

Pulmonary, gastrointestinal and urogenital pharmacology

Anti-allergic and anti-inflammatory properties of a potent histamine H₁ receptor antagonist, desloratadine citrate disodium injection, and its anti-inflammatory mechanism on EA.hy926 endothelial cellsQiong Jie^a, Nandani Darshika Kodithuwakku^a, Xin Yuan^a, Guangwei He^b, Meiling Chen^a, Shuhong Xu^a, Yulin Wu^{a,*}^a Department of Physiology, China Pharmaceutical University, Nanjing 210009, Jiangsu, China^b Hefei Yigong Medicine Co., Ltd, Hefei 230088, Anhui, China

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ABSTRACT

The present study, demonstrates that, desloratadine citrate disodium injection (DLC) possesses antihistaminic, anti-allergic and anti-inflammatory properties and elucidates its molecular mechanisms of anti-inflammatory properties. In vitro antihistamine activity of DLC was determined in guinea pig isolated tissues. In vivo antihistamine effects were evaluated after following intravenous administration of DLC in mice with histamine- induced paw edema and in rats with increased capillary permeability. Anti-allergic effects were assessed through passive cutaneous anaphylactic (PCA) reactions in sensitized rodents and ovalbumin-induced allergic rhinitis in rats. Anti-inflammatory properties and molecular mechanisms of DLC were determined on histamine- and lipopolysaccharide (LPS)-induced EA.hy926 endothelial cells. DLC exhibited significant and reversible inhibition of histamine-induced contractions of isolated guinea pig ileum with pA₂ value of 8.88. Histamine-induced paw edema and increased capillary permeability were notably inhibited by DLC intravenous administration. In the model of PCA reactions, DLC showed significant activity in a dose-dependent and potently inhibited both the early-phase and late-phase allergic reaction of ovalbumin-induced allergic rhinitis in rats. DLC alleviated the rhinitis symptoms and inhibited inflammatory cell infiltration, IL-4 and protein leakage in nasal lavage fluid (NLF). In EA.hy926 cells, DLC significantly inhibited the histamine- and LPS- induced IL-6 and IL-8 production and P-selectin and intercellular cell adhesion molecule-1 (ICAM-1) expression. Moreover, DLC reduced translocation of nuclear factor-kappaB (NF-κB) to the nucleus in activated EA.hy926 cells. These results provide evidence that DLC possesses potent antihistaminic, anti-allergic and, anti-inflammatory properties via suppressing IL-6, IL-8, P-selectin and ICAM-1 expression.

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1. Introduction

Histamine is a primary mediator in the pathogenesis of the allergic disease, including seasonal and perennial allergic rhinitis (SAR and PAR, respectively) and chronic idiopathic urticaria (CIU) (Mygind, 2014; Wedi et al., 2014). Histamine is stored in mast cells and basophils, and released due to immunological or non-immunological stimuli. After its release, histamine reactions are occurred through the mediation of multiple receptors including histamine H₁ receptor, histamine H₂ receptor, histamine H₃ receptor or histamine H₄ receptor. Histamine, acting via H₁ receptor, plays a key role in the induction of early-phase allergic symptoms (EPR) (Cataldi et al., 2014). However, histamine has also been suggested to play an important role in the late phase allergic reaction (LPR) and protracted allergic inflammation. It

has been shown to upregulate the release of cytokines, chemokines and adhesion molecules by inflammatory cells, epithelium and endothelium (Vo and Kim, 2013; Yamauchi et al., 2007). These activities lead to an influx of leukocytes to the site of allergic inflammation, especially influx of eosinophils. In the process of inflammatory cell infiltration, the first step is that the activation of endothelial cells (EC) and then the adherence of leukocytes. Recently, endothelial cells are recognized as important cells in the implication of early and late events of the allergic reaction (Barnes, 2000).

H₁ antihistamines have been the first line treatment of the allergic disease. Recently, H₁ antihistamines are recognized as inverse agonists that combine and stabilize with the inactive conformation of H₁ receptor and, thus, inhibit this constitutive H₁ receptor signaling (Church and Maurer, 2014). First generation H₁ antihistamines, some of which have been in clinical use since the 1940s, provide symptomatic relief of allergic rhinitis and urticaria. However, they are associated with adverse anticholinergic effects and undesirable central nervous system effects. Second generation H₁ antihistamines such as loratadine, astemizole and

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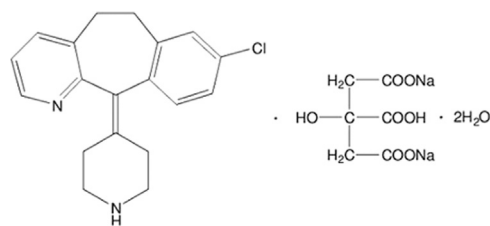


Fig. 1. Chemical structure of desloratadine citrate disodium (DLC).

terfenadine exhibit fewer anticholinergic and sedative effects, but astemizole and terfenadine have been associated with adverse cardiovascular effects (Church and Maurer, 2014). New generation H_1 antihistamines include desloratadine, fexofenadine and levocetirizine. Most of the new therapies for allergic diseases under development are aimed at both relieving early allergic symptoms and inhibiting components of the allergic inflammatory response (Barnes, 2000; Mandhane et al., 2011), including inhibition of eosinophilic inflammation. However, the second generation H_1 antihistamines exhibited weak anti-inflammatory properties and had no effect on nasal congestion (Salmun, 2002).

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 H_1 antihistamine developed for symptomatic treatment of SAR, PAR and CIU for intravenous injection.

The present study describes the pharmacological properties of a potent antihistamine agent, DLC, with respect to its antihistaminic and anti-allergic effects in vitro and in vivo, and anti-inflammatory activities on EA.hy926 endothelial cells. Furthermore, our study elucidates the molecular mechanisms of anti-inflammatory properties of DLC. Chlorpheniramine maleate injection (CHM), a typical antihistamine injection, is used as a positive control drug in the studies of antihistamine and anti-allergic activity.

2. Materials and methods

2.1. Chemicals and reagents

Desloratadine citrate disodium (DLC) was supplied by Hefei Yigong Medicine Co., Ltd. CHM was purchased from Shanghai hefeng Medicine Co., Ltd. (Shanghai, China). Histamine was obtained from Sangon Biotech (Shanghai, China). Ovalbumin (chicken egg, Grade V) and Evans blue were purchased from Sigma (St. Louis, Mo., USA). Aluminum hydroxide gel was bought from Thermo Fisher Scientific, China. Antibodies used in this study were anti-P-selectin (1:5000, Santa Cruz Biotechnology, CA, USA), anti-ICAM-1 (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA) and anti- NF- κ B (1:5000, Cell Signaling Technology, Boston, USA). All other chemicals were of high purity reagent grade. Drug doses were based on clinical and experimental animal doses. Data of pilot studies carried out in our laboratories was also utilized in deciding the doses.

2.2. Animals care and use

All experimental procedures and animal care were performed in accordance with the guidelines issued by the Chinese Council on Animal Research and Care and the Ethics Committee of China Pharmaceutical University. Male Sprague-Dawley (SD) rats (150–200 g), ICR mice (18–22 g) and Male Dunkin–Hartley guinea pigs (300–400 g) were used for different studies and were housed under a temperature and light-controlled environment ($24 \pm 2^\circ\text{C}$ on a 12 h light/dark cycle at 50%–70% humidity). Animals were given free access to standard chow diet and water, except as noted below for fasted animals.

2.3. Antihistaminic activity

2.3.1. In vitro

2.3.1.1. Functional antagonism at H_1 receptor. The Male Dunkin–Hartley guinea pigs were fasted for 24 h, prior to sacrifice. After euthanasia, ileum sections were isolated and immersed in an ice-cold Krebs–Henseleit solution (K–H), containing (mM): NaCl, 120; NaHCO_3 , 20; $\text{MgCl}_2 \cdot 6H_2O$, 1.2; NaH_2PO_4 , 1.56; KCl, 5.5; Dextrose, 10.1; CaCl_2 , 2.5 and EDTA 0.03. Then, Ileum fragment (2–3 cm length) was connected to a force–displacement transducer in a tissue bath with 10 ml K–H solution, maintained at 37°C and continuously aerated with 95% O_2 and 5% CO_2 (Corcostegui et al., 2005). A resting tension of 1.0 g was applied to the ileum and equilibrated with the buffer for 1 h. Changes of isometric tension were recorded continuously on a two-channel physiological recorder (Chengdu Technology Co., Ltd., China). For each ileum fragment, consecutive histamine (1×10^{-9} M to 3×10^{-6} M) cumulative concentration–response curves were obtained. After washing, different concentrations of DLC and CHM (one per fragment) were added to the bath and 30 min later histamine cumulative concentration–response curves were again recorded. DLC was assayed at 2×10^{-9} , 1.2×10^{-8} and 2×10^{-8} M concentrations. CHM was assayed at 1×10^{-8} , 3×10^{-8} and 6×10^{-8} M concentrations. The extent of contraction was estimated from the maximal contraction of the isolated ileum after the addition of histamine alone. The pA_2 (Antagonism parameter) of DLC and CHM were calculated by BL-420 biological function experiment system. For each response, three independent experiments were performed.

2.3.2. In vivo

2.3.2.1. Histamine induced paw edema in mice. Male ICR mice were randomly segregated in five groups (10 mouse/group): control (saline only) group; DLC (1.6 mg/kg) group; DLC (0.8 mg/kg) group; DLC (0.4 mg/kg) group; CHM (0.4 mg/kg) group. Initial left hind paw volume was measured using a volume differential meter (model TM025-1012-02-005-003, Chengdu TME, China). Five minutes after the intravenous administration of drugs, freshly prepared 10 μl histamine (0.05% w/v, in normal saline) was injected into the sub-plantar region of left hind paw and the elevation of paw volume was measured at 15, 30, 45 and 60 min.

2.3.2.2. Histamine induced changes of capillary permeability (CP) in rats. Male SD rats were randomly segregated in five groups (8 rat/group): control group (saline only); DLC (1.2 mg/kg) group; DLC (0.6 mg/kg) group; DLC (0.3 mg/kg) group; CHM (0.6 mg/kg) group. Five minutes later of the drugs or saline intravenous administration, under light anesthesia, two papules were induced by intradermal injection of histamine solution (0.1 ml, 0.1 mg/ml), one on each side of the dorsal midline. Immediately afterwards, Evans blue solution (1% w/v, 2 ml/kg) was injected through the caudal vein. After 30 min, animals were killed by cervical dislocation and the dorsal blue spots were dissected and dissolved as described previously (Katayama et al., 1978). After shaking and centrifugation, the amount of dye leakage in the supernatant was determined colorimetrically at 620 nm. The mean value of the dye leakage for each treated group was compared with the mean value of the control group. The results were recorded as μg per site.

2.4. Anti-allergic effects

2.4.1. Homologous serum induced passive cutaneous anaphylaxis (PCA) reaction in rats

Male SD rats were randomly segregated in five groups (8 rat/group) as described in Section 2.3.2.2. DLC and CHM were intravenously injected 5 min before ovalbumin challenge. Besides the control animals received an intravenous dose of the saline alone, they were also treated

similarly to others. Rat anti-ovalbumin serum was prepared as described previously (Corcostegui et al., 2006; El-Agamy, 2012). After that, prepared rat anti-ovalbumin serum was diluted into four fold and injected intradermally into 2 sites (100 μ l/site) of the shaved dorsal skin of male SD rats. After 48 h, the rats were challenged with 1 ml of saline containing 2 mg of ovalbumin and 5 mg of Evans blue via the caudal vein. After 30 min, the animals were killed by cervical dislocation, and the dorsal blue spots were prepared as mentioned in Section 2.3.2.2. The mean value of dye leakage was determined as mentioned in Section 2.3.2.2. Figures in parenthesis show, % inhibition rate calculated as follows: Inhibition (%) = [(amount of Evans blue without DLC – amount of Evans blue with DLC)/amount of Evans blue without DLC] $\times$ 100.

2.4.2. Ovalbumin induced allergic rhinitis (AR) in rats

2.4.2.1. Sensitization protocols. A procedure for active sensitization was applied as described previously (Mandhane et al., 2010; Sugimoto et al., 2000) with some modification. Male rats were randomly divided into six groups, containing nonsensitized control (NSC) group, sensitized control (SC) group, DLC (0.3 mg/kg) group, DLC (0.6 mg/kg) group, DLC (1.2 mg/kg) group and CHM (0.6 mg/kg) group. Rats were sensitized by intraperitoneal injection of 1 ml of normal saline containing 300 μ g of ovalbumin and 5 mg of aluminum hydroxide gel on days 1, 3, 5, 7, 9, 11 and day 13. Aluminum hydroxide gel was used as an adjuvant. Nonsensitized control rats received suspension of aluminum hydroxide gel without ovalbumin. After seven days of systemic sensitization, overnight-fasted rats were topically sensitized by instilling ovalbumin solution (20 μ l/nostri, 60 mg/ml) into the bilateral nasal cavities using a micropipette. A week 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 (i.d.) injection of ovalbumin solution (200 μ g/ml, 25 μ l).

2.4.2.2. Evaluation of rhinitis symptoms. After the intranasal challenge, 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). Prophylactic treatment of DLC (0.3, 0.6 and 1.2 mg/kg) and CHM (0.6 mg/kg) was performed intravenously once daily for 3 consecutive days. The last treatment was given 30 min before the antigen challenge.

2.4.2.3. Evaluation of biochemical and biophysical parameters (Inflammatory cellular infiltration, total protein and IL-4 level) in nasal lavage fluid (NLF). Two hours after the intranasal challenge, described above, animals were anesthetized by chloral hydrate. Nasal lavage fluid (NLF) was collected using a cannula, which attached to the nasopharynx and connected to a perfusion pump (BT100-2J, LongerPump, China) with a delivery rate of 0.25 ml/min and perfused with Hanks Blanced Salt Solution (HBSS). After the NLF centrifugation (120g for 10 min at 4 $^{\circ}$ C), the cell pellet was suspended in 100 μ l of ice-cold HBSS and Leucocytes number was determined using hemocytometer. 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 Eosinophils/number of Leucocytes $\times$ 100. Furthermore, the supernatant after NLF centrifugation was used for assessing total protein and IL-4 level, using BCA protein assay kit (Beyotime, China) and ELISA kit (Joyee, China), respectively according to the manufacturer's protocol.

2.4.2.4. Histopathological evaluation of nasal mucosa. At the end of aforementioned procedures, rats were killed by an overdose of anesthesia. Nasal cavities were removed 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). The sections were examined under a light microscope to observe the histopathological features of the nasal mucosa.

2.5. Anti-inflammatory effect and its mechanism

2.5.1. Histamine- and LPS-induced activation of EA.hy926 endothelial cells

2.5.1.1. Cell culture. EA.hy926 endothelial cells (provided by department of physiology, China Pharmaceutical University, Nanjing, China) were maintained in Dulbecco's modified Eagle's medium (DMEM; Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, China,) at 37 $^{\circ}$ C in a humidified atmosphere of air/CO₂ (19:1, v/v). Passage was carried out once or twice a week at a ratio of 1:3 and cells between the 20th and 30th passage in an active growing phase were used for the study.

2.5.1.2. MTT assay. MTT assay was used to measure the cell viability as described previously (Kang et al., 2010). In brief, 200 μ l of EAhy926 cell suspension was cultured in 96-well plates for 24 h after treatment with each final concentration of DLC (10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} and 10^{-10} 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 (0 M DLC group) cells was taken as 100% viability.

2.5.1.3. Enzyme-linked immunosorbent assay (ELISA) and Western blot analysis. At confluence, cells were washed with DMEM medium and treated with various concentration of DLC (10^{-8} , 10^{-9} and 10^{-10} M) for 3 h and cells were stimulated in the presence or the absence of histamine (10^{-4} M) and LPS (50 ng/ml) for 10 min (P-selectin expression) (Foreman et al., 1994), 4 h (ICAM-1 expression, NF- κ B p65 expression and the inhibitor of nuclear factor-kappaB α (I κ B α) expression) (Wu et al., 2004) or 24 h (IL-6 and IL-8 secretion) (Liu et al., 2014). Then supernatants were collected accordingly and IL-6 and IL-8 were quantified using ELISA kits (Joyee, China) according to the manufacturer's protocol, respectively. Analogously, the cells were collected on ice and lysed in RIPA lysis buffer for 30 min, followed by centrifugation (14,000g, 20 min) to obtain a cytoplasm fraction. Nuclear protein was prepared using nuclear extraction reagents according to the manufacturer's protocol. Equal amounts of protein were separated on 6% to 12% SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane. After that, followed the instructions of conventional protocol and, the immunoreactive bands were visualized using the electrochemiluminescence detection system (Tannon-5200, Shanghai, China). Band intensities were measured using Quantity One software. The experiment was repeated at least three times.

2.6. Statistical analyses

All experiments were carried out at least in triplicate. pA₂ was calculated by BL-420 biological function experiment system. All other quantitative data were presented with mean $\pm$ standard deviation (mean $\pm$ S.D.). Statistical differences among different groups were assessed by one-way analysis of variance (ANOVA) with Dunnett's multiple comparison tests. A difference with *P*-value less than 0.05 (*P* < 0.05) was considered as statistically significant. Statistical analysis was performed with SPSS 17.0 software (SPSS Inc.).

3. Results

3.1. Antihistaminic activity

3.1.1. In vitro

3.1.1.1. Functional antagonism at H₁ receptor. Desloratadine citrate disodium injection (DLC) was evaluated for histamine H₁ receptor

antagonistic activity on histamine-induced guinea pig isolated ileum. As shown in Fig. 2, DLC inhibited histamine-induced contractions on guinea pig isolated ileum in a concentration-dependent manner as a competitive antagonist. pA_2 values were expressed based on the expression of potency. DLC (pA_2 value of 8.88) was about 3 times more potent than CHM and the positive control drug in the study (pA_2 value of 8.35). The pA_2 is an index of the antagonist, which indicates the intensity of the antagonist. Bigger pA_2 shows a higher affinity of a drug antagonism with a receptor. $pA_2 = ([\log [CR - 1] - \log [B]])$, where CR is the concentration ratio of the EC₅₀ values in the presence and absence of antagonist and [B] is the molar concentration of the antagonist) was calculated by BL-420 biological function experiment system.

3.1.2. In vivo

3.1.2.1. Effect on histamine-induced paw edema in mice. Antihistaminic activity of DLC in vivo was assessed by evaluating the inhibition of paw edema in mice, caused by the intraplantar injection of histamine. As shown in Fig. 3, maximal edema was attained 15 min after the histamine injection. DLC caused dose-dependent reduction in histamine-induced mice paw edema. There was an extremely significant reduction of histamine-induced reaction after the intravenous administration of DLC (0.8 and 1.6 mg/kg), and comparatively less significant effect at the doses of 0.4 mg/kg of DLC and 0.8 mg/kg of CHM than compared with control, $**P < 0.01$, $*P < 0.05$, respectively. Moreover, the inhibitory effects of DLC (0.4–1.6 mg/kg) were persisted more than 1 h. However, the effect of CHM (0.8 mg/kg) gradually declined during the latter minutes of the 1 h, especially after 45 min. Hence, DLC possessed strong inhibitory effect on the edema induced by histamine, and the inhibitory effects of DLC (0.8 and 1.6 mg/kg) were higher than that of CHM (0.8 mg/kg).

3.1.2.2. Effect on histamine-induced increased capillary permeability (ICP) in rats. The effects of antihistaminic activity of DLC on increased capillary permeability induced by histamine in rats were evaluated by dye leakage ($\mu\text{g}/\text{site}$) of the dorsal skin. As shown in Fig. 4, DLC dose-dependently inhibited ICP induced by histamine. Reduction of increased dye leakage by DLC (0.3 mg/kg, 0.6 mg/kg and 1.2 mg/kg) was extremely significant compared with the control ($**P < 0.01$). The positive control agent, CHM (0.6 mg/kg) showed a similar profile with DLC (0.6 mg/kg).

3.2. Anti-allergic effects

3.2.1. Effect on passive cutaneous anaphylaxis (PCA) reaction induced by homologous serum in rats

Passive cutaneous anaphylaxis (PCA), is one of the most important in vivo models of local allergic reaction. We examined the anti-allergic activity of DLC by using the PCA. DLC, which was administered intravenously 5 min before induction of an IgE-dependent PCA reaction in rats, those who were sensitized 48 h previously to the study, showed significant and dose-dependent activity. DLC (0.3, 0.6 and 1.2 mg/kg) inhibited PCA in rats by 47.4%, 59.2% and 68.2%, respectively and CHM (0.6 mg/kg) inhibited PCA by 61.9% (Fig. 5). DLC (1.2 mg/kg) had a much greater anti-allergic effect than that of CHM (0.6 mg/kg). However, DLC at the doses of 0.3 mg/kg and 0.6 mg/kg had a less potent anti-allergic effect than that of CHM (0.6 mg/kg).

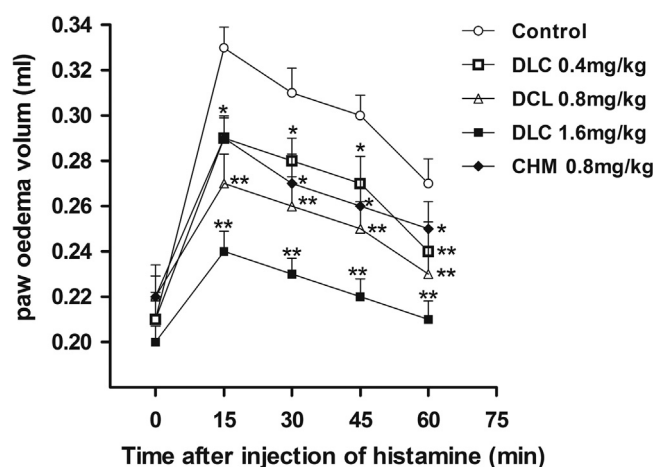


Fig. 3. Effect of DLC on histamine-induced paw edema in mice. DLC (0.4 mg/kg, 0.8 mg/kg and 1.6 mg/kg) and CHM (0.8 mg/kg) were intravenously administered 5 min before the subcutaneous injection of 10 μl histamine (0.05% w/v, in normal saline) in the sub-plantar region of left hind paw. Each value represents the mean $\pm$ S.D. of ten animals. $**P < 0.01$, $*P < 0.05$ compared with control group. $\circ$ control group, $\square$ DLC (0.4 mg/kg) group, $\triangle$ DLC (0.8 mg/kg) group, $\blacksquare$ DLC (1.6 mg/kg) group, $\blacklozenge$ CHM (the positive control drug)(0.8 mg/kg) group.

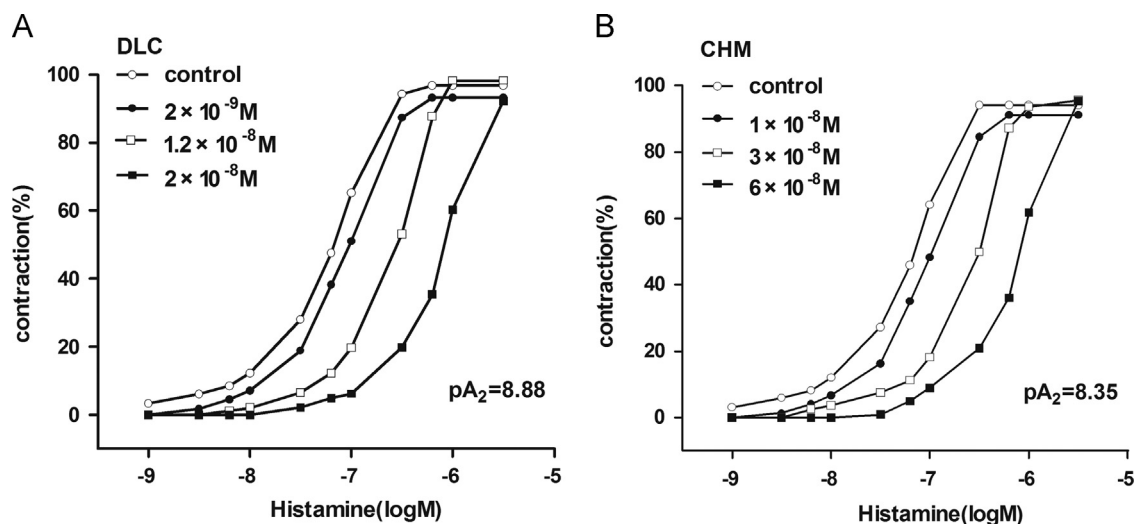


Fig. 2. Effect of (A) desloratadine citrate disodium injection (DLC, $\bullet$ 2×10^{-9} M, $\square$ 1.2×10^{-8} M, $\blacksquare$ 2×10^{-8} M) and (B) chlorphenamine maleate injection (CHM, $\bullet$ 1×10^{-8} M, $\square$ 3×10^{-8} M, $\blacksquare$ 6×10^{-8} M) on histamine-induced contraction ($\circ$ control) on guinea pig isolated ileum. The pA_2 is an index of the antagonist, which indicates the intensity of the antagonist. The pA_2 value was calculated by BL-420 biological function experiment system. For each response, 3 independent experiments were performed.

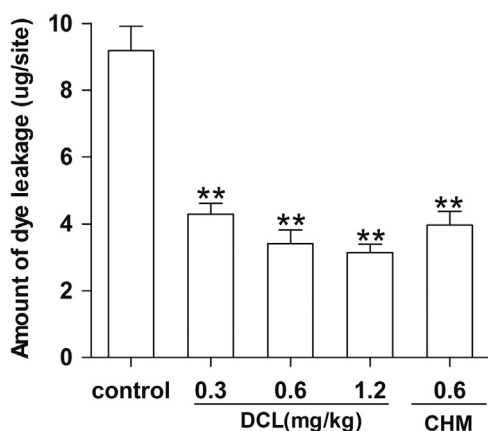


Fig. 4. Effect of DLC on histamine-induced increased capillary permeability in rats. The animals were administered intravenously by DLC (0.3 mg/kg, 0.6 mg/kg and 1.2 mg/kg) and CHM (0.6 mg/kg). 5 min later, rats were injected with histamine (10 µg/site) via the caudal vein. Each column represents the mean ± S.D. of eight animals. *** $P < 0.01$, ** $P < 0.05$ compared with control group.

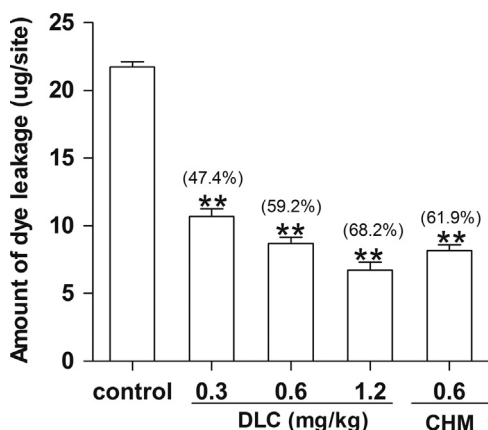


Fig. 5. Effect of DLC on passive cutaneous anaphylaxis (PCA) reaction induced by homologous serum in rats. For experimental details refer to Section 2.4.1. Each amount of dye leakage is presented as mean ± S.D. of eight animals. *** $P < 0.01$, ** $P < 0.05$ compared with control group.

3.2.2. Effect on allergic rhinitis (AR) induced by ovalbumin in rats

3.2.2.1. Effect of DLC on ovalbumin-induced rhinitis symptoms. As shown in Fig. 6, to evaluate the effect of DLC, the number of sneezing and nasal rubbing were counted continuously for 30 min and they were markedly increased by topical ovalbumin challenge, $P < 0.01$. DLC attenuated ovalbumin-induced rhinitis symptoms in a concentration-dependent manner. Moreover, DLC at 0.6 and 1.2 mg/kg doses were strikingly effective than CHM at 0.6 mg/kg dose in reducing sneezing and nose rubbing.

3.2.2.2. Effect of DLC on biochemical and biophysical parameters (inflammatory cellular infiltration, total protein, IL-4) in nasal lavage fluid (NLF). Results are summarized in Fig. 7A–C. Nasal ovalbumin challenge induced a marked inflammatory cellular infiltration and notable elevation of total protein and IL-4 levels in the NLF of sensitized rats, $P < 0.01$. As shown Fig. 7A, the rate of Eosinophils in the Leucocyte increased by 53.68% after ovalbumin challenged. Rats treated with DLC once daily for 3 days showed a marked reduction in Leucocyte and Eosinophil infiltration in a dose-dependent manner. DLC also caused a significant reduction in total protein and IL-4 level. DLC at 0.6 mg/kg and 1.2 mg/kg dose showed higher effects than the standard drug CHM at 0.6 mg/kg dose.

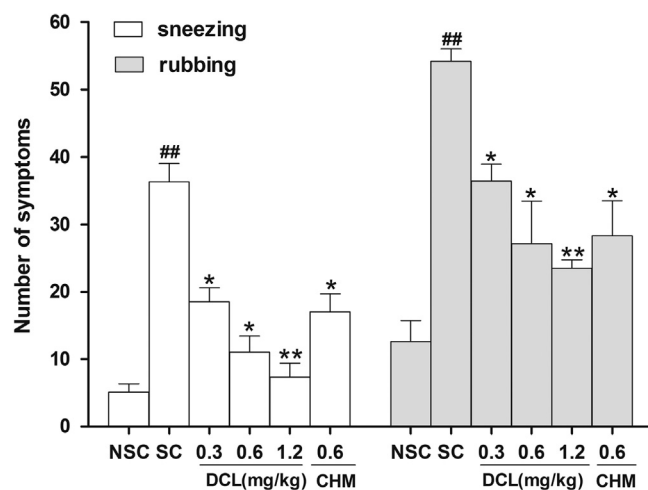


Fig. 6. Effect of DLC on ovalbumin-induced sneezing and nasal rubbing in rats. NSC=nonsensitized ovalbumin challenged control. SC=ovalbumin-sensitized and challenged control. For experimental details refer to Section 2.4.2.1. Each value represents the mean ± S.D. of eight animals. *** $P < 0.01$, ** $P < 0.05$, as compared with sensitized control. $P < 0.01$ and $P < 0.05$, as compared with nonsensitized control.

3.2.2.3. Effect of DLC on leukocyte infiltration in the nasal mucosa: Histopathological evaluation. Ovalbumin challenge in sensitized rats evoked a potent inflammatory cellular infiltration in the nasal mucosa. After ovalbumin challenge, predominant presence of eosinophils was noted in the interstitial tissues between the blood vessels and surface epithelium. In addition neutrophils, lymphocytes and mast cells were also infiltrated to the nasal mucosa of ovalbumin-challenged rats compared with nonsensitized ovalbumin challenged rats. DLC treatment showed a dose-dependent reduction of eosinophils. However, the reductions were most marked at 0.6 and 1.2 mg/kg doses of DLC. Nasal mucosa of DLC-treated rats also showed a significant reduction in lymphocytes and mast cell infiltrations. CHM at 0.6 mg/kg dose caused a mild reduction of eosinophil infiltration. The photomicrographs of sections of nasal mucosa are shown in Fig. 8.

3.3. Study of anti-inflammatory effect and its mechanism on EA.hy926 endothelial cells

3.3.1. Effect of DLC on cell viability

MTT assay was performed for assessing the effect of DLC on cell viability in EA.hy926 cells. As shown in Fig. 9, treatment with DLC at concentrations of 10^{-7} , 10^{-8} , 10^{-9} and 10^{-10} M did not affect in cell viability. Therefore, DLC at the concentration of 10^{-8} , 10^{-9} and 10^{-10} M were used during follow-up experiments.

3.3.2. Effect of DLC on histamine- and LPS- induced cytokines production and, P-selectin and cellular adhesion molecule expression of EA.hy926 cells

Stimulation of EA.hy926 cells with histamine and LPS caused a significant elevation of IL-6 and IL-8 production (Fig. 10A: $P < 0.01$). However, the effect of histamine and LPS on EA.hy926 cells were significantly inhibited by DLC and effectiveness was dose dependent (Fig. 10A: *** $P < 0.01$ in all concentration). Similarly, the exposure of EA.hy926 cells to histamine and LPS also increased the expression of cellular adhesion molecules. The expression of P-selectin and ICAM-1 were enhanced after a stimulation of EA.hy926 cells for 10 min and 4 h, respectively (Fig. 10B and C, $P < 0.01$). When EA.hy926 cells were pretreated for 3 h with the increasing concentrations of DLC (from 10^{-8} to 10^{-9} to 10^{-10} M), DLC potentially inhibited the histamine- and LPS-induced P-selectin and ICAM-1 expressions in a dose-dependent manner (** $P < 0.01$ for 10^{-8} M, * $P < 0.05$ for 10^{-9} M and 10^{-10} M).

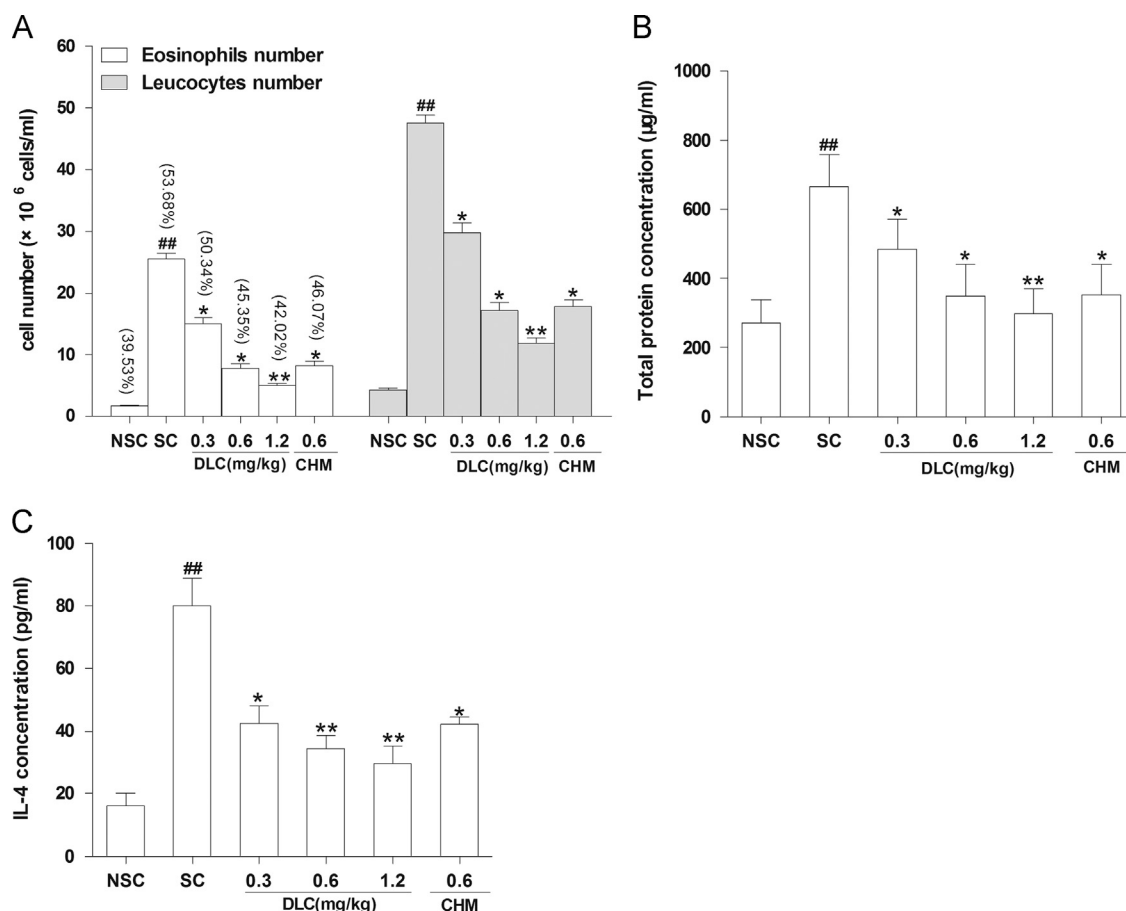


Fig. 7. Effect of DLC on inflammatory cellular infiltration, total protein and IL-4 level in nasal lavage fluid (NLF). NSC=nonsensitized ovalbumin challenged control. SC=ovalbumin-sensitized and challenged control. NLF was collected as described in Section 2.4.2.2. (A) Leucocytes number and Eosinophil number in the NLF were determined using hemocytometer, based on normal morphological criteria after stained with Giemsa. (B) Total protein in the NLF was determined using BCA protein assay kit. (C) IL-4 level in the NLF was estimated using ELISA kit. Each value represents the mean $\pm$ S.D. of eight animals. $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with sensitized control. $^{*}P < 0.01$ and $^{*}P < 0.05$ compared with nonsensitized control.

3.3.3. Effect of DLC on histamine- and LPS-induced nuclear factor- κ B (NF- κ B) activation of EA.hy926 cells

To clarify the anti-inflammatory mechanism of DLC on activated EA.hy926 cells, we performed Western blot to investigate the expression of NF- κ B p65 in EA.hy926 cells. As shown in Fig. 11, treatment with DLC resulted in inhibiting Histamine- and LPS-induced degradation of I κ B α in the cytoplasm and translocation of NF- κ B p65 to the nucleus significantly in a concentration-dependent manner, $^{*}P < 0.05$ (Fig. 11A and B). DLC may suppress NF- κ B activation via reduction of translocation of NF- κ B p65 to the nucleus, which, in turn, could reduce the activation of a variety of genes coding for inflammatory factors, such as IL-6, IL-8, P-selectin and ICAM-1.

4. Discussion

Allergic diseases are complex conditions that histamine is playing a crucial role in the progression of pathogenesis of the illnesses. Desloratadine citrate disodium injection (DLC) is considered as a non-sedating H_1 antihistamine developed for symptomatic treatment of SAR, PAR and CIU for intravenous injection. In order to get a better insight of the DLC in the management of aforesaid conditions we performed our study to evaluate the antihistaminic activity, anti-allergic effects, and anti-inflammatory properties of DLC on EA.hy926 endothelial cells. Furthermore, we elucidated the anti-inflammatory

mechanism of DLC on endothelial cells via its suppressive capability of proinflammatory cytokines.

Allergic diseases are characterized by expression and activation of inflammatory mediators by immune cells (e.g. mast cells, basophils and T cells). Histamine is the most important and abundant mediator during the allergic response. Allergic response is composed of three distinct immunological phases: sensitization, early-phase allergic reaction (EPR), and late-phase allergic reaction (LPR) (Mygind, 2014; Pearlman, 1999). Histamine, acting via H_1 receptor, plays a key role in the induction of early-phase allergic reaction. The symptoms of EPR include smooth muscle contraction, edema formation and mucus hyper secretion, etc.

In the present study, DLC seemed to exert an antihistaminic activity and was notably more potent than chlorpheniramine maleate injection (CHM), a typical antihistamine reference injection drug. In guinea pig ileum, the pA_2 value for this effect was comparable to that of CHM. In contrast, DLC (pA_2 value of 8.88) exhibited significant and reversible H_1 receptor antagonistic activity with superior potency to CHM (pA_2 value of 8.35). In mice, the maximal edema was reached 15 min after histamine injection. However, the edema was suppressed by intravenous DLC in a dose-dependent manner and the inhibitory effect of DLC (0.4–1.6 mg/kg) was persisted more than 1 h. However, the effect of CHM (0.8 mg/kg) gradually declined during the later minutes, especially after 45 min. Hence, DLC possessed strong inhibition of edema induced by histamine, and the inhibitory effects of DLC (0.8 and 1.6 mg/kg) were

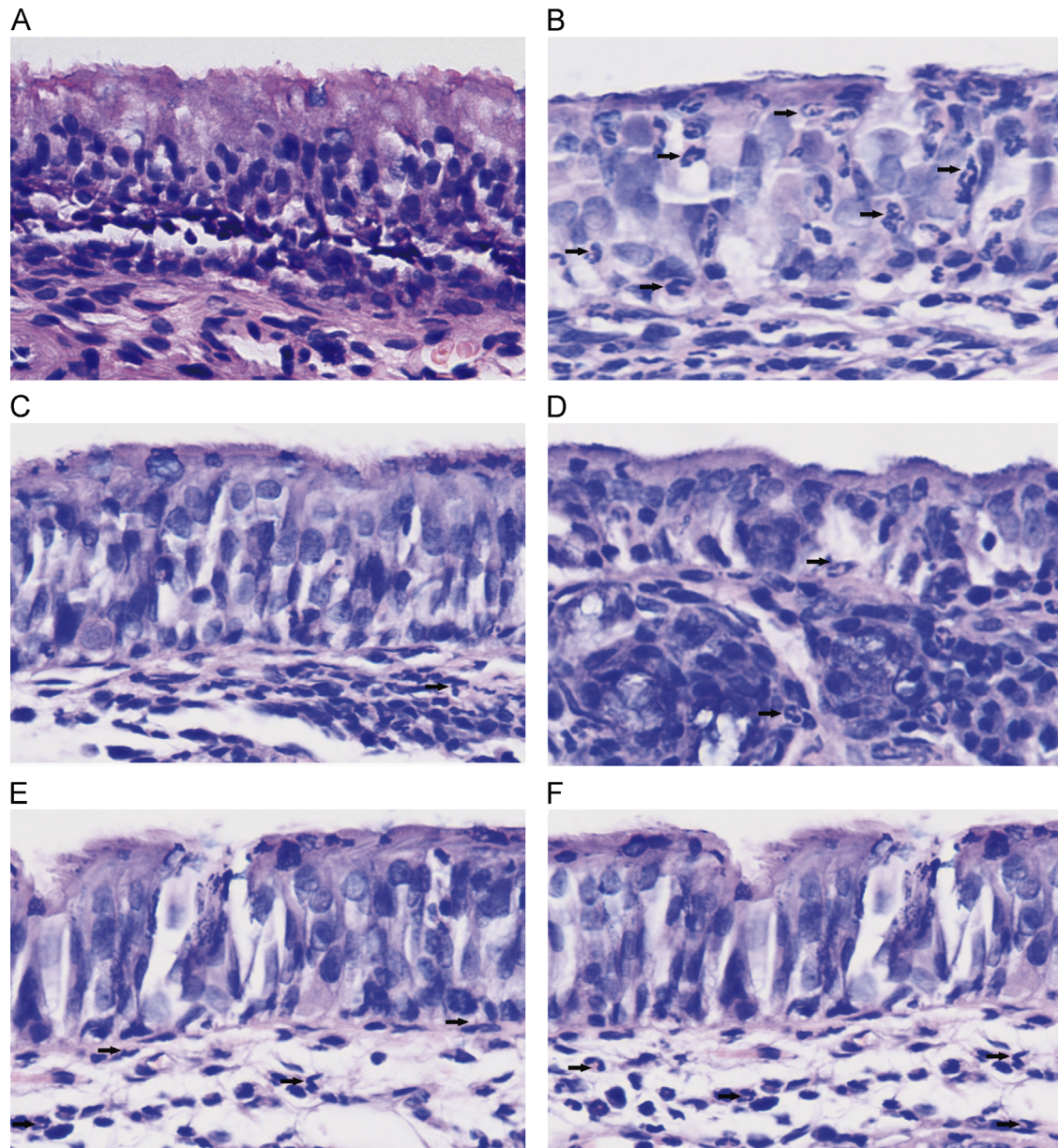


Fig. 8. Light photomicrographs of hematoxylin-eosin (H&E) stained sections of nasal mucosa from the nonsensitized (A) and sensitized rats (B) after ovalbumin challenge. Leukocytes were not observed in nasal mucosa of nonsensitized rats while there was a remarkable infiltration of leukocytes, particularly eosinophils in sensitized rats. Results shown in these images indicated reduced infiltration of eosinophils, which were pretreated with DLC 1.2 mg/kg (C), DLC 0.6 mg/kg (D), DLC 0.3 mg/kg (E) and CHM 0.6 mg/kg (F) by intravenous administration, once daily for 3 days. Original magnification $\times 400$.

higher and longer than that of CHM (0.8 mg/kg). Moreover, DLC was able to reduce the increase of dye leakage significantly compared with the control group of the guinea pig model of increased capillary permeability induced by histamine. These results demonstrated the effectiveness of antihistaminic action of DLC in vitro and in vivo.

In addition to histamine, other inflammatory mediators (e.g. prostaglandins, kininogenase and leukotrienes) also play important roles in the EPR (Howarth, 2000; Skoner, 2001). These mediators including histamine are collectively responsible for increasing the vascular permeability and local blood flow, and stimulating excessive secretion of mucus. In allergic rhinitis, this process is manifested as a rapid onset of sneezing, itching, rhinorrhoea and nasal obstruction. In ovalbumin-induced allergic rhinitis rat model, the frequency of sneezing and nasal rubbing behaviors were markedly increased by topical ovalbumin challenge, but DLC attenuated the rhinitis symptoms in a concentration-dependent manner. Moreover,

DLC at 0.6 and 1.2 mg/kg doses were strikingly effective than CHM at the 0.6 mg/kg dose. DLC also reduced the release of prostaglandin D2 (PGD2) and leukotriene C4 (LTC4) in ovalbumin sensitized and challenged rat serum (data not shown). It is suggesting that, DLC could be able to reduce the early phase reaction of allergic response.

Late-phase allergic reactions (LPR) are noted approximately 4–6 h following the EPR and, are characterized as a cellular inflammatory response (Walsh, 2000). These responses result in the accumulation of inflammatory granulocytes (such as eosinophils, neutrophils, basophils and mononuclear cells) (Ciprandi et al., 2005). Of these cells, eosinophils are considered as hallmark of allergic inflammatory diseases. Histamine is also recognized to play an important role in the LPR and protracted allergic inflammation (Akdis and Simons, 2006). Histamine has been suggested to upregulate the release of cytokines (such as IL-6, IL-8), chemokines (such as Monocyte chemoattractant protein-1 (MCP-1), Monocyte chemoattractant protein-3

(MCP-3), Regulated on activated normal T expressed and secreted (RANTES) and eotaxin) and adhesion molecules (such as P-selectin, E-selectin, ICAM-1 and Vascular cell adhesion molecule-1 (VCAM-1)) via inflammatory cells, epithelium and endothelium (Cataldi et al., 2014; Church and Maurer, 2014; Igaz et al., 2001). These activities lead to an influx of leukocytes to the site of allergic inflammation, especially influx of eosinophils (Barnes, 2000; Okamoto et al., 2009). In ovalbumin-induced allergic rhinitis rat model, NLF of rats treated with DLC once daily for 3 days showed a marked reduction in Leucocytes and Eosinophils infiltration. IL-4 has been suggested to be associated with eosinophil recruitment (Bahekar et al., 2008; Okamoto et al., 2009). NLF total protein contains serum proteins, such as albumin, which provide the evidence for nasal vascular permeability in the animals challenged with ovalbumin were increased (Mandhane et al., 2010). DLC also caused a significant reduction in

total protein and IL-4 level. DLC at 0.6 mg/kg and 1.2 mg/kg doses showed better effects than the standard drug CHM at 0.6 mg/kg dose. Moreover, according to Histopathological Evaluation of the nasal mucosa, DLC treatment showed a dose-dependent reduction of eosinophil infiltration. However, the reduction was most marked at 0.6 and 1.2 mg/kg doses of DLC. Nasal mucosa of DLC-treated rats also showed a significant reduction in lymphocytes and mast cell infiltrations. CHM at 0.6 mg/kg dose caused a mild reduction of eosinophil infiltration. These results revealed that DLC could be able to reduce the late phase reaction of allergic response.

Passive cutaneous anaphylaxis (PCA), induced by an injection of IgE and antigen, is one of the most frequent *in vivo* models of allergic inflammation characterized by formation of local edema at the site of antigen challenge. Our results have shown significant, moderate-to-potent and dose-dependent activity with the intravenous administration of DLC 5 min before induction of an IgE-dependent PCA reactions in rats who were sensitized for 48 h. These results revealed that DLC possessed anti-allergic properties and effectiveness of it was dose dependent.

Recently, endothelial cells have been recognized as important cells implicated in the allergic response in addition to its well-recognized function as a selective barrier between blood and tissues. Activated endothelial cells were participated in early and late events of the allergic reaction. In the LPR, endothelial cells express adhesion molecule (such as E-selectin and ICAM-1) and secrete several proinflammatory mediators (such as IL-6, IL-8 or MCP-1). The proinflammatory mediators and adhesion molecule are of special importance as they are implicated in inducing inflammatory responses and recruiting eosinophils, neutrophils and monocytes. Moreover they are leading to the adhesion and further transvascular migration of leucocytes. LPS is able to amplify the inflammatory effect of the histamine-induced endothelial cells. The co-stimulation of histamine and LPS on endothelial cells *in vitro* probably reflected its *in vivo* activity at the inflammatory sites. As shown in a previous report (Li et al., 2001), histamine and LPS are able to enhance IL-6 and IL-8 secretion by

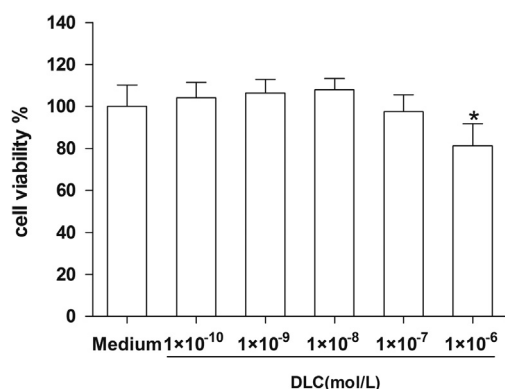


Fig. 9. Effect of DLC on cell viability in EA.hy926 cells. Cells were treated with various concentration of DLC (10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} and 10^{-10} M) for 24 h and followed by determination of cell viability using the MTT assay. Each value was expressed as means $\pm$ S.D. of three independent experiments. ** $P < 0.01$, * $P < 0.05$, compared with Medium group (0 M DLC).

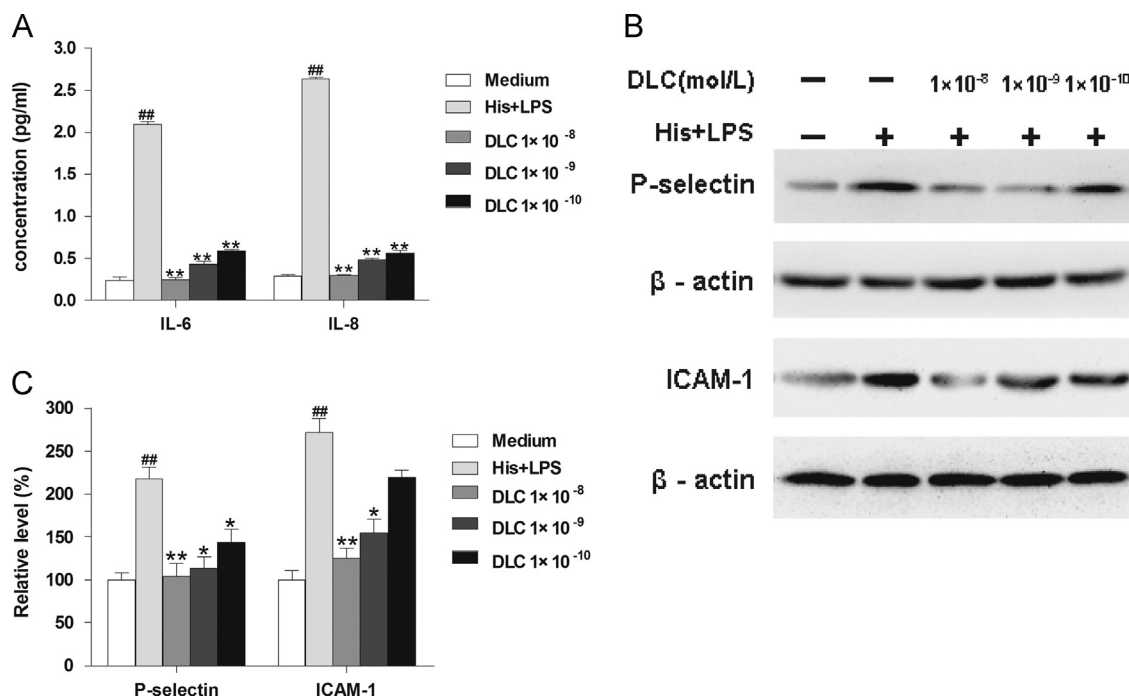


Fig. 10. Effect of DLC on expressions of cytokines and cellular adhesion molecule on EA.hy926 cells induced by histamine and LPS. EA.hy926 cells were pretreated as described in Section 2.5.1.3. (A) IL-6 and IL-8 were measured in the supernatants using ELISA kits, which were obtained 24 h after the stimulation. (B) The protein expression of P-selectin and ICAM-1 were assessed by Western blot after stimulation for 10 min and 4 h, respectively. β -actin was used as the internal controls. (C) Relative level (%) was calculated as the ratio of P-selectin or ICAM-1 expression to β -actin expression. Data are expressed as means $\pm$ S.D. ($n=3$). $P < 0.01$, $P < 0.05$ compared with Medium (0 M DLC), ** $P < 0.01$, * $P < 0.05$ compared with His+LPS alone.

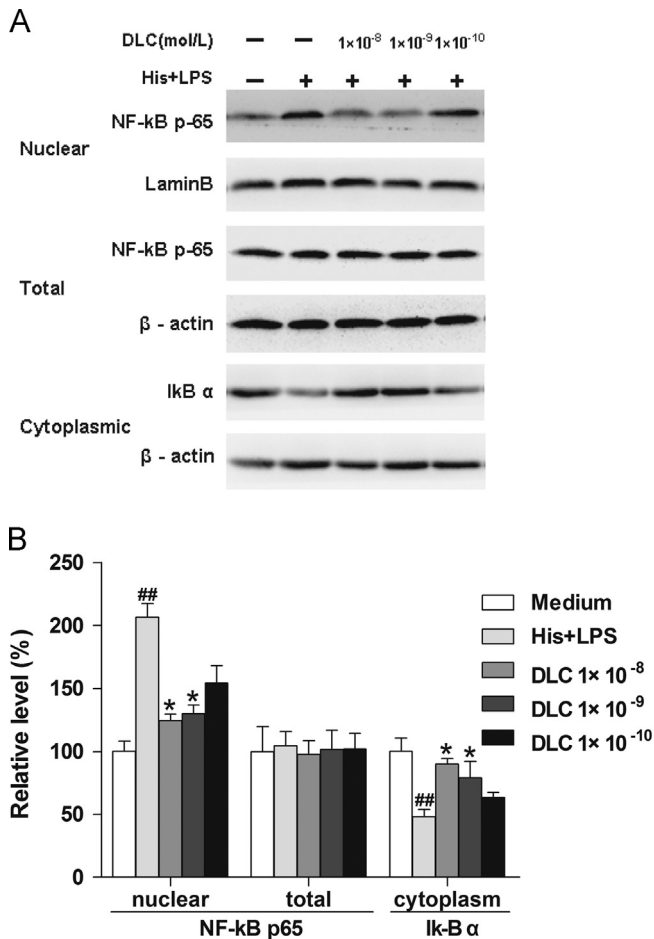


Fig. 11. Effect of DLC on nuclear factor-kappaB (NF-κB) of EA.hy926 cells induced by histamine and LPS. EA.hy926 cells were pretreated as described in Section 2.5.1.3. (A) Nuclear protein, cytoplasmic protein and total protein were prepared and analyzed for NF-κB p65 and IκBα by Western blot as described in Section 2. Lamine B and β-actin were used as internal controls, respectively. (B) Relative level (%) was calculated as the ratio of NF-κB expression to β-actin or Lamine B expression and IκBα expression to β-actin expression. Data are expressed as means ± S.D. (n = 3). $P < 0.01$, $P < 0.05$ compared with Medium (0 M DLC), $^{***}P < 0.01$, $^{*}P < 0.05$ compared with His+LPS alone.

endothelial cells. This observation was confirmed in this study. In the studies of EA.hy926 cells, a fusion of human umbilical vein endothelial cells (HUVEC) and the permanent lung carcinoma cell line A549 cells, induced by histamine and LPS, DLC potently inhibited the histamine- and LPS-induced IL-6 and IL-8 production and P-selectin and ICAM-1 expression.

Although histamine and LPS are known to induce proinflammatory cytokine production and cellular adhesion molecule expressions through endothelial cells, the inhibitory mechanism of DLC has not been established. Previous reports discover that antihistamines down-regulate the activation of nuclear factor-kappaB (NF-κB) to inhibit inflammatory cell accumulation and activation (Matsubara et al., 2005; Vena et al., 2008), which may explain for most of these extra anti-inflammatory activities. Our results were in agreement with the previous reports. Heterodimers composed of p50 and p65 subunits and homodimers composed of p50 subunits are the key forms of the NF-κB, that exist in the cytoplasm as a dormant complex integrated with the particular inhibitor, IκB. Phosphorylation and subsequent degradation of IκB result in the translocation of NF-κB to the nucleus. The exposure of EA.hy926 cells to histamine and LPS for 4 h substantially increased the translocation of NF-κB p65 proteins to the nuclei. In our studies, treatment with DLC resulted in a significant inhibition of histamine- and LPS-induced degradation of IκBα in the

cytoplasm and suppressed the translocation of NF-κB p65 to the nucleus in a concentration-dependent manner. Hence, DLC could reduce the translocation of NF-κB p65 to the nucleus, which, in turn, could diminish the activation of a variety of genes coding for inflammatory factors, such as IL-6, IL-8, P-selectin and ICAM-1.

Overall, these preclinical studies provide evidence that DLC possesses potent and long-acting antihistaminic activity and anti-allergic effects with a superior potency to CHM. Moreover, DLC not only blocks the H₁ receptor, but also prevents the proinflammatory cytokine production and cellular adhesion molecule expressions by endothelial cells, contributes to its anti-allergic activity. Therefore in conclusion, it is noteworthy that DLC may suppress NF-κB activation via reduction of translocation of NF-κB p65 to the nucleus, which, in turn, could reduce the activation of a variety of genes coding for inflammatory factors, such as IL-6, IL-8, P-selectin and ICAM-1.

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