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Jin Gui Shen Qi Wan, a Traditional Chinese Medicine, Alleviated Allergic Airway Hypersensitivity and Inflammatory Cell Infiltration in a Chronic Asthma Mouse Model

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Conflict of interest

Declarations of conflicts of interest: none

ABSTRACT

Ethnopharmacological relevance: In Asia, Jin Gui Shen Qi Wan (JGSQW) has been used for hundreds of years to treat asthma.

Aim of the study: The traditional Chinese medicine JGSQW is composed of *Rehmannia glutinosa*, *Dioscorea opposita*, *Cornus officinalis*, *Poria cocos*, *Paeonia suffruticosa*, *Alisma*

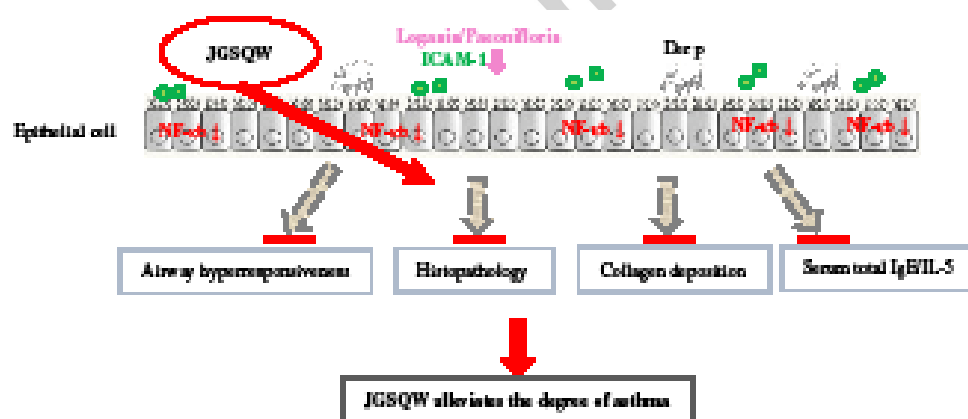
orientalis, *Aconitum carmichaelii* and *Cinnamomum cassia*. However, the immunological mechanism underlying the effect of JGSQW treatment on asthma remains unclear. This study examined whether JGSQW has the potential to reduce asthma symptoms in mice with chronic asthma induced by recurrent *Dermatophagoides pteronyssinus* (Der p) stimulation, as well as its immunoregulatory mechanisms.

Materials and methods: The airways of BALB/c mice were stimulated with Der p (i.t.) once per week (50 μ L, 1 mg/mL) for 6 consecutive weeks, and the mice were fed JGSQW (1 g/kg) 30 min prior to the Der p stimulation. Three days after the last stimulation, the mice were sacrificed to evaluate the airway remodelling, infiltration of inflammatory cells, lung histological features, and total IgE in the blood. Additionally, after A549 cells were treated with JGSQW, loganin, or paeoniflorin for 30 min, 10 ng/mL IL-1 β was added to stimulate the A549 cells to evaluate the effect of the medicine on the ICAM-1 gene expression after IL-1 β stimulation.

Results: JGSQW significantly reduced the Der p-induced infiltration of inflammatory cells into airways and decreased the total IgE and Der p-specific IgG1 in serum. Collagen assays and histopathological examinations showed that JGSQW reduced lung airway remodelling. Additionally, an electrophoretic mobility shift assay and immunohistochemical staining verified that JGSQW inhibited the NF- κ B expression in airway epithelial cell nuclei. JGSQW, loganin, and paeoniflorin inhibited the ICAM-1 gene expression caused by the IL-1 β stimulation of A549 cells, and loganin and paeoniflorin had the maximum inhibitory effect when mixed according to the combination of doses in JGSQW.

Conclusion: These results indicated that in the chronic asthma mouse model, JGSQW inhibits the infiltration of inflammatory cells into the airways and airway remodelling and exhibits specific immunoregulatory effects. JGSQW with certain doses of loganin and paeoniflorin inhibited ICAM-1 gene expression in epithelial cells.

Graphical abstract:



Keywords:

Traditional Chinese Medicine, Jin Gui Shen Qi Wa, Chronic asthmatic mouse model, *Dermatogoides pteronyssinus*, Inflammation

Abbreviations used: Der p, *Dermatophagoides pteronyssinus*; Dex, dexamethasone; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; FBS, foetal bovine serum; GMCSF, granulocyte-macrophage colony-stimulating factor; GMP, good manufacturing practice; HPLC, high-performance liquid chromatography; ICAM-1, intercellular adhesion molecule 1; Ig, immunoglobulin; IHC, immunohistochemical; IL, interleukin; i.t., intratracheal; JGSQW, Jin Gui Shen Qi Wan; moAb, monoclonal antibodies;

MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); MUC5AC, mucin5AC; P.S, Penicillin/Streptomycin; TCM, traditional Chinese medicine; Th2, type 2 T helper cell.

1. Introduction

Asthma is a chronic inflammatory disease of the airways that involves a variety of cells (e.g., eosinophils, T-lymphocytes, and mast cells) and factors (e.g., cytokines, chemokines, histamine, prostaglandin and leukotrienes). The incidence and mortality rates of asthma are increasing steadily each year (Hammad and Lambrecht, 2011; Makino and Sagara, 2010). The clinical symptoms of asthma include recurrent attacks of wheezing, shortness of breath, chest tightness, or coughing; during attacks, scattered or diffused wheezing can be heard in both lungs.

Many studies have indicated that lung epithelial cells are the first line of defence against allergens or environmental substances, such as pollutants and viruses, through mechanisms involving inflammation, immune regulation, and regeneration. Therefore, lung epithelial cells play a crucial role in the development of the asthma disease process (Loxham et al., 2014). In addition, studies have indicated that NF- κ B is the central regulator of the generation of numerous responses by epithelial cells. An increase in NF- κ B activity changes the structure of epithelial cells and the membrane permeability in the airways; when epithelial cells lose their barrier function, NF- κ B causes the gene expression of several inflammatory cytokines and induces inflammatory reactions in epithelial cells (Heijinkwt al., 2014). The NF- κ B signalling pathway has been considered a crucial target for interventional asthma treatment. Some studies have indicated that NF- κ B plays a crucial role in Th2 cell differentiation and the production of proinflammatory cytokines. For example, the inhibition of NF- κ B strongly reduces lung inflammatory responses, airway hyperresponsiveness, and increased mucus levels (Poynter et al., 2002).

In a pathological state, NF- κ B-regulated ICAM-1 is abundantly expressed in lung epithelial and vascular endothelial cells to attract numerous white blood cells, particularly eosinophils, to the lungs. These leukocytes release cytokines, chemokines, nitric oxide, and reactive oxygen species in the lungs to increase the degree of inflammation and oxidative stress, eventually leading to various pulmonary diseases, such as asthma or lung injury (Mukhopadhyay et al., 2014). Therefore, several studies have indicated that inhibiting the expression or effects of ICAM-1 can reduce inflammatory responses (Krakauer, 2000), inflammatory cell infiltration (Huang et al., 2013) and the number and activity of asthma eosinophils (Pazdrak et al., 2008). Thus, interfering with ICAM-1 can be a strategy for treating asthma.

Jin Gui Shen Qi Wan (JGSQW) originates from *Synopsis of Prescriptions of the Golden Chamber* by Zhang Zhongjing of the Eastern Han dynasty, and it is a combination of yin-nourishing and yang-strengthening medicine (Zhang, Zhongjing, 2016). Its compatibility mechanism is “obtaining yang from yin,” that is, the *qi* function of the kidney can be naturally recovered through yin and yang coordination (Hua and Zhang, 2005). Modern studies have confirmed that JGSQW significantly restores the immunocompetence of immunosuppressed mice and increases the immune function of patients (Liu, et al., 2011; Chen et al., 2007). Furthermore, JGSQW has been used clinically to treat diseases such as

urinary difficulty with oedema (Scheid et al., 2015), hypertension (Xiong et al., 2015), and asthma (Ji et al., 2018; Deng, Shimin., 2012; Wang, Zhongf., 2010; Gan et al., 2016; Han, Ruifeng., 2007).

Past generations of TCM practitioners often used prescriptions to treat diseases. The composition of prescriptions is based on certain principles; medicinal materials are adequately combined according to specific principles to form prescriptions. Various medicines can form a variety of prescriptions and have varied primary therapeutic effects. However, simply combining several medicines does not make a prescription; instead, a prescription can be formed only by combining medicines according to certain rules and methods. For prescriptions, suitable medicines and usage amounts should be selected and determined after dialectical treatment; the medicines should be then adequately combined according to the requirements for the roles of the components (*jun*, *chen*, *zuo*, and *shi*, or sovereign, minister, assistant, and messenger, respectively) to prepare a suitable dose for clinical use (Yang Yifan, 2010). JGSQW is a prescription composed of eight types of medicinal materials according to certain proportions. However, no scientific theory yet exists regarding how it achieves asthma improvement with a clinical dose. This study verified that the clinical dose of JGSQW mitigates asthma symptoms and that JGSQW and its compounds inhibit the NF- κ B activity of lung epithelial cells, thereby affecting the ICAM-1 gene expression related to the inflammatory responses of epithelial cells regulated by NF- κ B.

2. Materials and methods

2.1. Mice and reagents

BALB/c male mice free of pathogens and aged 6–8 weeks were purchased from the National Laboratory Animal Center in Taiwan. The feeding and experimental treatment of the mice were in accordance with the guidelines of the National Science Council of the Republic of China. This animal experiment was reviewed and approved by the Institutional Animal Care and Use Committee of China Medical University (No. 104-71-N). Dust mites were purchased from Allergon (Sweden) and extracted using ether; after extraction, sterile double-distilled water was used for dialysis (Lin et al., 2012). Loganin (36483, $\geq 97\%$ HPLC, molecular weight 390.38, Pubchem ID 329755352) and paeoniflorin (P0038, $\geq 98\%$ HPLC, molecular weight 480.46, Pubchem ID 329819989) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of a JGSQW extract

The JGSQW comprised *Rehmanniae Radix* (*Rehmannia glutinosa* Libosch.), *Dioscoreae Rhizoma* (*Dioscorea opposita* Thunb.), *Corni Fructus* (*Cornus officinalis* Sieb. et Zucc.), *Poria* (*Poria cocos* (Schw.) Wolf), *Moutan Cortex* (*Paeonia suffruticosa* Andr.), *Alismatis Rhizoma* (*Alisma orientalis* (Sam.) Juzep.), *Aconiti Lateralis* (*Aconitum carmichaelii* Debx.) and *Cinnamomi Cortex* (*Cinnamomum cassia* Presl) purchased from a good manufacturing practice (GMP) pharmaceutical company, Koda Pharmaceuticals Co., Ltd. (Taoyuan, Taiwan), and the voucher specimens were deposited at Koda Pharmaceuticals Co., Ltd for future reference. The trait description and identification tests (such as TLC) of the medicinal materials were all identified according to Taiwan Herbal Pharmacopeia and were also identified by Dr. Chang Jinming, a Quality Assurance Manager from Koda Pharmaceuticals Co., Ltd.. The preparation of a JGSQW clinical drug extract (also named BA WEI DI HUANG WAN) was entrusted to a GMP pharmaceutical company, Koda Pharmaceuticals Co., Ltd., in accordance with the license number approved by the Ministry of Health and Welfare

of the Republic of China: DOH Manufacturing No. 028491. The Taiwan Herbal Pharmacopeia records for the reference standard of Shan Chu Yu and Mou Tan Pi are paeoniflorin and loganin, respectively, according to the JGSQW quality control as described in this license.

The JGSQW extract was prepared from eight commonly seen traditional Chinese medicinal materials according to certain weight proportions (Table 1). The total amount of medicinal materials used in this study was 2.5 kg. After 17.5 L of water was added for decoction for 1 h and the decocted extract was obtained, 12.5 L of water was added to the medicinal materials for a second decoction for 1 h. The decocted extracts from the first and second decoctions were sieved through a 100-mesh sieve, and the sieved extracts were collected for concentration into an extract. The total weight of the medicinal materials was 2.5 kg, and a 779.5-g dry extract was obtained for a yield of 31.18% (W/W). The JGSQW extract was dissolved in sterile water and prepared to a specific concentration to feed mice.

2.3. High-performance liquid chromatography for identification and content determination

The high-performance liquid chromatography (HPLC) system comprised a Hitachi D-7000 interface, L-7100 pump, L-7420 UV/VIS detector, and L-7200 autosampler; in addition, the chromatographic column used was a Mightysil RP-18 GP column (250 × 4.6 mm, 5 μm). The mobile phase conditions are presented in the following table:

Time (min)	Acetonitrile (%)	0.03% H ₃ PO ₄
0	12	88
5	12	88
30	18	82
40	50	50
50	12	88
60	12	88

The flow velocity was 1.0 mL/min, the detection wavelength was 245 nm, and the sample injection volume was 10 μL. The loganin standard was prepared at 0.0093, 0.0186, 0.0372, and 0.0744 mg/mL using 70% methanol, and the paeoniflorin standard was prepared at 0.1496, 0.0748, 0.0374, and 0.0187 mg/mL using 70% methanol. Each standard contained 0.25226 mg/mL guaiacol glyceryl ether as an internal standard. Approximately 1 g of JGSQW extract was added to 10 mL of MeOH, and the mixture was placed in an ultrasonic oscillator for 60 min of oscillation extraction. After filtering, methanol was used to reach a quantified volume of 10 mL. Subsequently, 2.5 mL of filtrate was placed in a 5-mL volumetric flask, and guaiacol glyceryl ether internal standard was added to obtain a concentration of 0.25226 mg/mL. Methanol was again used to reach a quantified volume of 5 mL, and finally, a suitable amount was passed through a 0.45-μm filter membrane to be used as a test liquid. The HPLC figure was provided by Koda Pharmaceuticals Co., Ltd. (Fig. 1a). The loganin and paeoniflorin contents in the sample were calculated as follows (Fig. 1b):

Loganin content (mg/g) = test liquid concentration × quantified volume/dilution factor/amount of test sample used

$$0.0343757 \times 10 \times 5/2.5 \div 1.0630 = \mathbf{0.6468 \text{ mg/g}}$$

Paeoniflorin content (mg/day) = test liquid concentration × quantitated volume/dilution factor/amount of test sample used

$$0.0161272 \times 10 \times 5/2.5 \div 1.0630 = \mathbf{0.3034 \text{ mg/g}}$$

2.4. *Dermatophagoides pteronyssinus*-induced model of chronic asthma

A total of 18 BALB/c male mice were randomly divided into three groups. Mice in the Der p group were administered a stimulus of 50 μ L of 1 mg/mL *Dermatophagoides pteronyssinus* (Der p) in their trachea once per week for a total of seven treatments in 6 consecutive weeks to induce a chronic asthma mouse model. The mice were fed sterile water 30 min prior to the Der p stimulus. The mice in the JGSQW group were fed 1 g/kg JGSQW extract 30 min prior to the Der p stimulus. The administered dose of JGSQW was based on the package instruction of Kodak (DOH Manufacturing No. 028441 in Taiwan). The daily dose for a 70-kg adult is 6 g of JGSQW extract, and the dose for human conversion to mice is 1g/kg (Nair and Jacob, 2016). Naïve mice were also included in the experiments for comparative purposes. At 72 h after the last stimulus, male mice were injected with xylazine (200 μ g/mouse) and ketamine (2 mg/mouse) in the abdominal cavity and sacrificed (Wang et al., 2012). Blood serum samples were collected and preserved at -80°C for subsequent analysis. Additionally, bronchoalveolar lavage fluid samples were collected for calculating the total and differential leukocyte counts, and lung tissue was used as a pathological section for the collagen analysis and the electrophoretic mobility shift assay (EMSA).

2.5. Measurement of airway hyperresponsiveness

A single-chamber, whole-body plethysmograph (Buxco Electronics, Inc., Troy, NY) system was used to measure the airway resistance of male mice. Changes in the value of enhanced pause (Penh) represented the airway resistance. The operating procedures of the instrument followed the instructions in the operating manual; first, each male mouse was placed in a chamber, and ultrasonic oscillation was used to convert methacholine fluid into a spray. A connecting tube was used to inject the spray into the chamber, causing the male mice inside the chambers to inhale the spray. One dose of methacholine (Sigma-Aldrich, St. Louis, MO) was added every 3 min, and the methacholine doses were 0, 3.125, 6.25, 12.5, 25, and 50 mg/mL.

2.6. Measurement of the total IgE, Der p-specific IgG1, Der p-specific IgG_{2a/2b}, and IL-5 content in blood serum

In total, 100 μ L of serum was injected into 96-well assay plates, and the total IgE content was measured according to the operating instructions of an IgE-specific enzyme-linked immunosorbent assay (ELISA) kit (BD Pharmingen). The ELISA Ready-SET-Go! Kit (eBioscience, San Diego, CA) was used to measure the IL-5 content; all analytical procedures were performed according to kit usage instructions and included tests of standards. Prior to measuring Der p-specific antibodies, the surface of each 96-well assay plate was coated with 2 μ g/mL Der p antigen and placed in a 4°C refrigerator overnight. Unbound antigens were washed off thoroughly, and biotinylated rat anti-mouse IgG1 or IgG_{2a/2b} mAb (2 μ g/mL; BD Pharmingen) was added and cultivated for 1 h. After the binding reaction, a buffer solution was used to wash unbound monospecific antibodies. Subsequently, the TMB Microwell Peroxidase Substrate System (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added. After completion of the streptavidin-horseradish peroxidase conjugate (1:1000 dilution, BD Pharmingen) colour reaction, an ELISA reader was used for scanning and recording the OD₄₅₀ value.

2.7. Staining method

a. Haematoxylin and eosin staining

Mouse lungs were removed and preserved in 4% paraformaldehyde fixative. After progressive alcohol dehydration and paraffin embedding, a microtome was used to slice the lungs into 0.5-mm sections. The sections were fixed on glass slides and stained using brazilic acid and eosin.

b. Periodic acid–Schiff staining

After the fixed tissue sections were dewaxed and washed, they were immersed in distilled water and placed in 0.5% periodic acid for 15 min. Subsequently, the sections were washed with tap water for 5 min, immersed in distilled water, and reacted with Schiff's reagent for 5 min. The sections were washed in tap water for 5 min, drained, and then sealed. When observed under an optical microscope, the glycoprotein and neutral mucus appeared red, and the cell nuclei appeared blue.

c. Immunohistochemical staining

The tissue specimens were fixed with 10% neutral buffered formalin, embedded in paraffin, sliced to thicknesses of 4–5 μm , and then dewaxed. The glass slides were immersed in 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase activity and dipped in normal goat serum (1.5%) for 20 min to block nonspecific binding sites. Subsequently, a 1:150 dilution of rabbit polyclonal IgG (specific for the p65 subunit of NF- κB ; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at 4°C for overnight immunostaining. A 1:200 dilution of biotinylated secondary antibodies (Nichirei Co., Ltd., Tokyo, Japan) was used to treat the sections, followed by the use of an avidin–biotin–peroxidase complex solution and 3,3'-diaminobenzidine (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for colouring.

2.8. Collagen testing

After the addition of 2 mL of Hanks' balanced salt solution, 100 mg of mouse lung was homogenized using a homogenizer and centrifuged at 800 g for 10 min; the supernatant liquid was then collected. Separately, two 100- μL tubes of the Hanks' balanced salt solution and the sample were collected and serially diluted using collagen as the standard, followed by adding 1 mL of Sircol Dye Reagent (Sircol) to each tube. The tubes were placed on a table concentrator for 30 min and centrifuged at 10,000 g for 10 min to remove unbound dye solution, and 1 mL of alkaline reagent was added to each tube. After 10 min, the ELISA reader at a wavelength of 540 nm was used to determine the collagen concentration.

2.9. Nucleoprotein analysis

Nucleoprotein was extracted from mouse left lung tissue according to the operating manual of the Nuclear Extraction Kit (Panomics). The procedure is briefly described as follows: after 1.5 mL of Buffer A Mix was added to the left lung tissue, the lung tissue was homogenized, placed on ice for 15 min and then centrifuged at 850 g and 4°C for 10 min. The precipitate was mixed with 150 μL of Buffer B and dispersed, and the centrifuge tube was placed on ice and shaken at 200 rpm for 2 h on a rocking platform. The tube was then centrifuged at 4°C and 15,000 g for 5 min, and the supernatant liquid, which was a nucleoprotein extract, was collected and stored at -80°C .

2.10. EMSA

A LightShiftTM Chemiluminescent EMSA Kit (PIERCE) was used to perform the EMSA experiment. The procedure is briefly described as follows: a 6% nondenaturing polyacrylamide gel was prepared, and 5 μ L of 5X loading buffer was added to each tube of 20 μ L of binding reagent. After the samples were loaded on the gel, electrophoresis was performed at 120 V for 90 min, and the gel was blotted onto a nylon membrane. The nylon membrane was immersed in 20 mL of LightShift blocking buffer and shaken for 15 min. After the buffer was poured out, the conjugate/blocking buffer solution was added for 15 min of shaking. The nylon membrane was washed four times in 20 mL of 1X wash solution, and then, 30 mL of LightShift substrate equilibration buffer was added to the solution in a darkroom and shaken for 5 min. After the buffer was poured out, the LightShift substrate working solution was directly added to the nylon membrane for 5 min of reaction, after which, tableting was performed.

2.11. Cell survival test (MTT assay)

A549 cells were inoculated into 96 wells, and 5×10^4 cells/90 μ L of culture medium (F12 (Gibco)+10% FBS+1% penicillin-streptomycin (PS)) was used for each well. The cells were placed in a 5% CO₂ incubator at 37°C and cultivated for 24 h. Subsequently, the cells were removed and administered 10 μ L of medicine for stimulation for 24 h. Next, 5 μ L of PrestoBlue (Invitrogen) was added to each well; after 1.5 h of reaction, the optical density values at 570 and 600 nm were measured.

2.12. Cell cultivation and drug treatment

A549 cells were inoculated into 6 wells, with 1×10^6 cells/3 mL of culture medium (F12+10% FBS+1% PS) used for each well. The cells were placed in a 5% CO₂ incubator at 37°C and cultivated for 24 h. A549 cells were treated with the addition of various concentrations of JGSQW and compounds. After 30 min, 10 ng/mL IL-1 β was used to stimulate the A549 cells for 4 h, and each cell was collected for the evaluation of ICAM-1 gene expression. Subsequently, 100 nM dexamethasone was used in a control group.

2.13. Real-time polymerase chain reaction

Total RNA was extracted from the A549 cells using TRIzol reagent (Invitrogen Life Technologies), and subsequently, an ImProm-II Reverse Transcription System Kit (Promega) was used to convert total RNA into complementary DNA. For real-time polymerase chain reaction, a Roche LightCycler FastStart DNA Master SYBR Green I Kit was used to conduct the experiment according to the operating manual, and a Roche LightCycler 1.5 Instrument was employed to perform a real-time polymerase chain reaction. The results were analysed using crossing points values. β -Actin was used as a control. The gene-specific primers (sense and antisense) for each gene were used as follows: β -actin, 5'-GGAAATCGTGC GTGACA-3' and 5'-CACAGGATTCCATACCCAAG-3'; ICAM-1, 5'-TGACCGTGAATGTGCTCTCC-3' and 5'-TCCCTTTTGGGCCTGTT GT-3'.

2.14. Statistical methods

Student's *t*-test was employed for statistical analysis to compare the differences between each group. The experimental results were presented as the means $\pm$ SDs; a *P* value of less than .05 was considered statistically significant.

3. Results

3.1. JGSQW reduces airway hyperresponsiveness and inflammatory responses in mice with Der p-induced chronic asthma

The most dangerous clinical manifestation for patients with asthma is dyspnoea. Patients who develop such a symptom often die from an inability to breathe. Therefore, enabling patients with asthma to breathe smoothly is paramount to treating or alleviating asthma. To determine whether JGSQW alleviates dyspnoea, we repetitively administered a dust mite stimulus to mouse airways to establish a chronic asthma mouse model. The mice with chronic asthma that were obtained using such a method exhibited clinical symptoms similar to those of patients with asthma; these symptoms include airway hyperresponsiveness, airway inflammation, airway remodelling, and high total IgE levels in the blood (Yu et al., 1999). The male mice were randomly divided into three groups. With both the 25 and 50 mg/mL methacholine concentrations, the Der p group that was administered only the dust mite stimulus had a significant increase in Penh value compared with that of the naïve group. Moreover, the JGSQW group that had been orally fed JGSQW followed by dust mite stimulation showed a significant decrease in Penh value compared with that of the Der p group with 25 and 50 mg/mL methacholine (Fig. 2a). Furthermore, we analysed the total number of inflammatory cells in the bronchoalveolar lavage fluid of each group of mice, as well as the numbers of macrophages, lymphocytes, neutrophils, and eosinophils. The results showed that all values in the Der p group were significantly higher than those in the naïve group. However, the total numbers of inflammatory cells, macrophages, and eosinophils significantly decreased in the JGSQW group compared with those in the Der p group (Fig. 2b).

3.2. JGSQW alleviates the pathological characteristics of lungs in mice with Der p-induced chronic asthma

The lungs of patients with asthma have three major characteristics: infiltration of inflammatory cells in the lungs, deposition of collagen in the subepithelial regions, and excessive secretion of sputum by goblet cells. Therefore, to determine whether JGSQW alleviates the aforementioned three major pathological characteristics of lungs with asthma, haematoxylin and eosin staining and periodic acid–Schiff staining were separately performed on the pathological sections for analysis. The results indicated that the mice in the naïve group did not have infiltration of inflammatory cells in the lungs or excessive secretion of sputum (Fig. 3a and b, top panel). However, for the mice in the Der p group, infiltration of inflammatory cells surrounding the lung airway tissue was distinctly observed, as was excessive secretion of sputum by goblet cells (Fig. 3a and b, middle panel). However, compared with the Der p group, the JGSQW group had distinctly reduced infiltration of inflammatory cells in the lungs and production of sputum (Fig. 3a and b, bottom panel). Simultaneously, the collagen content was significantly lower in the lung tissue of the mice fed JGSQW (Fig. 3c).

3.3. Effect of JGSQW in regulating the total IgE, Der p-specific IgG1/IgG_{2a/2b}, and IL-5 content in the blood of mice with Der p-induced chronic asthma

Most patients with asthma have been shown to have a higher total IgE content in their serum than individuals without asthma. To understand whether JGSQW regulates humoral

immunity, we collected blood samples from the mice and analysed the samples using ELISA. The results indicated that the total IgE content in the serum of the Der p group mice was significantly higher than that of the mice in the naïve group and that feeding mice JGSQW could effectively reduce the total IgE content induced by dust mite stimulation. In addition, the mice with chronic asthma produced an overly high Der p-specific IgG1 level because of the dust mite stimulation; JGSQW feeding significantly decreased the ratio of Der p-specific IgG1 to IgG_{2a/2b} (Fig. 4). Moreover, IL-5 is known to increase the activity and lifespan of eosinophils and causes the infiltration of eosinophils into inflammatory sites. To determine whether JGSQW reduces the eosinophil content in the lungs by inhibiting IL-5 expression, we analysed the IL-5 content in serum. The results indicated that the IL-5 content in the serum of the Der p group mice was significantly higher than that of the naïve group and that feeding mice JGSQW could effectively reduce the IL-5 content.

3.4. JGSQW significantly inhibits the NF-κB expression in the lungs of mice with Der p-induced chronic asthma

The NF-κB signalling pathway has been considered a crucial target for intervention in asthma treatment. For example, inhibition of NF-κB expression strongly reduces the lung inflammatory responses, airway hyperresponsiveness, and increased mucus levels. Furthermore, the increase in NF-κB activity changes the structure of airway epithelial cells and the membrane permeability; the increased activity also induces an inflammatory response in epithelial cells. Therefore, the experimental results of the EMSA and immunohistochemical (IHC) staining showed that the NF-κB activity in lung cell nuclei and the NF-κB expression in airway epithelial cells in the Der p group, respectively, distinctly increased compared with those in the naïve group. In a comparison between the JGSQW and Der p groups, administering JGSQW distinctly inhibited NF-κB expression and activity (Fig. 5).

3.5. Effect of JGSQW on lung epithelial cells

The IHC experimental results showed that JGSQW reduces the NF-κB expression of epithelial cells. To determine whether JGSQW inhibits the NF-κB expression of epithelial cells, thereby reducing NF-κB-regulated gene expression, IL-1β was used to stimulate A549 lung epithelial cells to activate NF-κB expression. The results showed that JGSQW and its reference standards, paeoniflorin and loganin, exerted a dose-dependent effect that significantly inhibited the ICAM-1 gene expression regulated by NF-κB (Fig. 6a–c). Further examination revealed that JGSQW at a concentration of 5 mg/mL exhibited a 38% inhibition rate towards ICAM-1 gene expression (Fig. 6a) and that 5 mg/mL JGSQW contained 8.28 μM loganin and 3.16 μM paeoniflorin. However, when only paeoniflorin or loganin was used to achieve a similar inhibitory effect, 100 μM paeoniflorin (Fig. 6b) or 200 μM loganin (Fig. 6c) was required. Notably, the inhibitory effect of mixing 10 μM loganin and 3.85 μM paeoniflorin on ICAM-1 gene expression was similar to that of 5 mg/mL JGSQW, and a higher concentration in the mixture resulted in a poorer inhibitory effect (Fig. 6d).

4. Discussion

Two types of prescriptions exist for Shen Qi Wan in the ancient records of Chinese medicine. One is Gosha-jinki-gan (濟生腎氣丸), comprising 10 medicinal materials, and the other is the prescription used in this study, JGSQW, comprising 8 medicinal materials.

Gosha-jinki-gan has the addition of *Achyranthes bidentata* and psyllium seed and has been studied more frequently in clinical research. Gosha-jinki-gan has been proven to have several therapeutic effects, such as alleviating the frequent urination symptoms caused by drugs that treat lower urinary tract symptoms (Fujiuchi et al., 2008; Gotoh et al., 2004), treating the cold sensations or numbness associated with diabetic neuropathy (Hoshino et al., 2017), and treating low back pain (Hamaguchi et al., 2017). Gosha-jinki-gan can also delay the process of renal failure, relieving side pain or tenderness (Lin et al., 2016). By contrast, relatively few studies have been conducted on JGSQW (金匱腎氣丸); most have reported on clinical applications for the treatment of urinary difficulty with oedema (Scheid et al., 2015), hypertension (Xiong et al., 2015), and asthma (Ji et al., 2018; Deng, Shimin, 2012; Wang, Zhongf, 2010; Gan et al., 2016; Han, Ruifeng, 2007). We used a mouse model of chronic asthma induced by Der p, an allergen for 85% of patients with asthma, and verified for the first time that the clinical dose of JGSQW can effectively alleviate the symptoms of patients with allergic asthma; the alleviated symptoms include the high levels of IgE in serum, airway hyperresponsiveness, and airway inflammation and remodelling (Figs. 2, 3, and 4).

To clarify the mechanism of JGSQW in alleviating the symptoms of allergic asthma, we studied the effect of JGSQW on NF- κ B in epithelial cells. The literature has demonstrated that when epithelial cells contact pollutants, viruses, or allergens, the cells activate NF- κ B to transmit inflammatory information and perform immune responses (Loxham et al., 2014). However, most airway epithelial cells in patients with asthma do not have sufficient function and have overactivated NF- κ B (Heijink et al., 2014). This overactivated NF- κ B not only evokes excess gene expression of cytokines related to inflammation, which cause lung inflammatory responses (Heijink et al., 2014), but also leads to the differentiation of Th2 cells, airway hyperresponsiveness, and increased mucus levels. Inhibition of NF- κ B signalling pathways has been identified as a crucial target for intervention in asthma treatment. We used IHC and EMSA experiments and verified that JGSQW can effectively reduce the increased expression and activity of NF- κ B caused by dust mite stimulation (Fig. 5).

Moreover, studies have indicated that inhibiting NF- κ B-regulated ICAM-1 expression or effects can alleviate inflammatory responses (Krakauer, 2000) and the infiltration of inflammatory cells (Huang et al., 2013) or can reduce the number and activity of eosinophils in patients with asthma (Pazdrak et al., 2008). Additionally, IL-5 causes eosinophils to congregate at inflammation sites and increases their lifespan and activity (Akdis et al., 2011; Heijink et al., 2014). Our experimental results indicated that JGSQW reduces the infiltration of inflammatory cells and eosinophils into the lungs (Fig. 2b) and the content of IL-5 (Fig. 4). We inferred that JGSQW reduces the NF- κ B activity of epithelial cells, thereby decreasing ICAM-1 expression and reducing the IL-5 content in serum, ultimately leading to decreased infiltration of inflammatory cells into the lungs. Therefore, we used IL-1 β to stimulate A549 epithelial cells in the lungs to activate NF- κ B expression and activity (Molfino et al., 2011). The results showed that JGSQW inhibited the gene expression of ICAM-1 regulated by NF- κ B (Fig. 6a).

TCM prescriptions achieve therapeutic effects using multisystem, multitarget, and multichannel methods because TCM prescriptions are based on the concept of Chinese medicine combinations; namely, medicinal materials are combined according to certain proportions (Yang, Yifan, 2010). Therefore, we employed the TCM theory of medicine combination to study the molecular mechanism by which JGSQW inhibits ICAM-1 expression. The reference standards of JGSQW are paeoniflorin and loganin. Our experimental results revealed that JGSQW, paeoniflorin, and loganin inhibited ICAM-1 gene expression in a dose-dependent manner, and this inhibitory effect reached statistical significance (Fig. 6). Because TCM has a certain allocation for doses, 1 g of JGSQW contained 0.6468 mg of loganin and 0.3034 mg of paeoniflorin (Fig. 1b), and the

concentration ratio of loganin to paeoniflorin was 2.6. At a concentration of 5 mg/mL, JGSQW showed a 38% inhibition rate towards ICAM-1 gene expression and contained 8.28 μ M loganin and 3.16 μ M paeoniflorin; however, for loganin and paeoniflorin alone to achieve a similar inhibitory effect, concentrations of 200 μ M (Fig. 6c) and 100 μ M (Fig. 6b), respectively, are required. In addition, the inhibitory effect of the mixture of 10 μ M loganin and 3.85 μ M paeoniflorin on ICAM-1 gene expression is similar to that of 5 mg/mL JGSQW (Fig. 6a), and a mixture with a higher concentration resulted in a poorer inhibitory effect (Fig. 6d). The mixture of 10 μ M loganin and 3.85 μ M paeoniflorin can inhibit the NF- κ B-regulated gene expression of GMCSF, MUC5AC, and IL-8 (data not shown), in addition to inhibiting ICAM-1. Summarizing the aforementioned results, the inhibition of ICAM-1 expression by JGSQW is a result of the synergistic effects of paeoniflorin and loganin. Although mixing paeoniflorin and loganin inhibits the expression of the other three genes, JGSQW does not (data not shown). This result may be because the other components in JGSQW offset the effect of inhibiting these three genes (GMCSF, MUC5AC, and IL-8); however, further study is required to verify this possibility. Moreover, loganin and paeoniflorin can achieve maximum effectiveness only at doses of 10 μ M and 3.85 μ M, respectively. Higher doses result in lower effectiveness, which indicates that the medicinal materials in JGSQW must be combined in a specific proportion to maximize their treatment effects, verifying the theory of Chinese medicine combination.

In conclusion, JGSQW and its compounds inhibit the expression and activity of NF- κ B in lung epithelial cells and further affect NF- κ B-regulated expression of genes (e.g., ICAM-1) involved in the inflammatory responses of epithelial cells. JGSQW reduces the abnormal physiological functions caused by the overexpression of these genes by inhibiting their expression, consequently reducing the infiltration of monocytes/macrophages and leukocytes into the lungs and eventually slowing the asthma development process.

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Authors' Contribution

Professor Shung-Te Kao was responsible for the theory and experimental design of Chinese medicine. Dr. Shulhn-Der Wang and Dr. Chih-Che Lin were responsible performing the experiments. Associate Professor Li-Jen Lin was responsible for analysing the experimental results and writing the manuscript.

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Table legends

Table 1. JGSQW medicinal material combinations and doses

TCM materia medica	Botanic family	Botanical nomenclature	Pharmacognostic nomenclature	Amount (g)
1. Shou Ti Huang (熟地黄)	Scrophulariaceae	<i>Rehmannia glutinosa</i> Libosch.	<i>Rehmanniae Radix Praeparata</i>	8.0

2. Shan Yao (山藥)	Dioscoreaceae	<i>Dioscorea opposita</i> Thunb.	Dioscoreae Rhizoma	4.0
3. Shan Chu Yu (山茱萸)	Cornaceae	<i>Cornus officinalis</i> Sieb. et Zucc.	Corni Fructus	4.0
4. Fu Ling (茯苓)	Polyporaceae	<i>Poria cocos</i> (Schw.) Wolf	Poria	3.0
5. Mou Tan Pi (牡 丹皮)	Ranunculaceae	<i>Paeonia suffruticosa</i> Andr.	Moutan Cortex	3.0
6. Tse Hsieh (澤瀉)	Alismataceae	<i>Alisma orientalis</i> (Sam.) Juzep.	Alismatis Rhizoma	3.0
7. Pao Fu Tzu (炮附 子)	Ranunculaceae	<i>Aconitum carmichaelii</i> Debx.	Aconiti Lateralis Radix Praeparata	1.0
8. Jou Kuei (肉桂)	Lauraceae	<i>Cinnamomum cassia</i> Presl	Cinnamomi Cortex	1.0
Total amounts				27.0

Figure legends

Fig. 1. (a) HPLC chromatogram of JGSQW; (b) Content of the reference standards in JGSQW: loganin and paeoniflorin (units: mg/JGSQW; e.g., 1 g).

Fig. 2. Effect of JGSQW on the airway hyperresponsiveness and infiltration of inflammatory cells into the lungs of mice with Der p-induced asthma. (a) Airway hyperresponsiveness evaluation performed 2 days after last dust mite stimulus. (b) Mice were sacrificed 3 days after the last dust mite stimulus, and the total and differential leukocytes in bronchoalveolar lavage fluid were calculated. Values represent the means $\pm$ SDs of 6 mice. # $P < 0.05$ (versus the naïve group); * $P < 0.05$ (between the untreated and JGSQW-treated groups).

Fig. 3. Effect of JGSQW on the lung tissue of mice with Der p-induced asthma. (a) Histopathological study on the infiltration of inflammatory cells in the airways (haematoxylin and eosin staining; original magnification, $\times 100$ and $\times 400$). (b) Histopathological study of goblet cell proliferation and mucus secretion in the airways (periodic acid–Schiff staining; original magnification, $\times 100$ and $\times 400$). (c) Collagen deposition in the lungs. Values represent the means $\pm$ SD of 6 mice. # $P < 0.05$ (versus the naïve group); * $P < 0.05$ (between the untreated and JGSQW-treated groups). Values represent the means $\pm$ SD of 6 mice.

Fig. 4. Effect of JGSQW on antibodies and IL-5 in the serum of mice with Der p-induced

asthma. JGSQW significantly decreased the total IgE level in serum and the ratio of Der p-specific IgG1 to IgG_{2a/2b}. Furthermore, JGSQW significantly reduced the IL-5 expression in serum. #*P* < 0.05 (versus the naïve group); **P* < 0.05 (between the untreated and JGSQW-treated groups).

Fig. 5. Effect of JGSQW on NF-κB in the lungs of mice with Der p-induced asthma. (a) The EMSA results show that JGSQW reduced the NF-κB activity in the entire lung tissue; the arrow indicates the position of the specific DNA-probe complex. (b) In the IHC staining analysis, JGSQW inhibited the NF-κB expression in airway epithelial cells (p65; ×100). Data are representative of the results from three separate experiments.

Fig. 6. Effect of JGSQW, paeoniflorin, and loganin on A549 lung epithelial cells stimulated by IL-1β. (a) JGSQW has a dose-dependent inhibitory effect on the ICAM-1 gene expression. (b) Paeoniflorin and (c) loganin have significant inhibitory effects on ICAM-1 gene expression. (d) The mixture of 10 μM paeoniflorin and 3.85 μM loganin combined according to the weight combination principle for JGSQW exhibited the most favourable inhibitory effect. This gene profile is representative of three independent experiments. #*P* < 0.05 (versus the naïve group); **P* < 0.05 (between the untreated and drug-treated groups).

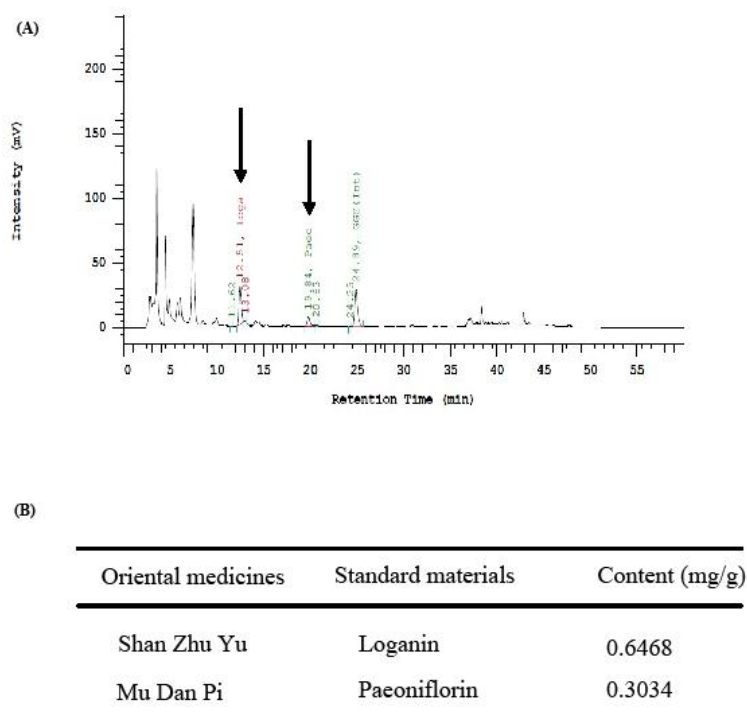


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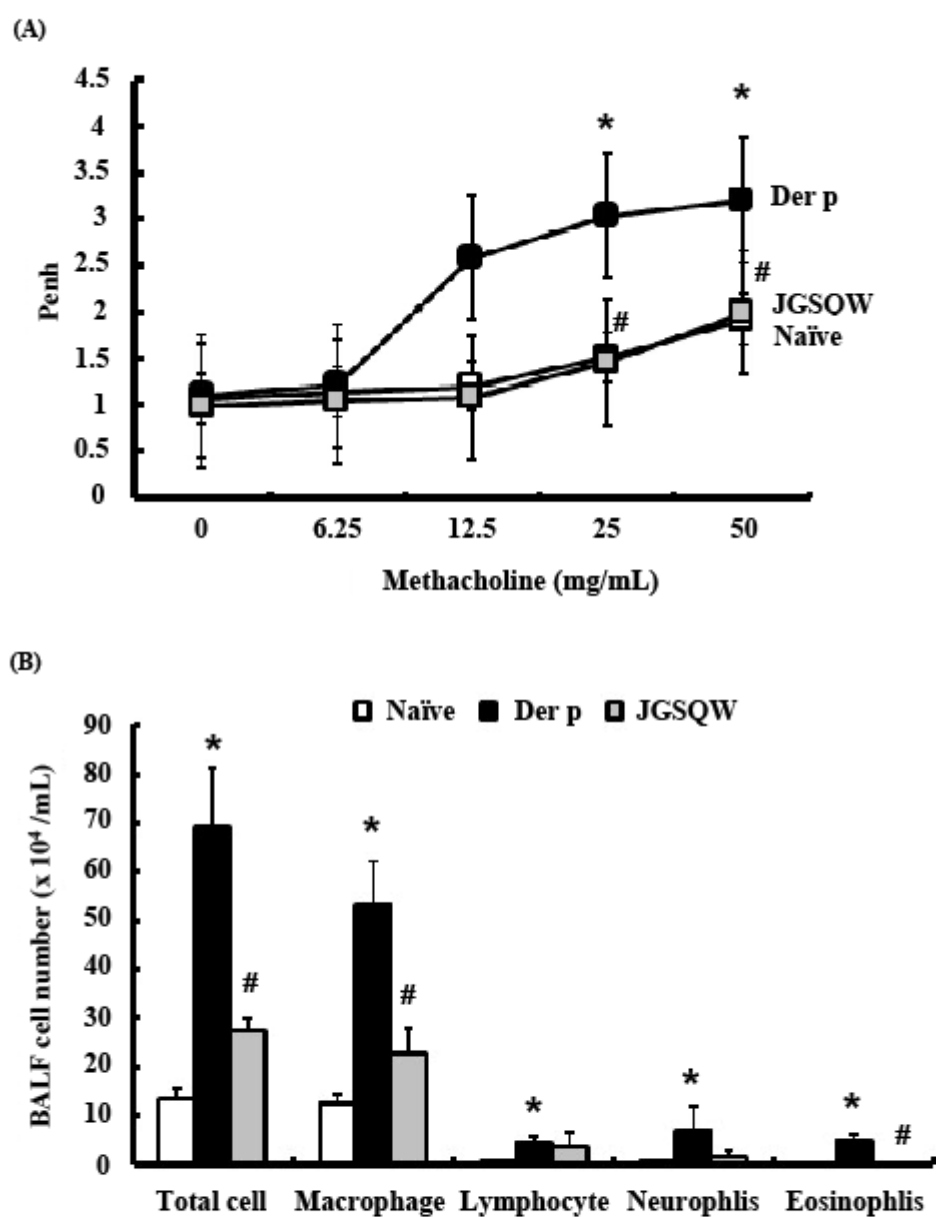


Fig. 2.

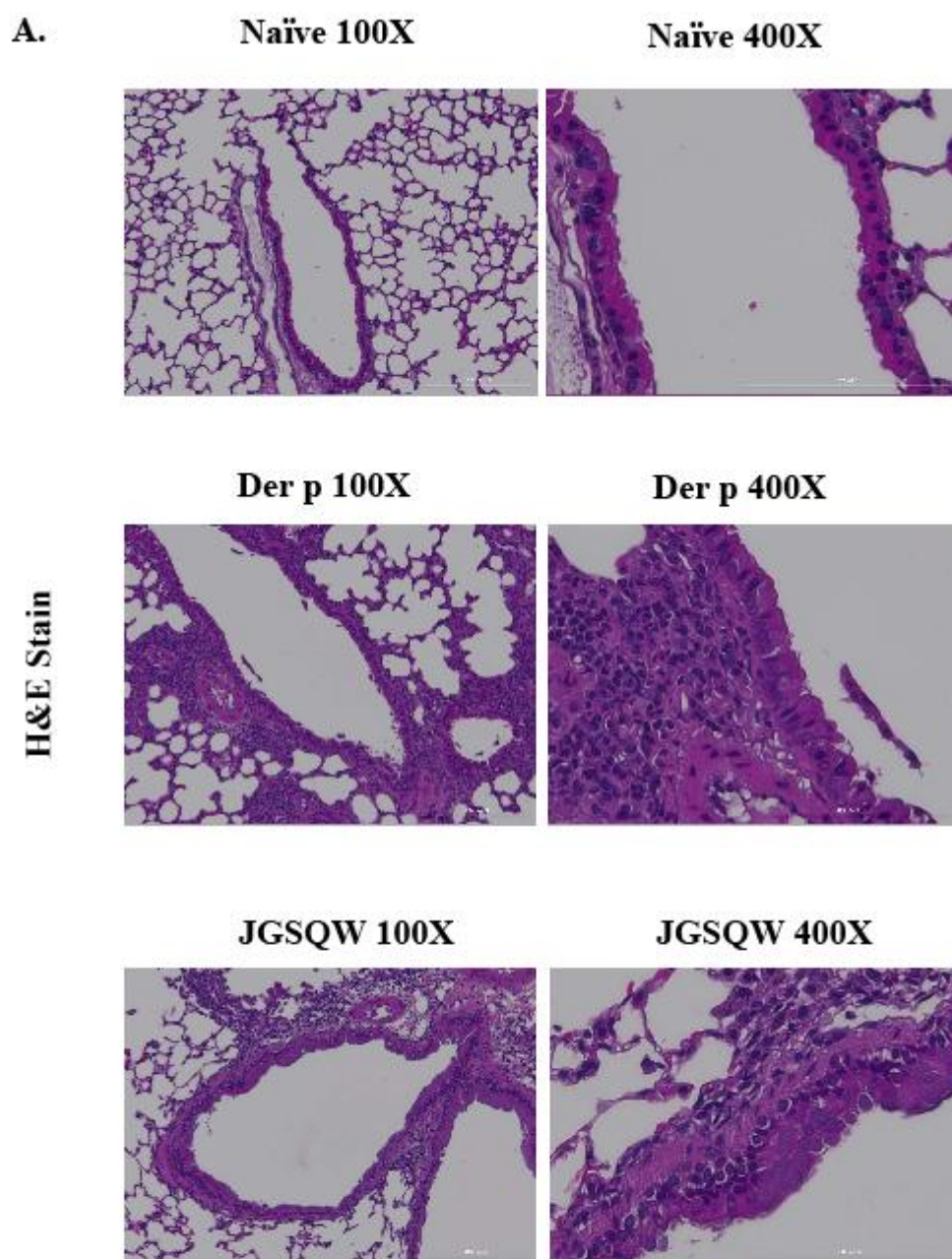


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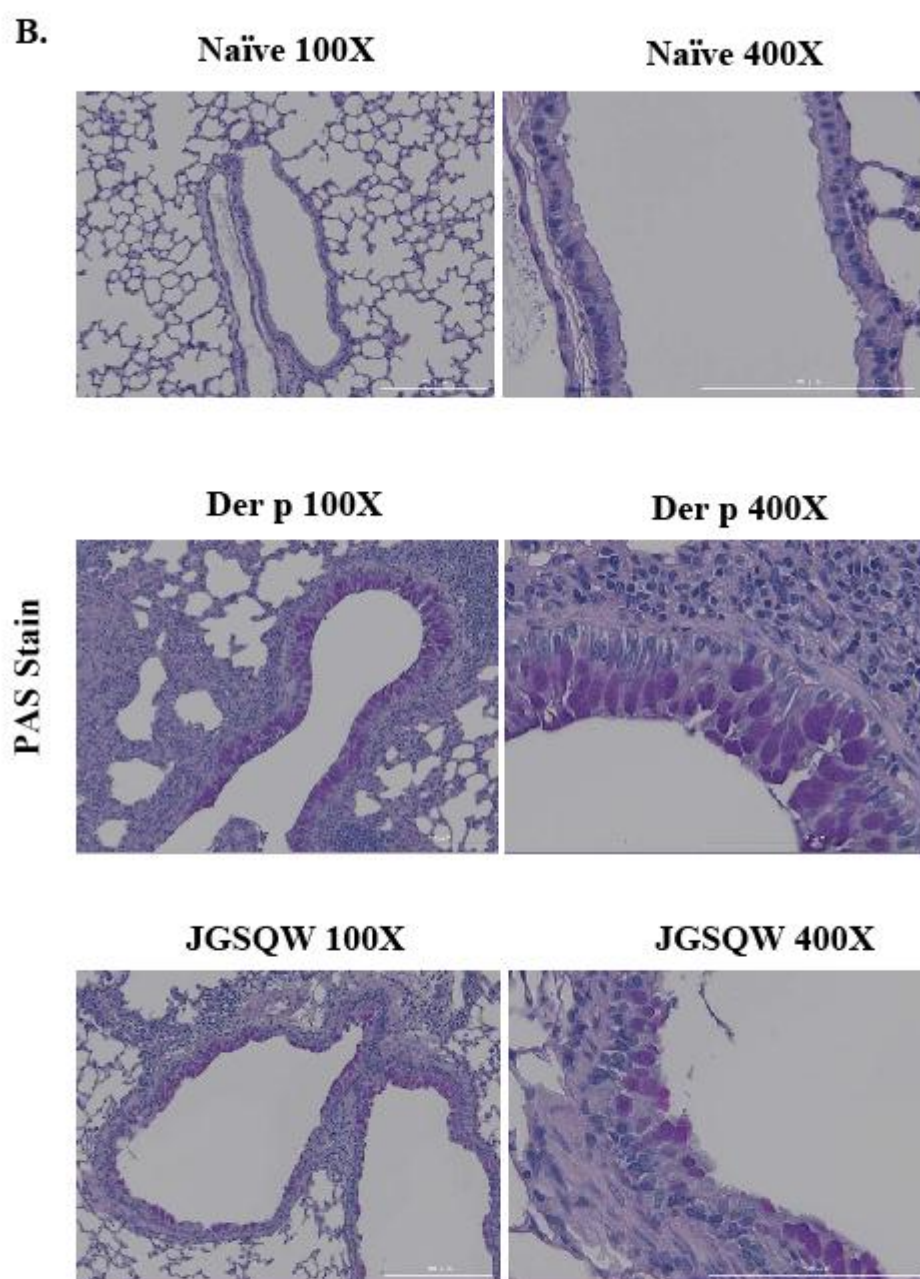


Fig. 3.

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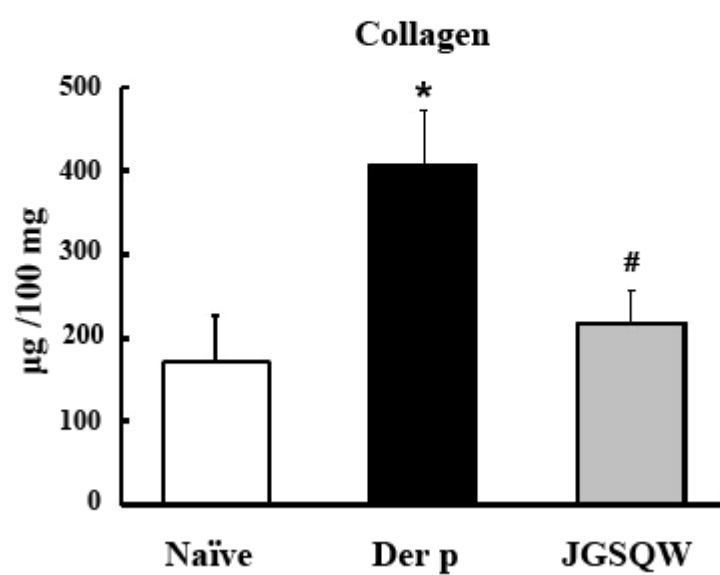


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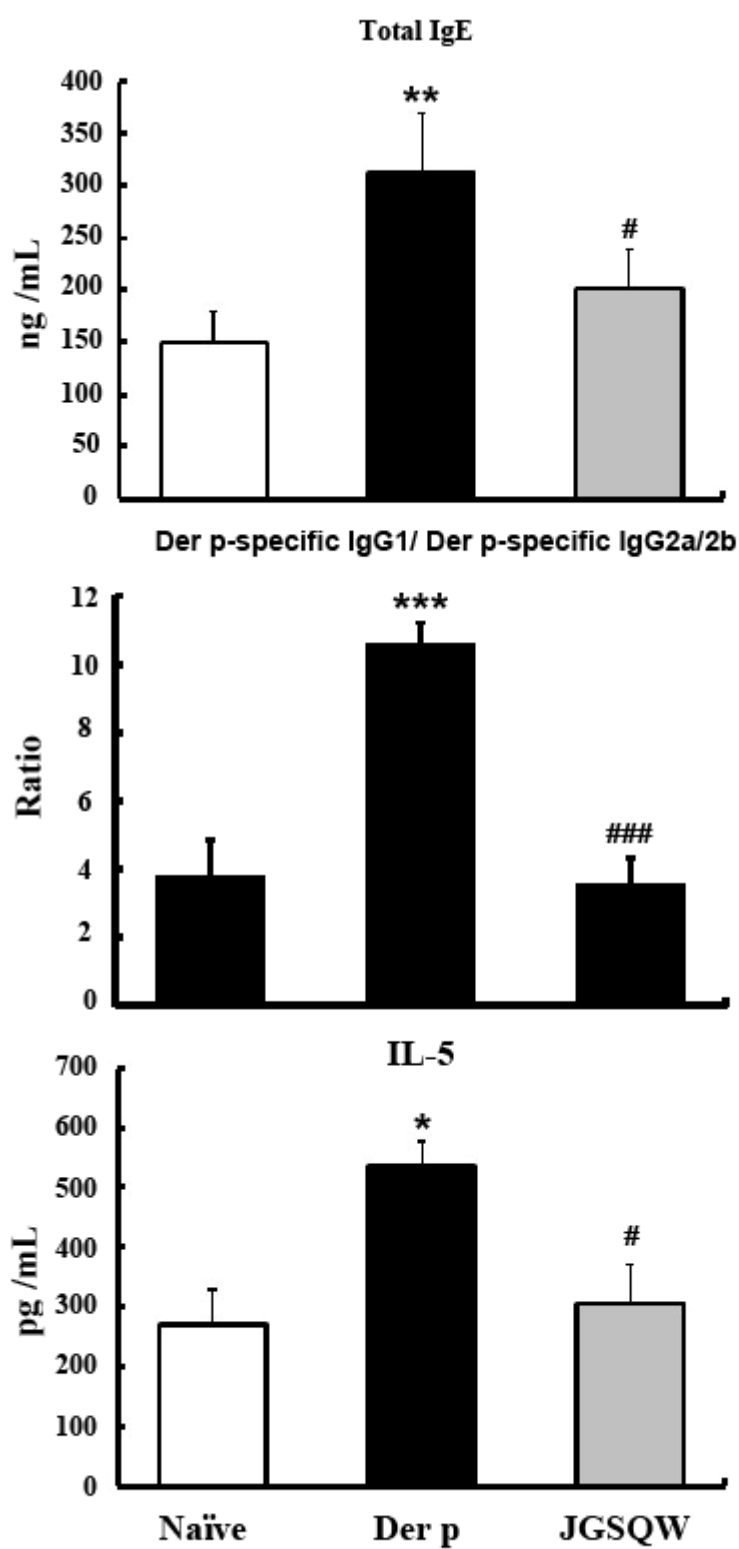


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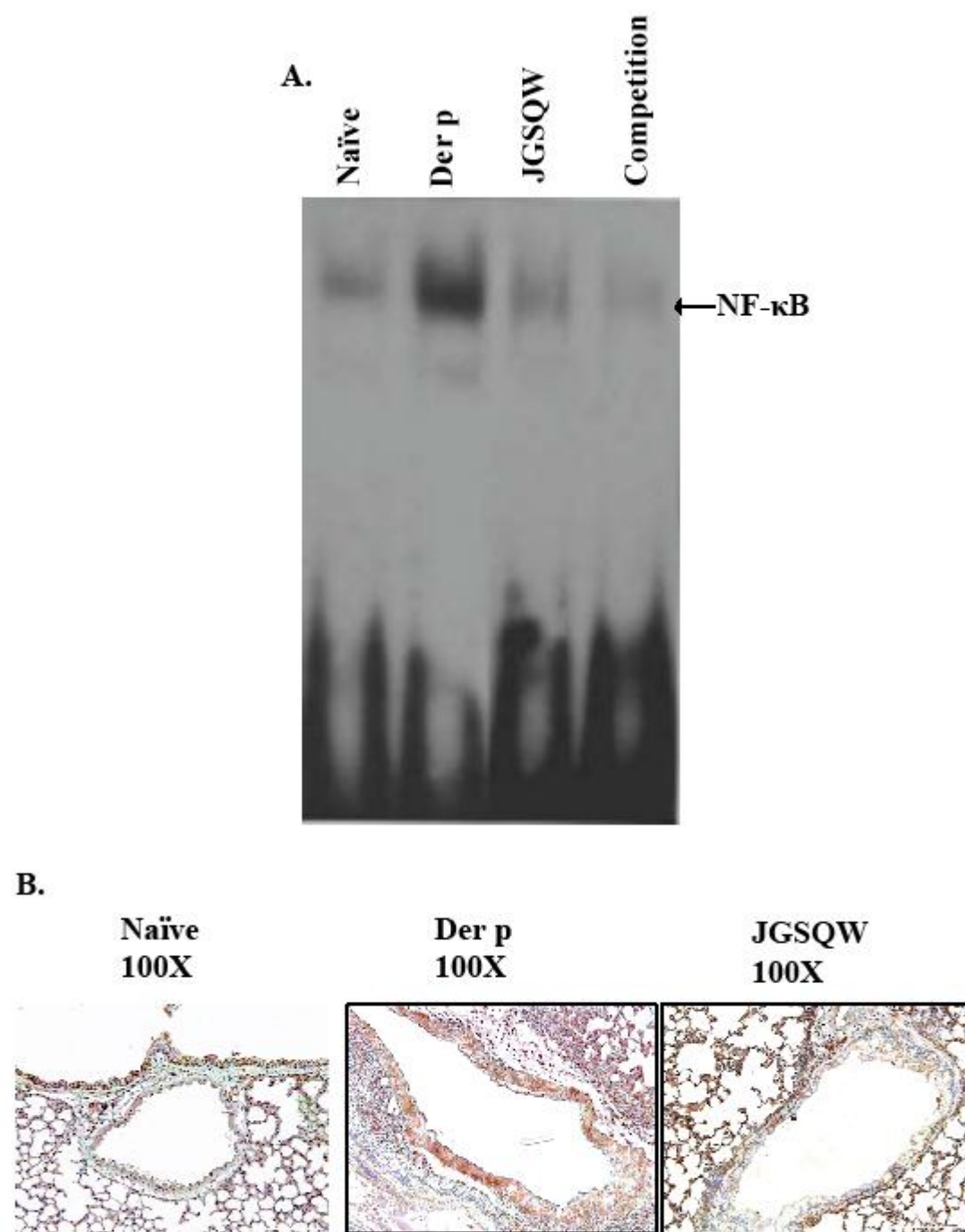


Fig. 5.

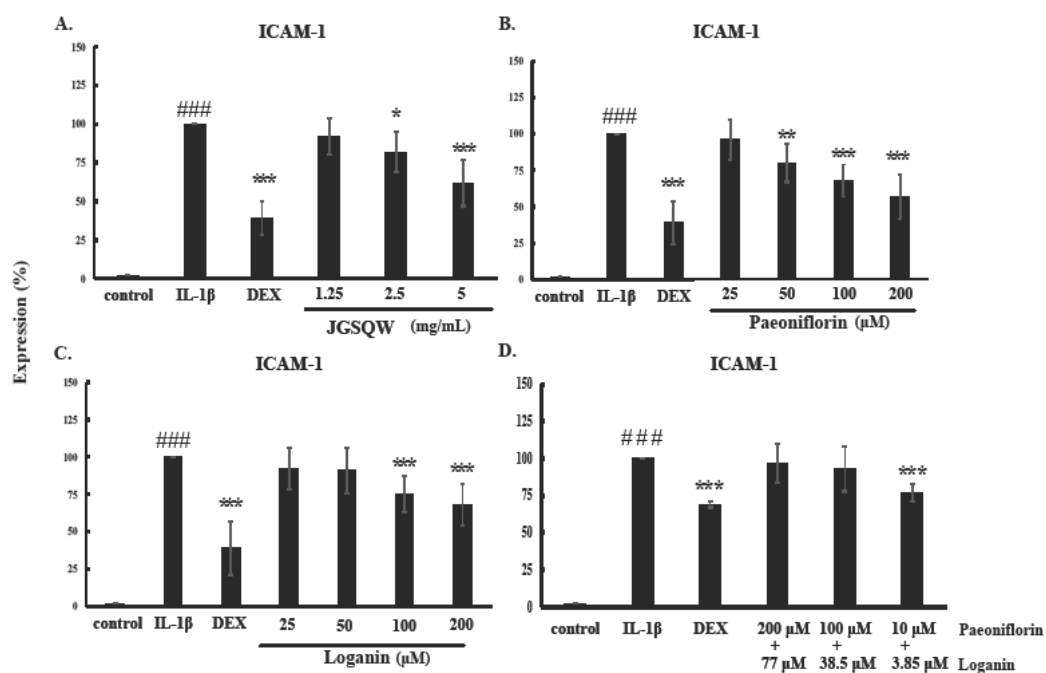


Fig. 6.