

Glucosamine has an antiallergic effect in mice with allergic asthma and rhinitis

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Background: Glucosamine (GlcN) is generally used as a dietary supplement because of its antiinflammatory effects. We evaluated the antiallergic effect of GlcN in mice with allergic asthma and rhinitis.

Methods: Thirty-two mice were allocated equally into 4 groups ($n = 8$). In group A (control), we performed intraperitoneal/intranasal challenge using sterile saline. In group B (asthma/rhinitis), we used ovalbumin for intraperitoneal/intranasal challenge to induce allergic asthma and rhinitis. In groups C and D (GlcN treatment), mice were given 1% and 5% GlcN throughout the period of ovalbumin challenge, respectively. We measured serum total and ovalbumin-specific immunoglobulin E (IgE), cytokine titers (interleukin-1, -4, -5, -6, -10, and -17; tumor necrosis factor- α ; and interferon- γ), and the number of inflammatory cells (eosinophils, neutrophils, lymphocytes) in bronchoalveolar lavage (BAL) fluid. We also performed histopathologic examination of the lung and nasal cavity. Finally, we performed real-time polymerase chain reaction for the genes *Bcl-2*, *EC-SOD*, *VEGF*, *caspase-3*, *Bax*, *COX-2*, *Hif-1 α* , and *heme oxygenase-1*.

Results: Compared with group B, group D had significant serum total and ovalbumin-specific IgE decreases after

GlcN treatment ($p < 0.05$). Titers for IL-4, IL-5, IL-6, and IL-17 in BAL fluid were significantly decreased in group D ($p < 0.05$). Eosinophils in BAL fluid were significantly decreased in group D compared with group B ($p < 0.05$). Groups C and D showed significant improvement of inflammation compared with group B. Group D had significant downregulation of *EC-SOD*, *Bax*, *Hif-1 α* , and *heme oxygenase-1* compared with group B.

Conclusion: GlcN had a significant antiallergic effect in mice with allergic asthma and rhinitis. © 2017 ARS-AAOA, LLC.

Key Words:

allergic rhinitis; asthma; heme oxygenase-1; glucosamine; hypoxia-inducible factor-1; superoxide dismutase

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Glucosamine (2-amino-2-deoxy-D-glucose; GlcN) is a sugar synthesized from glucose by the hexosamine biosynthetic pathway in nearly all types of cells in the

body.^{1,2} Exogenous GlcN can enter the cell by passing through glucose transporters (GLUT-1, GLUT-2, and GLUT-4) and induce an increase in UDP-N-acetyl-GlcN (UDP-GlcNAc). It sequentially causes O-GlcNAcylation of nuclear and cytoplasmic proteins.^{3,4} As this process is a kind of posttranslational modification of proteins, it can contribute to intracellular signaling and pathways (in other words, O-GlcNAcylation can enhance or inhibit activity of some enzymes).^{3,5}

O-GlcNAcylation induced by administration of GlcN has been shown to have antiinflammatory effects, including lipopolysaccharide-induced expression of inflammatory markers and nuclear factor- κ B pathway activation.⁶ Therefore, GlcN has been hypothesized to have a therapeutic effect on allergic diseases. Although some animal and human studies have examined the progression of atopic dermatitis after GlcN administration,⁷⁻⁹ no studies have evaluated

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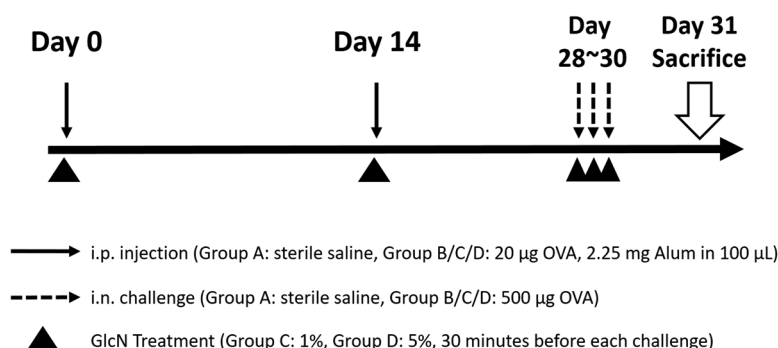


FIGURE 1. Study protocol for experiments. Group A: non-allergic group; group B: allergic group; group C: 1% GlcN treatment group; group D: 5% GlcN treatment group. GlcN = glucosamine; i.n. = intranasal; i.p. = intraperitoneal.

the therapeutic effects of GlcN administration on allergic asthma and rhinitis.

We evaluated the effects of GlcN treatment in a murine model of allergic asthma and rhinitis by measuring and evaluating: (1) serum total and ovalbumin-specific immunoglobulin E (IgE); (2) inflammatory cytokine titers (interleukin [IL]-1, IL-4, IL-5, IL-6, IL-10, IL-17; tumor necrosis factor [TNF]- α ; and interferon [IFN]- γ); (3) number of inflammatory cells (eosinophils, neutrophils, and lymphocytes) in bronchoalveolar lavage (BAL) fluid; (4) histopathologic findings in the lung and in the nasal cavity; and (5) real-time polymerase chain reaction (PCR) for the genes *Bcl-2* (B-cell lymphoma-2), *EC-SOD* (extracellular superoxide dismutase), vascular endothelial growth factor (VEGF), *caspase-3*, *Bax*, *COX-2* (cyclooxygenase-2), *Hif-1 α* (hypoxia-inducible factor-1- α), and *heme oxygenase-1*.

Materials and methods

Animals

We used 32 female BALB/c mice, 6 to 8 weeks old, and free of any murine-specific pathogens (Orient Bio, Seongnam, Korea). Mice were kept in a well-controlled environment with a regular 12-hour light-dark cycle and unrestricted access to ovalbumin (OVA)-free food and water. All mice were handled according to a protocol approved by the animal care and use committee of Inha University (INHA-150309-351). All experiments were performed in accordance with approved guidelines and regulations.

Systemic sensitization and intranasal challenge

To induce allergic asthma and rhinitis, we adopted a published protocol with slight modifications.^{10–12} Under pathogen-free conditions, 20 μ g OVA (Sigma-Aldrich, St. Louis, MO) diluted in sterile saline and 2.25 mg aluminum hydroxide gel (alum adjuvant; Thermo, Waltham, MA) was injected intraperitoneally at a total volume of 100 μ L on experimental days 0 and 14 for systemic sensitization. Thereafter, mice were locally challenged by intranasal instillation with 500 μ g OVA into their nostrils on days 28, 29, and 30.

Mice were divided into 4 groups ($n = 8$ mice/group). Negative control animals (group A, nonallergic group) were exposed to sterile saline, whereas positive control animals (group B, allergic group) were exposed to OVA intraperitoneal injection and intranasal challenge. The remaining groups (groups C and D) received either 1% or 5% (w/v) D-(+)-glucosamine hydrochloride (Sigma-Aldrich) intraperitoneally, 30 minutes before sensitization and intranasal challenge (Fig. 1).

Serum and BAL fluid harvest

After the last intranasal challenge, euthanasia is performed after 24 hours to ensure that the ovalbumin administered into the nose is sufficiently effective. We collected serum from the abdominal aorta using an aortic puncture technique. BAL fluid was collected by lavaging lungs with 3 mL of Hanks balanced salt solution (HBSS; Thermo) through an intratracheal tube. Total BAL cell numbers were determined in duplicate with a hemocytometer. Subsequently, a 100- to 200- μ L aliquot was centrifuged in a cytocentrifuge (Model 2 Cytospin, Shandon Scientific, Pittsburgh, PA). Differential cell counts for eosinophils, neutrophils, and lymphocytes were determined from centrifuged preparations stained with Diff-Quik stain kit (Sysmex Corp, Kobe, Japan) by counting ≥ 500 cells from each sample at $\times 200$ magnification.

Enzyme-linked immunosorbent assay

We evaluated the levels of total and ovalbumin-specific IgE in serum by enzyme-linked immunosorbent assay (ELISA), as described previously.¹³ Total IgE was measured and compared with a known concentration of mouse IgE standard (BD Biosciences, San Diego, CA). We used an optical density of 450 nm to measure ovalbumin-specific IgE instead of calculating the concentration with a standard solution. Cytokine titers (IL-1, IL-4, IL-5, IL-6, IL-10, and IL-17; TNF- α ; IFN- γ) in BAL fluid supernatants were measured using individual ELISA kits (BD Biosciences) according to the manufacturer's instructions.

TABLE 1. Primer sequence for real-time PCR

Gene		Primer sequence
<i>Bcl-2</i>	Forward	5'-GGA CTT GAA GTG CCA TTG GT-3'
	Reverse	5'-AGC CCC TCT GTG ACA GCT TA-3'
<i>EC-SOD</i>	Forward	5'-GTG TCC CAA GAC AAT C-3'
	Reverse	5'-GTG CTA TGG GGA CAG G-3'
<i>VEGF</i>	Forward	5'-CTT GTT CAG AGC GGA GAA AGC-3'
	Reverse	5'-ACA TCT GCA AGT ACG TTC GTT-3'
<i>Caspase-3</i>	Forward	5'-TGG GCC TGA AAT ACC AAG TC-3'
	Reverse	5'-AAA TGA CCC CTT CAT CAC CA-3'
<i>Bax</i>	Forward	5'-GGA TGC GTC CAC CAA GAA GC-3'
	Reverse	5'-GGA GGA AGT CCA GTG TCC AGC C-3'
<i>COX-2</i>	Forward	5'-TTC GGG AGC ACA ACA GAG TG-3'
	Reverse	5'-TAA CCG CTC AGG TGT TGC AC-3'
<i>Hif-1α</i>	Forward	5'-ACC TTC ATC GGA AAC TCC AAA G-3'
	Reverse	5'-CTG TTA GGC TGG GAA AAG TTA GG-3'
<i>HO-1</i>	Forward	5'-CCT CAC TGG CAG GAA ATC ATC-3'
	Reverse	5'-CCT CGT GGA GAC GCT TTA CAT A-3'

PCR = polymerase chain reaction.

Histopathology

After fixation in 4% paraformaldehyde solution for 24 hours, lung tissues were embedded in paraffin. Nasal tissues were decalcified for 3 to 4 weeks and embedded in paraffin. Tissue sections (4- μ m thickness) were stained using hematoxylin-eosin (for qualitative evaluation of histopathologic changes), periodic acid-Schiff (for mucus), and Sirius Red (for evaluation of eosinophilic infiltration). The number of eosinophils infiltrated into 1 mm² of pulmonary parenchyma was counted in 20 random high-power fields ($\times 200$ magnification). The number of eosinophils infiltrated into 1 mm² of lamina propria was counted in 10 high-power fields ($\times 400$ magnification) of the T1 area (the section was taken immediately caudal to

the upper incisor teeth). Two blinded researchers performed the histopathologic examinations and counted eosinophils in tissue specimens.

Quantitative real-time PCR

Total RNA was isolated from lung tissue with TRIzol reagent (Invitrogen Life Technologies, Waltham, MA) according to the manufacturer's recommended protocol. Complementary DNA was synthesized with 1 μ g of total RNA by using a PrimeScript RT reagent kit (Takara Bio, Inc, Shiga, Japan). Real-time PCR analysis was performed in duplicate using SYBR Green Master Mix ABI Prism in a PCR machine (7500 Real-Time PCR System; Applied Biosystems, Inc, Carlsbad, CA). We identified the highlight sequence features of the target gene from the National Center for Biotechnology Information site and primers were designed using Primer 3.0 software (Table 1). Primer for *GAPDH* was purchased from QuantiTect Primer Assays (QIAGEN, Venlo, The Netherlands). To calculate amplification efficiency, the relative expression of each target gene was normalized to the housekeeping gene *GAPDH* using the comparative $2^{-\Delta\Delta CT}$ method. Primer sequences are summarized in Table 1.

Statistical analysis

All statistical analyses were conducted with SPSS version 19.0 software (IBM, Armonk, NY). We used the Kruskal-Wallis test and Mann-Whitney *U* test for between-group comparisons. $P < 0.05$ was considered statistically significant.

Results

Compared with group A (nonallergic group), mice in group B (allergic group) had significantly elevated titers of serum total and OVA-specific IgE ($p < 0.001$). After GlcN treatment, mice in group C (1% GlcN) and those in group D (5% GlcN) had decreased total and OVA-specific IgE titers compared with those in group B. There were especially significant differences between groups B and D ($p < 0.05$). As GlcN concentration increased there was a trend for decreased total and OVA-specific IgE (Fig. 2).

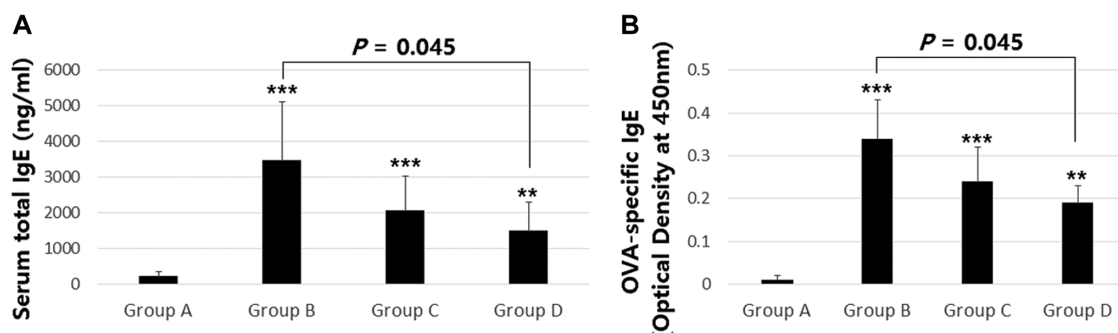


FIGURE 2. Effects of glucosamine (GlcN) treatment on serum (A) total IgE and (B) OVA-specific IgE levels. Group A: non-allergic group; group B: allergic group; group C: 1% GlcN treatment group; group D: 5% GlcN treatment group ($n = 8$ mice/group). ** $p < 0.01$ vs group A; *** $p < 0.001$ vs group A (Kruskal-Wallis test and Mann-Whitney *U* test). GlcN = glucosamine; OVA = ovalbumin; PCR = polymerase chain reaction.

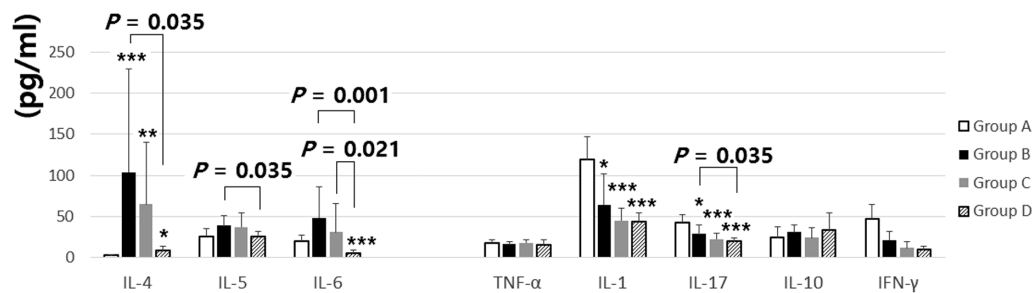


FIGURE 3. Effects of GlcN treatment on cytokine titers in BAL fluid. Group A: nonallergic group; group B: allergic group; group C: 1% GlcN treatment group; group D: 5% GlcN treatment group ($n = 8$ mice/group). * $p < 0.05$ vs group A; ** $p < 0.01$ vs group A; *** $p < 0.001$ vs group A (Kruskal-Wallis test and Mann-Whitney U test). BAL = bronchoalveolar lavage; GlcN = glucosamine.

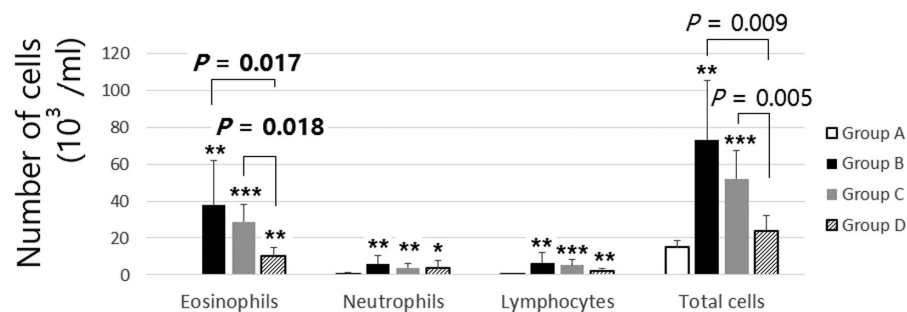


FIGURE 4. Number of inflammatory cells (eosinophils, neutrophils, lymphocytes, and total cells) in (BAL) fluid. Group A: nonallergic group; group B: allergic group; group C: 1% GlcN treatment group; group D: 5% GlcN treatment group ($n = 8$ mice/group). * $p < 0.05$ vs group A; ** $p < 0.01$ vs group A; *** $p < 0.001$ vs group A (Kruskal-Wallis test and Mann-Whitney U test). BAL = bronchoalveolar lavage; GlcN = glucosamine; PCR = polymerase chain reaction.

ELISA of BAL fluid revealed that group D showed a significant decrease in IL-4, IL-5, IL-6, and IL-17 compared with group B ($p < 0.05$). However, there was no significant difference between groups B and D in TNF- α , IL-1, IL-10, and IFN- γ (Fig. 3).

Through differential cell count analysis in BAL fluid, we found there were significant increases in eosinophils, neutrophils, and lymphocytes in group B compared with group A ($p < 0.01$). After GlcN treatment, group D had a significant decrease in eosinophils ($p = 0.017$) and total cell count ($p = 0.005$). Although there was also a trend toward decreased neutrophils and lymphocytes, these did not reach statistical significance ($p > 0.05$; Fig. 4).

Histopathologic examination of lung and nasal cavity revealed that mice in group B had significantly more inflammatory cell infiltration around bronchioles and within nasal mucosa. On the other hand, those in groups C and D had significantly decreased inflammatory infiltration (Fig. 5). The number of eosinophils in 1 mm² of lung parenchyma and lamina papyracea of nasal mucosa were all significantly decreased in group D compared with group B ($p < 0.05$; Fig. 6).

On real-time PCR, we found that mice in group D showed significant downregulation of *EC-SOD*, *Bax*, *Hif-1 α* , and *heme oxygenase-1* compared with group B ($p < 0.05$; Fig. 7).

Discussion

Studies have only recently begun to elucidate the link between GlcN and allergic diseases. Jin et al reported that crystallinity-controlled *N*-acetylGlcN reduced systemic anaphylactic shock, ear swelling response, and passive cutaneous anaphylaxis in experimental animals.¹⁴ In addition, the combination of GlcN and cyclosporine significantly reduced serum IgE, Th2 cytokines, and chemokines in spleen homogenate, and infiltration of eosinophils and mast cells in experimental animals with atopic dermatitis, when compared with cyclosporine alone.⁷ Concomitant use of GlcN and cyclosporine in patients with atopic dermatitis also showed significant improvement of clinical symptoms compared with cyclosporine-only treatment.⁸ According to our literature review, this study is the first to evaluate the antiallergic effects of GlcN administration in mice with allergic asthma, and especially rhinitis.

After administration of GlcN, serum IgE decreased significantly compared with allergic mice. Because IgE is an important antibody mediating the allergic reaction, the antiallergic effect of GlcN can be partially explained by IgE reduction. In addition, when analyzing BAL fluid cytokines we found that Th2 cytokine titers were significantly decreased compared with group B. This is consistent with previous findings. Shibata et al reported that *N*-acetyl-D-glucosamine polymer downregulated Th2 response.¹⁵ Kim et al also suggested that their cyclosporine/GlcN

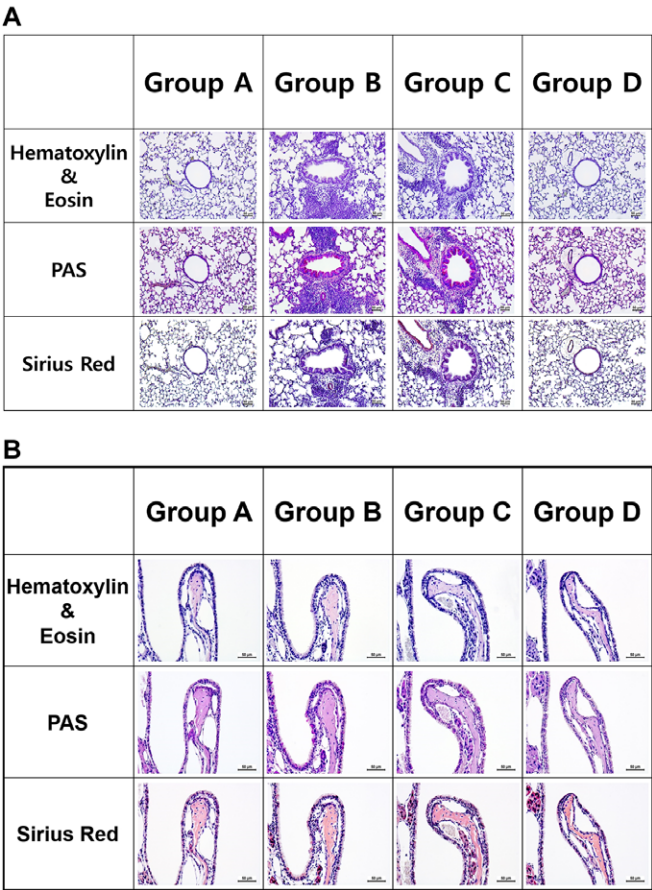


FIGURE 5. Histopathologic findings of (A) lung parenchyma and (B) lamina papyracea of nasal turbinate. Group A: nonallergic group; group B: allergic group; group C: 1% GlcN treatment group; group D: 5% GlcN treatment group (n = 8 mice/group). GlcN = glucosamine.

combination treatment group had a significant decrease in serum IgE and Th2 cytokines, including IL-4, IL-5, and IL-13, compared with their cyclosporine monotherapy group.⁷ Unexpectedly, we found that concentration of IL-17, a cytokine involved in the Th17 response, also decreased significantly after GlcN administration. Therefore, additional studies are needed to clarify the association between GlcN and the Th17 pathway in a murine allergic model.

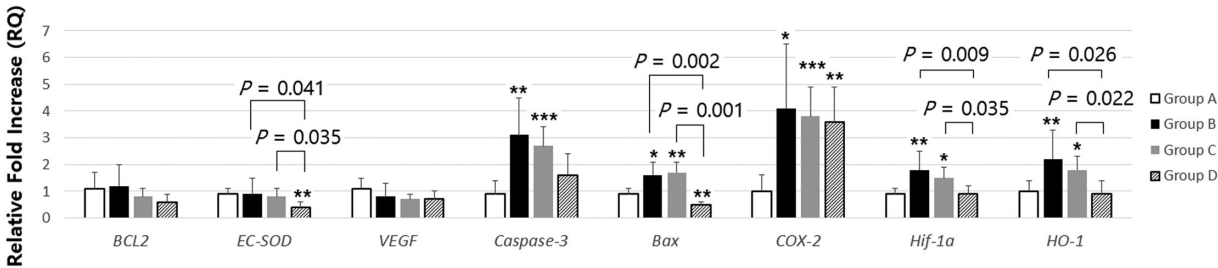


FIGURE 7. Effects of GlcN treatment on expression of genes evaluated by real-time PCR. Group A: non-allergic group; group B: allergic group; group C: 1% GlcN treatment group; group D: 5% GlcN treatment group (n = 8 mice/group). **p* < 0.05 vs group A; ***p* < 0.01 vs Group A, ****p* < 0.001 vs group A (Kruskal-Wallis test and Mann-Whitney *U* test). GlcN = glucosamine; PCR = polymerase chain reaction.

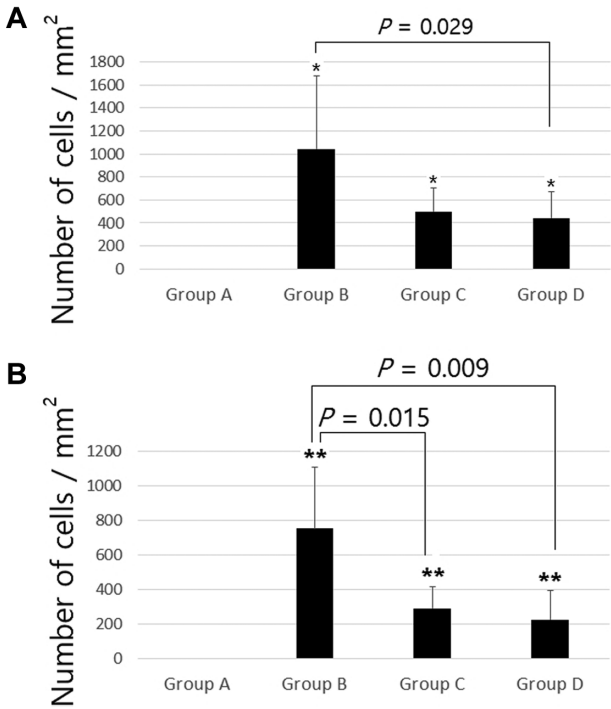


FIGURE 6. Number of eosinophils infiltrated into 1 mm² of (A) lung parenchyma and (B) nasal turbinate. Group A: nonallergic group; group B: allergic group; group C: 1% GlcN treatment group; group D: 5% GlcN treatment group (n = 8 mice/group). **p* < 0.05 vs group A; ***p* < 0.01 vs group A (Kruskal-Wallis test and Mann-Whitney *U* test).

When we evaluated number of inflammatory cells in BAL fluid, we found that the total number in the GlcN treatment group was significantly reduced compared with the allergic group. Of special note, the number of eosinophils was significantly decreased in BAL fluid. To confirm this histologically, we evaluated infiltration of inflammatory cells in lung tissue and the nasal cavity. We found that infiltration of inflammatory cells (eosinophils in particular) in the lung parenchyma and lamina papyracea of the nasal cavity was significantly decreased in the GlcN treatment group. To the best of our knowledge, this is the first study to identify the antiallergic effect of GlcN histologically in lung and nasal tissue.

To identify the GlcN antiallergic mechanism, we performed real-time PCR. The GlcN treatment group showed a significant decrease in *Bax* (pro-apoptosis gene) compared with the allergic group. On the other hand, there was no significant change in the gene expression of *Bcl-2* (anti-apoptosis gene). Hao et al also reported that *Bax* was significantly inhibited, but there was no change in *Bcl-2* expression after chitosan oligosaccharide treatment in a glutamate-induced cell death model in a PC12 cell line.¹⁶ Therefore, it is possible that cells related to the allergic response undergo apoptosis more frequently due to upregulation of pro-apoptotic gene.

During allergic responses, the *ec-sod* gene controls the maturation of dendritic cells, regulates the activation/proliferation of T lymphocytes, and regulates the Th2 and Th17 pathways. Thus, activation of the *ec-sod* gene appears to weaken the allergic response.¹⁷ In our study, *ec-sod* expression was significantly upregulated in the allergic group and suppressed in the GlcN treatment group. These results suggest that, as allergic reactions and oxidative stress decrease after GlcN treatment, *ec-sod* expression is also suppressed. In fact, expression of *superoxide dismutase 2* was significantly suppressed in human articular chondrocytes after treatment with chondroitin and/or glucosamine sulfate.¹⁸

HO-1 exhibits protective effects against allergic airway inflammation and hyperresponsiveness by inhibiting degranulation of mast cells and synthesis of Th2 cytokines.¹⁹ In our study, the expression of *HO-1* was significantly upregulated in allergic mice and was suppressed significantly after GlcN treatment. Yu et al reported that OVA-sensitized animals demonstrated upregulation of *HO-1*, which was significantly suppressed after dexamethasone treatment.²⁰ Elhini et al also reported that *HO-1* was significantly upregulated in patients with allergic rhinitis.²¹ Therefore, we postulate that *HO-1* is another potential target for treatment of allergic disorders.^{19,22,23} In this study, suppression of *HO-1* may have contributed to the antiallergic effect of GlcN.

Under conditions of hypoxia, HIF upregulates expression of several target genes, including pro-inflammatory cytokines and chemokines, adhesion molecules, and VEGF. Expression of *HIF-1 α* is upregulated in mice with allergic airway inflammation.²⁴ Inhibitors of *HIF-1 α* (eg, 2-methoxyestradiol and HIF-1 siRNA) could ameliorate allergic airway responses, infiltration of inflammatory cells, airway hyperresponsiveness, and Th2 cytokines.^{25,26} In our study, *HIF-1 α* expression was also significantly suppressed after GlcN treatment. These results are in good agreement with previous studies. Jo et al reported that the expression of *HIF-1 α* was inhibited when human tongue cancer cells were treated with glucosamine for a short period of time. Jung et al also reported that *HIF-1 α* was downregulated by glucosamine treatment in human oral cancer cells. Park et al reported similar results in prostate cancer cells.^{27–29} Therefore, HIF-1 α suppression is a plausible GlcN antiallergic mechanism.

Despite the excellent efficacy of GlcN, careful consideration is needed for commercial drug applications. In patients with chronic liver disease, GlcN is often taken for health promotion purposes. However, aspartate transaminase/alanine transaminase (AST/ALT) levels increased in 2 of 15 patients treated with GlcN. Thus, Cerda et al suggested that GlcN should be more carefully administered in patients with chronic liver disease.³⁰ On the other hand, Chung et al found no significant difference in liver enzyme levels between experimental animals treated with GlcN and nontreatment animals.³¹ Other studies have reported that gastrointestinal side effects are similar to those of placebo, and very rare cases of asthma exacerbation or immediate hypersensitivity have been reported.^{32,33} Thus, overall, GlcN is regarded as a safe drug.

In conclusion, GlcN had a significant antiallergic effect in mice with allergic asthma and rhinitis through inhibition of Th2 and Th17 cytokines, less infiltration of eosinophils, and suppression of pro-inflammatory genes, including *Bax*, *Hif-1 α* , and *HO-1*.

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