured in the tissue homogenate were presented as a ratio to the total protein level in tissue. $^{\#}p < 0.05$; significantly different from the OVA-unsensitized mice. $^{*}p < 0.05$; significantly different from the OVA-sensitized mice. N = 5. DEX, dexamethasone.

OVA challenge in the OVA-sensitized mice was significantly higher than those in the OVA-unsensitized mice. Increased rub scores were inhibited by the treatment of JW (Fig. 1A). The spleen weights after the OVA challenge in the OVA-sensitized mice were significantly higher than those in the OVA-unsensitized mice. Increased spleen weight was attenuated by JW administration (Fig. 1B). Histamine level in the serum was reduced by JW (Fig. 1C). Level of IgE in the AR mice was significantly higher than those in the serum, spleen, and nasal mucosa tissues of the OVA-unsensitized mice (Fig. 1D and E). The IgE level increased by OVA was inhibited by JW.

To identify the Th1/Th2 immune reaction in JW-administered mice, we measured IL-4 and IFN-γ levels in the spleen. As shown in Figs. 1F and G, the level of IL-4 in the OVA-sensitized was significantly increased compared to those in the OVA-unsensitized mice. IL-4 level was significantly decreased in the JW-administered AR mice. IFN-g level was significantly increased in the JW-administered AR mice. The protein level of IL-1β in the serum was increased in the OVA-sensitized mice compared to those levels in the OVA-unsensitized mice (Fig. 1H). However, serum level of IL-1β was significantly inhibited by JW administration.

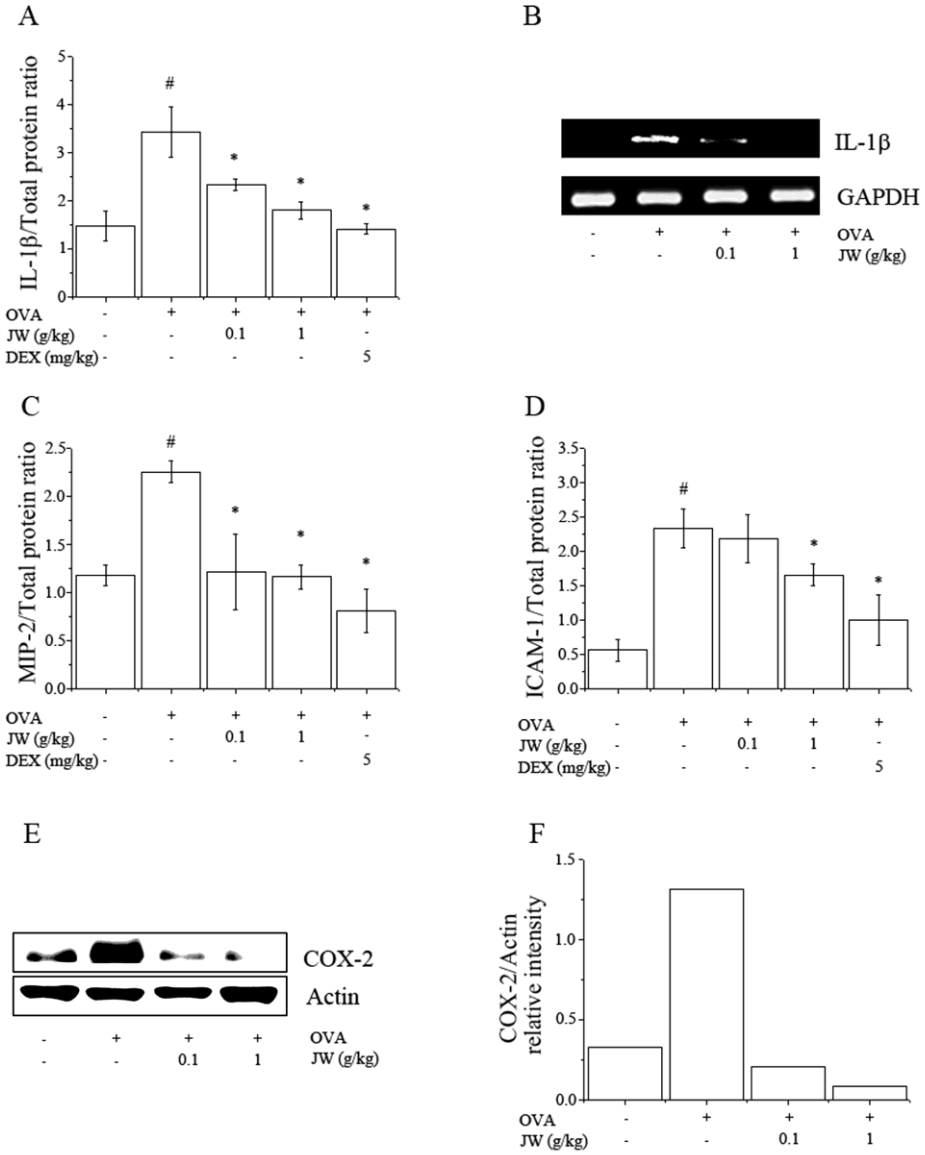

Fig. 2. Effects of JW on IL-1β, MIP-2, ICAM-1, and COX-2 Expressions in Nasal Mucosa of The AR Mice. We sensitized mice on days 1, 5, and 14 by i.p. injections of 100 μg OVA emulsified in 20 mg of aluminum hydroxide and we challenged mice with 1.5 mg OVA. Mice received JW before the intranasal OVA challenge for 10 days. (A) IL-1β, (C) MIP-2, and (D) ICAM-1 were measured by the ELISA method in the nasal mucosa tissue. (B) Messenger RNA was measured using the RT-PCR method. (E) COX-2 protein expression was evaluated by using Western blot analysis. (F) The protein levels of COX-2 were quantified by densitometry. $^{\#}p < 0.05$; significantly different from the OVA-unsensitized mice. $^{*}p < 0.05$; significantly different from the OVA-sensitized mice. N=5. DEX, dexamethasone.

Effect of JW on IL-1β, MIP-2, ICAM-1, and COX-2 levels in the nasal mucosa tissue of AR model

To evaluate the regulatory effect of JW on IL-1β expression, we measured the protein and mRNA levels of IL-1β in the AR model. The protein and mRNA levels of IL-1β in the nasal mucosa tissue were increased in the OVA-sensitized mice compared to those levels in the OVA-unsensitized mice (Fig. 2A and B). However, protein and mRNA levels of IL-1β were inhibited by JW administration. Levels of MIP-2, ICAM-1, and COX-2 in the AR mice were significantly higher than those in the nasal mucosa tissues of the OVA-unsensitized mice (Fig. 2C-F). Increased levels of MIP-2, ICAM-1, and COX-2 were inhibited by JW administration (Fig. 2C-F).

Effect of JW on eosinophil and mast cell infiltration and IL-1β expression in the nasal mucosa tissue

The respective numbers of inflammatory cells (eosinophil and mast cell) in the nasal mucosa in the OVA-sensitized mice were significantly higher than those in the OVA-unsensitized mice. In the JW-administered mice, eosinophil and mast cell infiltration increased by OVA sensitization was inhibited (Fig. 3). Immunohistochemical analysis of the nasal mucosa sections in the OVA-sensitized mice revealed that IL-1β is highly expressed; whereas in the JW-administered mice it is decreased (Fig. 3).

Effect of JW on caspase-1 activation in the nasal mucosa tissues

Caspase-1 plays a key role in inflammatory responses by cleaving pro-IL-1β into secreted pro-inflammatory cytokines. To investigate the effect of JW on caspase-1 activation, caspase-1 assays were performed with the nasal mucosa tissues. As shown in Fig. 4A, JW (1 g/kg) inhibited OVA-induced caspase-1 activation. JW also reduced the expression of caspase-1 in the nasal mucosa tissue (Fig. 4B and C).

Effect of JW on PMACI-induced IL-1β expression in HMC-1 cells and histamine release in RPMCs

Mast cells play a major role in the inflammatory reaction of AR

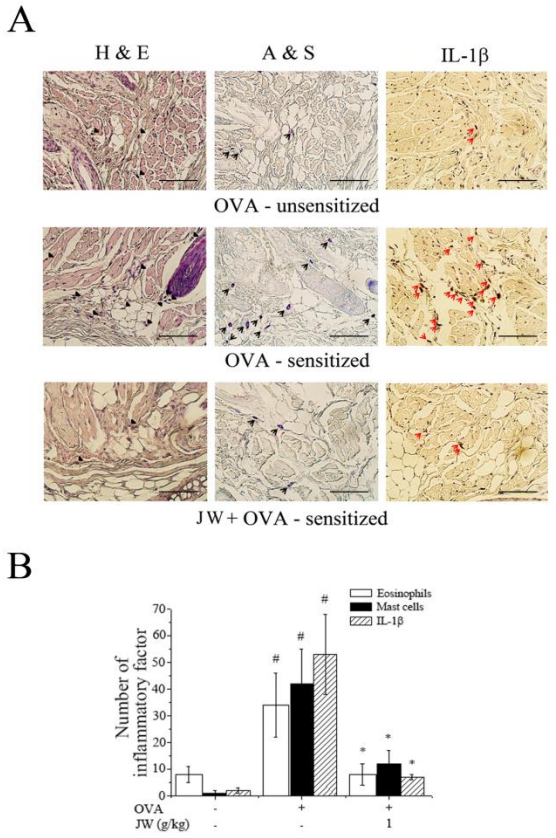

Fig. 3. Effects of JW on Eosinophil and Mast Cell Infiltration, and IL-1β Expression in The AR Nasal Mucosa Tissue. (A) Nasal mucosa stained with H&E (for eosinophils = arrow head), A&S (for mast cells = black arrow) and immunohistochemical DAB stain (for IL-1β = red arrow). (B) Eosinophil, mast cell, and IL-1β were counted by two individuals. Afterwards, five randomly selected tissue sections per mouse were counted. The absolute number of cell was counted as the mean ± standard error of the mean (S.E.M.). $^{\#}p < 0.05$; significantly different from the OVA-unsensitized mice. $^{*}p < 0.05$; significantly different from the OVA-sensitized mice. DEX, dexamethasone. (Original magnification × 400, scale bar = 100 μm).

(Pawankar et al., 2007). Since IL-1β is a major cytokine released from mast cells after allergic responses, we examined the effect of JW on the expression of IL-1β in human mast cells. The protein and mRNA levels of IL-1β were significantly inhibited by treatment with JW (Fig. 5A and B). Also, the inhibitory effect of JW on com 48/80-induced histamine release from RPMCs is shown in Fig. 5C. We examined cell viability using a MTT assay, and JW had no effect on cell viability (Fig. 5D).

Effect of JW on PMACI-induced NF-κB activation in HMC-1 cells

To assess the regulatory mechanism of JW on allergic inflammation in the in vitro model, we examined the effect of JW on PMACI-induced NF-κB activation, which is known to be important for cytokine expression in HMC-1 cells. Because the suppression of NF-κB is linked with anti-inflammation, we postulated that JW mediates its effects at least partly through the suppression of NF-κB activation. The pretreatment with JW (0.01, 0.1, and 1 mg/ml) inhibited PMACI-induced NF-κB/Rel A levels in the nuclear extract (Fig. 6A). As a marker of NF-κB activation, the degradation of IκB-α in cell lysates was detected. We showed that JW (0.01, 0.1, and 1 mg/ml) inhibited the PMACI-induced IκB-α degradation (Fig. 6A). Histone and actin expression levels were not changed by any treatment in the nuclear and cytoplasmic extracts. We next examined whether JW could modulate the luciferase expression specifically via NF-κB. NF-κB luciferase reporter gene constructs (pNF-κB-LUC, plasmid containing NF-κB binding site; STRATAGENE, La Jolla, CA, USA) were transiently transfected into HMC-1 cells, which were treated by JW and then stimulated by PMACI. As shown in Fig. 6B, PMACI

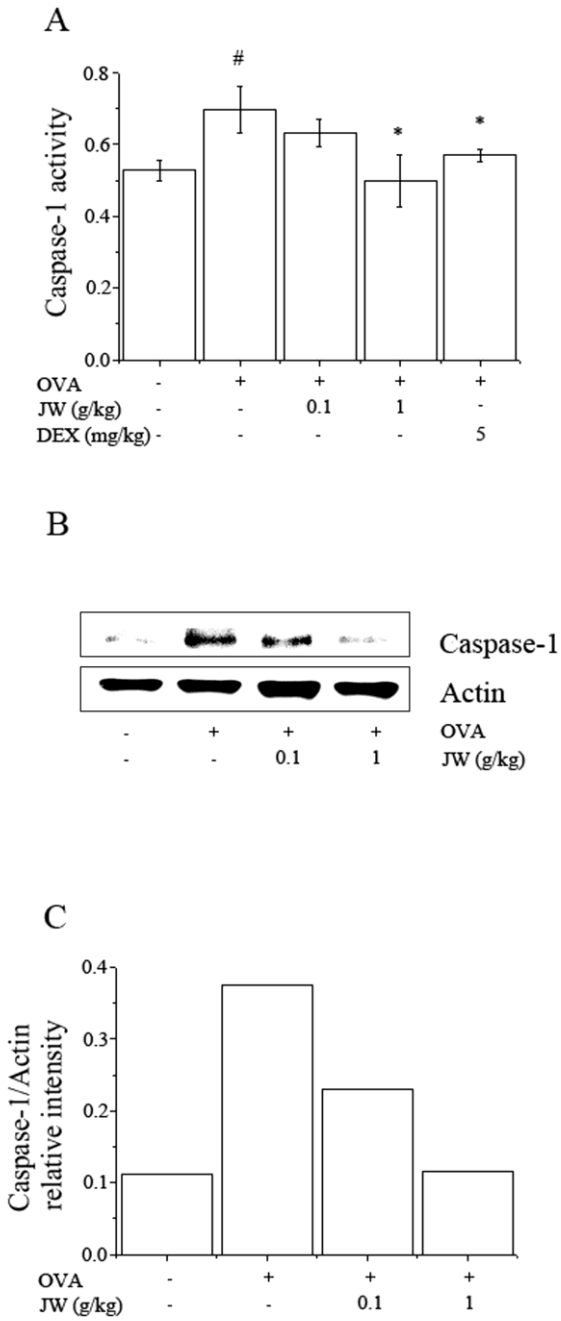

Fig. 4. Effect of JW on caspase-1 Activation in Nasal Mucosa of The AR Mice. We sensitized mice on days 1, 5, and 14 by i.p. injections of 100 μg OVA emulsified in 20 mg of aluminum hydroxide and we challenged mice with 1.5 mg OVA. Mice received JW before the intranasal OVA challenge for 10 days. (A) Protein was assayed about caspse-1. (B) Caspase-1 protein expression was evaluated by using Western blot analysis. (C) The protein levels were quantified by densitometry. Results are representative of three independent experiments. $^{\#}p < 0.05$; significantly different from the OVA-unsensitized mice. $^{*}p < 0.05$; significantly different from the OVA-sensitized mice. N = 5. DEX, dexamethasone.

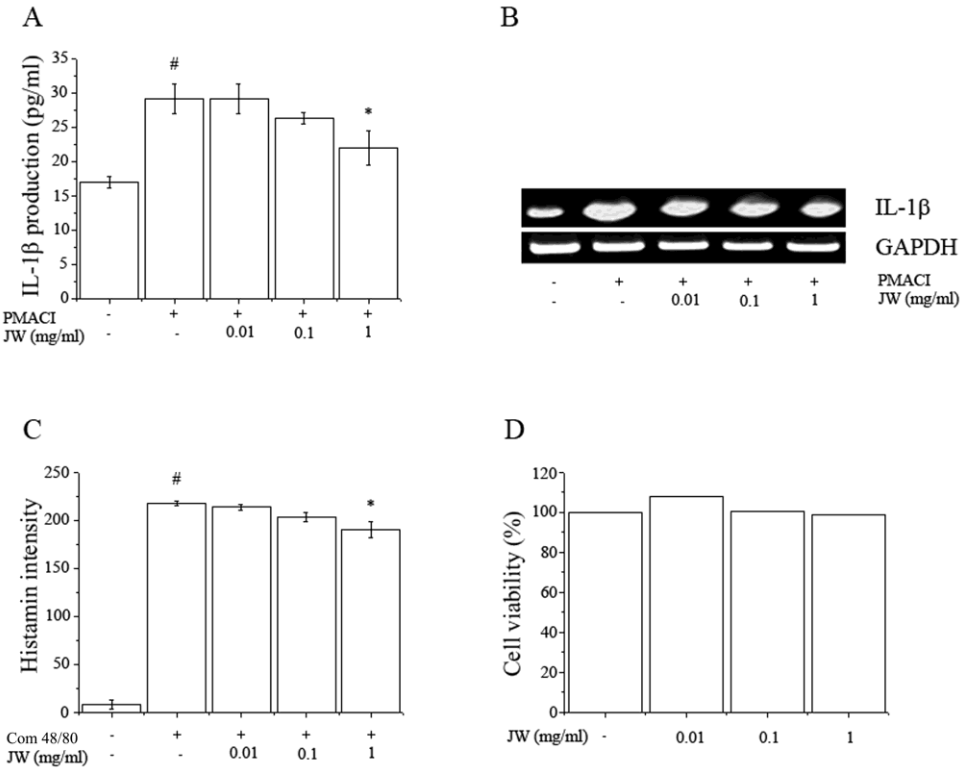

Fig. 5. Effects of JW on PMACI-Induced IL-1β Expression in HMC-1 Cells and Histamine Release in RPMCs. (A) HMC-1 cells were treated with JW (0.01, 0.1, and 1mg/ml) for 1 h and then stimulated with PMACI for 8 h. IL-1β was measured by the ELISA method. (B) Messenger RNA was measured using the RT-PCR method. (C) Secreted histamine was assayed by a histamine assay. (D) Cell viability was evaluated by a MTT assay. $^{\#}p < 0.05$; significantly different from the unstimulated cells. $^{*}p < 0.05$; significantly different from the PMACI-stimulated cells.

increased reporter gene activity. However, the increased activity was significantly inhibited by the treatment with JW ($p < 0.05$).

Effect of JW on PMACI-induced RIP2/IKK-β/caspase-1 activation in HMC-1 cells

RIP2 is a CARD-containing kinase that interacts with caspase-1 and plays an important role in NF-κB activation (Kelsall, 2005). Apoptosis-associated speck-like protein containing a CARD (ASC) is a PYRIN and CARD-containing molecule, important in the induction of apoptosis and caspase-1 activation (Sarkar et al., 2006). Expressions of RIP2 and IKK-β were inhibited by JW in HMC-1 cells (Fig. 7A). To evaluate whether JW regulated the caspase-1 activation, we performed caspase-1 assay and Western blot analysis. As shown in Figs. 7B and C, caspase-1 activity increased by PMACI was inhibited by treatment with JW.

DISCUSSION

In this study, JW reduced the allergic inflammatory reaction in the AR model. AR is characterized by a two-phase allergic reaction. In the early-phase inflammatory response, the allergen-IgE dependent activation of mast cells and basophils results in the production of pharmacologically active mediators such as histamine, prostaglandins, leukotrienes, and cytokines which produce sneezing, rhinorrhea, and itching (Jeong et al., 2009). The late-phase of AR shows accumulations of mast cells, eosinophils, and basophils in the epithelium and an accumulation of eosinophils in the deeper lamina propria (Fuentes-Beltrán et al., 2009). Recruitment of inflammatory cells, including eosinophils, mast cells, basophils, and T cells, results in further release of histamine and leukotrienes, as well as in the release of other compounds including proinflammatory cytokines, COX-2, and chemokines, sustaining the allergic response and promoting the late phase response (Fuentes-Beltrán et al., 2009; Fukui et al., 2009). In previous studies, polyphenolic phytochemicals including rosmarinic acid have been shown to inhibit IgE response (Makio et al., 2001; Tachibana et al., 2001) and inflammation characterized by polymorphonuclear leukocytes (eosinophils, and neutrophils) infiltration (Osakabe et al., 2002; Sanbongi et al., 2003). Glucocorticosteroid (GC) is the most effective drug for AR. GC inhibits the function of infiltrating inflammatory cells and their recruitment into the nasal mucosa. GC inhibits the maturation, cytokine production, COX-2 expression, FcεRI expression, and mediator release of mast cells (Takano et al., 2004; Smith et al., 2002). We observed that JW inhibited IgE production, inflammatory cytokine production, chemokine production, and COX-2 expression in the mice AR model. Dexamethasone also reduced the allergic and inflammatory reactions. Therefore, our results suggest that the effect of JW is similar to the mechanism of rosmarinic acid or GC. We can also deduce that JW has an anti-allergic effect.

Inflammasomes are multiprotein cytoplasmic complexes that mediate the activation of inflammatory caspase-1 (Woschnagg et al., 2009). Inflammasome regulates the activation and secretion of caspase 1-regulated IL-1β and IL-18. Caspase-1–/– mice have a decreased production of IL-6 after stimulation with lipopolysaccharide (Martinon, 2005). Grzegorczyk et al., have reported a significant increase in caspase-1 levels in the serum from allergic asthmatic patients as compared to a control group (Grzegorczyk et al., 2002). Caspase-1 activity was increased in patients with Netherton syndrome (Hosomi et al., 2008). In this study, we observed that caspase-1 was activated in the AR mice. We postulate that the inhibitory effect of JW on inflammatory cytokine production might be derived from the regulation of caspase-1 activation. Further studies will be needed to clarify precisely the mechanism of JW that has the effect on the relationship between caspase-1 activation and inflammatory cytokines

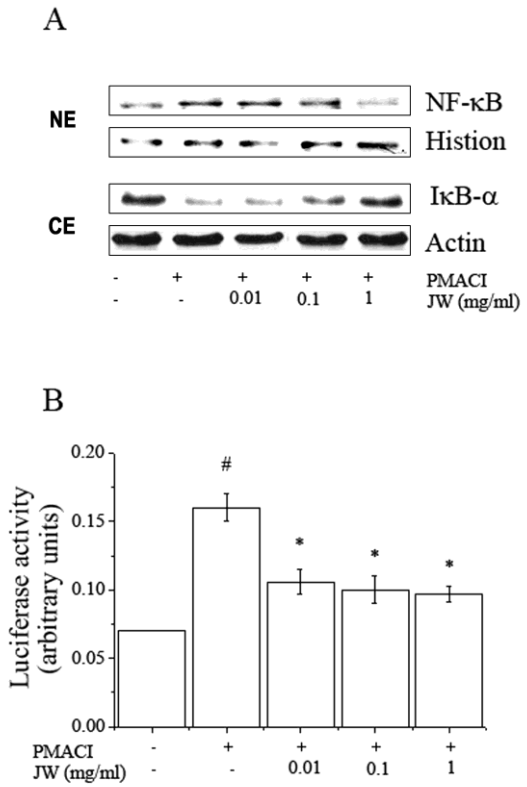

Fig. 6. Effect of JW on PMACI-Induced NF-κB Activation in HMC-1 Cells. (A) HMC-1 cells were treated with JW (0.01, 0.1, and 1 mg/ml) for 1 h and then stimulated with PMACI for 2 h. Nuclear protein and cytoplasmic protein were prepared and analyzed for NF-κB and IκB-α by Western blotting as described in the experimental procedures. Results are representative of three independent experiments. (B) The NF-κB activity was assayed by a luciferase assay. Values are the mean ± S.E.M. of duplicate determinations from three separate experiments. $^{\#}p < 0.05$: significantly different from the unstimulated cells. $^{*}p < 0.05$: significantly different from the PMACI-stimulated cells. NE, nuclear extract; CE, cytoplasmic extract.

production in the AR mice.

Mast cells arise from pluripotential stem cells and mature in the tissue. They have the ability to generate immune reactions following exposure to a variety of receptor-mediated signals initiated by both innate and acquired immune response mechanisms. Activated mast cells release a broad spectrum of mediators including cytokines such as IL-1β (Howarth, 2003; Johansson et al., 2001). Suppression of NF-κB activation has been linked with the inhibition of inflammation (Kelsall, 2005). Activation by RIP2 induces caspase-1 oligomerization and promotes caspase-1 activation, with the latter inducing cytokine stimulation (Kelsall, 2005). RIP2 and IKK complexes may play an important role for NF-κB activation (Inohara et al., 2000). Atractylodis Rhizoma Alba inhibited the production of pro-inflammatory cytokines through the inhibition of the NF-κB/IκB signal pathway in human mast cells (Hong et al., 2010). Limonene is another component of Ponciri Fructus Immaturus, it inhibits the production of MAPK through the inhibition of the NF-κB/IκB signal pathway in eosinophils (Hirote et al., 2010). In this study, we confirmed that JW suppressed the RIP2/IKK-β/caspase-1 activation induced by PMACI. This result suggested that the inhibitory effect of JW on allergic inflammatory reaction might be derived through the regulation of RIP2/IKK-β/caspase-1 activation.

JW, which this paper intends to research, is a prescription that is crudely known as a medicine that contains Atractylodis Rhizoma Alba and Ponciri Fructus Immaturus and that has an improving effect on the functions of the spleen and the stomach. However, there are 66 kinds of prescriptions in 'Dongeuibogam' that contain Atractylodis Rhizoma Alba and Ponciri Fructus Immaturus, and the number tops several hundred if all treatments in East Asian Medicine are included. Despite the importance of this combination of drugs, almost all medical documents use the explanation of symptoms as was written in 'Shanghanlun' in the 2nd century, when the prescription first emerged, without suggesting specific syndromes or prominent symptoms. In 'Bang-yak-hap-pyeon', which was published in the early 20th century, there is a slightly more detailed explanation about the prescription that says it helps digestion of food; however, the comprehensive meaning of 'tonifying the spleen and the stomach' is repeated. This tells us that this prescription had a low utilization rate. In fact, this prescription is rarely used as a digestive in clinics. This is because there are more effective digestives such as the Calm the Stomach Powder (Pyungwee-san), the Aucklandia and Amomum Decoction to Maintain the Stomach (Hyansapyungwee-san), or the Eliminate Stagnation Pill (Soche-whan. Rather, Atractylodis Rhizoma Alba and Ponciri Fructus Immaturus are more frequently used with other drugs. Among the 3917 prescriptions mentioned in Dongeuibogam, 160 contain Ponciri Fructus Immaturus and 621 contain Atractylodis Rhizoma Alba. Although Atractylodis Rhizoma Alba and Ponciri Fructus Immaturus are very meaningful drugs in Oriental Medicine, medicine in the form of JW, which contains only two ingredients, is rarely used because those problems that require a solution more than the mere combination of Atractylodis Rhizoma Alba and Ponciri Fructus Immaturus have been solved by adding different drugs to the combination, and by slightly improving the effects and precision.

As mentioned in this paper, the fact that JW, a prescription that rarely got attention in the ancient East Asian medicine because it has been thought that it could not function as more than a digestive, has an anti-allergic effect on AR suggests a possibility that Oriental Medical remedies could be applied in the future to newly developed diseases.

Certain ingredients in Atractylodis Rhizoma Alba and Ponciri Fructus Immaturus of JW may have restrained a certain allergen effectively. Or, a certain ingredient in the prescription could possibly have controlled a certain part of the immune system to remove allergic responses focused on the nasal cavity. However, these results of existing research contain concepts so different from the East Asian concepts when it comes to effectively utilizing the core of Oriental medicine. Moreover, this kind of approach is not a suitable one to find the reason Oriental Medical drugs were developed from simple drugs to combined drugs in order to cure diseases. The meanings the spleen and the stomach hold in Oriental Medicine are much more than that of the digestive organ system of modern medicine. This is because the spleen and the stomach are considered as the central axis of the human body and are thought to control resisting power and immunity. Therefore, in the current field of Oriental Medical clinics, many practitioners who treat allergic diseases apply treatments that improve the functions of the spleen and the stomach.

If one understands the context in which this prescription was created and used, this prescription should be interpreted as the following: many living beings, including humans, cannot synthesis living energy in the body and thus have to obtain it from the outside. The decomposing process of external energy that is composed of the act of 'eating' is expressed as the functions of the spleen and the stomach in Oriental Medicine.

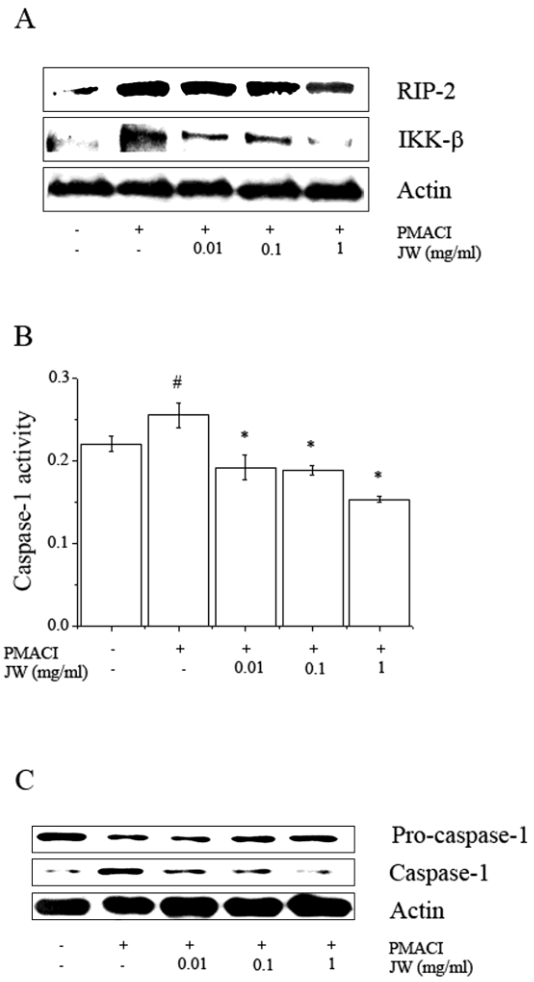

Fig. 7. Effect of JW on PMACI-Induced RIP2/IKK-β/caspase-1 Activation in HMC-1 Cells. HMC-1 cells were pretreated with JW for 1 h prior to PMACI stimulation for 2 h. (A) RIP2 and IKK-β protein expression were evaluated by using Western blot analysis. (B) The enzymatic activity of caspase-1 was tested by a caspase-1 colorimetric assay. (C) The level of caspase-1 was assayed by Western blot analysis. Data represent mean ± S.E.M. of three independent experiments. $^{\#}p < 0.05$: significantly different from the unstimulated cells. $^{*}p < 0.05$: significantly different from the PMACI-stimulated cells.

The act of eating is often affected by mental factors. Therefore, the expression that, 'Much thinking causes ailments in the spleen and the stomach.' appears in ancient medical records more than two thousand years ago.

Therefore, ailments of the spleen and the stomach mean that important parts of the body have been damaged. This damage gets transferred to many other body parts. Therefore, the state of the spleen and the stomach are directly related to the body's resistance and general life energy. In all cultures, be it Eastern, Western, modern, or ancient, there exist a proverb that says 'One has to eat well in order to be healthy' and this reflects the forementioned insight of humankind, which argues that organs related to "eating" are the most crucial body parts.

A response that AR patients show is an anaphylactic response. It is when the body responds over sensitively to a stimulus that could be perceived as mild to common people. It does not matter which kind the allergen is. This occurs because the body's resistance or immunity has become weak. JW, like described in ancient records, is a medicine that improves the functions of the spleen and the stomach. Improving the functions of the spleen and the stomach does not simply result in increased appetite or increased digestive power. It means that a central function of the human body has been restored, and at the same time, it means that the person can live on with more powerful life energy.

The fact that JW has an effect in AR does not mean that the spleen- and stomach-strengthening effect of this prescription can cure all allergic diseases. This is why there exist numerous spleen- and stomach-improving prescriptions in Oriental medical records, and why we have to step forward from component-oriented research to prescription-oriented research. Disorders in the spleen and the stomach are represented in the form of various symptoms depending on the individual, environment, time, or even one's psychological state. Numerous prescriptions may be the result of trying to confront such various changes.

Many prescriptions in Oriental Medicine have similar ingredients in common. It seems that practitioners were trying not to discover an effective drug, but to create effectiveness by changing the combinations of known drugs. The background for being able to continue on with this effort was not the perception that diseases are circumstances that certain factors create, but a concept that they are troubles caused by loss of the human body's resistance. Therefore, many practitioners focused on strengthening the spleen and the stomach which are the central axis of the body, and on leading the body to exhibit its self-healing power by improving resistance. However, improving the functions of the spleen and the stomach does not automatically cure all symptoms. Although JW is a representative combination of improving the functions of the spleen and the stomach, there are other such basic prescriptions as the Four Gentlemen Decoction that contains Ginseng Radix, Atractylodis Rhizoma Alba, Poria Sclerotium, and Glycyrrhizae Radix, and the Two Matured Substances Decoction that contains Pinelliae Tuber, Poria Sclerotium, Citrus Unshius Pericarpium, and Glycyrrhizae Radix.

JW has an improving effect on the spleen and stomach function is clear, and it is also clear that it is effective on AR, as displayed in this paper. However, many links have to be made between the functions of the spleen and the stomach and AR. It is because the question that states why among many other prescriptions the combination of Atractylodis Rhizoma Alba and Ponciri Fructus Immaturus has to be used has to be answered. As for now, there is no clear answer to this question. However, from the perspective of Oriental Medicine, there have been so many syndromes that could not be cured only with the combination of Atractylodis Rhizoma Alba and Ponciri Fructus Immaturus, and instead of discovering other combinations, many ancient practitioners accumulated experiences of precisely controlling similar syndromes by adding different drugs to the combination of Atractylodis Rhizoma Alba and Ponciri Fructus Immaturus. It is not important whether these efforts were successful in curing the said syndromes, as many medical texts say. It is because even though patients were cured of diseases at that time, it is meaningless if those methods are ineffective in the present time. On the other hand, if there is an effective treatment that is not mentioned in documents, it can be added to our knowledge in Oriental Medicine as a new experience.

The anti-allergic effect of JW on AR is meaningful in this way. In the past, accumulating remedial experiences was impossible because there was no AR to which treatments could be applied or because people could not perceive AR. However, with newly emerging diseases, it is meaningful to approach with an ancient Oriental Medical theory that basic resistance of the human body toward external environmental changes

originates from the spleen and the stomach. In this research, it has only been stated that JW has an anti-allergic effect. However, if we delve more into Oriental Medical theories, it cannot be said that JW has the same anti-allergic effect on all kinds of AR. It is because the anti-allergic effect mentioned in this paper is derived from an increased resistance of the human body induced by improved spleen and stomach functions; one should have continuous interest in the body's state in which the best anti-allergic effect can be displayed. Also, all possibilities have to be taken into account, including simple differences of state such as whether symptoms are acute or chronic, and whether the degree of AR is light or severe, and other factors such as whether there are other symptoms besides symptoms related to AR, and whether there is a relationship between the application of medicine and the psychological state of a person.

The body's resistance represents whether one's life is being conducted well, and is the ability to display all of its power toward the external environment. In East Asian Medicine, it is thought that the process by which an individual can display his/her vitality can vary. Starting from a simple difference of degree such as strength and weakness, the variation can reach constitutional differences as mentioned by the 19th century Korean medical scholar, Lee Jema (Cha et al., 2007; Choi et al., 2011; Park et al., 2011). East Asian medical scholars were curious about different responses displayed in the same environment, and made efforts to explain such differences. They even reflected their efforts in treatments. They found out that those who have strong vitality (so-called the healthy) may display allergic responses but only temporarily, and that allopathic methods of removing nasal congestion and runny nose, not improving the spleen and stomach functions, were effective enough. They also found out that those who suffered from rhinitis whenever seasons changed could not be cured only by removing the symptoms, but were to be treated by continuously improving the functions of the spleen and the stomach. Depending on the situation, there were those types that did not display effectiveness when their spleen and stomach functions were improved. Numerous prescriptions in Oriental Medicine resulted from such experiences of various possibilities.

Therefore, the anti-allergic effects of JW mentioned in this paper are to be thought of as the starting point for verifying the concept on which many East Asian medical practitioners have been focusing. The research is not to be concluded, thinking that an effective drug has been found for AR.

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CONFLICT OF INTEREST

The authors have no conflicting financial interests.

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