

HEPARIN PROTECTS BALB/c MICE FROM MITE-INDUCED AIRWAY ALLERGIC INFLAMMATION

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More and more studies have demonstrated the anti-inflammatory effects of heparin. However, in the aspect of allergic airway inflammation, data about its daily use in animal model is scarce. To evaluate the efficacy of 22-day intranasal heparin administration in mite-induced airway allergic inflammation in BALB/c mice, the murine model of house dust-mite allergen-induced asthma was used to assess the effect of heparin (h) and low molecular weight heparin (l mwh) administered intra-nasally (IN) throughout the full study period (22 days). Effects were monitored by histopathology, cell counts in broncho-alveolar lavage fluid (BALF), local cytokine production, serum, specific antibody levels, and airway resistance measurements. Compared to the positive control group, both hIN and lmwhIN groups had lower peri-bronchiolar/alveolar inflammatory pathology score and lower goblet cell scores ($p < 0.01$); lower eosinophil and neutrophil counts in BALF ($p < 0.0001$); and lower cytokine levels including IL-17A/F, IL-5, IL-13, IL-8 and eotaxin in lung tissue ($p < 0.001$). Serum Der p-specific IgE level was also lower in heparin-treated groups ($p < 0.004$). The two heparin-treated groups also revealed lower value of Penh after Mch stimulation. In conclusion, heparin and lmw heparin decrease serum Der p-specific IgE level and possess anti-inflammatory effects on mite-induced airway allergic inflammation model in BALB/c mice.

Besides anti-coagulation, heparin has been demonstrated to possess abundance of anti-inflammatory effects (1-5). Heparin and its derivatives inhibit complement activation (1), adhesion molecular expression (2), neutrophil function (3), and NF- κ B activation (4). Celasco et al. also showed the extended colon-release heparin tablet improved ulcerative colitis histologically (5).

Bronchial asthma is a chronic disease characterized

by airway obstruction, airway allergic inflammation, and enhanced bronchial responsiveness. The pathology in asthma occurs as a consequence of increased production of interleukin (IL)-4, IL-5 (6), and IL-13 (7) by allergen-specific CD4⁺ T helper (Th)2 cells. In addition, the risk of developing asthma is directly related to the acquisition of immediate hypersensitivity to environmental allergens (8).

Several studies have involved the anti-

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inflammatory effects of heparin and its derivatives on allergic airway inflammation (9-13). The studies were on short-term effects, usually one inhalation before challenge, such as allergen, exercise or methacholine. To the best of our knowledge, data on the mid- to long-term use of inhalation heparin in asthma patient or animal model is very limited. We therefore initiated this 23-day study including airway and lung histopathology, cytokine secretion, and mite-specific IgE level, to evaluate the efficacy of heparin administered intra-nasally on mite-induced airway allergic inflammation in BALB/c mice.

MATERIALS AND METHODS

Heparin

Heparin was purchased from China Chemical and Pharmaceutical company (Taipei, Taiwan). Each ml contains 1000IU heparin. Low molecular weight heparin (1 mwh) was from Sanofi Winthrop Industry (Maisons-Alfort Cedex, France). Each ml contains 1 mwh 100 mg, which is equivalent to 10,000 anti-Xa IU.

Animals

A total of 48 male BALB/c mice (6–8 weeks of age) were purchased from National Laboratory Animal Center (Nangang, Taipei, Taiwan) and were fed a commercial diet and water.

Study protocol

The mice were divided into four groups for 3 repeated experiments: i) control, PBS intranasal (IN) (number, n=10); ii) mites delivered intratracheally (mIT) alone (n=10); iii) mIT+ heparin intranasal (hIN) (n=14); and iv) mIT+ low molecular weight heparin intranasal (lmwhIN) (n=14). All groups receiving mIT were immunized with mite crude extract allergen on days 1 and 8 and then given mite crude extract allergen intratracheally on day 15. The control, mIT+hIN and mIT+lmwhIN groups received PBS IN, hIN and lmwhIN, respectively, from day 1 to day 22. All mice were sacrificed on day 23. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC Approval No: La-95278).

Mite protein preparation

Crude extract of mite *Dermatophoides p* (Der p) was purchased from Greer Lab (Lenior, NC, USA). The mite protein was isolated from the crude extract by homogenization with phosphate-buffered saline (PBS) using a glass homogenizer (Kontes Glass Company, Vineland, NJ, USA). The mite protein concentration

was then determined using the Bradford assay (Bio-Rad Protein Assay; Bio-Rad, Hercules, CA, USA).

Immunization

Der p (1.6 mg/ml) was prepared and emulsified in complete Freund's adjuvant (Sigma, St Louis, MO, USA) at a ratio of 1:1 [(v/v)] at 4°C. All groups except control were immunized subcutaneously with 50 µl (40 µg) emulsified Der p on days 1 and 8.

Intratracheal administration of mite protein

Der p (5 mg/ml) dissolved in PBS was given to each mouse on day 15. The mouse was placed in an upright position with its neck extended, and then 10 µl extract was directly administered to its trachea. Each mouse was anesthetized by inhaling isoflurane for ~1 min.

Intranasal administration of heparin and low molecular weight heparin

Ten micro-liter of PBS, heparin or 1 mw heparin was then administered intranasally daily. Heparin was in the saline solution at a final concentration of 0.8IU/µL; and low molecular weight heparin was 0.0015µg/µL. No anesthesia was used for IN.

Histopathological analysis

The formalin-fixed lung sections stained with hematoxylin and eosin (H&E) were assigned a unit value for alveolar, peri-bronchiolar, and total inflammation by computing the means of three independent scores for three randomly selected fields. Each section was independently and blindly interpreted by two physicians. We followed a previous scoring system for airway inflammation (14). We also looked for histopathological changes in the heart, intestine, kidney, spleen, and liver in these mice.

For evaluating the hyperplasia goblet cells, medium-sized airways were assessed in sections stained with Periodic Acid-Schiff (PAS) stain. Two reviewers independently and randomly scored 10 fields of each slide. Each lung was assigned a unit by computing the mean of the numerical scores. The numerical scores for the abundance of PAS-positive goblet cells (15) in each airway were determined as follows: 0, <5% goblet cells; 1, 5–25%; 2, 25–50%; 3, 50–75%; 4, 75%.

Bronchoalveolar lavage

The trachea was exposed and cannulated. Two washings, each consisting of 1 ml of PBS, were introduced into the lungs via the cannula and withdrawn to collect the cells. Bronchoalveolar lavage fluid (BALF) from each wash was placed in polypropylene tubes on ice. The collected BALF was then centrifuged at 2,000 revolution/min for 7 min at 4°C. After supernatant removal, cells were re-suspended in 1 ml of phosphate-

buffered saline (pH 7.4). Total cell numbers were counted on a hemocytometer, and $1-5 \times 10^3$ cells were spun onto glass microscope slides (cytospin 3; Shandon Scientific, Cheshire, UK). The cell slides were air dried for 24 h, fixed, and stained with Wright stain. Differential cell counts of at least 300 cells per slide were made according to morphological criteria. The number of cells recovered was calculated and expressed as absolute cell numbers.

Measurement of Der p-specific IgE

All BALB/c mice were sacrificed on day 23, and blood was obtained from the inferior vena cava. The serum Der p-specific IgE was measured by enzyme-linked immunosorbent assays (ELISAs). We coated the plates with mite crude extract allergen (50 µg/ml), after the wells were blocked with 10% FCS-PBS, mouse serum were added. IgEs were detected by biotinylated anti-mouse IgE mAb (BD Biosciences, San Diego, CA, USA) as previously described (15).

Preparation of lung tissue supernatant

The left lung was homogenized in 1 mL of cold phosphate buffered saline and preserved in an iced bath before use. Lung homogenates were centrifuged at 20,000 g for 5 min at 4°C. The lung tissue homogenate supernatants were then diluted in PBS to a final protein concentration of 500 µg/ml and stored at -80°C.

ELISA

The levels of IL-5, IL-10, IL-13, IL-17E, IL-17A/F, matrix metalloproteinase 9 (MMP9), eotaxin and interferon-γ (IFN-γ) in lung protein extract were examined using BD OptEIA™ Set Mouse IL-5, IL-10, IL-13, and IFN-γ (BD Bioscience, San Diego, CA, USA) and DuoSet mouse eotaxin, IL-17E, IL-17A/F, and MMP 9 kits (R&D, Minneapolis, MN, USA). The microplates were read at 450 nm with an ELISA reader (Thermo Labsystems, Waltham, MA, USA).

Measurement of pause enhancement (Penh)

Airway responsiveness was measured in mice on day 22 (*i.e.*, 1 hour after the last IN treatment) in conscious, spontaneously breathing mice using a whole-body plethysmography system (Buxco, Wilmington, NC, USA) as described previously (16). Briefly, mice were individually placed in the chamber and allowed to settle for 3–5 min. The chamber-pressure-time wave was continuously measured via a transducer connected to a computer data acquisition system. After a baseline Penh reading for >3 min, mice were serially exposed to increasing concentrations of nebulized methacholine (Mch) (0, 6.25, 12.5, and 25 mg/ml; Sigma, St Louis, MO, USA) in PBS for 1 min by inhalation. Penh values, which

are measured as changes in enhanced pause, tidal volume, and breathing frequency (breaths/minute) for the first 3 min after the end of MCh nebulization, were averaged and used to compare responses among all groups.

Measurement of coagulation-bleeding time (BT) and prothrombin time (PT)

We performed mice bleeding time on day 23 before scarification by a shallow cut on their tail, then gently wiped the bleeding site by cotton ball every 30 s until the site was totally dry. The prothrombin time was measured by a Coagulex 100 autoanalyzer (International reagent Co., Kobe, Japan).

Statistical analysis

Data were expressed as the mean ± standard deviation (SD). Analysis was performed with the Mann-Whitney U-test for comparison of two groups. Differences with a *p* value <0.05 were considered significant. Analysis was performed using the Statistical Package for the Social Sciences (version 10.1; SPSS, Chicago, IL, USA).

RESULTS

Histopathologic changes in lung tissues

To determine whether suppression of airway cellular inflammation was representative of a generally more-improved pathology, H&E staining of lung histological sections were compared among the four groups of mice in Fig. 1, A–D. The score of peri-bronchiolar and alveolar inflammation were shown in Fig. 1, E and F. We found layers of cell infiltration in the peri-bronchial and alveolar area in the lungs of the mIT group. There were significantly lower peri-bronchiolar and alveolar scores in mIT+hIN group ($p=0.007$ and $p=0.04$, respectively), and even lower scores in mIT+lmwhIN group ($p<0.0001$ and $p=0.007$, respectively) than in the mIT group. The PAS stain and score of goblet cell hyperplasia were shown as Fig. 2 A to D and 2E. Both heparin-treated groups showed lower goblet cell hyperplasia score, especially the lmwhIN-treated group ($p=0.00013$).

BALF cell count

As shown in Fig. 3, the mIT group did induce higher BALF eosinophil, neutrophil and lymphocyte counts than in the control group. The eosinophil counts were significantly lower in the hIN and lmwhIN-treated groups than in the mIT group (both $p<0.0001$). Neutrophil count showed similar results

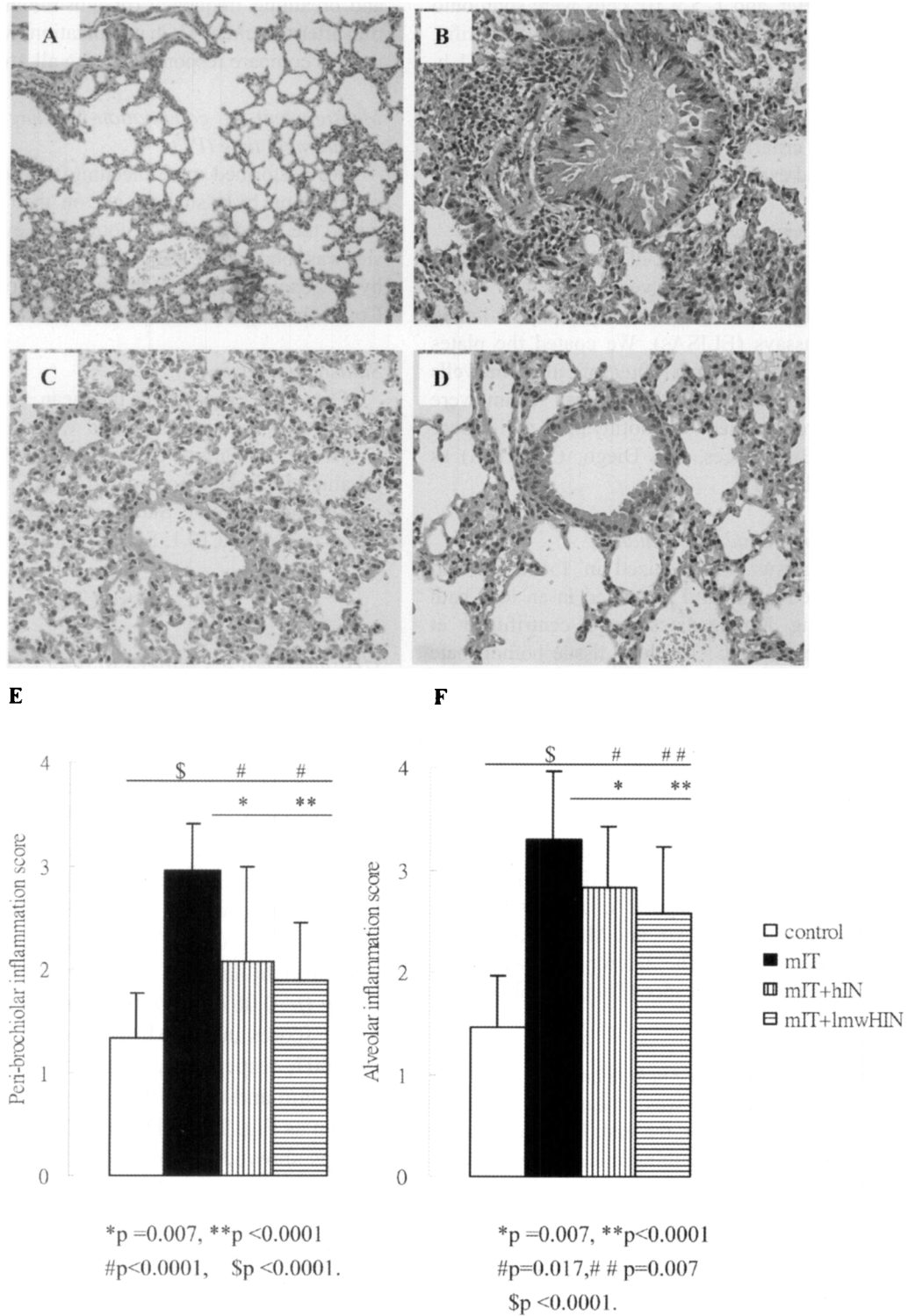


Fig. 1. Histopathology of lung tissues taken from the four groups (H&E stain): **A)** control; **B)** mIT; **C)** mIT+hIN; and **D)** mIT+lmwHIN. Scores expressed as mean±SD for peri-bronchiolar (**E**) and alveolar (**F**) inflammation were determined based on the histological sections from three independent experiments.

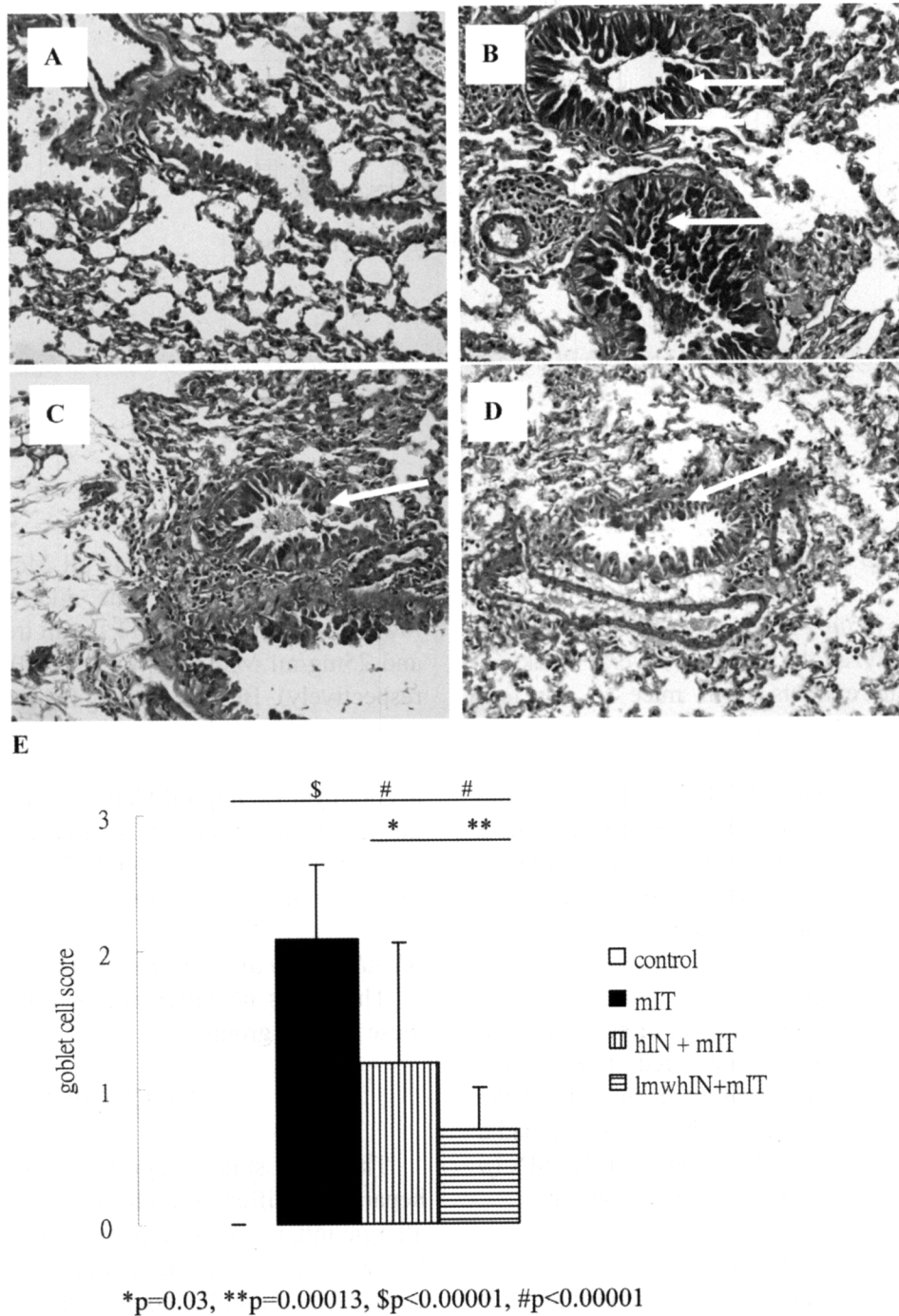


Fig. 2. Histopathology of lung tissues taken from the four groups (PAS stain). Positive areas of PAS stain were marked as white arrows. A) control; B) mIT; C) mIT+hIN; D) mIT+lmwhIN. Scores expressed as mean±SD for goblet cell stain (E) were determined based on the histological sections from three independent experiments.

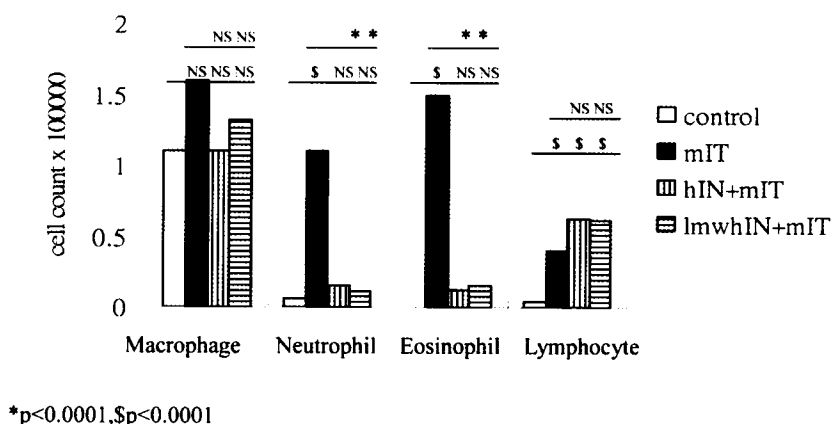


Fig. 3. Cell counts of bronchoalveolar lavage fluid in the four groups. Mean±SD values from three independent experiments are reported.

in these groups ($p<0.0001$).

Cytokine levels in lung protein extract

We next analyzed the levels of several cytokines in lung protein extracts from mice in the four treatment groups. As shown in Figs. 4A and 5B, there were no differences in MMP9, IFN- γ and IL-17E. The mIT+hIN and mIT+lmwhIN groups both had lower concentrations of IL-10, IL-13, IL-5, IL-8 and IL-17A/F than the mIT group. As to eotaxin, mIT+lmwhIN ($p=0.025$), but not mIT + hIN group had lower concentration than did the mIT group.

Mite-specific serum IgE level

To further analyze the inhibitory effect of heparin on mite sensitization, we measured mite-specific Der p IgE optical density values in mice from the four treatment groups (Fig. 5). The mIT group had a higher IgE reading than did the control, mIT+hIN ($p=0.0004$), and mIT+lmwhIN ($p=0.004$) groups.

Changes in Penh after Mch stimulation

The changes in Penh in these four groups after stimulation with increasing concentrations of Mch are shown in Fig. 6. The mIT group showed a much higher Penh/baseline ratio change after stimulation with 6.25, 12.5 and 25 mg/ml Mch than mIT+hIN group ($p\leq 0.007$). The mIT+lmwhIN group showed lower stimulation than mIT group

treated with 12.5 and 25mg/ml Mch ($p=0.009$ and $p=0.001$, respectively). The mIT+hIN group had lower stimulation than mIT group treated 6.25, 12.5 and 25mg/ml Mch ($p=0.025$, <0.000001 and 0.003 , respectively). Besides, the mIT+hIN group showed even lower Penh than control at 6.25 and 25mg/ml Mch stimulation ($p=0.02$); and no difference in Pneh to control at 12.5mg/ml Mch stimulation. Comparing the treatment groups, hIN+mIT group had lower Penh at 6.25 and 12.5mg/ml Mch stimulation than hIN+lmwmIT group ($p=0.02$).

Bleeding time and Prothrombin time

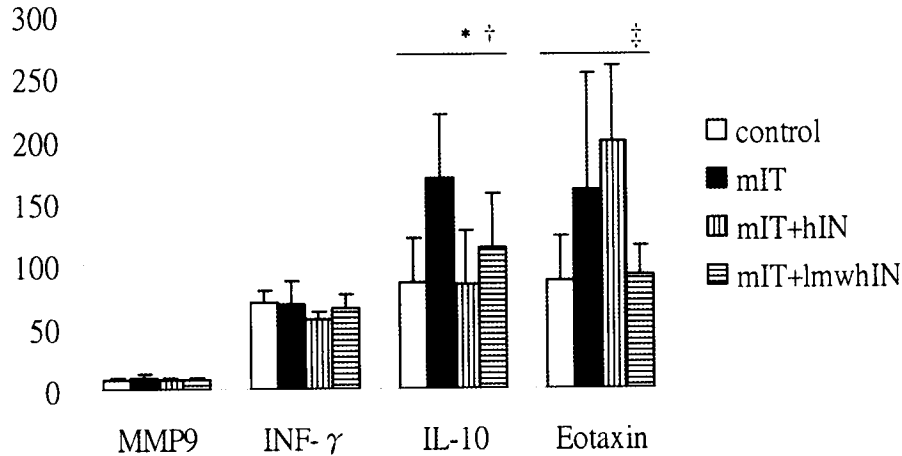
There was no difference in BT or PT between these 4 study groups.

DISCUSSION

This pilot study suggests a protective effect of heparin of different molecular weight on airway allergic inflammation in mite-treated BALB/c mice. Heparin decreased the pathology scores; BALF eosinophil/neutrophil count, serum Der p-specific IgE levels; and cytokines including IL-5, IL-10, IL-13, IL-17A/F, and eotaxin in lung protein extract. The Penh value stimulated by Mch also decreased in the presence of heparins.

Heparin is a highly acidic polymer and its biological effects depend on both specific and

A



B

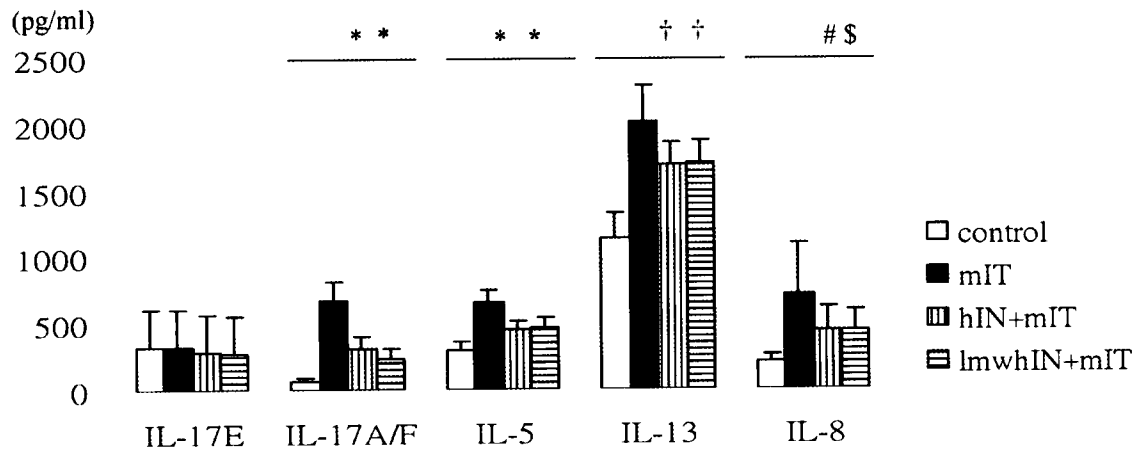
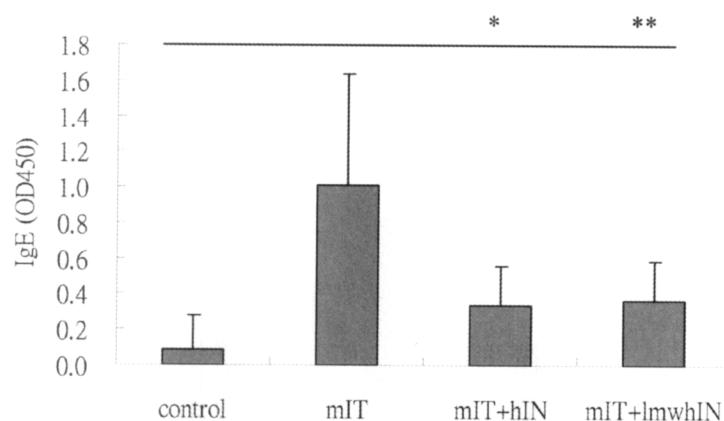
* $p < 0.0001$, † $p = 0.003$, ‡ $p = 0.025$ * $p < 0.000001$, † $p < 0.0001$, # $p = 0.03$, \$ $p = 0.026$

Fig. 4. Levels of MMP9, IFN- γ , IL-10, eotaxin (A) and IL-17E, IL-17A/F, IL-5, IL-13 and IL-8 levels (B) in lung protein extracts from mice from the four groups. Mean $\pm$ SD values from three independent experiments are reported.

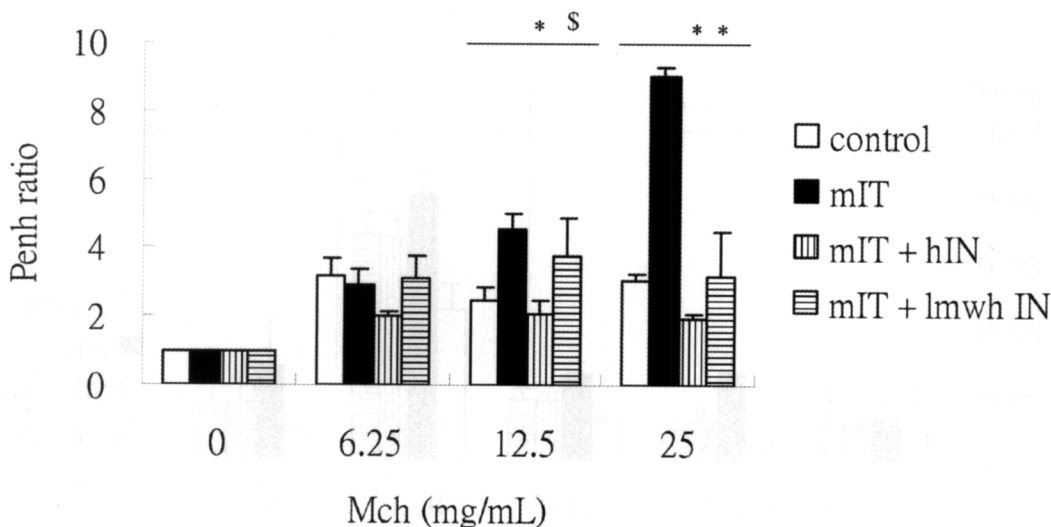
nonspecific ionic interactions that are mediated by sequence composition, charge density, charge distribution and molecular size. In a previous study, we demonstrated internalization of ECP to Beas-2B cells via an HS-facilitated, non-clathrin, non-caveolin macropinocytic route; and both high and low molecular weight heparin can hinder such binding (17). Therefore, heparin hinders the

macropinocytosis of cationic proteins secreted by eosinophils and other inflammatory cells, consequently blocking the cytotoxic effects of these proteins. According to the study by Clark et al. (18), degranulation of eosinophils primarily occurs in the airway lumen, which then induces AHR. The effect of heparin on AHR induction was shown in this study by a decrease in Penh after Mch stimulation.



*p=0.0004, **p=0.004

Fig. 5. IgE levels in serum on day 23 from mice in the four groups. Mean±SD values from three independent experiments are reported.



*p<0.000001, \$p=0.04.

Fig. 6. The effect of Mch on Penh in mice exposed to mite in the presence and absence of heparin. Penh was measured in the four groups after stimulation with 6.25, 12.5, or 25 mg/ml methacholine (Mch) for 1 min. The values are presented as mean±SD values from three independent experiments for each group.

Heparin has also been shown to bind acute phase (19) and complement proteins (20) and this property may contribute to the anti-inflammatory activity of heparin.

There was also evidence showing that heparin

and its low molecular weight derivatives can inhibit neutrophil adherence to endothelial cells by binding to P-selectin (21), and CD11/CD18 complex (22), known heparin-binding proteins, then, decreases neutrophil migration to inflamed tissue, as well as

other inflammatory function of neutrophil.

Besides, heparin and related molecules can bind electrostatically to the positively charged nuclear localization sequence of NF- κ B, a highly cationic domain of nine amino acids (VQRDRQKLM) that targets nuclear translocation (23), and prevent it from translocating to the nucleus. Blocking of this transcriptional factor can potentially reduce inflammatory gene activation and regulate the gene expression and production of proinflammatory cytokines, chemokines and adhesion molecules (24). We also demonstrated lower IL-8 level in lung protein extract; further studies on the mechanism(s) are warranted.

Another interesting finding is that heparin decreased IL-17A/F in lung protein extracts. Recent studies have demonstrated that allergic airway inflammation is mediated by both Th2 and Th17 in the BALB/c model of asthma (25, 26). IL-17A/F strongly induces CXC chemokines and neutrophil responses, as illustrated in many mouse models of asthma (27). In concert with Th2 cells, Th17 cells are important during the development of AHR. In human, increased expression of IL-17A/F occurs in the bronchial submucosa of patients with moderate-to-severe asthma (28). Moreover, allergic sensitization within the airway promotes Th17 responses, and IL-17F-deficient mice have an impaired neutrophil response to allergens (29). Thus, the correlation between heparin and IL-17 regulation suggests an attractive therapeutic and/or prophylactic approach for asthma (30).

Besides the anti-inflammatory effects on airway, the heparin-treated groups also decreased Der p-specific IgE. To the best of our knowledge, it has not been reported in humans or mice, that airway inflammation was induced by mite or ovalbumin. Further studies are needed on the mechanism of heparin effect(s) in this aspect.

The daily intranasal administration of heparin in healthy volunteers was demonstrated to be safe in previous studies (31). The subjects received daily dosage of heparin 150,000 IU up to 28 days. In another study on sheep model of airway burn injury the study animals were given heparin 10,000 IU every 4 h up to 48 h (32). Neither study showed local or systemic adverse effect related to heparin. We found normal BT and PT in all four groups on day 23-24 h after

the last intranasal heparin administration. Besides, in our study, tissues including lung, heart, liver, kidney, spleen, and brain revealed no gross or microscopic bleeding.

In summary, daily intranasal administration of heparin of various doses, showed several anti-inflammatory functions as well as the ability to decrease serum mite-specific IgE levels in airway allergic inflammation in the mite-induced BALB/c model. These results merit further investigations including the different treatment protocol, dosage and more detailed mechanism(s) in its immunomodulation.

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