Effects of 4-DAMP on allergic rhinitis in guinea pigs and its potential mechanism

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Background: Allergic rhinitis (AR) is a non-infectious chronic inflammatory disease of the nasal mucosa mainly mediated by immunoglobulin E (IgE), which seriously affects the life quality of affected patients. This study aimed to observe the effects of 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP; a selective M3 receptor antagonist) on ovalbumin (OVA)-induced AR guinea pigs, and to explore its potential mechanism of involvement.

Methods: An AR model was established by inducing male guinea pigs (4–6 weeks of age) with OVA. AR guinea pigs were randomly divided into a model group, 0.6 mg/kg ipratropium bromide (IB) group, 0.12 and 0.6 mg/kg 4-DAMP group (n=18). The 0.6 mg/kg IB group, 0.12 and 0.6 mg/kg 4-DAMP group animals were treated with IB (0.6 mg/kg) and 4-DAMP (0.12 or 0.6 mg/kg) by intranasal instillation per nostril daily. Animals in the model group and normal group were treated with saline as control. The AR symptom scores were counted and nasal secretion weights were measured. Histopathological methods were used to observe nasal mucosa. Enzyme-linked immunosorbent assay (ELISA) was used to detect the levels of histamine and cytokines. Western blot and quantitative real-time polymerase chain reaction (qRT-PCR) were used to detect the expressions of mucin 5AC (MUC5AC), matrix metalloproteinase 9 (MMP9), and epidermal growth factor receptor (EGFR).

Results: Compared with model group animals, the AR symptom scores and nasal secretion weights of animals treated with 4-DAMP were reduced significantly, goblet cell metaplasia was reversed, and eosinophil infiltration was visibly alleviated. The levels of histamine and cytokines in nasal lavage fluid (NLF) were decreased, and the protein and messenger RNA (mRNA) expressions of MUC5AC, MMP9, and EGFR were inhibited.

Conclusions: Treatment with 4-DAMP has a certain effect on AR, especially for mucus hypersecretion, which provides a new idea for clinical treatment of AR.

Keywords: 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP); allergic rhinitis (AR); M3 receptor; hypersecretion; chronic inflammatory

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Introduction

Allergic rhinitis (AR) is a non-infectious chronic inflammatory disease of the nasal mucosa that is mainly mediated by immunoglobulin E (IgE) after exposure to allergens (1). The predominant symptoms of AR include nasal itching and sneezing, rhinorrhea, and nasal obstruction, and some patients may also experience allergic conjunctivitis and asthma (2). About 40% of the global population are affected by AR, and the incidence is increasing year by year (3). Although it is not a life-threatening illness, the professional and personal lives of patients are impacted by AR, which increases economic burden and reduces quality of life (4). Therefore, it is critical to seek an effective treatment for AR.

At present, antihistamines and topical glucocorticoids are the most commonly used drugs to treat AR (5). Although most AR patients can be treated with these drugs, they remain unsuitable for some patients (6). For these patients, muscarinic (M) receptor antagonists can be used as an alternative treatment. The non-selective M receptor antagonist ipratropium bromide (IB) nasal spray has been marketed as a drug to treat AR (7). Studies have found that the M1/M3 receptor antagonist bencycloquidium bromide can effectively treat AR (8,9), but there are no relevant reports on the treatment of AR with highly selective M receptor antagonists.

There are 3 main types of M receptors in the nasal mucosa: M1, M2, and M3, among which, M3 receptors are the most widely distributed (10). The M3 receptors are distributed on the cell membranes of epithelial, glandular, and arteriovenous vascular endothelial cells. The M1 receptors are also distributed on the above-mentioned cell membranes, but the numbers are far less than those of the M3 receptors (11,12). The dilation of nasal vessels was mainly mediated via M1 and M3 receptor, and M2 receptor is an autoreceptor located in the presynaptic membrane and plays a negative feedback role in the regulation of acetylcholine release. The mucus secretory response to cholinergic stimulation is largely via muscarinic M3 receptors, with water secretion mediated via M1 receptors. In addition, It is reported that M3 receptors are related to chronic inflammation and immune response (10). Therefore, we suspected that M receptor antagonists may treat AR by blocking M3 receptors.

As a kind of M receptor antagonist, 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP) has a high affinity for M3 receptors, about 17 times that for M2 receptors and about 10 times that for M1 receptors, which is called a highly selective M3 receptor antagonist (13). It has been reported that 4-DAMP inhibits lipopolysaccharide (LPS)- and tobacco-induced pulmonary inflammation and reduces mucin 5AC (MUC5AC), oligomeric mucus/gel-forming secretion (13,14). However, its effect on AR remains uncertain. In our study, we treated ovalbumin (OVA)-induced AR guinea pigs with 4-DAMP, observed its effects, and explored the potential mechanism. We present the following article in accordance with the ARRIVE reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-2998/rc).

Methods

Animals

We purchased 100 Hartley guinea pigs (male, 4–6 weeks of age, 250±50 g) from the Laboratory Animal Center of Chongqing Medical University (Chongqing, China). They were kept in a room with a temperature of 21–25 ℃, humidity of 45–65%, noise level below 60 dB, and a 12-hour light/dark cycle. All the animals were provided with food and tap water ad libitum. All protocols in this study were approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University under a project license (No. 20200301), in compliance with the National Research Council Guide for the Care and Use of Laboratory Animals. A protocol was prepared before the study without registration.

Establishment of AR model in guinea pigs

The AR model in guinea pigs was established as described by Chen *et al.* (15) and Long *et al.* (8). As shown in *Figure 1*, after acclimation for 3 days, guinea pigs were sensitized with 1 mL saline suspended with 0.3 mg OVA (as antigen, Sigma-Aldrich, St. Louis, MO, USA) and 30 mg $Al(OH)_3$ (as adjuvant, Sigma, USA) by intraperitoneal injection for every other day (days 1–14). Then, guinea pigs were challenged with 20 μL 5% OVA saline by intranasal instillation per nostril daily (days 15–21). Normal control animals were repeatedly sensitized and challenged with normal saline.

Passive cutaneous anaphylaxis test

As described by Hu *et al.* (16), 1 week after the last sensitization (day 21), animals were shaved on the dorsal

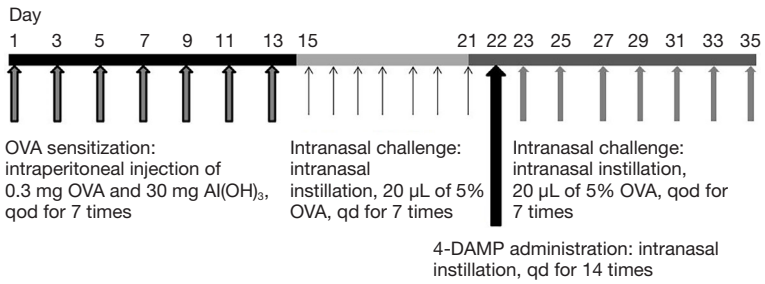

Figure 1 Experimental protocol of modeling and administration. OVA, ovalbumin; 4-DAMP, 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide; qd, quaque die; qod, quaque omni die.

Table 1 Symptom scores of allergic rhinitis

Scores	Number of nose scratches (16)	Number of sneezes	Symptoms of rhinorrhea
0	0	0	No rhinorrhea
1	1–3	1–3	Presence of mucous in the nostrils
2	Between 1–3 points	4–10	Presence of mucous outside the nostrils
3	Continually	>10	Presence of mucous on the face

skin area with an electric razor and intracutaneously injected with 25 μL OVA saline (200 μg/mL). At 20–40 minutes after injection, successful sensitization was confirmed by the presence of ≥1.0 cm diameter of redness and edema at the injection site.

Symptom scores of AR

The predominant symptoms of AR include nasal itching, sneezing, and rhinorrhea. According to the methods described by Hu *et al.* (16), after intranasal challenge with 5% OVA saline, the number of nose scratches and sneezes within 30 minutes were counted, and the severity of rhinorrhea was observed in guinea pigs. Then, scores were obtained according to *Table 1*. The cumulative scores were added together. An excess of 5 points indicated the successful establishment of an animal AR model. The observer was blinded to the experiments to avoid bias. Symptoms scores of AR were calculated on days 21 and 35, respectively, to screen out AR model animals and evaluate the effect of drugs.

Groups and administration

A total of 72 successfully modelled AR guinea pigs were selected and randomly divided into a model group, 0.6 mg/kg IB group, 0.12 mg/kg 4-DAMP group, and 0.6 mg/kg 4-DAMP group (n=18 for each group). Then, on days 22–35, the 0.12 mg/kg 4-DAMP group and 0.6 mg/kg 4-DAMP group animals were treated with 4-DAMP (0.12 or 0.6 mg/kg; Chongqing Maiyun Technology and Trade Co. Ltd., Chongqing, China) dissolved in 20 μL dimethyl sulfoxide (DMSO) by intranasal instillation per nostril daily. The 0.6 mg/kg IB group animals were treated with IB (0.6 mg/kg, Sigma, USA) dissolved in 20 μL saline. Animals in the model group and normal group were treated with an equal volume of saline as control. During this stage, after 30 minutes of administration, 20 μL 5% OVA saline was dripped into the nostril as a nasal challenge every other day (*Figure 1*).

Measurement of nasal secretion weight

After being challenged with 5% OVA saline, the guinea pigs were anesthetized with chloral hydrate (10%, 0.04 mL/100 g) by intraperitoneal injection. After anesthesia, a filter paper strip (2 mm × 60 mm) was inserted into one nostril of the guinea pig. After 30 minutes, the filter paper was removed and weighed. The difference between the front and back of the filter paper strip was recorded as the weight of the nasal secretions within 30 minutes after challenge. The weight of guinea pig nasal secretions was measured before

administration (day 21) and after administration (day 35), respectively, to evaluate the effect of 4-DAMP on the weight of nasal secretions.

Histopathology

After the last administration and challenge (day 35), the guinea pigs were euthanized by overdose of anesthetics. Nasal tissues were obtained and fixed in 4% paraformaldehyde immediately for 48 hours. The fixed nasal tissues were decalcified in 10% ethylene diamine tetraacetic acid (EDTA) for 8 weeks. Then, tissues were dehydrated in ethanol, embedded in paraffin, and sliced (4 μm). The sections were stained with hematoxylin and eosin (HE), Alcian Blue periodic acid-Schiff (AB-PAS) and immunohistochemistry [matrix metalloproteinase 9 (MMP9) and epidermal growth factor receptor (EGFR)]. Then, sections were observed under a microscope. The numbers of eosinophils were counted. The 2 observers were blinded to the experiment.

Measurement of histamine, interleukin (IL)-4, IL-13, and tumor necrosis factor-α (TNF-α) in nasal lavage fluid (NLF) by enzyme-linked immunosorbent assay (ELISA)

At 6 hours after the last administration and challenge (day 35), the guinea pigs were anesthetized with chloral hydrate (10%, 0.04 mL/100 g). The guinea pigs were turned upside down after anesthesia. Then, the nasopharynx was intubated with a catheter, and flushed with 2 mL phosphate-buffered saline (PBS) within 5 minutes. The collected NLF was centrifuged at 3,000 r/min for 15 minutes at 4 ℃, and the supernatant was removed. The concentrations of histamine, IL-4, IL-13, and TNF-α in the NLF were detected by the ELISA kit, and the assay was performed in accordance with the manufacturers' instructions. The tests were performed in duplicate for each sample.

Collection of nasal mucosa

At 24 hours after the last challenge, guinea pigs were euthanized by intraperitoneal injection of excess anesthetics. The nasal mucosa was separated, washed in PBS, then the surface liquid was removed, and stored in the refrigerator at –80 ℃.

Western-blot analysis

Nasal mucosa samples were grinded into a powder at a low temperature. Radio immunoprecipitation assay (RIPA) tissue lysis buffer was added to lyse tissues and the total protein was extracted. The Coomassie brilliant blue method was used to measure the concentration of total protein. An equal amount of protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis, and then transferred onto polyvinylidene difluoride (PVDF) membranes. After blocking with 5% skimmed milk in tris-buffered saline with Tween 20 (TBST), the membranes were incubated with anti-MUC5AC antibody (1:500, Abcam, Cambridge, MA, USA), anti-MMP9 antibody (1:1,000, Abcam, USA), and anti-EGFR antibody (1:1,000, Abcam, USA) at 4 ℃ overnight. After washing 3 times in TBST, the membranes were incubated with horseradish-peroxidase (HRP)-conjugated secondary antibody for 1 hour, and processed with and enhanced chemiluminescence (ECL) reaction kit. We used β-actin was used as the internal control. The band density of the target protein relative to β-actin was quantified by Image J software (National Institutes of Health, Bethesda, MD, USA).

Real-time reverse transcriptase polymerase chain reaction analysis

Nasal mucosa tissues were ground into a powder at low temperature, and then TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was added to isolate total RNA. The RNA extracts were reverse transcribed to complementary DNA (cDNA) using PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Shiga, Japan). Real-time polymerase chain reaction (RT-PCR) was performed using the TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) kit (TaKaRa, Japan) according to the manufacturer's instructions. We used β-actin as an endogenous control. The primers were synthesized by Genewiz Biotech (Suzhou, Jiangsu, China) and the sequences were as *Table 2*. Relative quantification of the different transcripts was determined with the $2^{-\Delta\Delta Ct}$ method using β-actin as an endogenous control. Data were expressed as fold-increase in RNA expression compared with control animals, which were set at a value of one.

Statistical analysis

Data were presented as mean ± standard deviation (SD), analyzed by SPSS 20.0 software (IBM Corp., Armonk, NY, USA), and graphed by GraphPad Prism 8.0 software

Table 2 All sequences of primers

Target gene	Forward	Reverse
MUC5AC	5'-GGCTTCGTGCCAGTGTCCAT-3'	5'-ACTGGCTTGGGCAACAGTCC-3'
EGFR	5'-GTGCGCTTCAGCAACAACCC-3'	5'-ACACTTTTGGCAGCCGCTCT-3'
MMP9	5'-GTCCAAGCCGTCT-3'	5'-TCGCCTTCGGCCCTCAGAAA-3'
β-actin	5'-CGGTGCTGTCCCTCTATGCG-3'	5'-AGACGCATGATGGCATGGGG-3'

MUC5AC, mucin 5AC; EGFR, epidermal growth factor receptor; MMP9, matrix metalloproteinase 9.

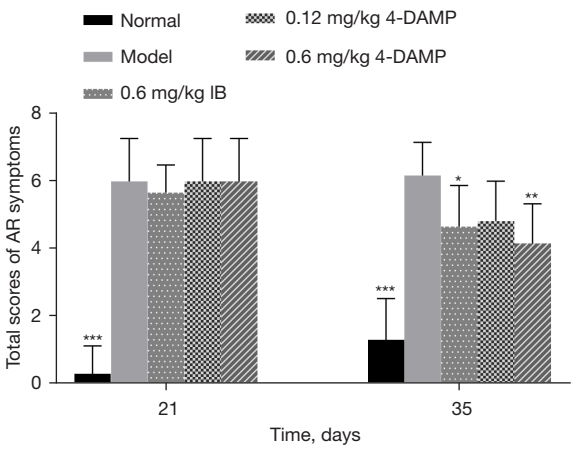

Figure 2 Effects of 4-DAMP on the total symptom scores of AR in guinea pigs. *, P<0.05; **, P<0.01; ***, P<0.001, *vs.* model group (n=6). AR, allergic rhinitis; 4-DAMP, 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide; IB, ipratropium bromide.

(GraphPad Software Inc., La Jolla, CA, USA). Statistical analysis was performed by the one-way analysis of variance (ANOVA) followed by Bonferroni and Games-Howell post-hoc tests. A P value <0.05 was considered statistically significant.

Results

Effects of 4-DAMP on the total symptom scores of AR

Before administration (day 21), the total AR symptom scores of model group animals were significantly increased compared with that of the normal group animals (P<0.001). No significance was detected among scores of the groups except the normal group, and the scores of all of them were all over 5 points, indicating a successful establishment of AR model guinea pigs. When finished administration (day 35), the total AR symptom scores of animals were significantly decreased in the 0.6 mg/kg IB group (P=0.033) and 0.6 mg/kg 4-DAMP group (P=0.006) compared with the model group. However, there was no significance between the scores of 0.12 mg/kg 4-DAMP group and model group animals (*Figure 2*). These results suggested that high-dose 4-DAMP could significantly reduce the total symptom scores of AR guinea pigs.

Effects of 4-DAMP on the weights of nasal secretions

Before administration (day 21), the nasal secretions in the model group animals were heavier than those of normal group animals (P<0.001), while there was no significance among weights of secretions in groups except the normal group. When administration was finished (day 35), compared with the model group, the weights of secretions were significantly decreased in the 0.6 mg/kg IB group, 0.12 mg/kg 4-DAMP group, and 0.6 mg/kg 4-DAMP group (all P<0.001) (*Figure 3*). These results suggested that 4-DAMP could reduce the weights of nasal secretions in AR guinea pigs.

Effects of 4-DAMP on the histopathology of nasal mucosa

According to the results of HE staining, the nasal mucosal epithelium of the normal group guinea pigs was intact without obvious pathological changes, which were pseudostratified ciliated columnar epithelial cells. In the model group animals, there were visible goblet cell metaplasia and eosinophil infiltration (*Figure 4*), and the number of eosinophils was larger than normal group (P<0.001) (*Figure 5A*). Compared with the model group, goblet cell metaplasia of the animal nasal mucosal epithelium was reversed partly in the 0.12 mg/kg 4-DAMP group, and visibly in the 0.6 mg/kg IB group and 0.6 mg/kg 4-DAMP group (*Figure 4*). Meanwhile, the eosinophils were also significantly reduced in the 0.12 mg/kg 4-DAMP group (P=0.001), 0.6 mg/kg IB group (P<0.001), and the 0.6 mg/kg 4-DAMP group (P<0.001) (*Figure 5A*).

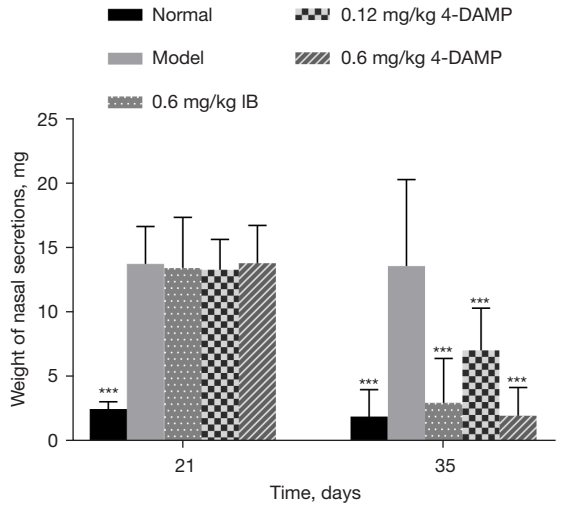

Figure 3 Effects of 4-DAMP on the weights of nasal secretions in AR guinea pigs. ***, P<0.001, *vs.* model group (n=6). AR, allergic rhinitis; 4-DAMP, 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide; IB, ipratropium bromide.

According to the results of AB-PAS staining, there were more goblet cells (blue-purple) in the nasal mucosal epithelium in model group guinea pigs than in normal group animals (P<0.001). The numbers were significantly decreased in the 0.6 mg/kg IB group (P=0.001), 0.12 mg/kg 4-DAMP group (P=0.001), and 0.6 mg/kg 4-DAMP group (P=0.001) compared with in the model group (*Figures 4,5B*).

The results of HE staining and AB-PAS staining of nasal mucosa suggested that 4-DAMP could reverse goblet cell metaplasia and alleviate eosinophilic infiltration of the nasal mucosa in AR guinea pigs.

Effects of 4-DAMP on MUC5AC in the nasal mucosa

Western-blot analysis was used to test the expression of MUC5AC protein of guinea pig nasal mucosa. The MUC5AC protein level of guinea pig nasal mucosa in the model group was significantly higher than that in the normal group (P<0.001). Compared with the model group, the expressions of MUC5AC protein were inhibited in the 0.6 mg/kg IB group (P=0.005), 0.12 mg/kg 4-DAMP group (P=0.001), and 0.6 mg/kg 4-DAMP group (P<0.001) (*Figure 6A*).

In addition, the results of RT-PCR, which were used to measure the levels of MUC5AC messenger RNA (mRNA) in the guinea pig nasal mucosa, were consistent with the above. The MUC5AC mRNA level of guinea pig nasal mucosa in the model group was significantly higher than that in the normal group (P<0.001). Compared with the model group, the levels of MUC5AC mRNA in guinea pig nasal mucosa were inhibited in 0.6 mg/kg IB group (P<0.001), 0.12 mg/kg 4-DAMP group (P<0.001), and 0.6 mg/kg 4-DAMP group (P<0.001) (*Figure 6B*).

These results indicated that 4-DAMP could significantly inhibit the levels of MUC5AC protein and mRNA in the AR guinea pig nasal mucosa.

Effects of 4-DAMP on the levels of histamine and cytokines IL-4, IL-13, and TNF-α in NLF

The levels of histamine and cytokines IL-4, IL-13, and TNF-α in guinea pig NLF were tested by ELISA. The level of histamine in the model group was significantly higher than that in the normal group (P<0.001). Compared with the model group, the levels of histamine in the NLF were reduced significantly in the 0.6 mg/kg IB group, 0.12 mg/kg 4-DAMP group, and 0.6 mg/kg 4-DAMP group (all P<0.001) (*Figure 7A*).

Similarly, the levels of cytokines IL-4, IL-13, and TNF-α in the in the model group NLF were significantly higher than those in the normal group (all P<0.001). The levels of IL-4, IL-13, and TNF-α in the 0.6 mg/kg IB group, 0.12 mg/kg 4-DAMP group, and 0.6 mg/kg 4-DAMP group were significantly reduced compared with those in the model group (all P<0.001) (*Figure 7B-7D*).

These results indicated that 4-DAMP could significantly reduce the levels of histamine and cytokines IL-4, IL-13, and TNF-α in AR guinea pig NLF.

Effects of 4-DAMP on MMP9 in the nasal mucosa

Western-blot analysis was used to test the expression of MMP9 protein in guinea pig nasal mucosa. The MMP9 protein level in nasal mucosa of the model group was significantly higher than that in the normal group (P<0.001). Compared with the model group, the expressions of MMP9 protein were inhibited in the 0.12 mg/kg 4-DAMP group (P=0.009) and 0.6 mg/kg 4-DAMP group (P=0.001). However, there was no significant difference between the 0.6 mg/kg IB group and model group (*Figure 8A*).

Similarly, the results of RT-PCR, which was used to measure the levels of MMP9 mRNA in the guinea pig nasal mucosa, were consistent with the above. The MMP9 mRNA level of guinea pig nasal mucosa in the model group was significantly higher than that in the normal group

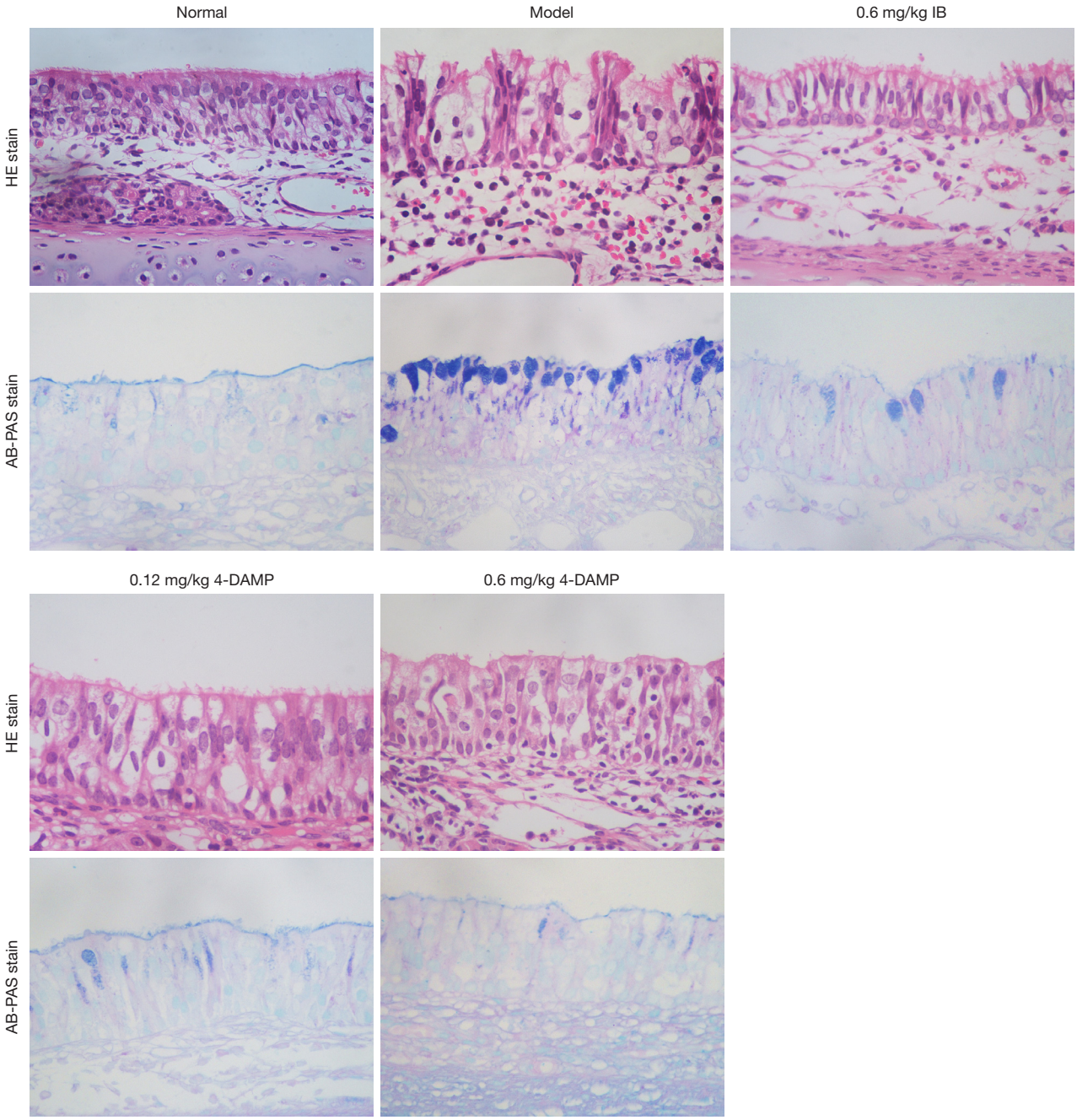

Figure 4 Effects of 4-DAMP on the histopathology of nasal mucosa in AR guinea pigs (original magnification ×400). AR, allergic rhinitis; HE, hematoxylin and eosin; AB-PAS, Alcian blue periodic acid-Schiff; 4-DAMP, 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide; IB, ipratropium bromide.

(P=0.005). Compared with the model group, the levels of MMP9 mRNA in guinea pig nasal mucosa were inhibited in the 0.12 mg/kg 4-DAMP group (P=0.025) and 0.6 mg/kg 4-DAMP group (P=0.006). However, there was no significant difference between the 0.6 mg/kg IB group and model group (*Figure 8B*).

These results indicated that 4-DAMP could significantly inhibit the levels of MMP9 protein and mRNA in the AR

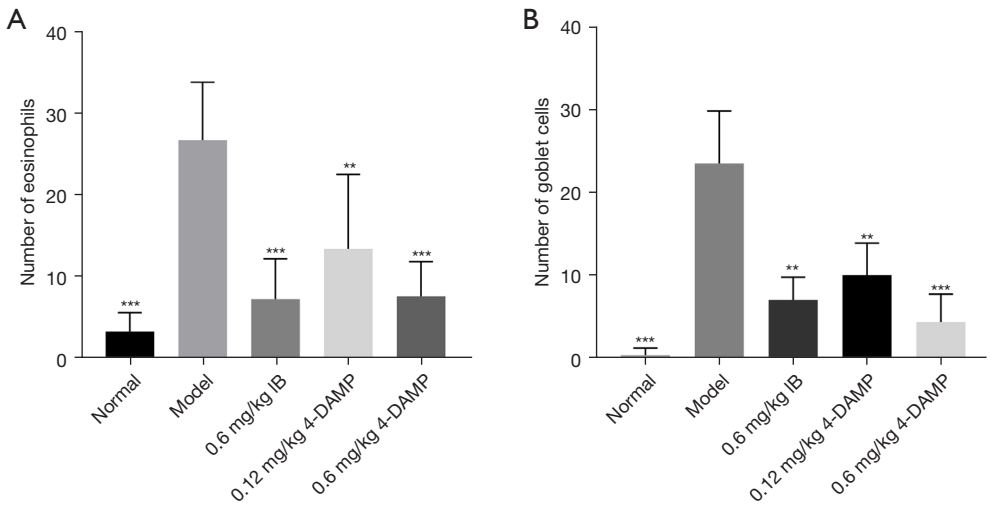

Figure 5 Effects of 4-DAMP on the numbers of eosinophils and goblet cells in the nasal mucosa of AR guinea pigs. **, P<0.01; ***, P<0.001, *vs.* model group (n=6). AR, allergic rhinitis; 4-DAMP, 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide; IB, ipratropium bromide.

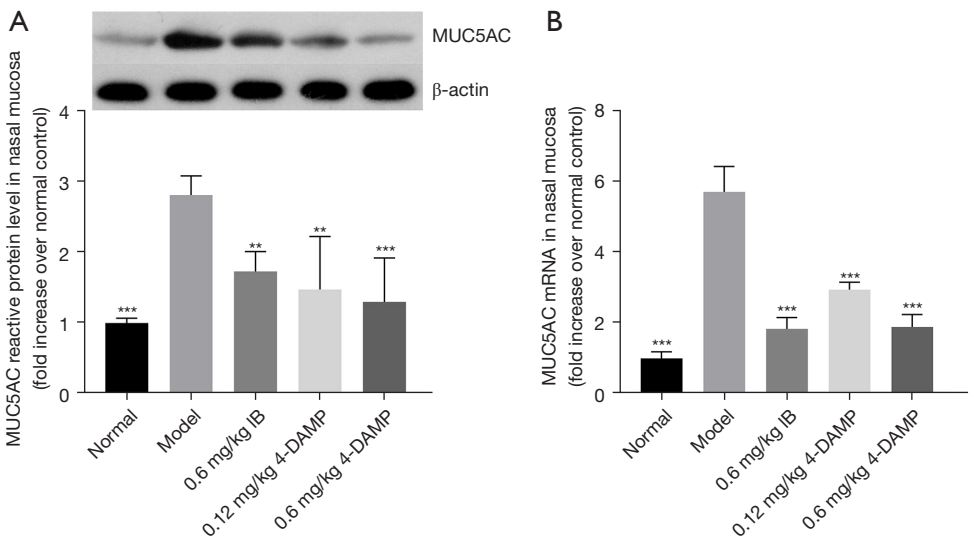

Figure 6 Effects of 4-DAMP on MUC5AC in the nasal mucosa. (A) MUC5AC protein level in the nasal mucosa was measured by Western-blot analysis (n=3). (B) MUC5AC mRNA levels in the nasal mucosa were measured by RT-PCR (n=4). **, P<0.01; ***, P<0.001, *vs.* model group. mRNA, messenger RNA; RT-PCR, real-time polymerase chain reaction; MUC5AC, mucin 5AC; 4-DAMP, 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide; IB, ipratropium bromide.

guinea pig nasal mucosa. This was also confirmed by the results of immunohistochemical examination. Compared with the normal group, more MMP9-positive cells (brown in the cytoplasm) were observed in the guinea pig nasal mucosa in the model group. The MMP9-positive cells in the 0.6 mg/kg IB group, 0.12 mg/kg 4-DAMP group, and 0.6 mg/kg 4-DAMP group were notably less than those in the model group (*Figure 9*).

Effects of 4-DAMP on EGFR in nasal mucosa

RT-PCR analysis was used to test the levels of EGFR mRNA in guinea pig nasal mucosa. The EGFR mRNA level of guinea pig nasal mucosa in the model group

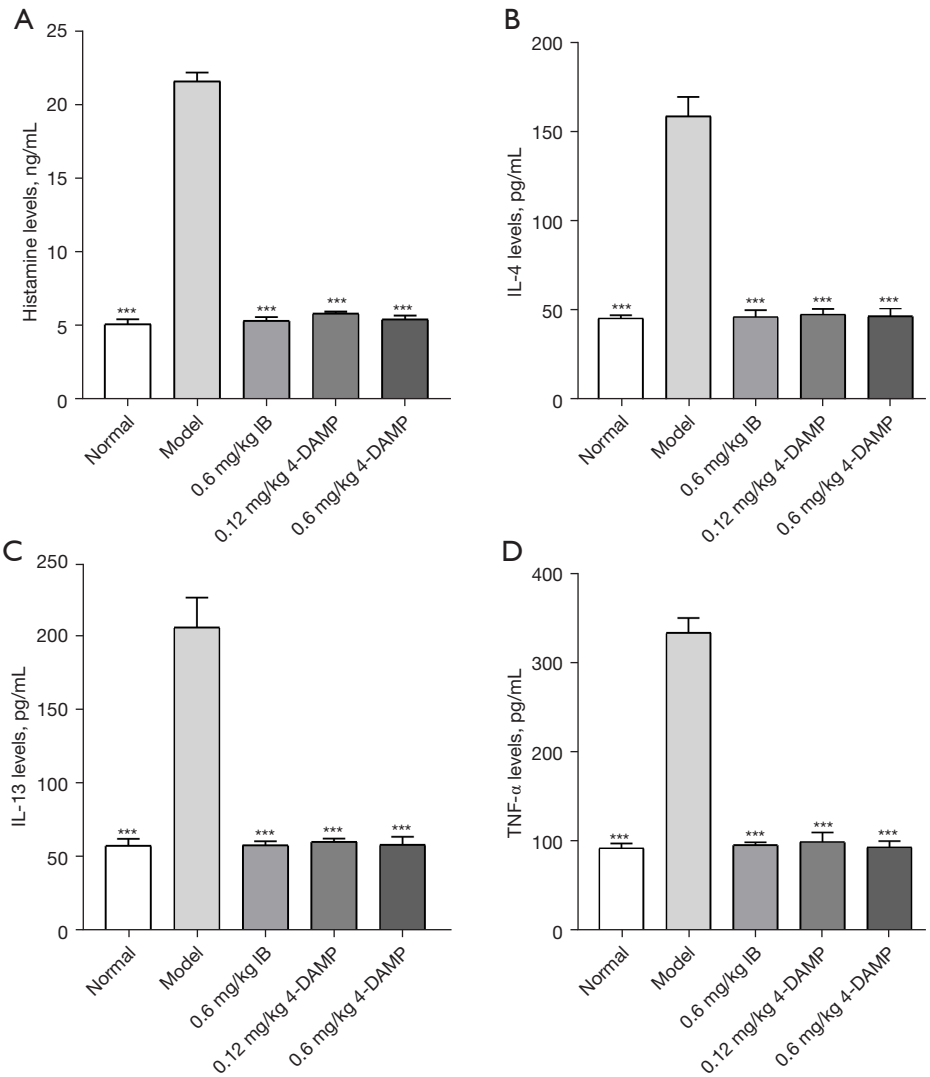

Figure 7 Effects of 4-DAMP on the levels of histamine and cytokines IL-4, IL-13, and TNF-α in guinea pig NLF. The levels of histamine (A) and cytokines IL-4 (B), IL-13 (C), and TNF-α (D) were measured by ELISA (n=6). ***, P<0.001, *vs.* model group. IL-4, interleukin 4; IL-13, interleukin 13; TNF-α, tumor necrosis factor-α; ELISA, enzyme-linked immunosorbent assay; 4-DAMP, 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide; IB, ipratropium bromide.

was significantly higher than that in the normal group (P=0.001). Compared with the model group, the levels of EGFR mRNA in guinea pig nasal mucosa were inhibited in the 0.6 mg/kg IB group (P=0.003), 0.12 mg/kg 4-DAMP group (P=0.004), and 0.6 mg/kg 4-DAMP group (P=0.001) (*Figure 10A*).

Similarly, Western blot was used to measure the levels of EGFR protein in the guinea pig nasal mucosa. The EGFR protein level of guinea pig nasal mucosa in the model group was significantly higher than that in the normal group (P<0.001). Compared with the model group, the expressions of EGFR protein were inhibited in the 0.12 mg/kg 4-DAMP group (P=0.033) and 0.6 mg/kg 4-DAMP group (P=0.002). However, there was no significant difference between the 0.6 mg/kg IB group and model group (*Figure 10B*).

These results indicated that 4-DAMP could significantly inhibit the levels of EGFR protein and mRNA in the AR guinea pig nasal mucosae. The results of immunohistochemical examination confirmed these findings. Compared with the normal group, more EGFR-

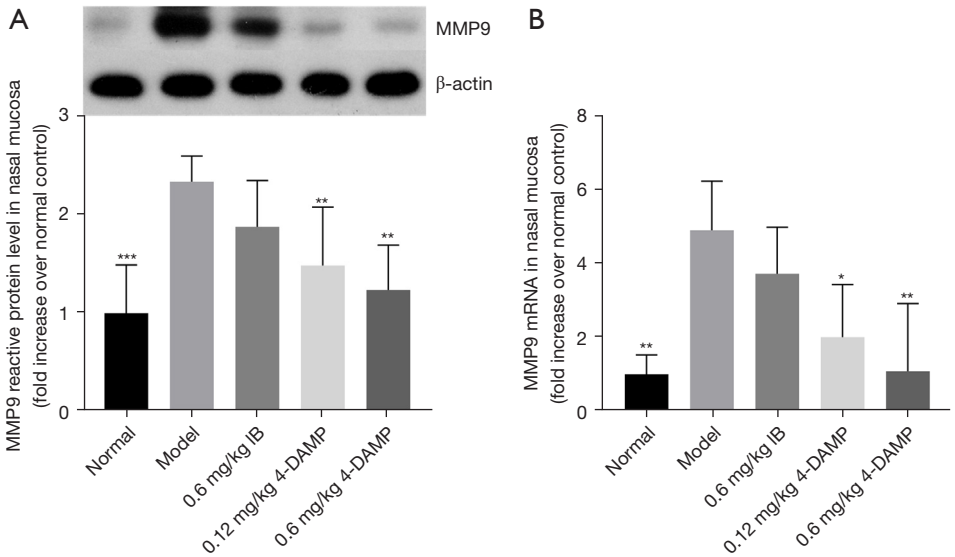

Figure 8 Effects of 4-DAMP on MMP9 in the nasal mucosa. (A) MMP9 protein level in the nasal mucosa was measured by Western-blot analysis (n=3). (B) MMP9 mRNA level in the nasal mucosa was measured by RT-PCR (n=5). *, P<0.05; **, P<0.01; ***, P<0.001, *vs.* model group. mRNA, messenger RNA; RT-PCR, real-time polymerase chain reaction; 4-DAMP, 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide; IB, ipratropium bromide; MMP9, matrix metalloproteinase 9.

positive cells (brown on the cytomembrane) were observed in the nasal mucosa of the model group guinea pigs. The EGFR-positive cells in the 0.6 mg/kg IB group, 0.12 mg/kg 4-DAMP group, and 0.6 mg/kg 4-DAMP group were markedly less than those in the model group (*Figure 9*).

Discussion

In this study, a guinea pig model of AR in was successfully established via OVA induction. The AR guinea pigs were treated with 0.6 mg/kg IB, 0.12 mg/kg 4-DAMP, and 0.6 mg/kg 4-DAMP for 2 weeks. We found that compared with the model group guinea pigs (treated with saline), the AR symptom scores of the treated guinea pigs were significantly decreased. The weights of nasal secretions were also significantly reduced. Histopathological results of the nasal mucosa revealed that goblet cell metaplasia was reversed and eosinophil infiltration was visibly alleviated after drug treatment. Meanwhile, the levels of histamine and cytokines IL-4, IL-13, and TNF-α in NLF also decreased markedly. The mRNA and protein expressions of MUC5AC were significantly inhibited. These results suggest that the M3 receptor antagonist 4-DAMP could reduce mucus secretion and relieve chronic inflammation, indicating that 4-DAMP plays a therapeutic role in AR. In addition, the study also found that the mRNA and protein levels of MMP9 and EGFR were significantly increased in the model group compared with the normal group. After 2 weeks of treatment with selective M3 receptor antagonist 4-DAMP, the mRNA protein levels of MMP9 and EGFR were decreased compared with the model group. It was speculated that the selective M3 receptor antagonist treating AR may inhibit the MMP9-EGFR pathway by blocking M3 receptors. This study was the first to investigate the therapeutic effects of selective M3 receptor antagonist 4-DAMP on AR.

Mucus hypersecretion and non-infectious chronic inflammation are the main pathophysiological characteristics of AR (17). Mucus hypersecretion consists of rapid serous hypersecretion and delayed mucinous hypersecretion (18). Mucin is the main component of mucus. At present, there are 12 main recognized types of mucins in the airway, among which MUC5AC is the most important and most closely related to AR (18-20). Therefore, we selected MUC5AC as a target protein in the treatment of mucus hypersecretion. In addition, AR is a non-infectious chronic disease involving a variety of cells, cytokines, inflammatory mediators, adhesion molecules, and so on, in which histamine and the cytokines IL-4, IL-13, and TNF-α play significant roles (3,8,15). Hence, we selected histamine

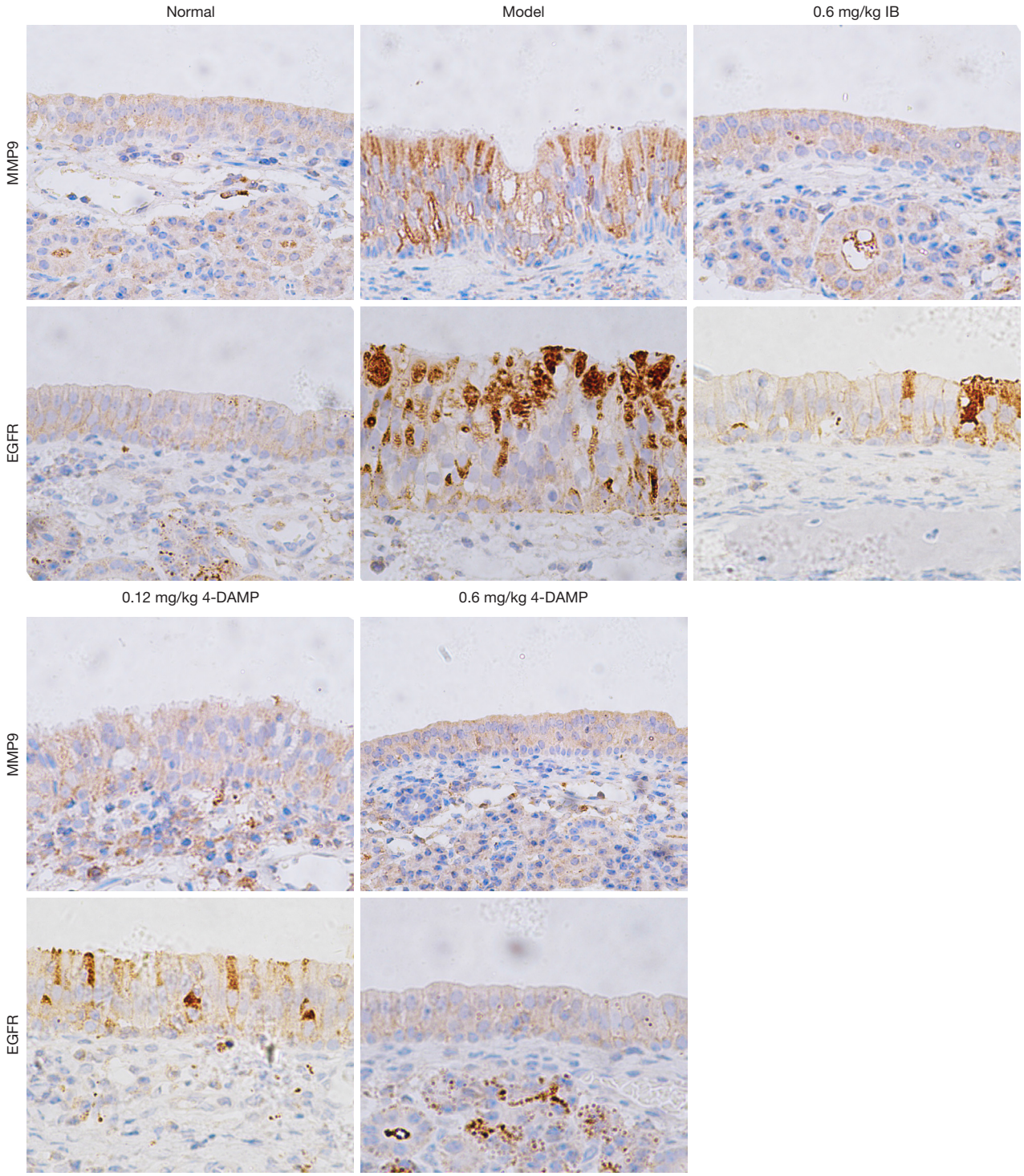

Figure 9 Immunohistochemistry of MMP9 and EGFR in nasal mucosa (original magnification ×400). EGFR, epidermal growth factor receptor; MMP9, matrix metalloproteinase 9; 4-DAMP, 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide; IB, ipratropium bromide.

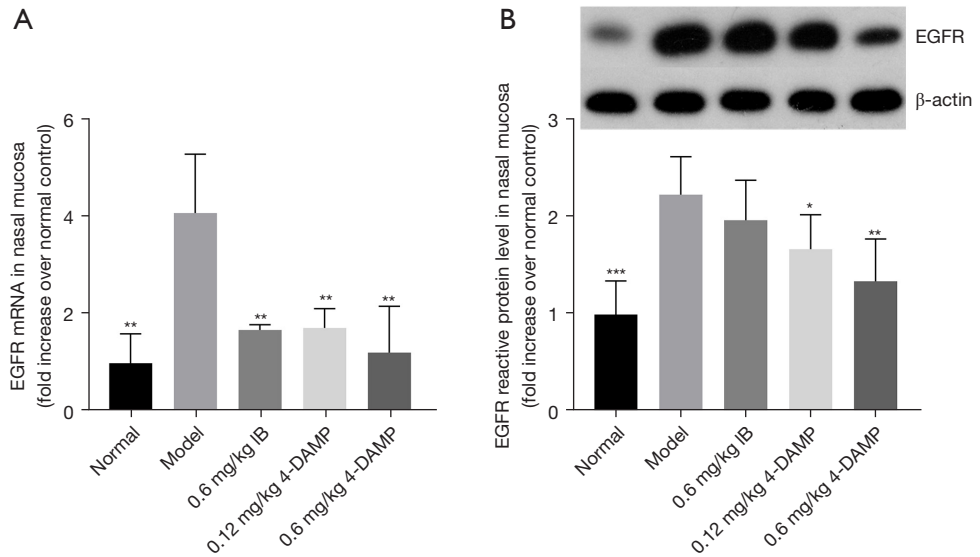

Figure 10 Effects of 4-DAMP on EGFR in the nasal mucosa. (A) EGFR mRNA level in the nasal mucosa was measured by RT-PCR (n=5). (B) EGFR protein level in the nasal mucosa was measured by Western-blot analysis (n=3). *, P<0.05; **, P<0.01; ***, P<0.001, *vs.* model group. mRNA, messenger RNA; RT-PCR, real-time polymerase chain reaction; EGFR, epidermal growth factor receptor; 4-DAMP, 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide; IB, ipratropium bromide.

and IL-4, IL-13, and TNF-α levels to assess the severity of inflammation in AR.

M receptor antagonists are effective drugs for AR, because they exert an anticholinergic effect that results in reduction of glandular secretion and contraction of blood vessels by blocking vagal cholinergic tone. At present, IB, a M receptor antagonist in clinical use for the treatment of AR, has been certified and listed by the Food and Drug Administration of the United States (21). Long *et al.* reported that the M1/M3 receptor antagonist bencycloquidium bromide could effectively treat OVA-induced AR rats, decrease nasal secretion, reduce the levels of inflammatory mediators histamine and cytokines IL-6, IL-13, and TNF-α, reverse goblet cell metaplasia, alleviate eosinophil infiltration, and inhibit mucin MUC5AC secretion (7,8). Furthermore, phase III clinical trial results of bencycloquidium bromide have confirmed its effects on AR (9). In addition, 4-DAMP was shown to effectively inhibit the IL-6 and TNF-α levels of LPS-induced lung inflammation in mice (14). At the same time, our previous study found that 4-DAMP could significantly inhibit the protein expression of MUC5AC in carbachol (choline receptor agonist)-induced human nasal mucosal epithelial cells, although pirenzepine was ineffective.

In this study, the selective M3 receptor antagonist 4-DAMP was used to treat OVA-induced AR in guinea pigs. We found that 4-DAMP had effects on AR symptoms, nasal secretion, goblet cell metaplasia, eosinophil infiltration, histamine and cytokines IL-4, IL-13, and TNF-α levels, and expression of MUC5AC. These consequences were consistent with related reports, suggesting that the selective M3 receptor antagonist plays a therapeutic role in AR.

The M3 receptor, a kind of G protein-coupled receptor, is widely distributed on the cell membrane of nasal mucosa epithelial cells, submucosal gland cells, and even nasal arteriovenous endothelial cells, closely related to chronic inflammation of the airway, mucus secretion and immune response, among others (10). As a kind of tyrosine kinase receptor, EGFR is widely distributed on the cell membrane of mammalian epithelial cells. It is mainly composed of extracellular ligand binding domain, transmembrane domain, and intracellular kinase domain. The extracellular ligand binding domain is phosphorylated and activated after binding to the ligand (such as EGF), and generally forms a dimer, causing the activation of the intracellular kinase domain, thereby causing a series of reactions in the cell. The EGFR participates in cell proliferation, differentiation, and functional expression, among other roles (22). Meanwhile, MMP9, a kind of MMPs, is closely related to airway diseases. The MMPs are secreted to the outside of cells

under the influence of stimulating factors, and activated by hydrolyzing amino terminal. They can degrade various proteins in the extracellular matrix and play an important role in the process of tissue repair and remodeling (11,23).

As a G protein-coupled receptor, M3 choline receptor can mediate the activation of EGFR and trigger downstream signal cascade reaction (24). Cortijo reported for the first time that M3 receptor up regulates MUC5AC expression by activating EGFR in human bronchial epithelial cells (25). It was reported that long-acting M receptor antagonists tiotropium, glycopyrrolate, and aclidinium could inhibit the secretion of MUC5AC in patients with chronic obstructive pulmonary disease, of which the mechanism was mainly to block M3 receptors and inhibit EGFR phosphorylation (26). Substantial preclinical evidence supports the beneficial effects of tiotropium on airway inflammation (27). Moreover, many articles have pointed out that the high expression of MUC5AC caused by various reasons was related to the phosphorylation of EGFR (22,24). These findings suggest that EGFR pathway is an important mechanism for M3 receptor to regulate AR mucin secretion.

The specific regulatory mechanism of EGFR activation mediated by M3 choline receptor on mucin hypersecretion is a hot topic at present. Studies have shown that matrix metalloproteinase (MMP) plays an important role as a bridge (24). Hodges *et al.* reported in cultured goblet cells EGF stimulated an increase in $[Ca^{2+}]_i$ in a concentration-dependent manner. Carbachol-stimulated increase in $[Ca^{2+}]_i$ was blocked by inhibitors for the M1 muscarinic acetylcholine receptors (M1AchRs), MMPs, and EGF receptors (22). Meanwhile, Deshmukh *et al.* found that acrolein induced MUC5AC hypersecretion in human airway epithelial cells mainly through stimulating MMP9 to activate the EGFR/MAPK signaling pathway (23). Similarly, Wang *et al.* also found that LPS induced MUC5AC hypersecretion in human airway epithelial cells mainly by activating MMP9 to activate EGFR (11). Feng *et al.* showed that the expression of MMP in nasal mucosa of patients with mild and severe AR was significantly higher than that of the control group (28). These studies indicated the existence of the M receptor-MMP9-EGFR-MUC5AC pathway. However, there is no study about M3-MMP9-EGFR-MUC5AC pathway in AR.

In our study, the expressions of MMP9, EGFR, and MUC5AC in the model group animals were increased (compared with those in the normal group), while these were decreased after treatment with 4-DAMP. These consequences were consistent with previous reports, speculating that 4-DAMP may inhibit MUC5AC secretion through the MMP9-EGFR pathway. The M3 receptor was blocked by 4-DAMP to inhibit the activation of MMP9, after which the inhibition of EGFR phosphorylation will ultimately reduce the MUC5AC secretion. However, the specific mechanism needs further research.

In conclusion, selective M3 receptor antagonists can effectively alleviate mucus hypersecretion and ease chronic inflammation in guinea pigs with AR. This study found that the therapeutic effect of 4-DAMP on AR guinea pigs was equivalent to that of the marketed drug IB, especially in the treatment of chronic inflammation of AR. We speculate that 4-DAMP, a selective M3 receptor antagonist, has a good prospect of clinical application.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-2998/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Animal experiments were performed under a project license (No. 20200301) granted by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University, in compliance with the National Research Council Guide for the Care and Use of laboratory Animals.

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