# Hydrogen-rich saline reduces airway remodeling via inactivation of NF-κB in a murine model of asthma

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**Abstract.** – BACKGROUND AND OBJECTIVES: Recent studies suggest that hydrogen has great therapeutic and prophylactic potential against organ injury caused by oxidative stress and inflammation. Here we investigated the effect of hydrogen-rich saline on airway inflammation and remodeling in a murine model of asthma.

MATERIALS AND METHODS: Asthma was induced by ovalbumin (OVA) sensitization and challenge. Then mice were treated with normal saline or hydrogen-rich saline at low and high doses. Cell counts and cytokine levels in bronchoalveolar lavage fluid (BALF) were determined, bronchial tissue was analyzed for pathology, and expression of MUC5AC, collagen III, VEGF, and total and phosphorylated NF-κB p65 was measured. Immunohistochemistry was used to identify levels and localization of VEGF expression in lung.

RESULTS: The results showed that hydrogen-rich saline reduced cell counts and levels of cytokines IL-4, IL-5, IL-13 and TNF-α in BALF. Hydrogen-rich saline treatment also significantly decreased mucus index, collagen deposition, and expression of MUC5AC, collagen III and VEGF. The ratio of phospho-NF-κB p65 to total NF-κB p65 was much lower in mice treated with hydrogen-rich saline than in untreated mice. These effects of hydrogen-rich saline on airway inflammation and remodeling were dose-dependent.

CONCLUSIONS: These findings suggest that hydrogen-rich saline reduces airway inflammation and remodeling in OVA-exposed mice by inhibiting NF-κB.

*Key Words:*
   Asthma, Airway remodeling, Airway inflammation, Ovalbumin (OVA), Hydrogen-rich saline, NF-κB.

## Introduction

Airway remodeling is a feature of chronic asthma in which the airway is partially and reversibly obstructed due to chronic inflammation. Recent evidence indicates that this remodeling is a dynamic process involving mucous hypersecretion, collagen deposition, wall thickening, myocyte hypertrophy and hyperplasia, myofibroblast hyperplasia, vascular proliferation and alterations in airway elastic fibers, all of which culminate in persistent structural alterations of the airway[1-8].

NF-κB, a well-studied transcriptional factor that plays numerous important roles in signaling biology, also plays a pivotal role in airway remodeling in asthma[9-11]. Cytokines and other factors triggered as part of inflammatory processes activate NF-κB via several signaling pathways, leading to a signaling cascade that amplifies the inflammation[9,10]. Several studies have associated NF-κB overactivation with airway remodeling[9-11].

Recent research has shown that hydrogen gas, seldom used in medicine, nevertheless shows strong therapeutic and prophylactic potential against organ injury, including lung injury, caused by oxidative stress and inflammation[12-16]. The molecular mechanism of these effects remain unclear. A recent study examining the therapeutic effects of hydrogen gas on a model of Alzheimer's disease suggests that the gas works by inhibiting NF-κB[17].

Therefore, the aim of our present study was to examine whether hydrogen-rich saline can ameliorate airway inflammation and remodeling in a mouse model of ovalbumin (OVA)-induced asthma, and if so, whether it works by inactivating NF-κB.

## Materials and Methods

### Animals

Six-week-old female BALB/c mice (16-20 g) were obtained from Animal Center of Sichuan University and maintained in a pathogen-free environment in the animal facilities of West China

Medical School of Sichuan University (Chengdu, China). Mice were kept in a temperature-controlled room with 12-h dark/light cycles, and allowed food and water *ad libitum*. The present study was performed according to NIH Guide for the Care and Use of Laboratory Animals (1985 revision) and the Helsinki Convention on the Use and Care of animals.

### Hydrogen-rich Saline Production

Hydrogen-rich saline was prepared as described[16]. Briefly, high-purity $H_2$ gas was injected at 0.4 MPa into saline for 6 h until the $H_2$ concentration reached a maximum. Then the hydrogen-rich saline was sterilized with gamma radiation and maintained under atmospheric pressure at 4°C in a dark color syringe with no dead volume. The actual concentration of hydrogen in saline was confirmed by gas chromatography as described[15].

### Treatment Groups

Asthma and airway remodeling were induced in this mouse model as described[4]. Briefly, mice were sensitized intraperitoneally with 10 $\mu$g of OVA (grade V, Sigma-Aldrich Chemical, St. Louis, MO, USA) in 100 $\mu$g of Al (OH)$_3$ on days 0, 7 and 14. The mice were challenged with 5% OVA aerosol for 1 h once a day from day 15 to 75. Then mice were randomly divided into the following three groups (n = 10 each) and treated in the indicated way from day 15 to day 75: the OVA group was given 0.5 ml d$^{-1}$ of phosphate-buffered saline (PBS; Sigma-Aldrich, USA); the OVA+Hydrogen-L group, hydrogen-rich saline at a dose of 5 ml kg$^{-1}$ d$^{-1}$; and the OVA+Hydrogen-H group, hydrogen-rich saline at a dose of 10 ml kg$^{-1}$ d$^{-1}$. Control mice (n = 10) were sensitized, challenged and treated with PBS in parallel with the three intervention groups.

### Bronchoalveolar Lavage Fluid (BALF) Collection and Differential Cell Counts

On day 75, mice were sacrificed by pentobarbitone (50 mg/kg i.p.). The thoracic cavity was carefully opened, the trachea exposed, and bronchoalveolar lavage fluid (BALF) collected by cannulating the upper part of the trachea and performing lavage twice with 1 ml and then with 0.8 ml of PBS; 85-90% of the total input volume was recovered. BALF samples were kept on ice during collection, then centrifuged at 400 $g$ for 5 min at 4°C. Pellets were resuspended in 100 []l PBS, and total viable cell numbers were counted

by trypan blue exclusion using a hemacytometer. Cells were centrifuged at 400 $g$ for 3 min, then stained with Diff-Quik (International Reagents Corp., Chuo-ku Kobe, Japan) for cytospin preparations.

### Measurement of Cytokine and Chemokine Levels in the Lung

BALF supernatant was collected as described above and stored at –70°C for use in cytokine assays. Commercial ELISA kits to measure TNF-$\alpha$, IL-4, IL-5 and IL-13 (R&D Systems, Carlsbad, CA, USA) were used according to the manufacturer's specifications.

### Quantitation of mRNA Expression by Real-Time Reverse Transcription-Polymerase Chain Reaction

Real-time-PCR (RT-PCR) was used to measure expression of collagen III, MUC5AC and VEGF mRNAs. Total RNA was isolated from the right upper lung tissue using Trizol (Invitrogen Co, Carlsbad, CA, USA). PCR reactions (50 $\mu$l) contained 5 $\mu$l 10× Taq Buffer, 4 $\mu$l 2.5 mM dNTP, 4 $\mu$l 25 mM MgCl$_2$, 2 $\mu$l each of forward and reverse primers, 0.5 $\mu$l Taq polymerase and 2 $\mu$l cDNA template. Reactions were amplified for 35 cycles of 45°C for 15 min, 97°C for 5 min, and 5°C for 5 min using a GeneAmp 9700 PCR system (Applied Biosystems, Foster City, CA, USA). The following primers were used: collagen III (forward), 5'-GTTCTAGAGGATGGCT-GTACTAAACACA-3'; collagen III (reverse), 5'-TTGCCTTGCGTGTTTGATATTC-3'; MUC5AC (forward), 5'-CTCTCCCACAAAA-GACACCAGT-3'; MUC5AC (reverse), 5'-CCTCCATCTCTCTCTCAGGGTAG-3'; VEGF (forward), 5'-CTGCTGTACCTCCACCATGC-CAAGT-3'; VEGF (reverse), 5'-CTGCAAG-TACGTTCGTTTAACTCA-3'. As an internal control, mouse $\beta$-actin mRNA levels were quantitated using the following primers: $\beta$-actin (forward), 5'-CGAGCGGGCTACAGCTTC-3'; $\beta$-actin (reverse), 5'-GTCACGC ACGATTCC-CTCT-3'.

### Quantitation of Protein Expression by Western Blotting

Left upper lung tissues were frozen in liquid nitrogen, then homogenised in PBS with protease inhibitor cocktail (Roche, South San Francisco, CA, USA) using a tissue grinder. Homogenates were centrifuged at 14000 rpm for 15 min at 4°C. Supernatants were collected and assayed for total

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protein using the bicinchoninic acid assay kit (Pierce, Rockford, IL, USA) and bovine serum albumin as the calibration standard. Equal amounts of protein (125 $\mu$g) were resolved on 10% Tris-glycine SDS (sodium dodecyl sulphatase) polyacrylamide gels. Protein bands were blotted onto nitrocellulose membranes. After blocking in 5% dried milk in Tris-buffered saline containing Tween-20 for 1 h at room temperature, membranes were incubated for 24 h at 4°C with one of the following antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA): anti-collagen III (1:1000), anti-MUC5AC (1:1000), anti-VEGF (1:1000), anti-NF-κB p65 (1:700) or anti-phospho-NF-κB p65 (1:700). Membranes were also incubated with mouse anti-β-actin antibody (1:5,000; Santa Cruz) to verify protein loading. Membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin (1:10,000; Santa Cruz). Peroxidase labeling was detected using the enhanced chemiluminescence Western blotting detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and analyzed by densitometry.

### Hematoxylin and Eosin Staining and Immunohistochemistry

The right lower lung from each mouse was fixed in 10% formalin, embedded in paraffin, cut into 5 $\mu$m sections, and stained with hematoxylin and eosin (HE). For immunohistochemistry analysis, tissue sections were deparaffinised and rehydrated. Then sample was treated at 95°C with Target Retrieval (Dako, Glodtrup, Denmark), blocked at room temperature using Serum-Free Protein Block (Dako), and incubated with anti-VEGF (1:700; Santa Cruz). After washing, sections were incubated for 30 min at room temperature with biotin-conjugated anti-rabbit IgG (H+L, Jackson ImmunoResearch, Suffolk, UK). Biotinylation was detected using the ABCcomplex HRP (Dako). Negative control staining was performed by using normal bovine serum instead of primary antibody. Two blinded investigators analyzed the sections using the Image-Pro Plus system (Media Cybernetics, Silver Spring, MD, USA).

### Periodic Acid-Schiff (PAS) Staining and Masson's Trichome (MT) Staining

The level of mucus secretion in the airways was assessed by periodic acid-Schiff (PAS) staining. The histological mucus index was cal-culated as the percentage of entire bronchial epithelium that was mucus-positive; at least 10 bronchioles were counted in each sample. Masson's trichrome (MT) staining was used to detect collagen deposition around the airway. The area of peribronchial trichrome staining in paraffin-embedded lung was outlined and quantified using a light microscope. Collagen deposition was quantitated as the area of trichrome staining per micron of basement membrane of bronchioles with an internal diameter of 150-200 $\mu$m; at least 10 bronchioles were counted in each sample. Two blinded investigators analysed the sections using Image-Pro Plus.

### Statistical Analysis

Statistical analyses were performed using SPSS 11.0 (SPSS Inc., Chicago, IL, USA). Results are reported as mean ± SD. Differences were analyzed for significance by one-way or two-way analysis of variance (ANOVA) and $p < 0.05$ was considered to be statistically significant.

## Results

### Hydrogen-Rich Saline Reduces Cellular Infiltration In Asthma

Airway inflammation was assessed based on HE staining and cell counts in BALF. OVA-induced mice showed extensive infiltration by inflammatory cells around the pulmonary blood vessels and airways, while such effects were not observed in control animals (Figure 1A). OVA-induced mice also showed significantly higher cell counts in BALF than did control mice (Figure 1B). After hydrogen-rich saline treatment, histological symptoms of asthma were much improved, and cell counts in BALF were significantly decreased (Figure 1).

### Hydrogen-rich Saline Decreases Cytokine Levels in BALF

Many cytokines and chemokines, particularly the helper T cell (Th2) cytokines IL-4, IL-5 and IL-13, as well as the pro-inflammatory mediator TNF-α play important roles in airway inflammation in asthma. Therefore, we studied the effects of hydrogen-rich saline on several cytokines and chemokines in BALF using ELISA. Levels of IL-4, IL-5, IL-13 and TNF-α were significantly higher in lungs after OVA challenge and sensitization. Then, hydrogen-rich saline markedly decreased levels of all cytokines (Figure 2).
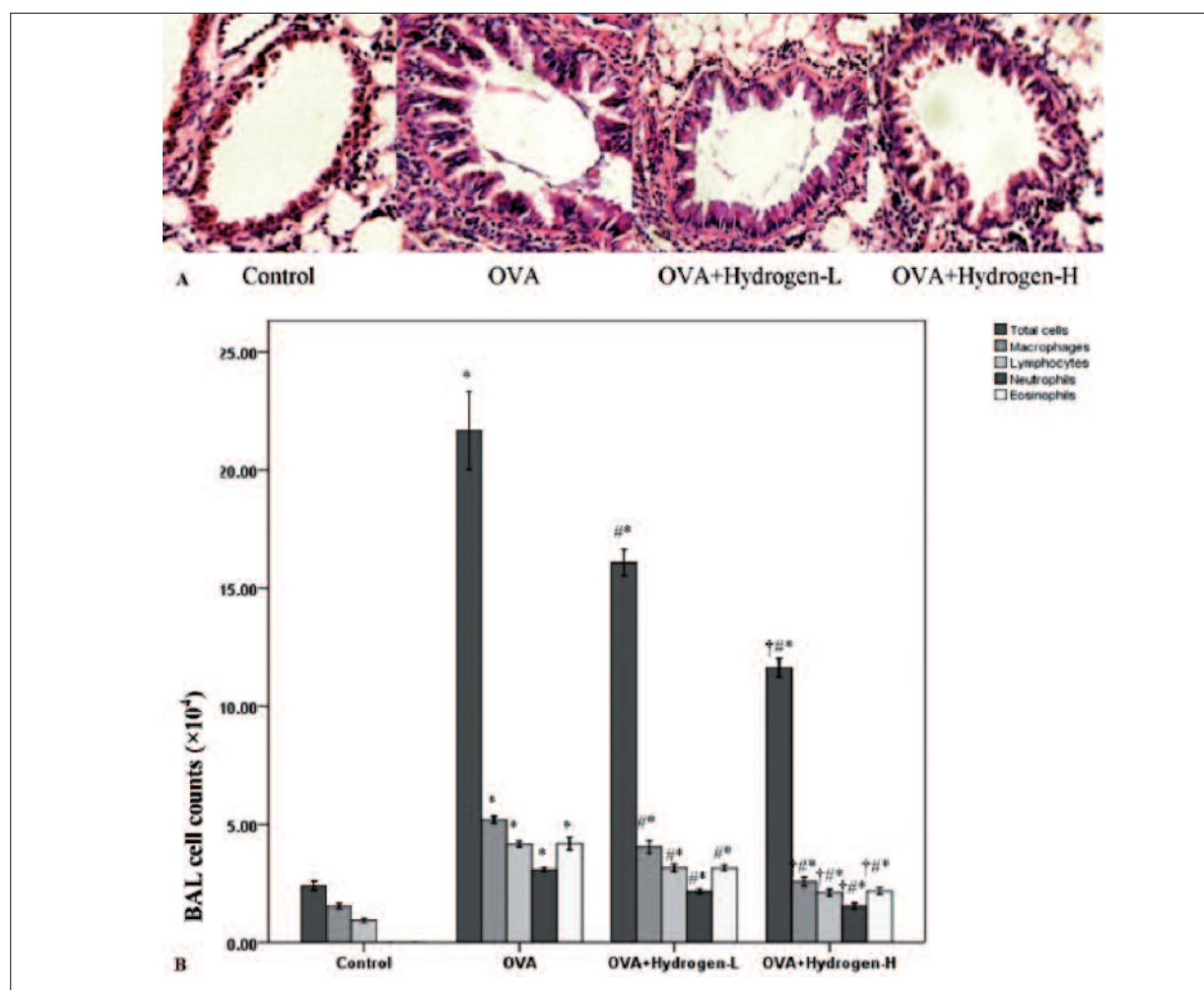
**Figure 1.** Hydrogen-rich saline reduces cellular infiltration, airway inflammation and cell counts in BALF in a mouse model of OVA-induced asthma. *A,* Representative hematoxylin and eosin-stained views (×400) from each treatment group. *B,* Mice were sacrificed and cell counts in BALF were measured. Bars show the means ± SD of 10 mice. *$p < 0.01$ compared with the control group. #$p < 0.01$ compared with the OVA group. †$p < 0.01$ compared with the OVA+Hydrogen-L group.

### Hydrogen-Rich Saline Attenuates Airway Remodeling in Asthma

One of the characteristics of chronic asthma is persistent inflammation that leads to airway remodeling, which results in partial and reversible airway obstruction.[18, 19] Lung tissues in the OVA group showed typical airway remodeling, including mucus hypersecretion, goblet epithelial metaplasia, collagen deposition, smooth muscle hypertrophy and angiogenesis (Figure 1). In contrast, slight airway remodeling was observed in hydrogen-rich saline treatment groups. By means of PAS staining, less mucus index was found in hydrogen-rich saline treatment groups (Figure 3A). Meanwhile, after hydrogen-rich saline treatment, a less mRNA and protein expression of

MUC5AC was observed (Figure 3B and C). Furthermore, collagen deposition and the expression of collagen III and VEGF in lung were also noticeably compromised by hydrogen-rich saline administrations (Figures 4 and 5).

### Hydrogen-rich Saline Inhibits NF-κB p65 Activation

NF-κB p65, one of the five members of the NF-κB family of transcription factors (p105/p50, p100/p52, p65, RelB and c-Rel), plays a key role in airway inflammation and airway remodeling[19-21]. Mice in the OVA group showed much higher activation of NF-κB p65, measured as phosphorylation of the protein, than did the control group, in which levels of phosphorylated NF-κB p65
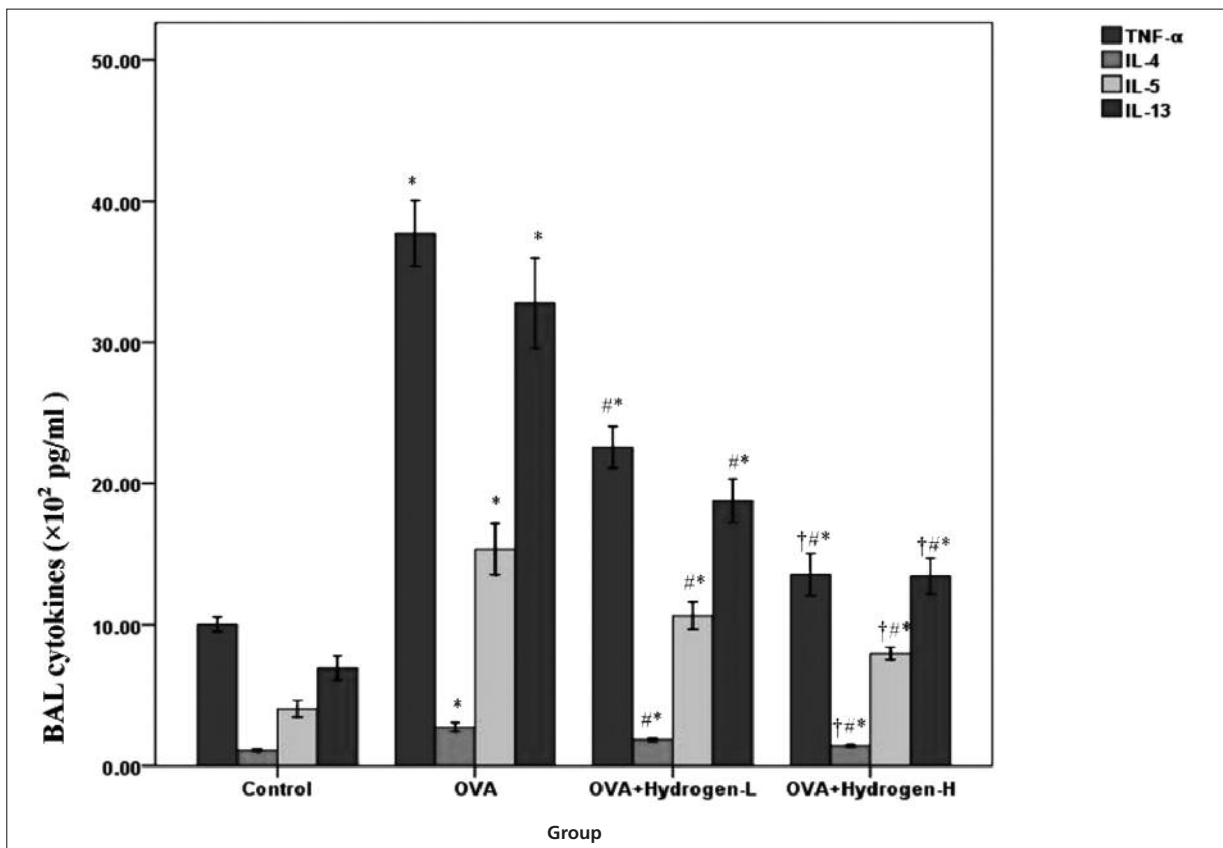
**Figure 2.** Hydrogen-rich saline reduces the levels of cytokines in BALF. Mice were sacrificed and levels of IL-4, IL-5, IL-13 and TNF-α in BALF were measured by ELISA. Bars show the means ± SD of 10 mice. *$p < 0.01$ compared with the control group. #$p < 0.01$ compared with the OVA group. †$p < 0.01$ compared with the OVA+Hydrogen-L group.

were barely detectable. This activation was significantly lower in the OVA+Hydrogen-L group than in the OVA group, and lower still in the OVA+Hydrogen-Hgroup than in the OVA+Hydrogen-L group, indicating that hydrogen-rich saline solution inhibited NF-κB p65 activation in a dose-dependent manner (Figure 6).

## Discussion

The present study provides evidence that hydrogen-rich saline can attenuate OVA-induced airway inflammation and remodeling in a dose-dependent manner in a mouse model of asthma. Our findings further suggest that hydrogen-rich saline exerts this effect by inhibiting NF-κB activation.

Asthma, which is causing increasing mortality and morbidity,is defined as chronic airway inflammation induced by allergens and cholinergic stimulation[1]. This inflammation involves inflammatory cells infiltration such as Th2 cells, mast cells, macrophages, lymphocytes,eosinophils and neutrophils, as well as release of cytokines, particularly IL-4, IL-5, IL-13 and TNF-α[1, 3]. Our results indicate that hydrogen-rich saline reverses or prevents several of these effects in a dose-dependent manner. Counts of total cells, eosinophils, macrophages, lymphocytes and neutrophils in BALF were much lower in OVA-induced mice treated with hydrogen-rich saline than in the OVAgroup. We obtained similar results for IL-4, IL-5, IL-13 and TNF-α. Consistent with these biochemical findings, HE staining showed that hydrogen-rich saline treatments ameliorated airway inflammation.

Pathology studies in patients with fatal asthma have shown that most bronchi and bronchioles are blocked by mucus plugs[3,5,7]. In fact, mucus hypersecretion by goblet cells is believed to be the major cause of fatal and refractory asthma. Using PAS staining, we found the mucus index in OVA mice to be much higher than that of con-
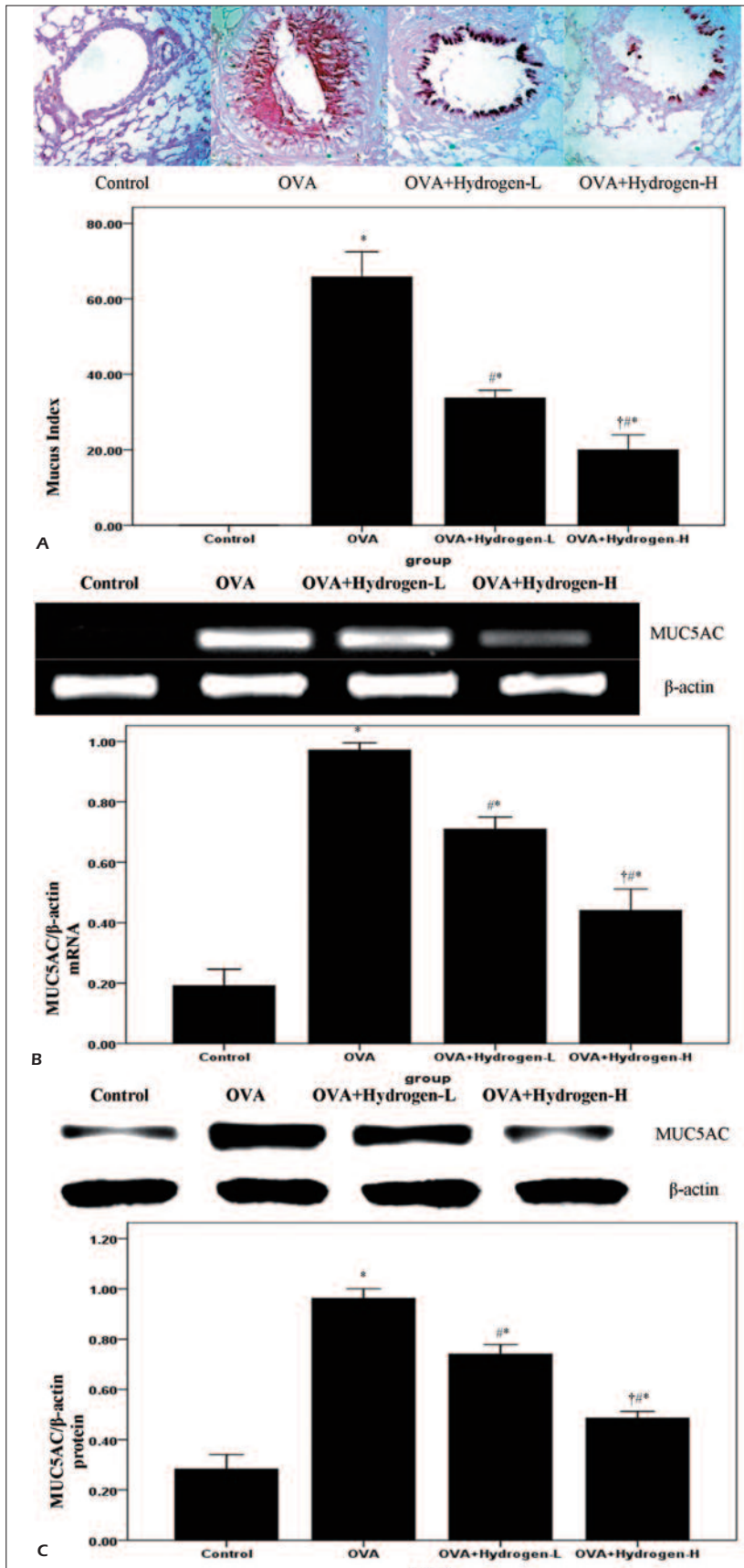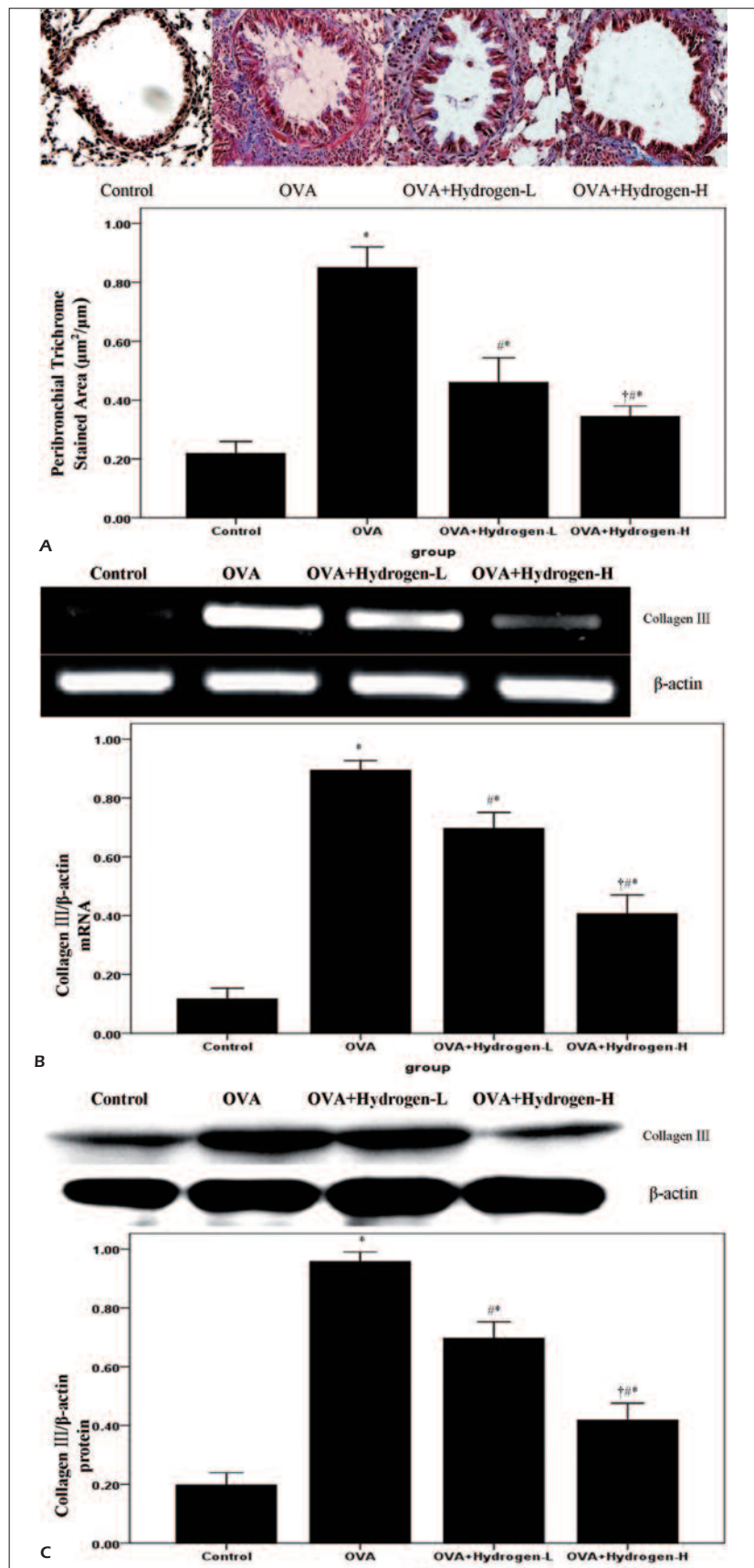
**Figure 3.** Hydrogen-rich saline inhibits mucus hypersecretion. *A,* Representative PAS-stained tissue (×400) from each treatment group. The mucus index was calculated as the percentage of entire bronchial epithelium that was mucus-positive. *B,* Expression of MUC5AC mRNA in lung was analyzed by RT-PCR. *C,* Expression of MUC5ACprotein in lung was analyzed by Western blotting. Expression of MUC5AC mRNA or protein in each sample was expressed as a ratio of MUC5AC level to β-actin level. Data shown are means ± SD of 10 mice. *$p$ < 0.01 compared with the control group. #$p$ < 0.01 compared with the OVA group. †$p$ < 0.01 compared with the OVA+Hydrogen-L group.

**Figure 4.** Hydrogen-rich saline inhibits collagen deposition. **A,** Representative MT-stained views (×400) for each treatment group. Peribronchial-trichrome-stained area per micron length of basement membrane of bronchioles with an internal diameter of 150-200 $\mu$m ($\mu$m$^2$/$\mu$m). **B,** Expression of collagen III mRNA in lung was analyzed by RT-PCR. **C,** Expression of collagen IIIprotein in lung was analyzed by Western blotting. Expression of collagen III mRNA or protein in each sample was expressed as a ratio of collagen III level to β-actin level. Data shown are means ± SD of 10 mice. *$p$ < 0.01 compared with the control group. #$p$ < 0.01 compared with the OVA group. †$p$ < 0.01 compared with the OVA+Hydrogen-L group.
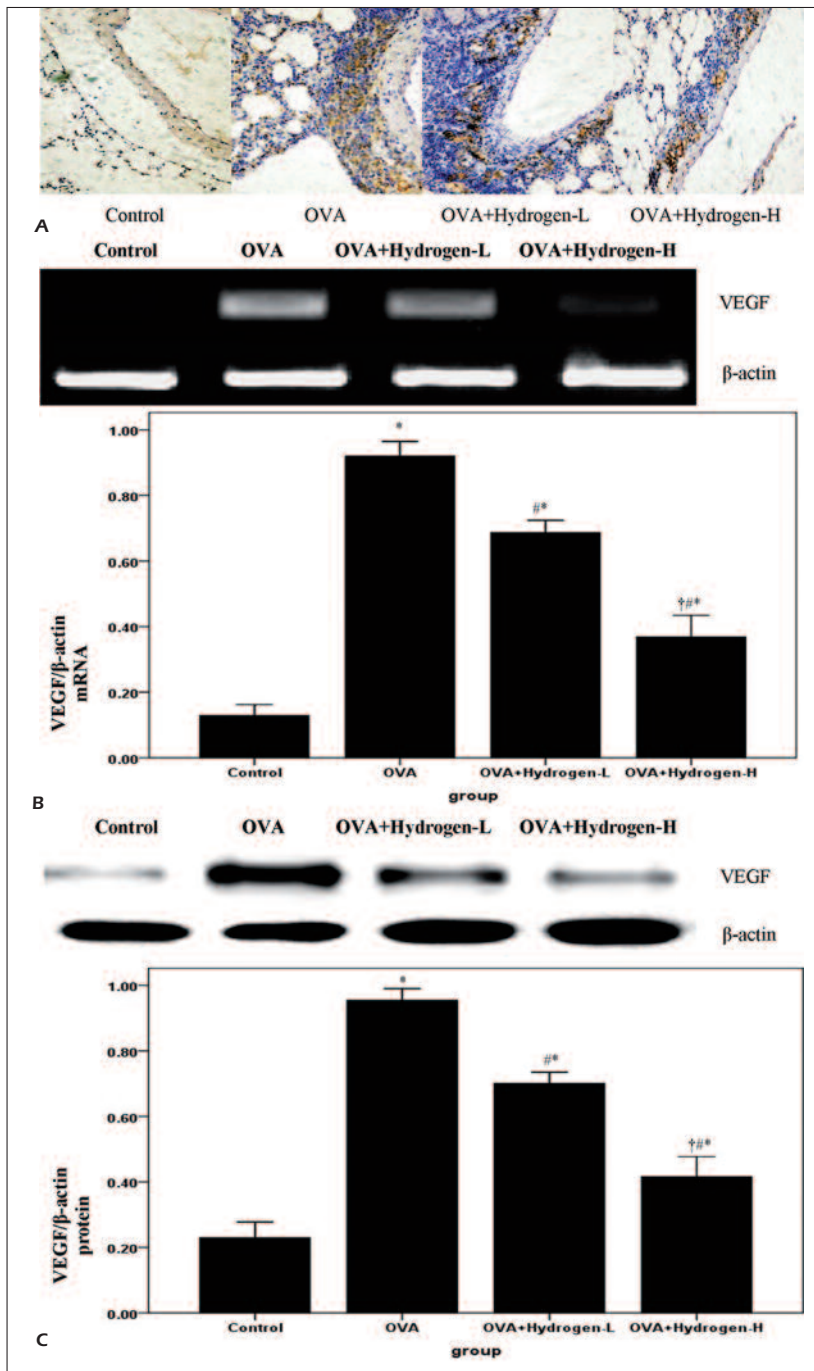
**Figure 5.** Hydrogen-rich saline inhibits VEGF expression in a mouse model of OVA-induced asthma. **A,** VEGF expression around blood vessels in the lung was examined by immunohistochemistry. This figure shows a representative view (×400) for each treatment group. **B,** Expression of VEGF mRNA in lung was analyzed by RT-PCR. **C,** Expression of VEGF protein in lung was analyzed by Western blotting. Expression of VEGF mRNA or protein in each sample was expressed as a ratio of VEGF level to β-actin level. Data shown are means ± SD of 10 mice. *$p$ < 0.01 compared with the control group. #$p$ < 0.01 compared with the OVA group. †$p$ < 0.01 compared with the OVA+Hydrogen-L group.

trol mice or mice in the OVA+Hydrogen-L and OVA+Hydrogen-H groups. The reduction of mucus by hydrogen-rich saline was dose-dependent, with the greater effect observed in the OVA+Hydrogen-H group. Since the main component of mucus is MUC5AC, we examined the effects of hydrogen-rich saline treatment on MUC5AC expression using RT-PCR and Western blotting. Hydrogen-rich saline suppressed OVA-induced expression of MUC5AC mRNA and protein, and this effect was dose-dependent.

Autopsies of patients with fatal asthma and chronic asthma have revealed collagen deposition around the small airway[28]. In fact, collagen deposition in the peribronchial area is the main culprit behind the partial reversible airway obstruction in chronic asthma[6-8]. To examine collagen deposition in our mouse model, we deter-
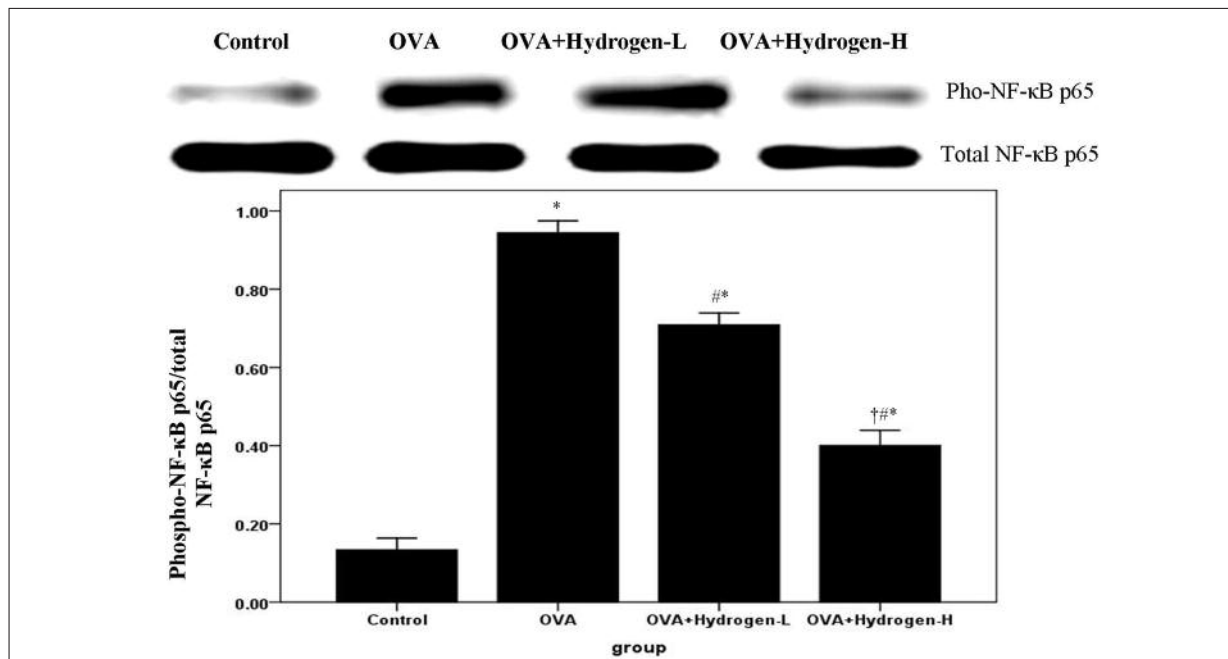
**Figure 6.** Hydrogen-rich saline inhibits NF-κB p65 activation, measured as the level of phospho-NF-κB p65 on Western blots. NF-κB p65 activation in each sample was expressed as the ratio of phospho-NF-κB p65 level to total NF-κB p65 level. Data shown are means ± SD of 10 mice. *$p < 0.01$ compared with the control group. #$p < 0.01$ compared with the OVA group. †$p < 0.01$ compared with the OVA+Hydrogen-L group.

mined the surface area of peribronchial area that was MT-positive per unit length of bronchiole basement membrane ($\mu m^2/\mu m$). This value was significantly higher in the OVA mice than in the control mice, and it was reduced in a dose-dependent manner by hydrogen-saline. To explore the origin of this effect on collagen deposition, we used RT-PCR and Western blotting to analyze the effects of hydrogen-rich saline on collagen III expression and found that hydrogen reduced it in a dose-dependent way.

Since studies have reported that VEGF contributes substantially to angiogenesis in airway remodeling in asthma[18,29], we used immunohistochemistry, RT-PCR and Western blotting to detect VEGF expression with and without hydrogen-rich saline treatment. OVA induced VEGF expression, and hydrogen-rich saline treatment reduced it, in a dose-dependent manner.

Since overactivation of NF-κB has been observed in asthma induced airway remodeling[9-11], we examined levels of total and phospho-NF-κB p65 with and without hydrogen-rich saline treatment in our mouse model. The ratio of phosphorylated to total NF-κB p65 was significantly higher in the OVA group than in the control group. Hydrogen-rich saline modified this ratio

in a dose-dependent manner. These findings suggest that the anti-inflammatory effects of hydrogen-rich saline in our mouse model are possibly due to inhibition of NF-κB activation.

Our findings that hydrogen-rich saline can attenuate airway inflammation adds to the growing literature on the potential therapeutic effects of hydrogen gas. Several years ago, Japanese researchers showed that treating rats with hydrogen gas protected them from cerebral ischaemia/reperfusion (I/R) injury by selectively reducing levels of cytotoxic oxygen radicals[15]. More recently, several reports showed that hydrogen gas and hydrogen-rich saline can effectively inhibit inflammation and oxidative stress[22-27]. Our results suggested that hydrogen gas exerts these effects by inhibiting the activation of NF-κB, as proposed in a recent investigation of hydrogen gas in a rat model of Alzheimer's disease[17].

## Conclusions

Our data demonstrate that hydrogen-rich saline attenuates airway inflammation and remodeling possibly through inhibiting the activation of NF-κB. However, more investigations

should be carried out to identify the underlying mechanisms of the signaling pathways in hydrogen-mediated inactivation of NF-κB property in future.

### Conflict of Interest Statement

None declared.

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