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Desloratadine Citrate Disodium Injection, a potent histamine H₁ receptor antagonist, inhibits chemokine production in ovalbumin-induced allergic rhinitis guinea pig model and histamine-induced human nasal epithelial cells via inhibiting the ERK1/2 and NF-kappa B signal cascades.

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Abstract

Chemokines have chemotactic properties on leukocyte subsets whose modulation plays a pivotal role in allergic inflammatory processes. Our present study was designed to investigate the anti-allergic and anti-inflammatory properties of desloratadine citrate disodium injection (DLC) and elucidate the molecular mechanisms of its anti-inflammatory properties. The anti-allergic effects of DLC were evaluated based on allergic symptoms, serological marker production and histological changes of the nasal mucosa in guinea pigs model of allergic rhinitis. The anti-inflammatory properties and molecular mechanisms of DLC were explored by studying the regulation of a set of chemokines and extracellular signal-regulated kinase (ERK)1/2 and nuclear factor- kappa B (NF- κ B) pathways, after DLC treatment in guinea pigs model of allergic rhinitis in vivo and histamine-activated human nasal epithelial cells (HNECs) in vitro. In vivo model in guinea pigs , DLC alleviated the rhinitis symptoms, inhibited inflammatory cells infiltration in nasal lavage fluid (NLF) and histamine, monocyte chemotactic protein (MCP)-1, regulated on activation normal T cell expressed, and presumably secreted (RANTES) and interleukin (IL)-8 release in sera and P-ERK1/2 and NF- κ B activation in nasal mucosa. In vitro, DLC markedly inhibited histamine-induced production of MCP-1, RANTES and IL-8 and suppressed c-Raf, mitogen-activated

protein/extracellular signal-regulated kinase kinase (MEK) and ERK1/2 activation in HNECs. These results provide evidence that DLC possesses potent anti-allergic and anti-inflammatory properties. The mechanism of action underlying DLC in allergic inflammation appears to be inhibition of the phosphorylation of ERK1/2, in addition to blocking of the NF- κ B pathway.

Key words: Allergic rhinitis, Desloratadine citrate disodium injection (DLC), Anti-allergic, Anti-inflammatory, Chemokine

1. Introduction

Allergic rhinitis (AR) is characterized by expression and activation of inflammatory mediators by immune cells (e.g. mast cells, basophils and T cells)(Al Suleimani and Walker, 2007; Baraniuk, 1997; Mandhane et al., 2011). The allergic response comprises three distinct phases: sensitization, early-phase response and late-phase response (Mygind, 2014; Pearlman, 1999). The early-phase response during allergic rhinitis is associated with IgE-mediated mast cell activation and the release of primary inflammatory mediators(e.g. histamine, leukotriene C4, prostaglandin D2), which may cause nasal rubbing, sneezing, rhinorrhea, lacrimation and nasal congestion(Salmun, 2002; Scadding, 2002). Late-phase response is characterized by an intense influx of inflammatory cells, particularly eosinophils and basophils, which ultimately disrupts the mucosal epithelium of the airway, resulting in clinical symptoms associated with the late-phase response (Pearlman, 1999).

Chemokines, including MCP-1, MCP-3, RANTES, IL-8 and eotaxin, are effective leukocyte chemoattractants, which play an important role in mediating leukocyte recruitment (Conti and DiGioacchino, 2001; Wong et al., 2005; Zimmermann et al., 2003). Furthermore, chemokines are potent cellular activating factors and histamine-releasing factors, which make them particularly important in the pathogenesis of allergic inflammation (Zimmermann et al., 2003). In the late-phase response of

nasal allergy, chemokines are recognized to have a great influence on protracted allergic inflammation (Fujikura et al., 2001).

Desloratadine citrate disodium

(8-chloro-6,11-dihydro-11-(4-piperidinylidene)-5H-benzo[5,6]cyclohepta[1,2,b] pyridine citric acid trisodium salt dehydrate) (DLC) (Fig. 1), molecular formula: $C_{25}H_{25}ClN_2O_7Na_2 \cdot 2H_2O$), is a potent non-sedating histamine H_1 receptor antagonist. The effective metabolite of DLC is desloratadine. Significant anti-allergic effects of DLC were assessed through passive cutaneous anaphylactic reactions (PCA) in sensitized rodents and ovalbumin-induced allergic rhinitis in rats by the previous work in our laboratory (Jie et al., 2015).

In the recent past, numerous in vitro studies have demonstrated that desloratadine possesses anti-allergic and anti-inflammatory properties by suppressing the synthesis and release of inflammation mediators through inhibition of NF- κ B activation (Mullol et al., 2011; Mullol et al., 2006; Roumestan et al., 2008; Scadding, 2005; Traidl-Hoffmann et al., 2006; Wu et al., 2004). Our previous studies also indicated that DLC suppresses NF- κ B activation and decreases the release of a variety of inflammatory factors (Jie et al., 2015). In order to better understand the possible anti-inflammatory effects of DLC in allergic rhinitis, we studied the expression of a set of chemokines known to be involved in the eosinophils-basophils activation as well as recruitment and T-cell

signaling events and explored inhibitory mechanism of DLC on chemokines expression.

So far, the anti-inflammatory effects of desloratadine on depression of production of MCP-1, RANTES and IL-8, have been shown in several in vitro experiments, but to date, no in vivo studies with respect to these findings have been done. In this study we sought to determine whether DLC are able to repress chemokines release in ovalbumin-induced allergic rhinitis in guinea pig in vivo and histamine-activated HNECs in vitro and explore the mechanism of signal transduction pathways of DLC leading to the induction of chemokines expression.

2. Materials and methods

2.1 Animals care and use

The care and treatment of animals were in accordance with the guidelines established by the Chinese Council on Animal Research and Care and the Ethics Committee of China Pharmaceutical University. Healthy male Dunkin–Hartley guinea pigs (250–300g) used for the study were housed under controlled environmental conditions at a temperature of $23 \pm 2^{\circ}\text{C}$ with a relative humidity of $55 \pm 5\%$ and 12 h light/dark cycle throughout the study. Animals were given free access to standard laboratory diet and water, except as noted below for fasted animals.

2.2 Chemicals and reagents

DLC was supplied by Hefei Yigong Medicine Co., Ltd. Chlorpheniramine maleate injection (CHM) was purchased from Shanghai Hefeng Medicine Co., Ltd. (Shanghai, China). Histamine was purchased from Sangon Biotech (Shanghai, China). Ovalbumin (chicken egg, Grade V) was bought from Sigma. The MEK-specific inhibitor PD98059 was purchased from Abcam. Aluminum hydroxide gel was purchased from Thermo Fisher Scientific, China. Antibodies used in this study were anti-phospho-c-Raf (Ser338) antibody (Abcam), anti-phospho-MEK1/2 (Ser217/221) antibody (Abcam), anti-p44/42 MAP kinase (ERK1/2) antibody (Abcam), anti-phospho-p44/42 MAP kinase

(Thr202/Tyr204) antibody (Abcam). All other chemicals were of high purity reagent grade. The decision about the drug doses were based on clinical and experimental animal doses. Data of previous studies carried out in our laboratories was also utilized in deciding the doses.

2.3 Ovalbumin induced allergic rhinitis in guinea pig

2.3.1 Sensitization protocols.

A procedure for active sensitization was applied as described previously by Mandhane et al (Bahekar et al., 2008) with some modification. Guinea pigs were randomized and made into different groups of each 12 animals, containing non sensitized control (NSC) group, sensitized control (SC) group, DLC (0.25 mg/kg) group, DLC (0.5 mg/kg) group, DLC (1.0 mg/kg) group and CHM (0.5 mg/kg) group. Guinea pigs were sensitized by intraperitoneal injection of ovalbumin (80 ug/animal) and aluminium hydroxide (5mg/animal) dissolved and suspended in 0.9% saline solution respectively on days 1, 3, 5, 7, 14 and 21. Aluminum hydroxide gel was used as an adjuvant. Non sensitized animals received suspension of aluminum hydroxide gel without ovalbumin. After 6 days of systemic sensitization, guinea pigs were sensitized by instilling ovalbumin solution (20 µl/nostril, 60 mg/ml) into the bilateral nasal cavities using a micropipette for 7 consecutive days. After the final sensitization, successful

sensitization was confirmed in a few animals in each group by observing the redness at the site on the shaved dorsal skin due to intradermal injection of ovalbumin solution (200 µg/ml, 25 µl).

2.3.2 Evaluation of rhinitis symptoms.

Nine successful sensitized guinea pigs were selected from each group. Treatment of DLC (0.25, 0.5 and 1.0 mg/kg) and CHM (0.5mg/kg) was performed intravenously once daily for 5 days. In addition, antigen challenge was performed sequentially. The last treatment was given 30 min before the antigen challenge. After the final intranasal challenge of 20 µl of ovalbumin (60mg/ml), symptoms of rhinitis were observed and the numbers of sneezes and nasal rubbing movements were counted continuously for 30 min in accordance with the previously published methods (Mizutani et al., 2003; Sugimoto et al., 2000)

2.3.3 Measurement of concentrations of Histamine, RANTES and MCP-1, IL-8 in the serum.

Four hours after the last treatment, overnight-fasted animals were anesthetized by chloral hydrate (300mg/kg). Overnight-fasting was performed to reduce the impact on anesthesia and cytokine levels. Blood of each guinea pig was collected from aorta abdominalis (Vigano et al., 2012). The serum was separated by centrifugation at 1000g for 10 min at

4 °C. Serum was prepared and frozen at -70 °C prior to analysis.

Histamine, RANTES, MCP-1 and IL-8 level were measured using the enzyme-linked

immunosorbent assay (ELISA) kit(Calvin biology, China), according to manufacturer's procedure.

2.3.4 Evaluation of inflammatory cellular infiltration in nasal lavage fluid.

Nasal lavage fluid (NLF) was collected using a cannula attached to the nasopharynx and connected to a perfusion pump (BT100-2J, Longer Pump, China) with a delivery rate of 0.25 ml/min and perfused with 0.9% saline solution. After the NLF centrifugation (120g for 10 min at 4 °C), the cell pellets were suspended in 100 µl of 0.9% saline solution. The total leucocytes number was determined by standard hem cytometer methods. To perform Eosinophil count, aliquots of the cell suspension were stained with Giemsa, and eosinophil count was done using normal morphological criteria. Figures in parenthesis show rate (%) calculated as follows: Rate (%) =number of Eosinophil/number of Leucocytes × 100.

2.3.5 Histopathological analysis and immunohistochemistry of nasal cavity.

Histopathological studies were performed as per the method described previously by Bahekar et al. and Yamasaki earlier (Bahekar et al., 2008; Yamasaki et al., 2001). Nasal cavities of guinea pigs were separated for histopathological analysis. After a routine process including fixation, dehydration, hyalinization and paraffin embedding, each section of the nasal mucosa (4- μ m thick) was stained with hematoxylin-eosin (H&E). Immunostaining for P-ERK and NF- κ B was performed by using immunohistochemistry. A procedure for immunohistochemistry was applied as described previously by Zhipeng Li et al (Li et al., 2014).

2.4 Histamine-induced HNECs activation

2.4.1 Cell culture.

The HNECs were purchased from Guangzhou Jini Ou biological Ltd. The HNECs were cultured in formulated RPMI-1640 medium (IMDM; Gibco BRL, Grand Island, NY, USA) supplemented with 15% fetal bovine serum (FBS), 100 U/ml of penicillin and 100 mg/mL of streptomycin at 37 °C in a humidified incubator with an atmosphere containing 5% CO₂. The medium was changed every 2 days. Each co-culture experiment was performed three times for validating the results.

2.4.2 Cytotoxicity assay.

Cell viability was assessed by the MTT reduction assay to measure the cytotoxic response to DLC. MTT assay was performed as described previously (Kang et al., 2010). In brief, 200 μ l of HNECs suspension was cultured in 96-well plates for 24 h after treatment with each final concentration of DLC (10^{-10} , 10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} and 10^{-5} M) and followed the conventional procedure. The optical density (OD) of 96-well culture plates was measured at 490 nm. The OD of formazan formed in Medium cells was taken as 100% viability.

2.4.3 Measurement of chemokines production.

HNECs cells were pretreated with various concentration of DLC and PD98059 at 37 °C for 1h and then stimulated with histamine (final concentration: 10^{-3} M) at 37 °C for 24h. RANTES, MCP-1 and IL-8 levels in the supernatant were measured using the ELISA Kit (Calvin biology, China)according to the manufacturer's instructions.

2.4.4 Immunocytochemistry evaluation.

Immunocytochemistry evaluation was performed according to the procedure described by Masahiro Matsubara (Matsubara et al., 2005). Confluent cells in 6-well plates were pre-incubated with RPMI-1640 without fetal bovine serum for 8 h. The cells were treated

with or without DLC (10^{-7} , 10^{-8} , 10^{-9} M) and PD98059 (10uM) for 1 h, and then stimulated with 10^{-3} M histamine for 7.5 min. After incubation, the cells were fixed with 4% (w/v) paraformaldehyde and permeabilized with PBS and then blocked with 5.5% (w/v) goat serum for 1 h at room temperature. A primary antibody to detect phosphor-ERK antibody was incubated at 4 °C overnight. Thereafter, addition of secondary antibody and staining were carried out as the manufacturer's instructions.

2.4.5 Western blot analysis.

After the same incubation process (DLC or PD98058 for 1h and 10^{-3} M histamine for 7.5 min), the cell pellets in 6-well plates were collected on ice and lysed in RIPA Lysis buffer for 30 min. Insoluble materials were removed by centrifugation (14,000g, 20 min) and the supernatant was boiled with Laemmli's sample buffer containing dithiothreitol for 10min . Equal amounts of protein in each sample were separated by 6% to 12% SDS-PAGE and then blotted onto a polyvinylidene difluoride membrane. After that, the immunoreactive bands were visualized using the electrochemiluminescence detection system (Tannon-5200, Shanghai, China) followed the instructions of conventional protocol.

2.5 Statistical analyses

All experiments were carried out at least in triplicate. All quantitative data were presented as mean $\pm$ standard deviation (mean $\pm$ S.D.). Statistical differences among different groups were analysed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests. P-value less than 0.05 ($P < 0.05$) was considered as statistically significant. Statistical analysis was performed by using SPSS 19.0 software (SPSS Inc.)

3. Results

3.1 Ovalbumin induced allergic rhinitis in guinea pig

3.1.1 Effect of DLC on the nasal symptoms in ovalbumin-sensitized /challenged guinea pig.

As shown in Fig.3A, the number of sneezes and nasal rubbing in guinea pig was markedly increased by topical ovalbumin challenge. DLC attenuated ovalbumin-induced rhinitis symptoms in a concentration-dependent manner. Moreover, At doses of 0.5 and 1.0mg/kg, DLC was more effective than CHM at 0.5 mg/kg dose in reducing sneezing and nose rubbing. The histological observation revealed epithelial disruption, goblet cell hyperplasia, mucosa edema, and infiltration of numerous inflammatory cells such as eosinophils in the nasal mucosa in the ovalbumin-sensitized control mice(Fig.3B).

However, DLC and CHM protected the nasal mucosa against damages and greatly reduced infiltration of eosinophils, especially for DLC at a dose of 1.0mg/kg.

3.1.2 Effect of DLC on cellular infiltration in nasal lavage fluid.

As shown in Fig.4, nasal ovalbumin challenge induced a marked inflammatory cellular infiltration in the NLF of sensitized guinea pigs, $P < 0.01$. The rate of eosinophils in the leucocyte increased by 51.63% after ovalbumin challenge (Fig.4). Guinea pigs treated with DLC once daily for 5 days showed a marked reduction in leucocyte and eosinophil number in a dose-dependent manner (Fig.4).

3.1.3 Effect of DLC on the serum levels of histamine, RANTES, MCP-1 and IL-8 in ovalbumin -sensitized /challenged guinea pig.

The administration of DLC significantly decreased the levels of histamine, MCP-1 and IL-8 in the sera compared to that in control group given ovalbumin sensitization alone in a concentration-dependent manner, $P < 0.01$ (Fig. 5A, B and D). Enhanced RANTES level by the sensitization was also significantly reduced by DLC administration at dose of 1.0mg/kg, $P < 0.01$ (Fig.5C). In addition, DLC at 0.5 mg/kg and 1.0 mg/kg dose showed higher effects than the standard drug CHM at 0.5 mg/kg dose, in reduction of the level of allergic mediators.

3.1.4 Effect of DLC on the Immunohistochemical evaluation of p-ERK1/2 and NF- κ B in ovalbumin-sensitized /challenged guinea pig.

To investigate the effect of DLC on MAPK and NF- κ B pathways in vivo, the phosphorylation of MAPK molecules ERK1/2 and NF- κ B were analyzed by Immunocytochemistry in the nasal mucosa of six groups of guinea pigs. The expression of ERK1/2 and NF- κ B were quantified by using an image analysis and measuring system (Image Pro-plus 6.0). As shown in Fig.6A, B and C, ovalbumin challenge markedly increased the expression of p-ERK and NF- κ B p65 in the nasal mucosa of sensitized guinea pigs, $P < 0.01$. And the administration of DLC significantly decreased the protein expression in a concentration-dependent manner (Fig.6C). Moreover, DLC at 0.5 mg/kg and 1.0 mg/kg showed higher effects than the positive control drug CHM at 0.5 mg/kg dose in reduction of the protein expression.

3.2 Effect of DLC on histamine-induced chemokines production and its mechanism on HNECs

3.2.1 Effect of DLC on cell viability.

By MTT assay, we found that treatment with DLC at concentrations of 10^{-10} , 10^{-9} , 10^{-8} , 10^{-7} and 10^{-6} M did not affect cell viability (Fig.7). Therefore, DLC at the concentration of 10^{-9} , 10^{-8} and 10^{-7} M were used

during follow-up experiments.

3.2.2 Effects of DLC and PD98059 in histamine-induced chemokines production in HNECs.

As shown in Fig.8A, treatment with histamine enhanced production of MCP-1, RANTES and IL-8 in dose and time dependent manner. DLC markedly suppressed histamine-induced production of MCP-1, RANTES and IL-8 in a concentration-dependent manner (Fig.8B). Moreover, at doses of 10^{-7} and 10^{-8} M, DLC were more effective than CHM at 10^{-8} M dose in reducing the production of RANTES and IL-8 (Fig. 8B). PD98059, a specific inhibitor of MEK1, suppressed histamine-induced RANTES and IL-8 production in concentration-dependent manner. However, it inhibit MCP-1 production at a low concentration of 3 μ mol/l (Fig.8B).

3.2.3 Effect of DLC on histamine-induced Raf/MEK/ERK activation of HNECs.

To investigate the effect of DLC on ERK1/2 MAPK signaling pathway in activated HNECs cells, the phosphorylation of three MAPK molecules c-Raf, MEK1/2 and ERK1/2 were analyzed by Western blot and P-ERK1/2 was analyzed by Immunocytochemistry in HNECs cells. As shown in Fig.9B, treatment with DLC significantly suppressed histamine-induced Raf/MEK/ERK activation of HNECs. The

immunocytochemical analysis also showed that DLC completely inhibited the phosphorylation of ERK (Fig.9A).

4. Discussion

Desloratadine has demonstrated significant clinical efficacy against symptoms of allergic rhinitis and consequently remains a first-line therapy for many allergic conditions (Berger, 2005; Reinartz et al., 2005). It is third-generation antihistamine, which lacks central nervous system and severe cardiovascular side effects (Bachert and Maurer, 2010; Berger, 2005). Desloratadine citrate disodium injection is a potent non-sedating H₁ antihistamine. The effective metabolite of DLC is desloratadine. It was developed for symptomatic treatment of perennial and seasonal allergic rhinitis (PAR and SAR, respectively) and chronic idiopathic urticaria (CIU) for intravenous injection. Compared with conventional tablets, it has some good properties such as, its rapid effect and high efficacy. It is suitable for patients with acute allergy diseases or severe dysphagia. Our previous work demonstrated the significant anti-allergic effects of DLC through passive cutaneous anaphylactic reactions in sensitized rodents and ovalbumin-induced allergic rhinitis (Jie et al., 2015). Previous in vitro studies have demonstrated that desloratadine exerts anti-inflammatory properties by suppressing the synthesis and release of inflammation mediators through inhibition of

NF- κ B activation (Mullol et al., 2011; Mullol et al., 2006; Roumestan et al., 2008; Scadding, 2005; Wu et al., 2004). So far no studies have addressed the potential anti-inflammatory activities of desloratadine on regulation of chemokines and ERK1/2 MAPK pathways in vivo and vitro simultaneously. In the present study, we evaluated that DLC has a good therapeutic effect on allergic responses in ovalbumin -induced allergic rhinitis in guinea pigs and can inhibit the chemokines release such as MCP-1, RANTES and IL-8 and block the ERK1/2 MAPK and NF- κ B pathways in ovalbumin -induced allergic rhinitis in guinea pig in vivo and histamine-induced HNECs in vitro.

Allergic response is composed by three distinct immunological phases, which is sensitization, early-phase response, and late-phase response (Mygind, 2014; Pearlman, 1999). The early-phase response initiates degranulation of basophils and mast cells, thereby releasing de novo synthesized pro-inflammatory substances (prostaglandin D₂, leukotriene C₄) and preformed inflammatory mediators (histamine, tryptase, bradykinin) (Salmun, 2002; Scadding, 2002). In allergic rhinitis, nasal histamine challenge inducing many of the symptoms including sneezing, nasal itching, rhinorrhea, and nasal congestion, histamine has been implicated as a principal mediator involved in the allergic response (Skoner, 2001). We discovered that, DLC concentration-dependently attenuated the rhinitis symptoms. Histological

observation revealed DLC protected the nasal mucosa against damages such as epithelial disruption, mucosa edema, goblet cell hyperplasia and greatly reduced infiltration of eosinophils. Moreover, DLC significantly inhibited the release of histamine, prostaglandin D₂ and leukotriene C₄ (some data not shown) in sera of guinea pig. Suggesting that, DLC could be able to reduce the early phase reaction of allergic response.

Late-phase response is noted approximately 4–24 h following the allergen exposure and, is characterized by a cellular inflammatory response (Assanasen and Naclerio, 2002) (Walsh, 2000). This response results in intense influx of inflammatory granulocytes, particularly eosinophils and basophils, into the nasal mucosa and other affected tissues (Pearlman, 1999). Chemokines, including monocyte chemotactic protein MCP-1, MCP-3, RANTES, IL-8 and eotaxin, are thought to play an important role in inducing selective recruitment of these cells to the allergic inflammatory site (Kuna et al., 1996; Nickel et al., 1999; Teran and Davies, 1996). Furthermore, Chemokines are potent cellular activating factors and histamine-releasing factors, which make them particularly important in the late-phase response of allergic inflammation (Fujikura et al., 2001a). Our result showed that guinea pigs treated with DLC showed a marked reduction in nasal ovalbumin challenge induced eosinophils infiltration in the NLF of sensitized guinea pigs. In addition, DLC successfully inhibited the chemokines release such as MCP-1,

RANTES and IL-8 in sera of ovalbumin-induced allergic rhinitis in guinea pig. In order to further explore the regulation of chemokines of DLC, we examine the effect of DLC on MCP-1, RANTES and IL-8 on HNECs in vitro. We selected nasal epithelial cells because they represent the first barrier against allergens and these cells are major source of chemokines (Ohkuni et al., 2011; Zimmermann et al., 2003). Histamine, as a inducer, was chosen due to its significant role in allergic rhinitis and histamine receptors are present on HNECs (Matsubara et al., 2005; Terada et al., 1999). We found out that, DLC suppressed histamine-induced MCP-1, RANTES and IL-8 production in concentration-dependent manner in HNECs. This effect of DLC as a potent H₁ antihistamine, is in agreement with previous studies, which demonstrated that, histamine H₁ receptor antagonist inhibited chemokine upregulation induced by antigen or histamine in epithelial cells (Fujikura et al., 2001b; Traidl-Hoffmann et al., 2006). This results indicate that, DLC might target both early- and late-phase mediators of the allergic cascade and shorten late-phase response time, by inhibiting the release of chemokines in allergic rhinitis.

Although DLC is known to suppress chemokine production in vivo and vitro, the inhibitory mechanism of DLC has not been established. Previous studies demonstrated that desloratadine down-regulate the activation of NF- κ B to suppress the release of inflammatory mediators,

which may explain for majority of these extra anti-inflammatory activities (Mullol et al., 2011; Mullol et al., 2006; Roumestan et al., 2008; Scadding, 2005; Wu et al., 2004). In the Immunohistochemical evaluation of NF- κ B in ovalbumin-sensitized/challenged guinea pig, we found out that, DLC significantly decreased NF- κ B expression in nasal mucosa in a concentration-dependent manner. We next explore the effect of DLC on the ERK1/2 MAPK signal pathway. PD98059, a MEK1 selective inhibitor, concentration-dependently suppressed histamine-induced RANTES and IL-8 production, indicating that ERK activation is essential for histamine-enhanced certain chemokine production. The results was consistent with the findings of other studies, which showed that many chemokines can be regulated by ERK1/2 MAPK and NF- κ B pathway (Alcorn et al., 2001; Matsubara et al., 2005; Pazdrak et al., 2002). In addition, P38 MAPK pathway has also demonstrated to regulate chemokine production according to other published papers (Hong et al., 2015; Wong et al., 2005). In our study, DLC significantly inhibited histamine-induced phosphorylation of c-Raf, MEK1/2 and ERK1/2 in HNECs and p-ERK1/2 expression in nasal mucosa of ovalbumin-sensitized /challenged guinea pigs. The result indicates that, DLC demonstrates its anti-inflammatory effect by blocking the ERK1/2 MAPK and NF- κ B signal and subsequently inhibiting certain chemokine production. Previous studies have shown that, histamine H₁ receptor

antagonists prevented histamine-induced cytokine such as GM-CSF, IL-8 and IL-6 production through inhibition of both ERK and NF- κ B cascades (Matsubara et al., 2005). This suggest that, histamine may induce expression of numerous genes other than MCP-1, RANTES and IL-8 in HNECs. Probably, both ERK1/2 and NF- κ B cascades participate in the expression of other genes and DLC may exert anti-inflammatory properties by inhibiting P38MAPK pathway, thus all of this speculation requires further studies.

In conclusion, our observations suggest that DLC possesses potent anti-allergic effects for the treatment of allergic rhinitis in early-phase response and late-phase response, with a superior potency to CHM in reducing, rhinitis symptoms, eosinophil infiltration and release of allergic mediators such as histamine, prostaglandinD2 and leukotriene C4. Furthermore, DLC exerts anti-inflammatory activity and shorten late-phase response time by suppressing the production of chemokines, MCP-1, RANTES and IL-8, partialy via the ERK1/2 and NF- κ B pathways in ovalbumin-induced allergic rhinitis in guinea pigs in vivo and histamine-activated HNECs in vitro. This result strongly suggest that DLC might be a promising drug for the treatment of allergic inflammatory diseases such as allergic rhinitis.

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Conflict interest

The authors declare no conflict of interest.

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Legend

Fig.1 Chemical structure of desloratadine citrate disodium (DLC).

Fig.2 Study plan for the development of the experiment in allergic rhinitis model.

Fig.3. Effects of DLC on numbers of sneezes and nasal rubbing and histological changes of nasal mucosa in ovalbumin- sensitized/challenged mice (A) Nasal symptoms were measured by scoring the numbers of sneezes and nasal rubbing. (B) The histological changes of nasal mucosa were observed by microscope after H&E staining(200X). Results shown in these images indicated reduced infiltration of eosinophils. NSC= nonsensitized ovalbumin challenged control. SC= ovalbumin-sensitized and challenged control. Each value represents the mean $\pm$ S.D. of nine animals. ^{##}P<0.01, as compared with nonsensitized control, ^{**}P<0.01, *P<0.05, as compared with sensitized control.

Fig.4 Effect of DLC on inflammatory cellular infiltration in nasal lavage fluid. NSC= nonsensitized ovalbumin challenged control. SC= ovalbumin-sensitized and challenged control. Leucocytes number in the NLF were determined using hemocytometer and eosinophil number was based on normal morphological criteria after stained with Giemsa. Each value represents the mean $\pm$ S.D. of nine animals. ^{##}P<0.01 when compared with nonsensitized control. ^{**}P<0.01 and *P<0.05 when compared with sensitized control.

Fig.5 Effect of DLC on the serum level of Histamine, RANTES, MCP-1 and IL-8 in ovalbumin-sensitized /challenged guinea pig. NSC= nonsensitized ovalbumin challenged control. SC= ovalbumin-sensitized and challenged control. The levels of (A) histamine, (B) MCP-1, (C) RANTES and (D)IL-8 were measured in sera by ELISA. Each value represents the mean±S.D. of nine animals. ^{###}P<0.01 when compared with nonsensitized control. ^{**}P<0.01 and ^{*}P<0.05 when compared with sensitized control.

Fig.6 Effect of DLC on the Immunocytochemistry evaluation of p-ERK and NF-κB (A) Immunohistochemical analysis of p-ERK expression in ovalbumin-sensitized /challenged guinea pig.(B) Immunohistochemical analysis of NF-κB expression in OVA-sensitized /challenged guinea pig.(C) Density mean of p-ERK and NF-κB expression calculated by Image-Pro Plus 6.0, Each value represents the mean±S.D. of six animals. NSC= nonsensitized ovalbumin challenged control. SC= ovalbumin-sensitized and challenged control. ^{##}P<0.01 when compared with nonsensitized control. ^{**}P<0.01 and ^{*}P<0.05 when compared with sensitized control.

Fig.7 Effect of DLC on cell viability in HNECs cells. Cells were treated with various concentration of DLC (10^{-10} , 10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} and 10^{-5} M) for 24 h and followed by determination of cell viability using the MTT assay. Each value represents the mean±S.D. of three independent experiments. ^{*}P<0.05, as compared with Medium

group.

Fig.8 Effect of DLC on chemokine production in HNECs (A) Histamine-induced production of MCP-1, RANTES and IL-8 in HNECs in different time and dose (B) Effects of DLC and PD98059 in histamine-induced chemokine production in HNECs.

HNECs were pretreated with indicated concentrations of DLC or PD98059 for 1 h prior to stimulation with 10^{-3} M histamine for 24 h. Protein released into the supernatant was measured by ELISA. Each value represents the mean $\pm$ S.D. of three independent experiments. $^{##}P<0.01$, as compared with Medium group. $^{**}P<0.01$ and $^{*}P<0.05$, as compared with control group (Dunnett).

Fig.9 Effect of DLC on histamine-induced Raf/MEK/ERK activation in HNECs.(A). Histamine-stimulated HNECs were subjected to immunocytochemistry with anti-phospho ERK antibody. (B) Histamine-induced phosphorylation levels of c-Raf,MEK1/2 and ERK1/2 was analyzed by Western blotting using phospho-protein specific antibodies. HNECs were pretreated with indicated concentrations of DLC or PD98059 for 1 h prior to stimulation with 10^{-3} M histamine for 7.5min. Each experiment was performed three times for validating the results. One representative result of several images is shown.

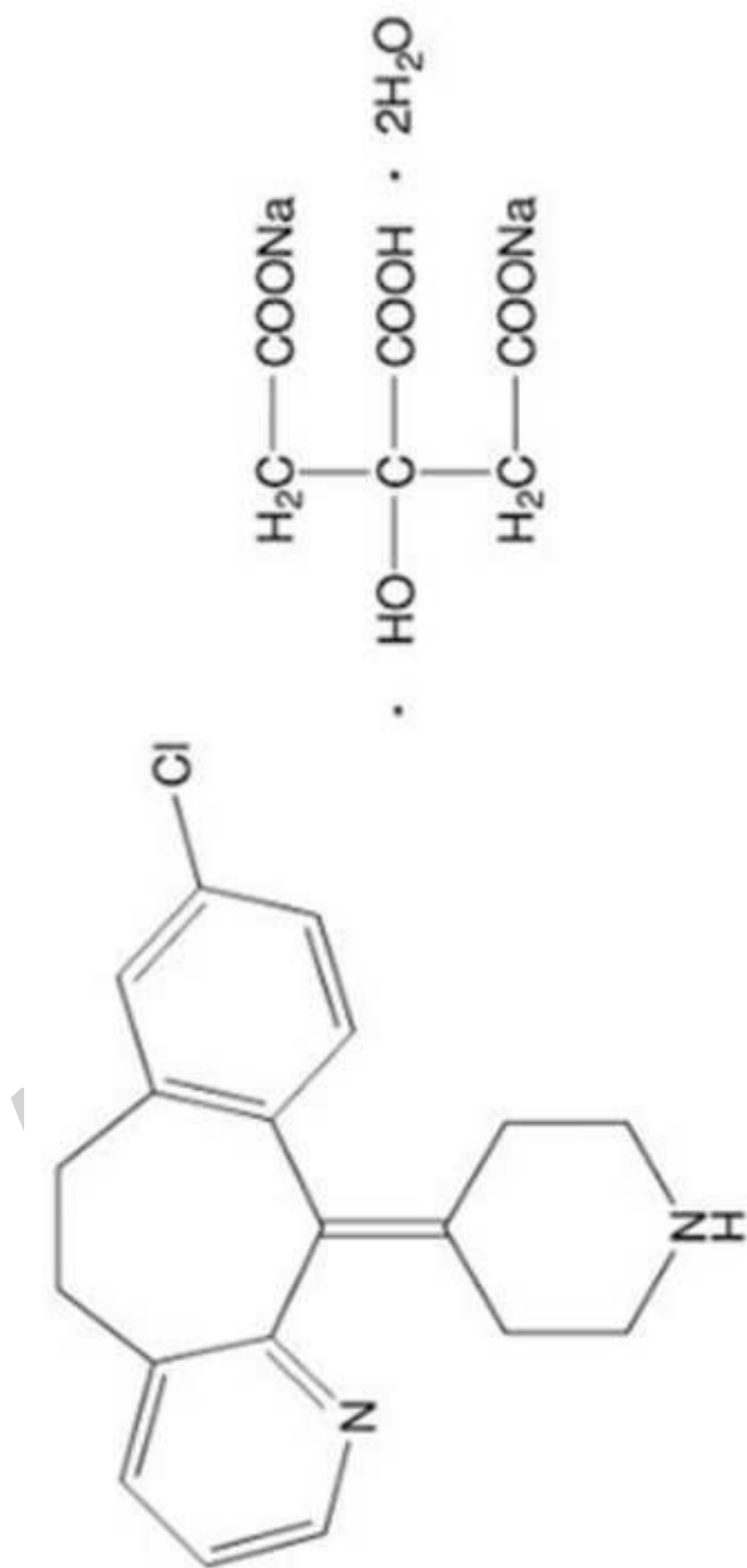


Figure1

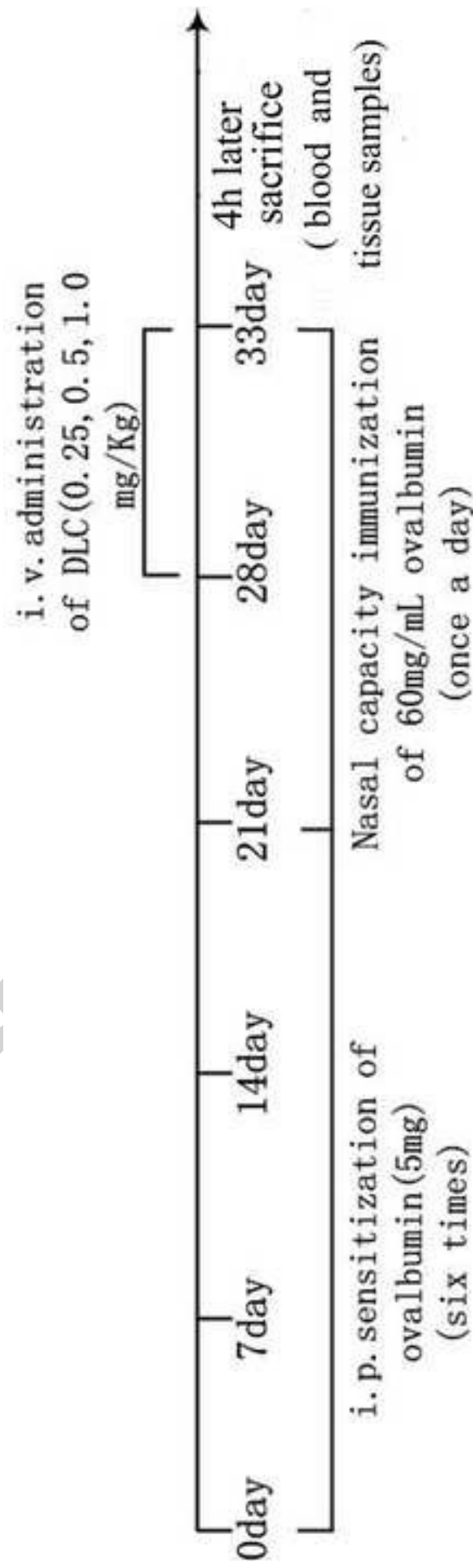
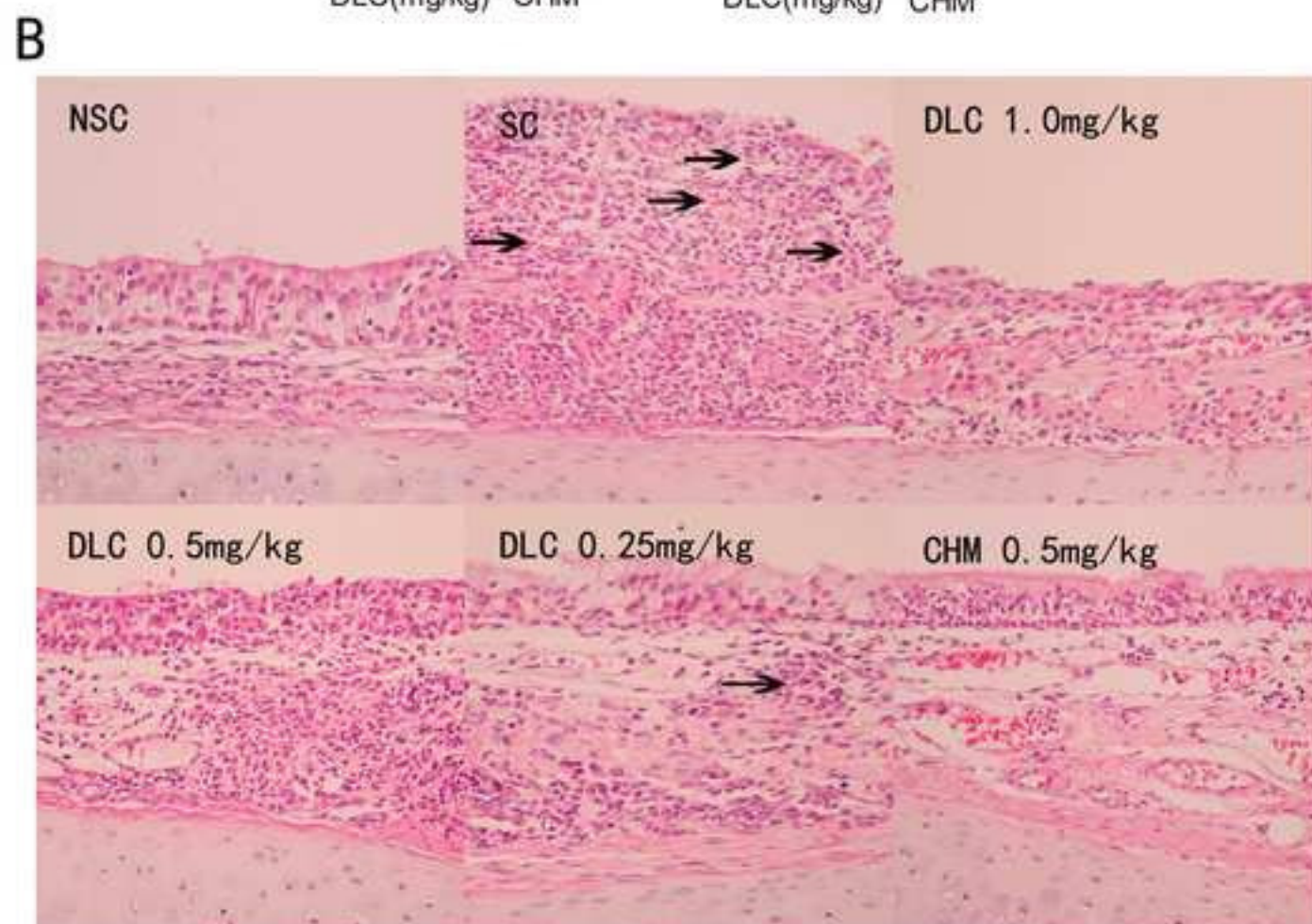
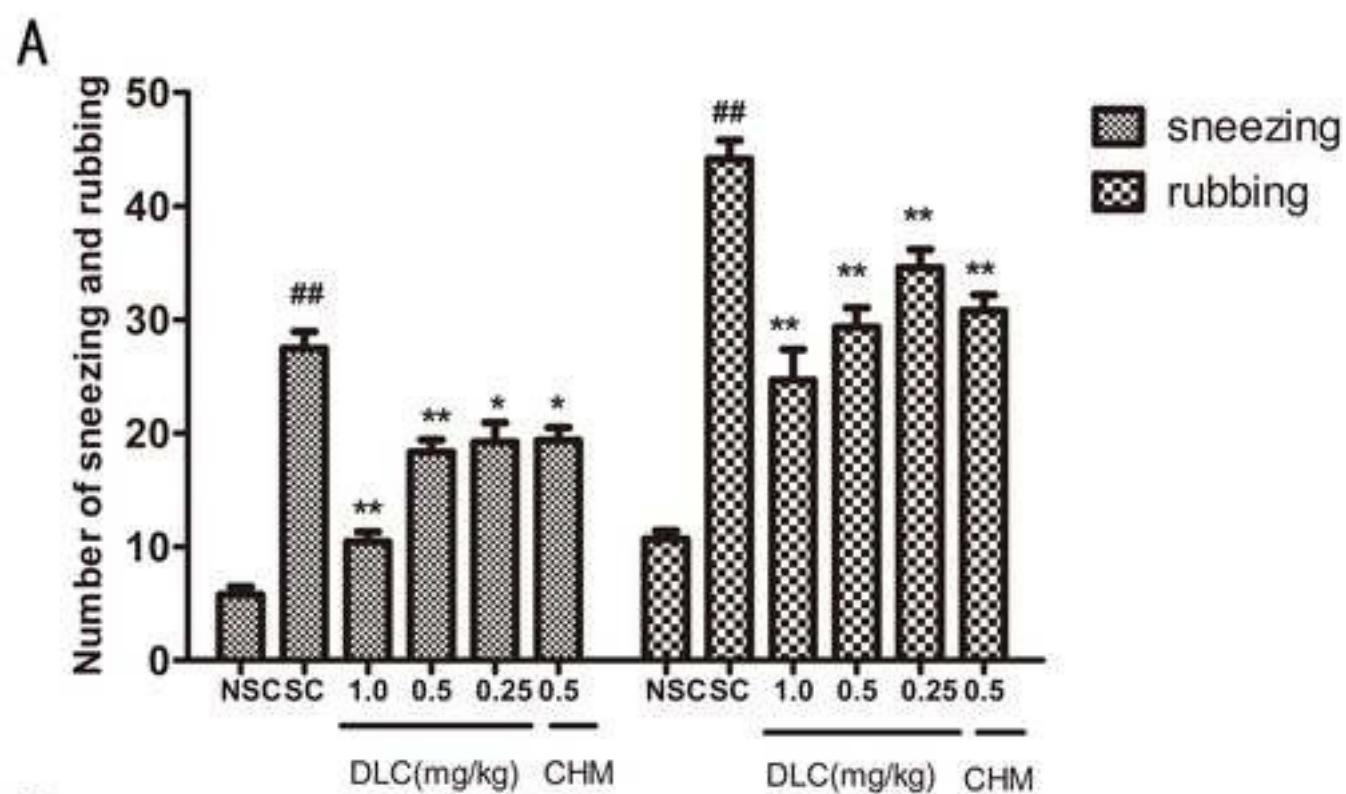


Figure 2



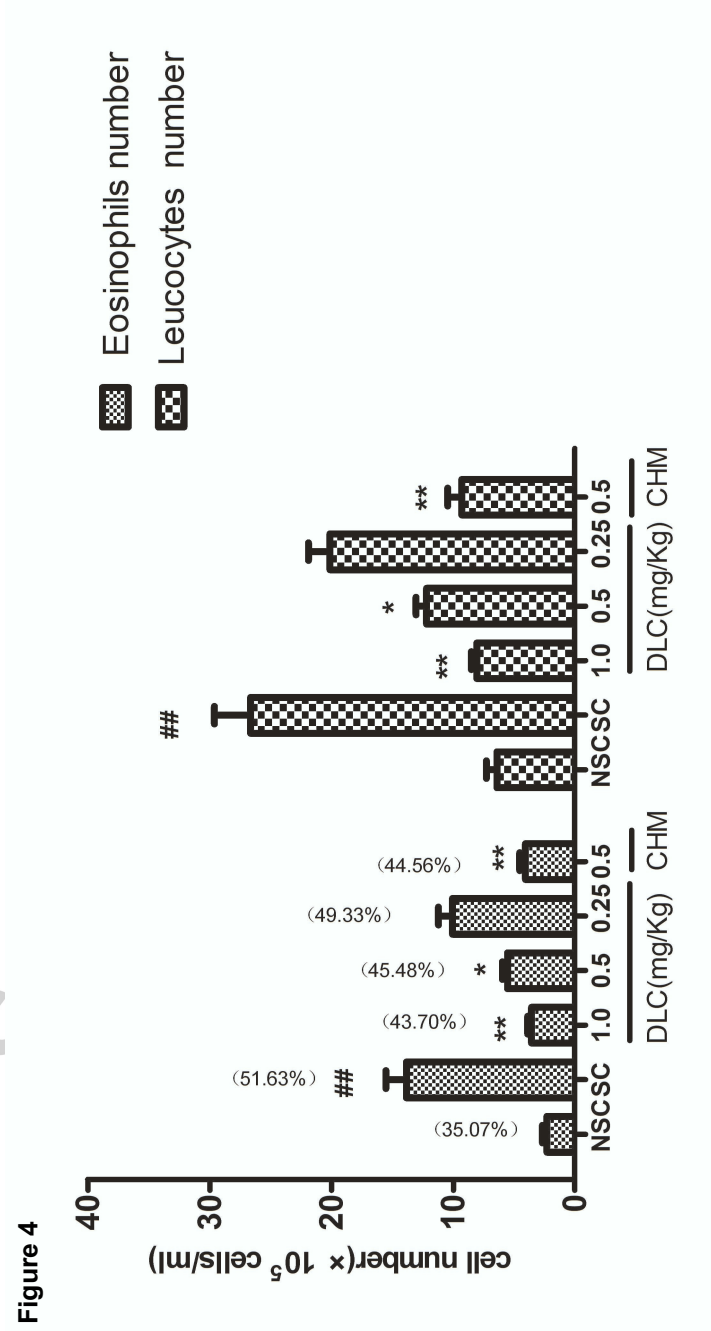
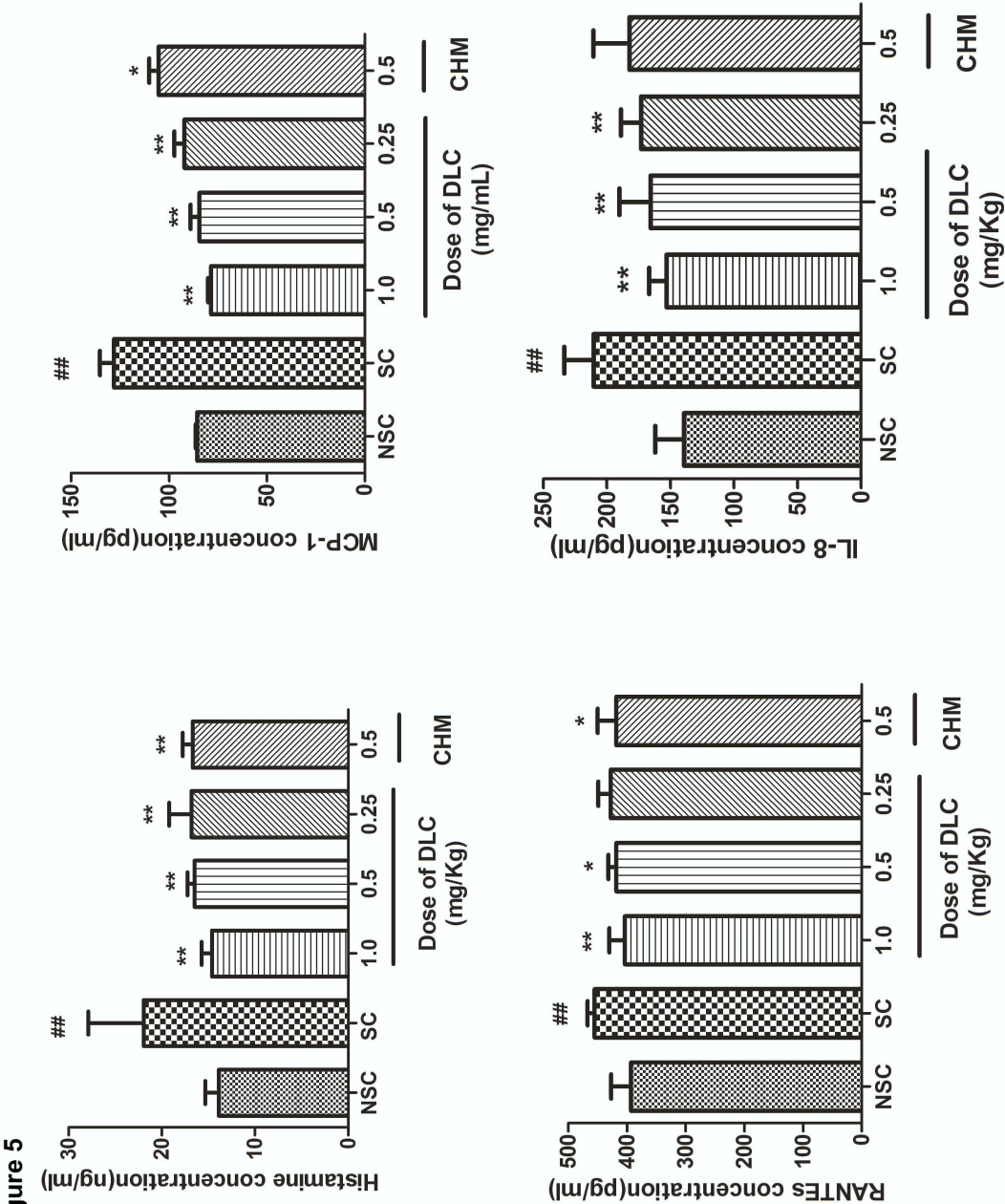


Figure 5



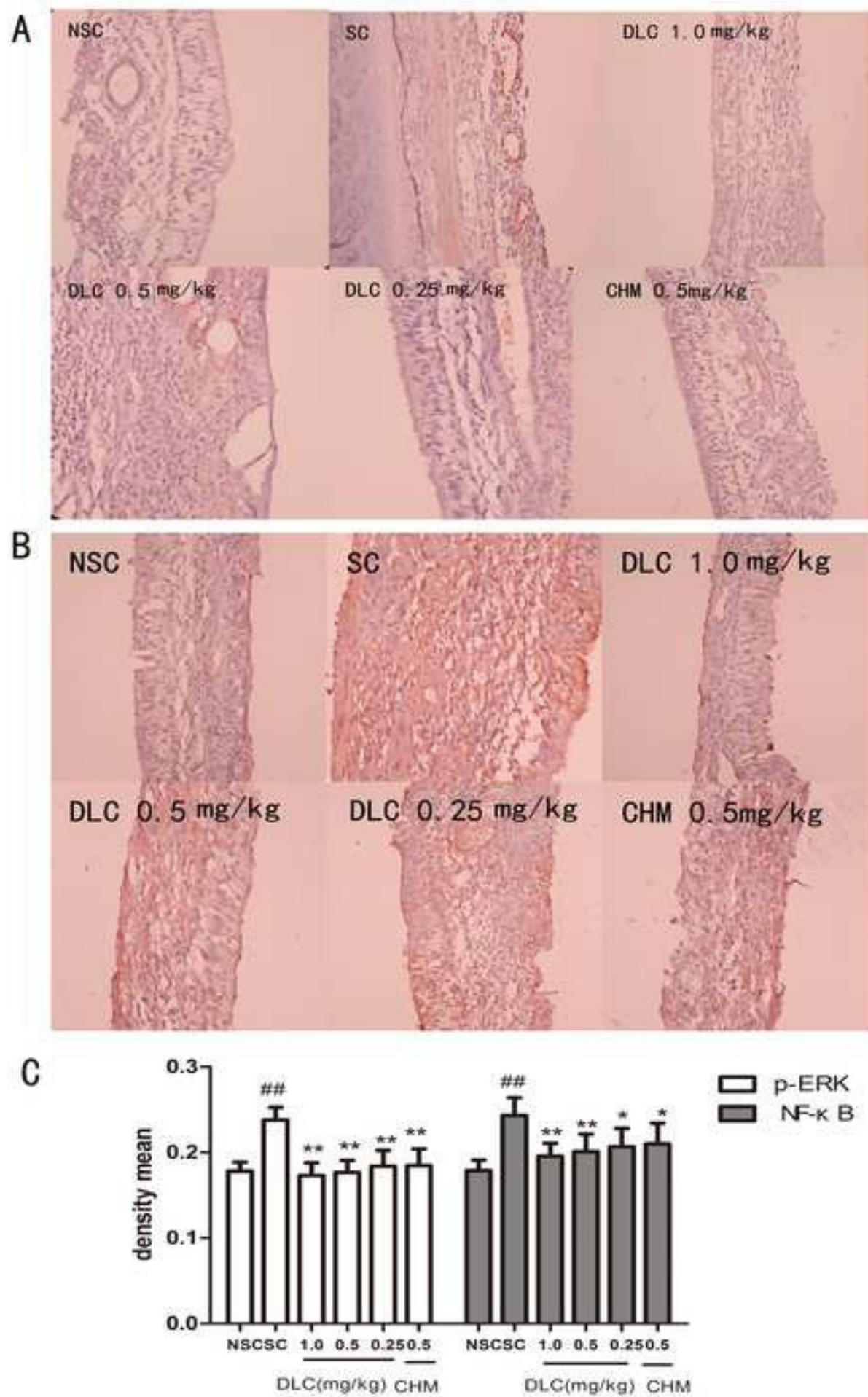


Figure 7

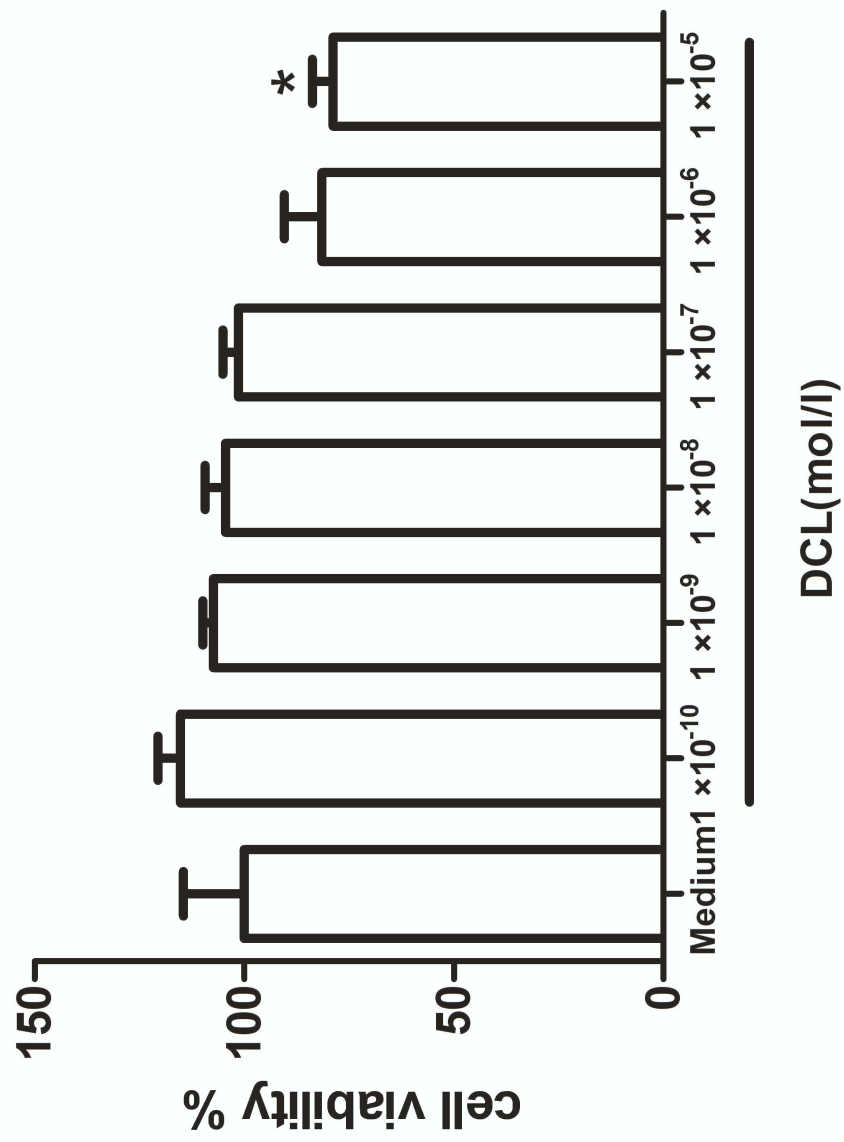


Figure.8

