Cheonggukjang Ethanol Extracts Inhibit a Murine Allergic Asthma via Suppression of Mast Cell-Dependent Anaphylactic Reactions

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ABSTRACT *Cheonggukjang* (CGJ), a traditional Korean fermented soybean food, exerts immunomodulatory effects. Asthma is the most common chronic allergic disease to be associated with immune response to environmental allergens. In the pathogenesis of asthma, histamine is one of the important inflammatory mediators released from granules of mast cells. In this study, we evaluated the therapeutic effect of CGJ on a mouse model of ovalbumin (OVA)-induced asthma via the suppression of histamine release. C57BL/6 mice were sensitized by intraperitoneal injection of OVA or a phosphate-buffered saline (PBS) control and then challenged with OVA inhalation. Mice were treated intraperitoneally with either 70% ethanol-extracted CGJ (CGJE) (100 mg/kg/day) or equivalent PBS. Asthma-related inflammation was assessed by bronchoalveolar lavage fluid cell counts and histopathological and immunohistochemical analysis of lung tissues. To elucidate the mechanisms of asthma inhibition by CGJE treatment, we also examined degranulation and histamine release of compound 48/80-induced rat peritoneal mast cells (RPMCs). Treatment with CGJE downregulated the number of eosinophils and monocytes in the lungs of mice challenged with OVA and suppressed histopathological changes, such as eosinophil infiltration, mucus accumulation, goblet cell hyperplasia, and collagen fiber deposits. Moreover, CGJE alleviated compound 48/80-induced mast cell degranulation and histamine release from RPMCs through inhibition of calcium (Ca^{2+}) uptake as well as ear swelling by infiltration of inflammatory cells. These findings demonstrated that CGJE can be used as an antiasthmatic dietary supplements candidate for histamine-mediated asthma.

KEY WORDS: • anaphylactoid reaction • asthma • cheonggukjang extract • degranulation • histamine

INTRODUCTION

CHEONGGUKJANG (CGJ) IS A CENTURIES-OLD, traditional Korean fermentation product, prepared from soybean, also called Natto, Tempeh, and Douchi in other Asian regions. CGJ contains microorganisms, enzymes, and diverse bioactive compounds, which are absent in unfermented soybean.¹ It has been previously reported that CGJ or its extract exerts immunomodulatory effects. Lee *et al.*² showed that mucosal immune activity in the gastrointestinal tract could be increased by CGJ intake. Kim *et al.*³ reported that oral administration of poly-gamma-glutamic acid (γ -PGA) derived from CGJ enhances natural killer cell-mediated antitumor activity. Kwon *et al.*⁴ showed that CGJ has po-

tential inhibitory properties on histamine-induced skin inflammation in humans. However, the mechanism for the suppressive effect of CGJ extracts on histamine-mediated allergic disease has not yet been fully elucidated.

Allergic asthma is the most common chronic disease among children and affects 235 million people according to the World Health Organization.⁵ Histamine is one of the important inflammatory mediators in the pathogenesis of asthma.^{6,7} Degranulation of mast cells, which frequently leads to histamine release, is dependent on the intracellular calcium (Ca^{2+}) levels. The enhancement of intracellular Ca^{2+} induces the movement of granules to the plasma membrane and the degranulation of mast cells or basophils.⁸ Released histamines could contribute to bronchoconstriction, airway hyperresponsiveness, eosinophilic infiltration, and mucus hypersecretion.^{9–11}

At present, antihistamines are commonly used as a therapy for asthma to produce acute bronchodilation.¹² However, these drugs tend to cause side effects and reduce receptor binding affinity and T-cell responses.¹³ Therefore, natural remedies derived from plants are attracting more and more interest as alternative therapies for respiratory disorders.

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In the present study, we investigated the inhibitory effect of 70% ethanol-extracted CGJ (CGJE) on ovalbumin (OVA)-induced asthma. Moreover, compound 48/80 was used to investigate the regulatory effects of CGJE on mast cell-dependent reaction. The aim of this study was, therefore, to evaluate the therapeutic efficacy of CGJ in allergic asthma via the suppression of histamine release from mast cells.

MATERIALS AND METHODS

Animals

C57BL/6 mice (aged 8 weeks) for a murine asthma model and Sprague–Dawley rats (aged 8 weeks) for acquirement of rat peritoneal mast cells (RPMCs) were purchased from Damool Science (Daejeon, Korea). They were housed in an air-conditioned room ($23^{\circ}\text{C} \pm 2^{\circ}\text{C}$), under standard 12 h light/dark cycles. They were allowed free access to food and tap water. All animal experiments were performed in compliance with the U.S. National Institutes of Health guidelines for the care and use of laboratory animals (NIH publication #85-23; revised in 1985) and were approved by the Animal Research Committee of Chonbuk National University (permission number: CBU 2013-0011).

Sample preparation

The CGJ used in this study was purchased from a local market (Seoul, Korea). The specimen (KFRI-SL-86) has been kept in the Functional Materials Research Group, Korea Food Research Institute. Samples of CGJ (100 g) were reflux-extracted twice in 1 L of 70% ethanol (EtOH) using a Soxhlet100 apparatus. EtOH extracts were dried under a vacuum in a rotary evaporator. Concentrated extracts were lyophilized, yielding a dried powder that was stored at 4°C . The yield (%) of each product was 19.69%. The dried EtOH extract was dissolved in dimethylsulfoxide (DMSO; AppliChem, Darmstadt, Germany) and freshly diluted in the HEPES-Tyrod buffer (136 mM NaCl, 5 mM

KCl, 2 mM CaCl_2 , 11 mM NaHCO_3 , 0.6 mM NaH_2PO_4 , 2.75 mM MgCl_2 , 5.4 mM HEPES, 1.0 mg/mL BSA, 1.0 mg/mL glucose, 0.1 mg/mL heparin, pH 7.4) before use.

Reagent

Compound 48/80, DMSO, HEPES, Folin–Ciocalteu reagent, and daidzein were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Percoll solution was purchased from Pharmacia (Uppsala, Sweden). Aluminum nitrate enneahydrate was purchased from Junsei Chemical Co., Ltd (Tokyo, Japan).

Induction of murine asthma model

Mice were divided into four groups according to the treatment: (1) phosphate-buffered saline (PBS; Sigma-Aldrich, St. Louis, MO, USA) as a vehicle control, (2) 100 mg/kg/day CGJE, (3) OVA-induced asthma mice, and (4) 100 mg/kg/day CGJE in OVA-induced asthma mice. Mice of groups 3 and 4 were immunized by intraperitoneal injection of 50 μg OVA and saline in a total volume of 200 μL on day 0 and 14. On day 21 after the beginning of the sensitization period, these mice received their first intranasal challenge of 30 μL of PBS containing 45 μg of OVA. After 7 days, the mice were challenged for 30 min with an aerosol of 5 g/mL OVA in saline using ultrasonic nebulization (NE-U12; Omron Corp., Tokoyo, Japan). On days 14–27, the treatment groups were also treated once daily with intraperitoneal doses of 100 mg/kg BW CGJE (Fig. 1A).

Collection and analysis of bronchoalveolar lavage fluid

Forty-eight hours after the final OVA challenge, bronchoalveolar lavage fluid (BALF) was collected by cannulating the upper part of the trachea and lavaging, as described previously.¹¹ The total number of viable cells in BAL fluid was determined by trypan blue exclusion using a hemocytometer. Differential cell counts were determined

FIG. 1. Effect of 70% ethanol extract of *cheonggukjang* (CGJE) on cellular changes in bronchoalveolar lavage fluid (BALF). (A) Schematic diagram of the asthma induction protocol. Mice were sensitized on day 0 and 14 and challenged on days 21 and 28 by ovalbumin (OVA). Mice in the control group were sensitized and challenged by saline, respectively. Also, mice in the CGJE treatment group were also treated once daily with intraperitoneal injection of 100 mg/kg BW. Infiltration of differential components and total cells (B) and inflammatory cells (C) in BALF. Values are presented as the mean $\pm$ SD ($n=10$ per group). Data were analyzed using analysis of variance (ANOVA) followed by Student's *t*-test. ** $P < .01$, significantly different from the value of the asthma group.

via cytopsin (Centrifuge 5403; Eppendorf, Hamburg, Germany) preparation, followed by Diff Quik staining (Sysmex Co., Kobe, Japan).

Histopathology and histochemistry

Histopathological analysis of lung tissue was performed as previously described.¹¹ Animals were sacrificed with an overdose of ether 48 h after the last OVA exposure, and histological specimens were collected. Using routine histological procedures for light microscopic evaluation, tissue specimens were taken from the mid-zone of the left lung of the mice and fixed in 10% formalin and embedded in paraffin. Serial sections measuring 5 μ m in thickness were then cut and stained with hematoxylin and eosin (H&E) for inflammatory cells, congo red for eosinophils, periodic acid-Schiff (PAS) for goblet cells and mucus, and trichrome for collagen fiber deposits.

Compound 48/80-induced ear swelling response

Ear swelling response was investigated by a method described previously.¹⁴ Compound 48/80 was intradermally injected into the left ear, and sham saline was injected into the right ear of each mouse (100 μ g/site, 20 μ L). Ear thickness was measured with a digital micrometer (No. 7326; Mitutoyo, Kawasaki, Japan) under mild anesthesia. Mice were immobilized during the measurement. Ear swelling response represented an increment in thickness above baseline control values. Ear swelling response was determined 1 h after the injection of compound 48/80 or vehicle. CGJE (1, 10, or 100 mg/kg/day) was dissolved in saline and administered intraperitoneally at 24, 12, and 1 h before injection of compound 48/80 ($n = 10$ /group).

Preparation of RPMCs

RPMCs were isolated as previously described.¹⁴ In brief, rats were anesthetized with ether by administering a peritoneal injection of 10 mL of Ca^{2+} -free HEPES-Tyrode buffer, following which the abdomen was gently massaged for ~ 90 s. The peritoneal cavity was opened, and the fluid was aspirated using a Pasteur pipette. RPMCs were purified using a Percoll density gradient, as described in detail elsewhere.¹⁴ RPMC preparation was $\sim 95\%$ pure as assessed by toluidine blue staining and at least 98% of these cells were viable as assessed by trypan blue exclusion.

Observation of degranulation by microscopy

Purified RPMCs (1×10^6 cells/mL) were resuspended in the HEPES-Tyrode buffer. The RPMCs were pretreated with CGJE (0–1 mg/mL) and the active compounds (0–1 mg/mL) for 10 min at 37°C and observed within 10 min of the addition of compound 48/80 (10 μ g/mL) under phase-contrast microscopy and photographed as described by Cochrane *et al.*¹⁵ The mast cells were classified ($\times 1000$) as follows: (1) extensively degranulated ($> 50\%$ of the cytoplasmic granules exhibiting fusion, staining alterations, and extrusion from the cell), (2) slight to moderately

degranulated (10–50% of the granules exhibiting fusion or discharge), or (3) normal. The number of granulocytes was quantitated, and the results were expressed as granulocytes per mm^2 , as previously described.¹⁶

Histamine assay

RPMCs (2×10^5 cells/well) were preincubated with CGJE (0–1 mg/mL) and the active compounds (0–1 mg/mL) at 37°C for 5 min and then incubated with compound 48/80 (0.25 μ g/mL) for 15 min. The cells were separated from the released histamine by centrifugation at 150 g for 10 min at 4°C. Residual histamine in the cells was released by boiling cells. After centrifugation, histamine content was measured by the radioenzymatic method described by Harvima *et al.*¹⁷ The percent inhibition of histamine release was calculated using the following formula: % inhibition = [(histamine release without CGJE – histamine release with CGJE)/histamine release without CGJE] $\times 100$.

Measurement of ^{45}Ca uptake

The Ca^{2+} uptake of mast cells was measured according to the method described by Chai *et al.*¹⁴ Purified mast cells were resuspended in the HEPES-Tyrode buffer containing ^{45}Ca (1.5 mCi/mL; 1 Ci = 3.7×10^{10} Bq; PerkinElmer Life Sciences, Waltham, MA, USA) and incubated for 10 min at 4°C. Mast cell suspensions were preincubated with CGJE (0–1 mg/mL) for 10 min at 37°C and then incubated with compound 48/80 (0.25 μ g/mL). The reaction was stopped by the adding 1 mM lanthanum chloride. The samples were centrifuged three times at 150 g for 10 min at 4°C, and then, RPMCs were lysed with 10% Triton X-100 and vigorously shaken. Radioactivity of the solution was measured in a scintillation β -counter (Liquid Scintillation Analyzer; Canberra Industries, Meriden, CT, USA).

Quantitative analysis of polyphenol and flavonoid

Polyphenol content was measured using Folin–Ciocalteu reagent.¹⁸ In detail, total levels of phenolic compounds were determined as follows: an aliquot of sample 1.6 mL was added to test tubes containing 0.1 mL of Folin–Ciocalteu's phenol reagent, and after 8 min at room temperature, 0.3 mL of 25% Na_2CO_3 was added. After 2 h at room temperature, the absorbance was determined spectrophotometrically at 765 nm (Epoch Microplate Spectrophotometer; BioTek Instruments, Winooski, VT, USA). Total amounts of phenolic compounds were calculated using tannic acid as a standard. Flavonoid content was measured using aluminum nitrate enneahydrate.¹⁹ In detail, flavonoid concentration was determined as follows: an aliquot of sample 0.5 mL was added to test tubes containing 0.1 mL of 10% aluminum nitrate, 0.1 mL of 1 M aqueous potassium acetate, and 4.3 mL of 80% EtOH. After 40 min at room temperature, the absorbance was determined spectrophotometrically at 415 nm (Epoch Microplate Spectrophotometer; BioTek Instruments). Total flavonoid concentration was calculated using quercetin as standard.

High-performance liquid chromatography analysis

Daidzein and genistein were assayed by High-performance liquid chromatography analysis. Briefly, extracts from soybean and CGJ were separated on a Jasco PU-980 liquid chromatography system (JASCO, Tokyo, Japan) equipped with multi wavelength detector Jasco MD-2010 plus (JASCO). The mobile phase was composed of 40% acetonitrile and 2% acetic acid in water. The mobile phase flow rate was 1 mL/min on a HC-C18(2) column (Agilent Technologies, Santa Clara, CA, USA), and the elution profiles were monitored by absorbance at 285 nm.

Statistical analysis

Each result is expressed as the mean $\pm$ SD. Differences were assessed by analysis of variance (ANOVA) followed by Student's *t*-test or Tukey *post hoc* test.

RESULTS

CGJE decreased the number of eosinophils and other inflammatory cells in BALF

In the mouse model of OVA-induced asthma, the number of inflammatory cells in BALF was significantly increased compared with that of mice treated with saline alone (Fig. 1B). In addition, 100 mg/kg/day CGJE treatment significantly lowered the number of eosinophils and monocytes (Fig. 1B) and had inflammatory cells counts similar to those of the normal controls on H&E staining of BALF (Fig. 1C).

CGJE inhibited OVA-induced histopathological changes in lung tissue

Histopathological analyses revealed typical pathological features of asthma in OVA-sensitized and OVA-challenged mice. In lung tissues of mice given OVA, increases were observed in infiltration of numerous inflammatory cells, such as eosinophils around bronchioles, accumulation of mucus in the lumen of bronchioles, and hyperplasia of goblet cells at the epithelium of large airways compared to saline-treated mice. Moreover, collagen fiber deposits between submucous and muscular layers were observed in lung tissues of OVA-sensitized and OVA-challenged mice (Fig. 2). However, CGJE treatment significantly attenuated the infiltration of inflammatory cells including eosinophils, the accumulation of mucus in the lumen of bronchioles, and the hyperplasia of goblet cells at the epithelium of airways induced by OVA. These data strongly suggest that CGJE may inhibit allergic asthma. These results showed a more efficient inhibitory effect than that of turmeric, which was used as a positive control on asthma (Fig. 2).²⁰

CGJE suppressed compound 48/80-induced passive cutaneous anaphylaxis as ear swelling response

We investigated the inhibitory effect of CGJE on mast cells activation with regard to certain parameters of allergic inflammation in eosinophil-infiltrated asthma. Infiltration of eosinophils and degranulation of mast cells were determined as a cellular mechanism underlying ear swelling response.¹⁴

FIG. 2. Effect of CGJE on histopathological changes in lung tissues. Mice were sensitized on day 0 and 14 and challenged on days 21 and 28 by OVA. Mice in the CGJE treatment group were also treated once daily with intraperitoneal injection of 100 mg/kg BW. Lung tissues from each group were stained with hematoxylin and eosin (H-E) for inflammatory cells (A–F), congo red for eosinophils (G–L), periodic acid–Schiff (PAS) for goblet cells (M–R) and mucus and trichrome for collagen fiber deposits (S–X). Pictures are representative of 1 experiment ($n = 10$ per group). Arrow in (D) indicates inflammatory cells infiltrated by OVA, and arrow in (I) shows eosinophils infiltrated by OVA. Each red and yellow arrow in (P) indicates goblet cells and mucus accumulated by OVA. Trichrome (blue) staining shows collagen type-1. Color images available online at www.liebertpub.com/jmf

TABLE 1. INHIBITORY EFFECT OF 70% ETHANOL EXTRACT OF *CHEONGGUKJANG* ON COMPOUND 48/80-INDUCED EAR SWELLING RESPONSE

CGJE (mg/kg per day)	Compound 48/80 (100 µg/site)	Ear thickness ($\times 10^{-4}$ inches)	Inhibition (%)
0	+	81.0 $\pm$ 3.6	0.0
1	+	76.0 $\pm$ 3.6	6.2
10	+	70.7 $\pm$ 2.5**	12.8
100	+	65.7 $\pm$ 4.0**	18.9

Groups of mice received 300 µL saline, CGJE (1, 10, or 100 mg/kg/day) by peritoneal administration at 24, 12, and 1 h before injection (+) of compound 48/80. Compound 48/80 (100 µg/site, 20 µL) was injected into the ears of mice ($n = 10$ per group). Each value represents the mean $\pm$ SD of three independent experiments.

Data were analyzed using analysis of variance followed by Student's *t*-test. ** $P < .01$, significantly different from the saline control value.

In mice, injections of compound 48/80 (100 µg per site) induced significant ear swelling response that returned to normal 24 h postinjection. CGJE (0–100 mg/mL) alleviated the ear swelling response induced by compound 48/80 (Table 1).

We also examined the effect of CGJE on compound 48/80-induced passive cutaneous anaphylaxis (PCA) response *in vivo*. Intradermal injection of compound 48/80 into the dorsal skin of rats elevates mast cell-dependent vascular permeability as documented by Evans blue extravasation.²¹

To evaluate the effect of CGJE in animals, a compound 48/80-induced systemic anaphylaxis response model was used in passively sensitized mice. Our results showed that compound 48/80 treatment induced a significant increase in Evans blue dye leakage into the mouse ears. However, CGJE (5 mg/mL) inhibited leakage of injected Evans Blue dye by about 58% by suppressing the compound 48/80-triggered vascular permeability (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/jmf). Taken together, these data clearly indicate that CGJE therapy is able to suppress anaphylactic reactions.

Treatment of CGJE suppressed the degranulation of mast cells and histamine release from RPMCs

The mast cell in asthma and anaphylaxis exerts its pathophysiological effects through histamine release induced by degranulation.²² To investigate whether CGJE inhibited the degranulation of mast cells, we observed degranulation of RPMCs by microscopy. RPMCs stimulated with compound 48/80 had cell swelling, cytoplasmic vacuoles, and extruded granules near the cell surface (Fig. 3B). When RPMCs were incubated with CGJE alone, RPMCs were similar to those seen in saline controls, regardless of the dose of the CGJE (Fig. 3C, E, G). However, treatment with CGJE (0.01–1 mg/mL) inhibited the degranulation of RPMCs stimulated with compound 48/80 (Fig. 3D, F, H, I).

FIG. 3. Suppressive effect of CGJE on compound 48/80-induced mast cell degranulation and histamine release of rat peritoneal mast cells (RPMCs). RPMCs were preincubated with CGJE (0.01–1 mg/mL) at 37°C for 10 min before incubation with compound 48/80. The RPMCs were micrographed within 10 min after the addition of saline or compound 48/80 by using inverted phase-contrast microscopy. (A) Controls: normal RPMCs in the HEPES-Tyrode buffer. RPMCs after the addition of CGJE (C) 0.01 mg/mL, (E) 0.1 mg/mL, and (G) 1 mg/mL. (B) Degranulated RPMCs after stimulation with compound 48/80 (10 µg/mL). RPMCs pretreated with CGJE (D) 0.01 mg/mL, (F) 0.1 mg/mL, and (H) 1 mg/mL and subsequently stimulated with compound 48/80 (10 µg/mL). The percentage of degranulation (I) is shown in the graph. Values are presented as the mean $\pm$ SD ($n = 5$ per group). Data were analyzed using ANOVA followed by Student's *t*-test. ** $P < .01$, significantly different from the value of compound 48/80 without CGJE.

Moreover, CGJE inhibited compound 48/80-induced histamine release at concentrations between 0 and 1 mg/mL from RPMCs (Fig. 4). These results indicate that CGJE may alleviate compound 48/80-induced histamine release by the inhibition of mast cell degranulation.

Treatment of CGJE inhibited Ca^{2+} uptake into RPMCs

Intracellular Ca^{2+} levels are augmented during mast cell degranulation.¹⁴ We examined whether CGJE can suppress Ca^{2+} uptake into RPMCs stimulated with compound 48/80. Treatment with CGJE alone did not affect Ca^{2+} uptake into RPMCs, whereas compound 48/80 increased Ca^{2+} uptake into RPMCs. However, pretreatment with CGJEs (0.01–1 mg/mL) inhibited compound 48/80-induced Ca^{2+} uptake into RPMCs (Fig. 5). These results suggest that CGJE could prevent histamine release from mast cells by suppressing Ca^{2+} uptake into mast cells.

DISCUSSION

The aim of the present study was to investigate immunobiological activity of the traditional Korean fermented food *cheonggukjang* and its potential efficacy as a therapeutic dietary remedy. We revealed an inhibitory effect of CGJE on allergic asthma exacerbated by histamine release from mast cells. CGJE treatment suppressed eosinophil infiltration into the lungs and blocked histopathological changes, such as airway remodeling, goblet cell hyperplasia, mucus hypersecretion, and collagen fiber deposits in the lungs of mice with OVA-induced asthma. Moreover, CGJE decreased compound 48/80-induced ear swelling response and PCA-like reaction in mice, probably through interference with the mast cell-histamine-calcium system. To the best of our knowledge, this is the first study about the alleviation of allergic asthma of CGJE, whose suppressive

FIG. 4. Inhibitory effect of CGJE on compound 48/80-mediated histamine release of RPMCs. RPMCs were preincubated with CGJE (0.01–1 mg/mL) at 37°C for 10 min before incubation with compound 48/80. CGJE inhibited compound 48/80-induced histamine release in a dose-dependent manner. Values are presented as the mean $\pm$ SD ($n=5$ per group). Data were analyzed using ANOVA followed by Student's *t*-test. ** $P<.01$, significantly different from the value of compound 48/80 without CGJE.

FIG. 5. Alleviative effect of CGJE on compound 48/80-induced calcium uptake into RPMCs. RPMCs were preincubated with CGJE (0.01–1 mg/mL) at 37°C for 10 min before incubation with compound 48/80. CGJE dose-dependently inhibited compound 48/80-induced calcium uptake into RPMCs. Data represent the mean $\pm$ SD ($n=5$ per group). Data were analyzed using ANOVA followed by Student's *t*-test. ** $P<.01$, significantly different from the value of compound 48/80 without CGJE.

mechanisms were considered to inhibit mast cell activation and mast cell mediated-anaphylactic response (Fig. 6).

Compound 48/80 is a mixed polymer of phenethylamine cross-linked by formaldehyde, which enhances the permeability of the lipid bilayer membrane and induces the release of histamine from mast cells through perturbation of the

FIG. 6. Schematic representation of possible inhibitory mechanism for allergic asthma by CGJE.

membrane.¹⁴ Increased release of histamine from mast cells is considered to cause compound 48/80-induced anaphylactic reactions.²³ Degranulation of mast cells with compound 48/80 is related to the synergistic activation of phospholipase, protein kinase C (PKC), and increases in the uptake of extracellular Ca^{2+} into RPMCs.²⁴ In general, reactive oxygen species (ROS) activate PKC leading to mast cell activation.²⁵ Moreover, compound 48/80 initiates the generation of superoxide anions, the main species of ROS, by activating cAMP-dependent protein phosphorylation in RPMC. Inhibition of superoxide anion generation delays IP_3 (inositol 1,4,5-triphosphate)- or GTP-induced Ca^{2+} release from the endoplasmic reticulum (ER). When the ER is filled with Ca^{2+} , its influx into RPMCs is blocked, consequently preventing histamine release. Hence, ROS scavenger materials are reported to have antihistaminic effects by suppressing degranulation of mast cells.^{21,26} CGJ is also rich in antioxidants and free radical scavengers. The major antioxidative component of CGJEs seems to be the aglycone form of natural phenols, such as isoflavones. During fermentation of CGJ, the conversion of isoflavone β -glycosides to aglycones in soybean increases, leading to an increase in biological activities.²⁷ Microbial sources have been shown to be a potential means of producing natural antioxidants in fermented products.²⁸ In the present study, high-performance liquid chromatography analyses of daidzein and other compounds of interest in CGJE revealed the presence of aglycones, such as daidzein and genistein at high concentrations (Supplementary Fig. S2). Moreover, genistein and daidzein are also inhibitors of tyrosine kinases, such as PKC. Wong *et al.*²⁹ and Duan *et al.*³⁰ demonstrated that genistein inhibits the release of mediators of anaphylaxis in asthma via its action on protein kinases and blocking Ca^{2+} channels. Gao *et al.*³¹ also reported that genistein attenuated airway inflammation and airway thickness in OVA-sensitized mice via skewing into Th1. The present study demonstrated that fermented soybean paste is rich in isoflavone aglycones, which strongly suppressed compound 48/80-induced degranulation of RPMCs and histamine release from RPMCs (Supplementary Fig. S3). Moreover, it was further demonstrated that CGJE inhibited the phosphorylation of PKC (Supplementary Fig. S4).

It has been reported that the active compounds of CGJE are γ -PGA, oligosaccharides as well as isoflavones.^{27,32} Gamma-PGA is mainly produced by *Bacillus subtilis* during fermentation processes of soybeans.³ Recently, several reports regarding the immunomodulatory activities of γ -PGA have been published.^{3,33,34} It is also apparent that enzymes from microorganisms such as β -glucanase in CGJ can convert polysaccharides into oligosaccharides,³⁵ which show stimulatory effects on immune cells in many studies.^{36,37} In our study, CGJE contained higher concentrations of daidzein and other compounds of interest than unfermented soybean extracts or water extracts of CGJ (Supplementary Table S1), but lower content of other components such as carbonates and amino acids (data not shown). Since it has been known that isoflavones are soluble in EtOH, whereas γ -PGA and oligosaccharides are water soluble and

insoluble in EtOH,³⁸ the results of the present study are in close agreement with those found in earlier studies.

In conclusion, we propose that CGJE treatment may lead to alleviation of allergic asthma symptoms and an improvement of anaphylactic reactions by decreasing Ca^{2+} influx into mast cells and subsequent reduction of degranulation and histamine release from mast cells. This study confirms that CGJEs may have efficacy as a dietary remedy for the therapy of histamine-mediated allergic diseases, and further studies using oral administration in animal and human studies are warranted.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflicts of interest.

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