

Ethyl Acetate Extract of Korean Rice Wine Lees Inhibits IgE-Mediated Degranulation in Rat Basophilic Leukemia RBL-2H3 Cells and Passive Cutaneous Anaphylaxis in Mice

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Mast cells, the central effector cells involved in the allergic response, release histamine, arachidonic acid, and proinflammatory cytokines. We investigated the effect of the ethyl acetate fraction (EA), derived from Korean rice wine lees, on RBL-2H3 cell degranulation and passive cutaneous anaphylaxis in an animal model. The EA fraction suppressed the release of beta-hexosaminidase, a marker of degranulation, and the mRNA expression of interleukin-3 (IL-3) and IL-13. EA also successfully suppressed the passive cutaneous anaphylaxis (PCA) reaction in mice in a dose-dependent manner. These results suggest that EA can inhibit mast cell degranulation through the inhibition of IL-3 and IL-13 mRNA expression, and that EA may potentially serve as an anti-allergic agent.

Key words : Korean rice wine lees, mast cells, allergic response, passive cutaneous anaphylaxis

Introduction

Korean rice wine, a traditional Korean alcoholic rice-source beverage, is fermented with yeast, *nuruk* a wheat-based source of the enzyme amylase. Lees, a Korean rice wine byproduct, are obtained during fermentation. The Korean rice wine lees are traditionally known to have a whitening effect on the skin as well as being a skin anti-wrinkle agent [10]. In this paper, we aimed to clarify the anti-allergic effect of Korean rice wine lees, especially the ethyl acetate (EA) fraction, in Type 1 hypersensitivity *in vitro* using mast cells and in passive cutaneous anaphylaxis (PCA) in mice *in vivo*.

Mast cells are central to the pathogenesis of immediate hypersensitivity and they are located in blood vessels and at epithelial surfaces such as the lung and intestine. It is this epithelial location that makes mast cells one of the first activators of the inflammatory response [13,17,19]. Mast cells have a high affinity for immune globulin E (IgE). They have a specific IgE receptor, FcεR1, on their surface. As the IgE-multivalent allergen complex binds to FcεR1, the mast cells become activated and release a range of preformed,

newly synthesized mediators and cytokines that trigger potent allergic reactions [2]. In normal conditions, the mast cells have pre-formed mediators such as histamine serine proteases and β-hexosaminidase in their cytoplasmic granules. The activated mast cells release these mediators in response to immune allergic reactions through IgE-FcεR1 binding [2,8,12,13,17,19,20].

β-hexosaminidase, a granule-associated exoglycosidase, has been used to monitor mast cell degranulation just as histamine has been used. β-hexosaminidase could act with trypsinases and chymases to induce degradation of the extracellular matrix. This extracellular matrix degradation is an important event during the remodeling of inflamed tissue [3].

The RBL-2H3 rat mast cell line exhibits homology with human mast cells, even though they are from rat basophilic leukemia tissue. For this reason, we assessed the level of degranulation and the interleukin-3 (IL-3) and interleukin-13 (IL-13) mRNA expression patterns using the RBL-2H3 cell line.

IL-3, one of the cytokines released by activated mast cells, acts as a multipotent hematopoietic growth factor inducing the mast cell to proliferate and encourage cell survival [16,24]. Cytokine, IL-13, plays an important role in encouraging epithelial cell proliferation and injury repair in airway epithelial cells. IL-13 also activates the cleavage of the matrix

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metalloproteinase 7-mediated FAS ligand in bronchial epithelial cells. Both cytokines can activate type 2 helper T cells during the inflammatory response. These cytokines are important mediators that interlink reactions between mast cells and T cells [21,23].

In this study, we examined the anti-allergic effect of EA using RBL-2H3 as the *in vitro* model and the *in vivo* anaphylactic mouse model. Our results indicate that EA not only effectively inhibits the degranulation and expression of cytokines involved in type 1 hypersensitivity in mast cells, but also suppresses hypersensitive reactions in the PCA mouse model.

Material and Methods

Materials

Mouse monoclonal anti-dinitrophenol (anti-DNP) IgE antibody, dinitrophenol-conjugated human serum albumin (DNP-BSA), and Evans blue were obtained from Sigma-Aldrich, (St. Louis, MO, USA). p-Nitrophenyl-N-acetyl- β -D-glucosaminide was obtained from MP Biomedicals, LLC (Solon, OH, USA).

Extraction and Isolation

Korean rice wine lees were cultivated and harvested according to the good agricultural practices method stipulated by the Korea Rural Development Administration. The Korean rice wine lees were subsequently extracted three times with ethyl acetate at room temperature for three days per extraction. The combined ethyl acetate extracts were then concentrated *in vacuo* at 40°C.

Cell culture

The rat mast cell line, RBL-2H3, was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were grown in Eagle's minimal essential medium (WelGENE, Inc., Daegu, Korea) containing 10% (v/v) fetal bovine serum and 100 units/ml penicillin-streptomycin (Lonza Walkersville, Inc., Walkersville, MD, USA) at 37°C in a humidified 5% CO₂ atmosphere. Cells were detached with trypsin-EDTA solution, washed with phosphate-buffered saline (PBS, pH 7.2), and resuspended in fresh medium for use in subsequent experiments.

Animals

Seven-week-old male BALB/C mice were purchased from

Orient Bio (Gangneung, Korea) and housed in wire cages at 20-22°C and a relative humidity of 40-50%. All animals were given access to standard laboratory chow and water *ad libitum*. The Institutional Animal Care and Use Committee (IACUC) at Yonsei University (Wonju, Korea) approved the protocol for this study.

Measurement of cell viability

The Ez-Cytox Enhanced Cell Viability Kit (Daeil Labservice, Seoul, Korea) was used to assess cell viability following the manufacturer's instructions. In brief, RBL-2H3 cells (2×10^4 cells/well) were seeded into 96-well plates and incubated overnight with 200 ng/ml anti-DNP-specific IgE. After incubation, the medium was replaced with serum free medium, and the cells were treated with graded concentrations of the EA fraction of Korean rice wine lees and 25 ng/ml DNP-BSA for 23 hr. The Ez-Cytox Kit reagent was then added to the medium and the cells were incubated at 37°C for 1 hr. Cell viability was determined based on absorbance at 450 nm measured with a microplate reader.

Measurement of β -hexosaminidase release

We measured the release of β -hexosaminidase as a marker for degranulation. RBL-2H3 cells were distributed in 24-well plates (2×10^5 cells/well) and were sensitized overnight with anti-DNP-specific IgE at 200 ng/ml. The IgE-sensitized cells were washed twice with PIPES buffer (25 mM PIPES, pH 7.2, 110 mM NaCl, 4 mM KCl, 0.4 mM MgCl₂, 40 mM HCl, 5.6 mM glucose, 1 mM CaCl₂, and 0.1% BSA). They were then treated for 30 min at 37°C with the EA fraction of the Korean rice wine lees in PIPES buffer at the indicated concentrations. The cells were subsequently stimulated with DNP-BSA (25 ng/ml) at 37°C for 15 min and chilled on ice to stop the stimulation. To measure the β -hexosaminidase release, the supernatant from the antigen (Ag)-stimulated cells in PIPES buffer and β -hexosaminidase substrate (p-nitrophenyl-N-acetyl- β -D-glucosaminide) in 0.1 M citrate buffer (pH 4.5) were mixed in 96-well plates and incubated at 37°C for 1 hr. This reaction was terminated by adding 0.1 M carbonate buffer (pH 10.5), and the absorbance was measured at 405 nm using a microplate reader.

Induction of mast cell-mediated passive cutaneous anaphylaxis in mice

An anti-DNP-specific IgE antibody (0.5 μ g) was injected intradermally into one ear of each mouse. After 24 hr, the

mice were challenged with an intravenous injection of DNP-BSA (200 μ g; antigen, Ag) in 200 μ l PBS 3% Evans blue. One hour later, the EA fraction of Korean rice wine lees was administered orally at a dose of 50 to 200 mg/kg. Two hours after the Ag challenge, the mice were euthanized and the treated ear was excised to measure the amount of dye that had extravasated in response to the Ag. The dye was extracted from the ear in 500 μ l formamide at 63°C overnight and quantified by measuring dye absorbance at 620 nm.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

The RBL-2H3 cells (8×10^5 cells/well) were seeded into six-well plates and incubated overnight in a medium containing 200 ng/ml anti-DNP-specific IgE. The cells were washed twice, resuspended in PIPES buffer and stimulated with DNP-BSA (25 ng/ml) for 1 h, with and without the EA fraction of Korean rice wine lees. After incubation, the cells were washed twice with ice-cold PBS. Total RNA was isolated using TRI reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. The concentration of total RNA was determined using a spectrophotometer. The total RNA (1 μ g) was used as the template for cDNA synthesis and PCR using the Accupower RT/PRC premix kit (Bioneer, Daejeon, Korea). The following primers were used: IL-3, forward 5'-GTATGCTGCTCCCGCTCCTGATG-3' and reverse 5'-CATTCCACGGTCATAGGGCGAAAG-3'; IL-13, forward 5'-GCTCTCGCTTGCCTGGTGGTC-3' and reverse 5'-CATCCGAGGCC TTTTGGTTACAG-3'; β -actin, forward 5'-atgccatctcgtctg-gacctggc-3'; and reverse 5'-agcatttgcgggtgcacgatggagg-3'. The denaturation, annealing, and extension conditions and number of cycles were 94°C for 60 sec, 60°C for 45 sec, 72°C for 45 sec and 25 cycles, respectively, for IL-3 (473 bp) and IL-13 (276 bp). The PCR products were electrophoresed on a 2% agarose gel and visualized by adding ethidium bromide. The gels were examined using a transilluminator (Vilber Lourmat, France).

Statistical analysis

Experimental results are expressed as the mean \pm SD. A one-way analysis of variance (ANOVA) with the Dunnett's test for multiple comparisons was performed. P-values <0.05 and <0.01 were considered statistically significant, as indicated.

Results

EA inhibits β -hexosaminidase release in IgE-sensitized RBL-2H3 cells

The RBL-2H3 cells were activated when the IgE-antigen complex bound their receptor, Fc ϵ R1. The aggregation of Fc ϵ R1 by the allergen-IgE complex induced mast cell degranulation and the release of mediators and cytokines [7,9,18,22]. β -hexosaminidase, one of these mediators, is a marker of mast cell degranulation [16]. To determine the anti-degranulation effect of EA, we measured the level of β -hexosaminidase release in IgE-sensitized RBL-2H3 cells. We pretreated the RBL-2H3 cells with different concentrations of EA (0-300 μ g/ml) for 30 min, followed by activation with DNP-BSA (50 ng/ml) for 15 min. The EA significantly inhibited DNP-BSA-induced mast cell degranulation (Fig. 1).

To confirm that the degranulation inhibition was indeed due to the activity of EA and not cytotoxicity, we assessed EA cytotoxicity in the RBL-2H3 cells using an Ez-cytox assay. We pretreated the mast cells overnight with different doses of EA. After 24 hr, cells were treated with the Ez-cytox solution for 5 min, and the absorbance of the cell culture plate was measured with a microplate reader. EA was not cytotoxic in the RBL-2H3 cell line at concentrations up to 300 μ g/ml (data not shown). These findings indicate that

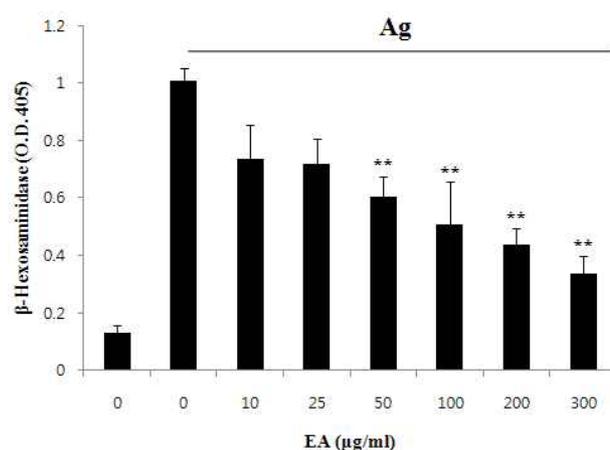


Fig. 1. Effect of the EA fraction of Korean rice wine lees on Ag-stimulated degranulation in mast cells. The cells were incubated overnight in 24-well plates in medium containing 200 ng/ml of DNP-specific IgE. The medium was replaced with PIPES buffer that contained the indicated concentration of EA fraction before stimulation with 25 ng/ml of DNP-BSA for 15 min. β -hexosaminidase was determined by measuring the absorbance at 405 nm (** <0.01).

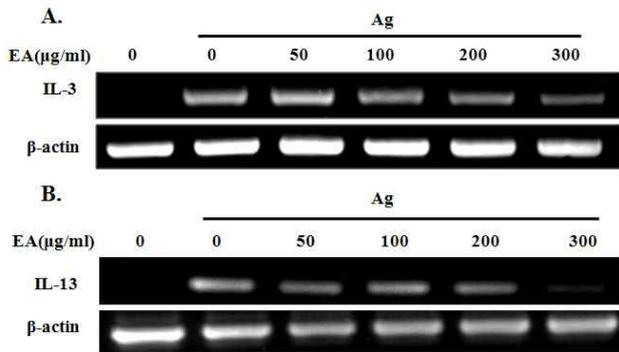


Fig. 2. Effect of the EA fraction of Korean rice wine lees on the expression of IL-3 and IL-13 mRNA in RBL-2H3 cells. The IgE-sensitized RBL-2H3 cells were treated with various doses of the EA fraction and then stimulated with 25 ng/ml DNP-BSA for 1 hr. Total RNA was isolated and used for RT-PCR analysis.

the reduction in degranulation of the activated RBL-2H3 cells was a result of the properties of the EA, and not cytotoxicity.

EA inhibits IL-3 and IL-13 mRNA expression levels in IgE-sensitized RBL-2H3 cells

IL-3 is a multipotent hematopoietic growth factor that acts on mast cells to induce cell proliferation and encourage cell survival [16,24]. IL-13 plays an important role in epithelial cell proliferation and injury repair in asthmatic airway epi-

thelial cells. IL-13 also activates the cleavage of the matrix metalloproteinase 7-mediated FAS ligand in bronchial epithelial cells [21,23]. To investigate the level of these cytokines involved in type I allergies mediated by mast cells, we examined IL-3 and IL-13 mRNA expression levels. We treated the RBL-2H3 cells with IgE (200 ng/ml) for 24 hr followed by treatment with EA at different concentrations (0-300 µg/ml). The cells were then activated with DNP-BSA (25 ng/ml) for 1 hr. We found that EA diminished the mRNA expression levels of IL-3 and IL-13 in a dose-dependent manner (Fig. 2). From these findings, we inferred that the anti-allergic effects of EA stem from suppression of the mRNA expression of these cytokines.

EA reduces anaphylactic shock in an *in vivo* mouse model

We examined the effect of EA in an *in vivo* allergic mouse model [15]. We induced anaphylaxis by injecting IgE intradermally into one ear of each mouse. One day after IgE administration, we injected a DNP-BSA-Evans blue mixture into tail veins of each mouse to induce a systemic allergic reaction. The IgE-sensitized mouse ears subsequently swelled and turned blue. However, in the mice that received EA orally, the intensity of the blue dye decreased with increasing EA concentration. EA also appeared to suppress anaphylaxis in the mice as evidenced by the results depicted in the

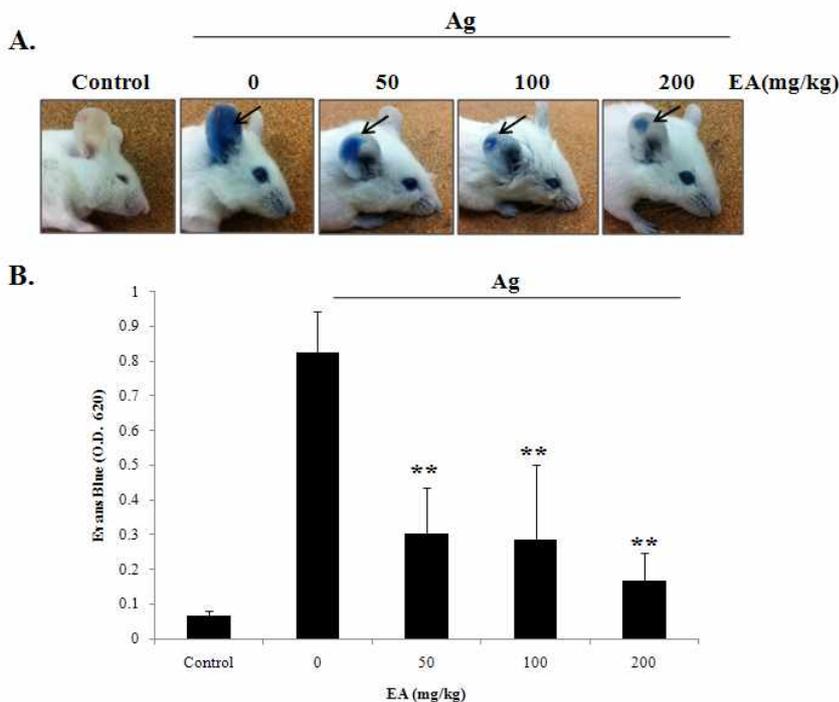


Fig. 3. Effect of the EA fraction of Korean rice wine lees on Ag-stimulated passive cutaneous anaphylaxis (PCA). Balb/c mice were intravenously injected with 200 µg antigen containing 3% Evans blue 24 hr after intradermal administration of DNP-IgE (0.5 µg) into the ear. The EA fraction was orally administered 1 hr before the administration of antigen. (A) Representative pictures of the ears are shown. (B) The dye was extracted overnight in 500 µl of formamide at 63°C, and the intensity was measured at 620 nm (** <0.01).

bar graph in Fig. 3B. These findings confirm the ability of EA to inhibit passive cutaneous anaphylaxis in an *in vivo* animal model just the same as in *in vitro* experiments (Fig. 3).

Discussion

Korean rice wine lees are a byproduct of Korean rice wine production. Recently, several research groups in Japan have begun to study the pharmacological benefits of Sake byproducts. They have found that Sake byproducts not only alleviate some of the symptoms of diabetes mellitus and osteoporosis, but also prevent some of the effects of arteriosclerosis [1,10]. There are few studies of the potential health benefits of Korean rice wine lees. In this study, we investigated the effects of the ethyl acetate fraction of Korean rice wine lees on type 1 hypersensitivity mediated by mast cells.

β -hexosaminidase, a granule-associated enzyme, can be released by mast cells when the crosslinking of Fc ϵ R1 with allergen-specific IgE leads to mast cell activation [14,15]. The β -hexosaminidase assay is an expedient method for monitoring the capacity of novel drugs to block mast cell activation and degranulation [3]. Using this method, we confirmed that EA inhibits degranulation in allergen-activated RBL-2H3 cells (Fig. 1).

Activated mast cells deliver allergic signals to other inflammatory cells including macrophages, T cells and B cells by releasing many mediators and cytokines via degranulation and other processes. One of these cytokines, IL-3, acts on the mast cells as a multipotent hematopoietic growth factor inducing cell proliferation and encouraging cell survival [16,24]. The another cytokine, IL-13 has an important role in the epithelial cell proliferation and injury repair in asthmatic airway epithelial cells. IL-13 activates the cleavage of the matrix metalloproteinase 7 - mediated FAS ligand in bronchial epithelial cells [21,23]. EA significantly inhibited both IL-3 and IL-13 mRNA expression (Fig. 2). We suspect that the anti-allergic effects of EA originate from its inhibition of IL-3 and IL-13 mRNA expression.

Next, we investigated how EA limits IgE-mediated passive cutaneous anaphylaxis (PCA) in mice. PCA is a well-established mast cell-dependent experiment model system for evaluating the localized IgE-mediated immediate allergic response *in vivo* [4,5,6,11]. Consistent with the *in vitro* observations, EA successfully reduced allergic inflammatory reactions in the PCA-induced mice (Fig. 3). We confirmed

that EA also alleviated type 1 hypersensitivity in mice with allergic reactions induced by DNP-BSA. We do not know which pathway is blocked by EA. In further studies, we plan to determine the proteins involved in the signaling pathway interrupted by EA.

In conclusion, the ethyl acetate fraction of Korean rice wine lees suppressed mast cell degranulation. EA also suppressed the expression of IL-3 and IL-13 mRNA in antigen-stimulated RBL-2H3 cells. However, EA also suppressed passive cutaneous anaphylaxis in a mouse model. Therefore, EA could be a useful candidate for anti-allergic drugs and may have fewer side effects than current allergy medications because of its natural origin.

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초록 : 주박 에틸아세테이트 추출물의 항알러지 효과

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제 1형 과민반응은 비만세포에 의해 매개되는 알러지 반응으로서, 예기치 못한 알러젠에 의해 비만세포가 활성화 되어 탈과립 되면서 히스타민등과 같은 여러 가지 매개체들을 분비하면서 과도하게 면역반응을 유도하게 된다. 주박(Korean rice wine lees)은 청주, 약주 등의 술을 걸러내는 과정에서 생기는 양조 부산물로서, 최근들어 청주를 걸러내는 과정에서 생기는 술 지게미의 생리기능에 대한 연구가 활발하게 진행되고 있다. 본 연구에서는 주박의 ethyl acetate (EA) 추출물을 이용하여 비만세포에서의 탈과립 억제 효과를 측정하였다. 그 결과 주박 EA 추출물이 탈과립의 표지인 β-hexosaminidase의 양을 농도 의존적으로 억제하는 것을 관찰하였고, 이러한 탈과립 억제 현상이 다양한 사이토카인 중 IL-3과 IL-13의 양을 감소시킴으로써 매개된다는 사실을 RT-PCR을 통해 확인하였다. 또한 쥐에서 passive cutaneous anaphylaxis (PCA)를 유도하여 EA를 경구투여 했을 때 anaphylactic 증상을 효과적으로 억제하는 것을 관찰하였다. 이러한 결과들은 주박 EA 추출물이 항 알러지 약물의 개발에 잠재적인 후보물질이 될 수 있음을 시사한다.