

Shufengjiedu capsules protect against neuronal loss in olfactory epithelium and lung injury by enhancing autophagy in rats with allergic rhinitis

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Summary

Shufengjiedu capsules (SFJDCs), a traditional Chinese medicine, have been widely used as an antiviral, antibacterial, antitumor, and anti-inflammatory drug. However, the roles of SFJDCs in allergic rhinitis remain unclear. The purpose of this study was to investigate the effects of SFJDCs in olfaction and lung injury in rats with allergic rhinitis. An animal model of allergic rhinitis was created by intraperitoneal injection and intranasal administration of ovalbumin to rats. All rats were divided into seven groups: a model group, a low-dose SFJDC group, a medium-dose SFJDC group, a high-dose SFJDC group, a cetirizine group, and a control group. Hematoxylin and eosin (HE) staining was used to observe pathological changes in rat lung and olfactory epithelium (OE) tissue, and peripheral blood was collected and subjected to an enzyme-linked immunosorbent assay (ELISA) to detect IgE, tumor necrosis factor alpha (TNF- α), and IL-1 β levels. Western blotting, immunohistochemistry staining, and immunofluorescence staining were performed to detect inflammatory cytokines and levels of the autophagy biomarker beclin1 and the apoptosis biomarker cleaved-caspase3 in lung and OE tissue. ELISA indicated that SFJDCs significantly decreased IgE, TNF- α , and IL-1 β levels in peripheral blood, the lungs, and OE tissue. In addition, Western blotting and staining indicated that SFJDCs repair lung injury, protect against neuronal apoptosis in OE, and rescue impaired autophagy in the lungs and OE tissue. In conclusion, results indicated that SFJDCs might protect against neuronal loss in the OE and lung injury by enhancing autophagy and decreasing apoptosis in rats with allergic rhinitis. Therefore, SFJDCs might serve as an alternative treatment for allergic rhinitis.

Keywords: Shufengjiedu capsules, allergic rhinitis, inflammation, autophagy, apoptosis

1. Introduction

Allergic rhinitis (AR) is the most common otorhinolaryngological disease (1). It is also one of

the main causes of olfactory dysfunction (2). The primary clinical manifestations of AR include nasal blockage, a runny and itching nose, sneezing, and olfactory dysfunction (1). AR is an IgE-mediated type I hypersensitivity inflammatory disease of the nasal mucosa (3). IgE bound to Fc ϵ RI on mast cells and eosinophils is cross-linked by allergens, resulting in the release of diverse preformed and newly synthesized mediators to promote the local recruitment and activation of leukocytes and the production of inflammatory cytokines and T helper 2 (T₂) cytokines, which contribute to the development of late-phase reactions (3). Current treatments for AR include

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antihistamines and hormones, but these drugs often have many untoward effects.

In China, traditional Chinese medicine (TCM) formulations, such as Radix isatidis and Radix bupleuri, have been extensively used to treat various respiratory infectious diseases. Shufengjiedu capsules (SFJDCs) are a TCM formulation consisting of eight medicinal herbs, *Polygonum cuspidatum* Sieb. et Zucc., *Forsythia suspense* (Thunb) Vahl, *Isatis indigotica* Fort., *Bupleurum chinense* DC., *Thlaspi arvense* L., *Verbena officinalis* (Officinalis) L., *Phragmites communistrin* Trin, and *Glycyrrhiza uralensis* Fisch., that are clinically effective at treating upper respiratory tract infections and ALI/ARDS (4). Since SFJDCs have antiviral action, the China Food and Drug Administration (CFDA) has recommended SFJDCs as a treatment for H1N1- and H5N9-induced acute lung injury (5). In China, SFJDCs have been listed as a drug to combat avian influenza (4,6-8). Studies have reported that active constituents of SFJDCs such as resveratrol and quercetin may alleviate inflammation by suppressing the expression of inflammatory factors *via* the mitogen-activated protein kinase (MAPK)/nuclear factor- κ B (NF- κ B) signaling pathway (9-11).

Autophagy is an intracellular self-degradative process that is responsible for the systematic degradation and recycling of cellular components such as misfolded or accumulated proteins and damaged organelles (12). One of the pivotal contributions of autophagy to immunity is the cell autonomous control of inflammation. This property leads to systemic consequences and thereby influences the development of innate and adaptive immunity, which promotes or suppresses pathology in various disease contexts. Autophagy can prevent excessive inflammasome activation and reduce the release of inflammatory factors such as TNF- α and IL-1 β (13).

Olfactory dysfunction is one of the most common symptoms of AR (14-16). Proinflammatory cytokines (IL-1 β , TNF- α , IL-18, IL-22, and IL-33) and OVA-specific IgE levels in peripheral blood are reported to be significantly increased in guinea pigs with ovalbumin (OVA)-induced AR (17). OVA can induce lung injury, which is characterized by acute hypoxemic respiratory failure with several potential causes that include trauma, shock, viruses, and bacterial endotoxins (18). Cells are increasingly exposed to intracellular oxidative stress and the downstream inflammatory pathway is triggered, leading to lung injury (19,20). When clinically used to treat influenza, SFJDCs can effectively improve symptoms such as nasal congestion, a runny nose, and headaches. Bupleurum, licorice, and other traditional Chinese medicines are important components of SFJDCs (4) and could be used to treat AR. However, few studies have examined the effect of SFJDCs on AR, especially in olfaction. The mechanism of SFJDCs on olfaction and lung injury remain unclear. Thus, the current study

sought to explore the effects of treatment with SFJDCs in olfactory epithelium (OE) and the lungs of rats with AR.

The aim of the current study was determine if SFJDCs significantly decrease TNF- α and IL-1 β levels and apoptosis, rescue impaired autophagy, and protect against neuronal loss in OE. If SFJDCs repair lung injury and improve olfactory function by enhancing autophagy in rats with AR, then SFJDCs might serve as an alternative treatment for AR.

2. Materials and Methods

2.1. Animals and Materials

SFJDCs were donated by Jiren Pharmaceutical (Anhui, China). Sixty specific-pathogen-free male Sprague-Dawley rats weighing 250 ± 20 g were purchased from Anhui Medical University's Animal Research Center (Anhui, China). These animals were fed in cages with a 12-hour light/dark cycle, constant temperature, and constant humidity in which mice were given food and water *ad libitum*. All animals involved in experiments were sacrificed in accordance with international standards. Animal care procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Anhui Medical University in accordance with the guidelines of the National Institutes of Health (Approval number: LLSC20190513).

2.2. An animal model of OVA-induced AR and drug administration

A previously reported protocol was followed to create an animal model of AR (21). The experiment timeline is shown in Figure 1A. The rats were randomly divided into six groups (6 mice per group per cage): a control group (Control), a model group (Model), a cetirizine (Yiling Pharmaceutical Company, Shijiazhuang, China) group (Lev, 0.75 g/kg), a high-dose SFJDC (Jiren pharmaceutical Company, Anhui, China) group (SFJDC-H, 0.18 g/kg), a medium-dose group (SFJDC-M, 0.09 g/kg), and a low-dose group (SFJDC-L, 0.045 g/kg) by gradient dilution. Rats were sensitized (every other day for 14 days) by intraperitoneal injection of 0.3 mg of OVA (Sigma A8040, USA) as an antigen and 30 mg of aluminum hydroxide as an adjuvant dissolved in 1 mL of saline. When *i.p.* immunization concluded, rats were administered 0.25% OVA dissolved in saline *via* aerosol (days 14-18). A nasal antigen challenge (days 21-27) was performed with intranasal dripping of 50 μ l of 5% OVA daily for 7 consecutive days. Animals in the control group were administered the same volume of saline. After OVA administration, animals in the cetirizine group and SFJDC groups were administered cetirizine or different doses of SFJDCs (twice/day for 14 consecutive days). The dosages of cetirizine and SFJDCs are the clinical recommended doses according to the

Chinese Pharmacopoeia. Moreover, combined symptom and behavior scoring was used to evaluate the model (21). Serum levels of cytokines were measured using ELISA performed with commercially available kits. Blood samples and nasal mucosa were collected for various assays.

2.3. Hematoxylin and eosin (H&E) Staining

Turbinate bone and lungs embedded in paraffin were sliced at a thickness of 4 μ m. Paraffin sections were deparaffinized in xylene, rehydrated in serial dilutions of alcohol, and immersed in distilled water for 30 sec. Sections were dipped in a hematoxylin solution and agitated for 10 min and then rinsed with distilled water for 1 min. Sections were subsequently stained with a 1% eosin solution for 10 sec with agitation. Stained sections were dehydrated with increasing concentrations of alcohol (70% and 90% alcohol) for 10 min at a time and then immersed in eosin for 3 min. The sections were dehydrated with alcohol and immersed in xylene. Stained sections were observed using an electron microscope (Olympus, Japan).

2.4. Toluidine blue staining

Turbinate bone embedded in paraffin was sliced at a thickness of 4 μ m. Paraffin sections were deparaffinized in xylene, rehydrated in serial dilutions of alcohol, and immersed in a toluidine blue solution for 30 min. Then sections were dipped in a glacial acetic acid solution and rinsed with distilled water for 1 min. The sections were dehydrated with alcohol and immersed in xylene. Stained sections were observed using an electron microscope (Olympus, Japan) (22).

2.5. Immunohistochemistry

For olfactory epithelium staining, animals were first sacrificed and decapitated. Skin and brain tissue were removed, and then the turbinate was removed and immersed in a 0.5 M EDTA (pH 8.0) solution at room temperature (RT) for 2 weeks. Paraffin-embedded OE tissue was sectioned into 4- μ m-thick slices. All tissue sections were mounted on glass slides. The slides were incubated for 12 hours at 4°C temperature with TNF- α (Abcam, ab62609) primary antibody in a humidified chamber, washed, and incubated for 50 min with goat anti-rabbit (074-1505, KPL) secondary antibody. Sections were analyzed with a microscope using a 20 and 40-fold magnification. Cell counting was performed with Image J whereby the cell number was normalized to the neuronal layer area (23).

2.6. Immunofluorescent staining

The OE and lung sections were incubated with beclin-1

(Affinity, AF5128), cleaved-caspase3 (Affinity, AF7022) and IL-1 β (Affinity, AF5103) primary antibodies overnight at 4°C, washed with PBS, and then incubated in second antibodies at 37°C for 50 min. After the sections were washing with PBS, they were incubated in DAPI Staining Solution (Beyotime, C1005) for 10 min. The sections were washed with PBS. Afterwards, the slides were mounted with Antifade Mounting Medium (Beyotime, P0126-5ml), viewed, and photographed under a fluorescence microscope (Leica, Germany), and the images were evaluated using the software ImageJ (NIH, Bethesda, MD, USA).

2.7. Western blotting

Rats were dissected on ice, and tissue was homogenized in lysis buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1% SDS P40, 5 mM EDTA, and protease inhibitors (Complete Mini; Roche). Cellular debris was removed by centrifugation at 14,000 rpm for 20 min at 4°C, and the supernatant was collected for analysis. Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 5% stacking gel and a 10% separation gel to separate 20 L of protein in running buffer. The stacking gel was run at 80 V for 0.5 h, and the separation gel was run at 100 V for 1.5 h. The separated proteins were then electrotransferred onto a nitrocellulose filter membrane in transfer buffer at 180 mA for 1.5 h. Protein blots were blocked with 5% defatted milk for 30 min and probed with specific antibodies against TNF- α (Abcam, ab62609), IL-1 β (Affinity, AF5103), Beclin-1 (Affinity, AF5128), and cleaved-caspase3 (Affinity, AF7022). Anti- α -tubulin antibody (Sigma, T6557) was used as a loading control. After three washes with TBST, secondary antibody (CWS) was added at room temperature for 1 h using 5% milk in TBST followed by three additional washes with TBST. Bands were visualized using the Immobilon Western ECL system and were analyzed with the software Gel Pro Analysis (24).

2.8. Statistical analysis

Data were analyzed using one-way ANOVA or a two-sample *t*-test, where $p < 0.05$ was considered statistically significant. All data are expressed as the mean \pm SEM.

3. Results

3.1. SFJDCs decreased increased serum IgE and mast cells in OE tissue

Nasal rubbing and watery rhinorrhea are two common symptoms of AR in rats (1). In order to detect the effects of SFJDCs on these symptoms, an experiment was performed along the timeline shown in Figure

1A. The frequency of sneezing and nasal rubbing was observed and recorded. All animals were closely observed for development of any nasal symptoms such as sneezing or watery rhinorrhea. In comparison to the control group, mice in the AR model group had clear nasal secretions and an increased frequency of nasal rubbing (data not shown). Figure 1B shows that rats with OVA-induced AR had more watery rhinorrhea. As shown in Figure 1C, IgE levels were lower in the SFJDC-M group than those in the model group but did not differ significantly from those in the SFJDC-L and SFJDC-H groups. As shown in Figure 1D, treatment with SFJDCs significantly reduced the number of mast cells in OE tissue. These findings indicate that SFJDCs alleviated allergic symptoms in rats with AR.

3.2. Morphological changes in lungs and OE tissue

As shown in Figure 2A, the model group displayed capillary congestion, obstruction of small airways by lymphocytic infiltrates, and widened alveolar septa in comparison to the control group. Treatment with medium-dose SFJDCs (0.09 g/kg) significantly reduced histological injury, such as decreasing obstruction of

small airways, to different degrees. As shown in Figure 2B and C, OE appeared thinner and had fewer neurons than that in the control group. The number of neurons in OE increased in the SFJDC-M and cetirizine groups.

3.3. Levels of $TNF-\alpha$ and $IL-1\beta$ in serum, the lungs, and OE tissues

As shown in Figure 3A, ELISA was used to determine levels of $TNF-\alpha$ and $IL-1\beta$ in serum in each group. Results indicated that $TNF-\alpha$ and $IL-1\beta$ levels increased significantly in the model group compared to those in the control group. Rats treated with SFJDCs had a marked decrease in levels of $TNF-\alpha$ and $IL-1\beta$. As shown in Figure 3B, Western blotting was used to detect levels of $TNF-\alpha$ and $IL-1\beta$ expression in lung tissue. Results indicated that levels of $TNF-\alpha$ and $IL-1\beta$ protein increased in the model group in comparison to those in the control group. SFJDCs caused a marked decrease in the levels of $TNF-\alpha$ and $IL-1\beta$ expression in each group. As shown in Figure 3C, immunohistochemistry staining was used to quantify the $TNF-\alpha$ expression in OE tissue in different groups. Results indicated that $TNF-\alpha$ levels decreased significantly in the SFJDC-H and

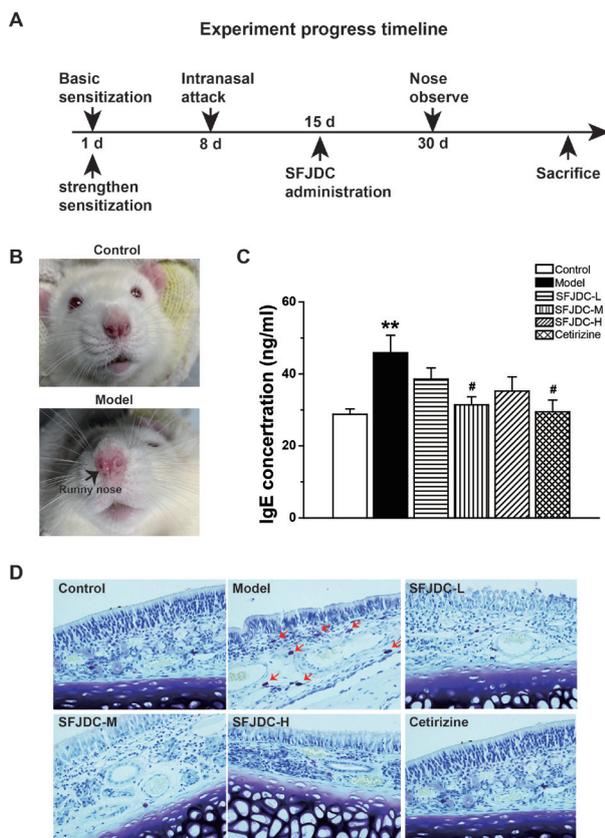


Figure 1. SFJDCs reduced levels of IgE and the number of mast cells in rats with AR. (A): Schematic overview of the study design. (B): Watery rhinorrhea in the model group. (C): Bar graph of the IgE level in serum according to ELISA. (D): Toluidine blue staining of OE from different groups. Arrows indicate mast cells. Data are representative of at least three separate experiments. (** $p < 0.01$ vs. control group; # $p < 0.05$ vs. model group).

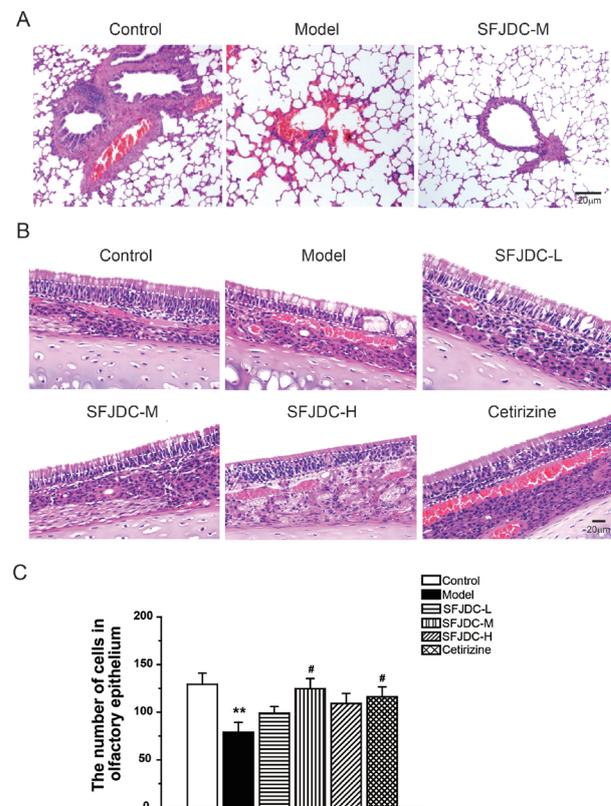


Figure 2. SFJDCs protect against lung injury and OE neuronal loss in rats with AR. (A): HE staining of lung tissue from the control, model, and SFJDC-M groups. (B): HE staining of OE from the control, model, SFJDC-L, SFJDC-M, SFJDC-H, and cetirizine groups. (C): Bar graph of cell numbers in OE. Data are representative of at least three separate experiments. (** $p < 0.01$ vs. control group; # $p < 0.05$ vs. model group).

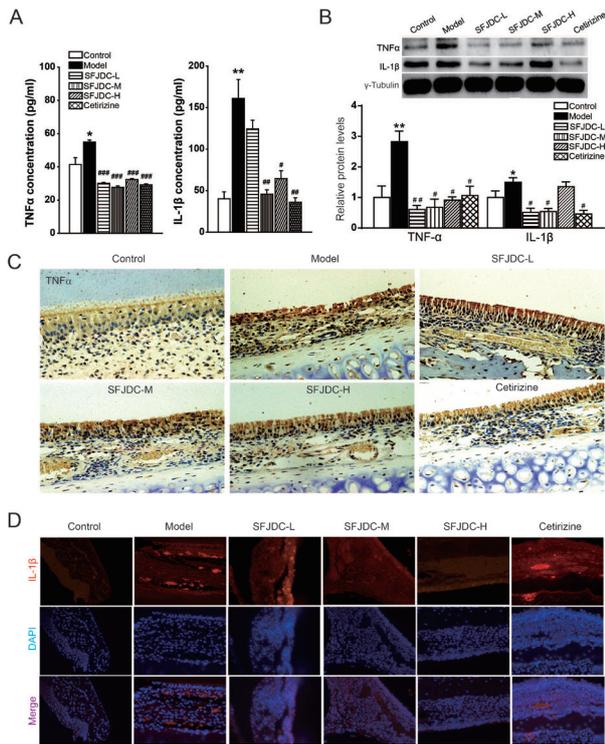


Figure 3. SFJDCs reduced increased TNF- α and IL-1 β levels in serum, the lungs, and OE. (A): Bar graph of TNF- α and IL-1 β concentrations in serum detecting with ELISA. **(B):** Western blot bands of TNF- α and IL-1 β protein in lung tissue. Bar graph of levels of TNF- α and IL-1 β protein in lung tissue. **(C):** Immunohistochemistry staining of TNF- α in OE tissue. **(D):** Immunofluorescent staining of IL-1 β in OE tissue. Data are representative of at least three separate experiments. (* $p < 0.05$, ** $p < 0.01$ vs. control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. model group).

cetirizine groups. There were no significant differences in those levels in the SFJDC-L and SFJDC-M groups. As shown in Figure 3D, immunofluorescent staining was performed to detect the level of IL-1 β in OE tissue. Red fluorescence for IL-1 β increased in the model group in comparison to that the control group, and red fluorescence decreased in the SFJDC-M and SFJDC-H groups. The results shown in Figure 3 indicate that SFJDCs decreased inflammation levels in rats with AR.

3.4. Levels of autophagy and apoptosis in the lungs and OE tissue

As shown in Figure 4, red fluorescence for beclin1 was not evident in the model group, indicating a very low level of autophagy. Green fluorescence for caspase3 increased in the model group in comparison to that in the control group, indicating that the level of apoptosis increased in the model group. When rats were treated with SFJDCs, the beclin1 level significantly increased in the SFJDC-M and SFJDC-H groups. A lower level of caspase3 was observed only in the SFJDC-L group. When Western blotting was used to detect levels of beclin1 and caspase3 protein expression, the same trend was observed, indicating that SFJDCs increased the level

of autophagy and decreased apoptosis. Since OE lesions also appeared, as shown in Figure 5, immunofluorescent staining was used to detect beclin1 and caspase3 levels in OE tissue. Results indicated that the beclin1 level decreased and the caspase3 level increased in the model group in comparison to levels in the control group. SFJDCs resulted in a marked increase in the level of beclin1 and a marked decrease in the level of caspase3 in the SFJDC-M and SFLDC-H groups.

4. Discussion

The current study found that SFJDCs reduce neuronal loss in OE and protect against lung injury induced by AR by increasing the level of autophagy and reducing apoptosis and inflammation factors in rats with AR. These findings are corroborated by several lines of evidence: (i) SFJDCs ameliorate the symptoms of AR, such as a runny or itching nose, sneezing, a decrease in the number of mast cells, and a lower level of IgE (which are markers of an allergy); (ii) TNF α and IL-1 β levels in serum, OE, and lung tissues are downregulated by treatment with SFJDCs; (iii) SFJDCs rescue the levels of beclin1 and cleaved-caspase3 protein, which are respective biomarkers of autophagy and apoptosis. Together, these findings indicate that SFJDCs may play a critical role in AR.

In order to study the effects of SFJDCs on AR, the first step in the current study was to create a reliable animal model of AR. OVA is mainly used as an allergen to create animal models of AR (25-27). The primary clinical manifestations of AR include nasal blockage, a runny and itching nose, sneezing, and olfactory dysfunction (1). In the current study, rats had a runny and itching nose and sneezing after OVA treatment (Figure 1B). A high level of IgE and an increase in mast cells are common hallmarks of AR. Mast cells release molecules that can lead to an allergic reaction. Treatment with SFJDCs decreased nasal rubbing and watery rhinorrhea in rats with AR. Together, these findings indicate that an animal model of AR was successfully created and that SFJDCs ameliorated some symptoms of AR.

OVA is mainly used as an allergen to create animal models of AR (21). In the current study, however, OVA-induced AR produced pathological changes in rats not only in olfactory tissue but also in the lungs (Figure 2A-C). Although several studies have found that asthma and rhinitis are characterized by a similar inflammatory process (28-30), pathophysiologic interactions between the upper and lower airways are not entirely understood. The condition of the upper airway definitely influences the lower airway. The current study considered whether lung injury was caused by AR. AR triggers a systemic increase in inflammation (31). Numerous studies have indicated that the active constituents of SFJDCs, such as resveratrol and other flavonoids, inhibit acute lung injury (ALI). In addition, the MAPK and NF-kB signaling

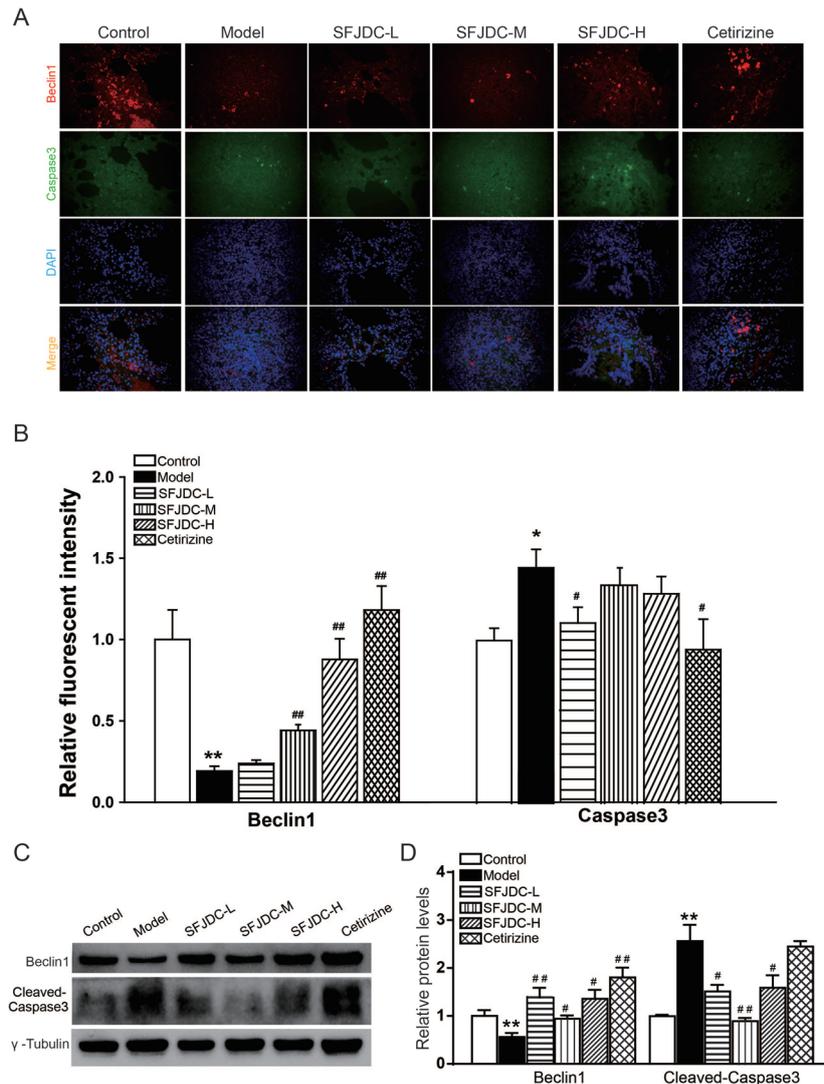


Figure 4. SFJDCs rescued impaired autophagy and levels of apoptosis in lung tissue. (A): Immunofluorescent staining of beclin1 and cleaved-caspase3 in lung tissue. **(B):** Bar graph of levels of beclin1 and cleaved-caspase3 expression in lung tissue. **(C):** Western blot bands of beclin1 and cleaved-caspase3 protein in lung tissue. **(D):** Bar graph of Western blot bands in Figure 5 (C). Data are representative of at least three separate experiments. (* $p < 0.05$, ** $p < 0.01$ vs. control group; # $p < 0.05$, ## $p < 0.01$ vs. model group).

pathways may be involved in ALI (32-38). NF- κ B is a key factor in the inflammatory response and is involved in the release of inflammatory factors and development of ALI (39,40). IL-1 β and TNF- α may serve as biomarkers of the NF- κ B inflammatory pathway. SFJDCs are reported to alleviate inflammation by inhibiting IL-1 β in the NF- κ B signaling pathway. The current study found that IL-1 β and TNF- α levels decreased significantly when rats were treated with SFJDCs, indicating that SFJDCs ameliorate AR by regulating inflammation levels. Autophagy can prevent excessive inflammasome activation and reduce the release of inflammatory factors such as TNF- α and IL-1 β (13). In a physiological state, autophagy can promote apoptosis. Examination of nasal lavage fluid from rats with AR has revealed apoptosis of eosinophils. However, the role of autophagy in AR still remain unknown. Measurement of beclin1 and caspase3 protein levels revealed that SFJDCs improved levels of

autophagy and inhibited apoptosis in OE and lung tissue. These findings indicate that SFJDCs may alleviate lung injury by mediating autophagy and apoptosis and by reducing the release of inflammatory factors in lung tissue.

Olfactory function is reported to be impaired in rats with AR (14). The current study used HE staining to observe the pathology of OE tissue in rats. Rats treated with SFJDCs had more neurons in OE tissue. The immunofluorescent staining of caspase3 indicated that SFJDCs markedly decreased apoptosis in OE tissue. Inflammatory factors such as TNF- α and IL-1 β can induce an increase in apoptosis. Like rats treated with cetirizine, rats treated with SFJDCs had a marked decrease in inflammatory factors and levels of apoptosis in OE tissue. The level of autophagy increased when rats were treated with SFJDCs. These findings indicate that SFJDCs may protect against neuronal loss in OE

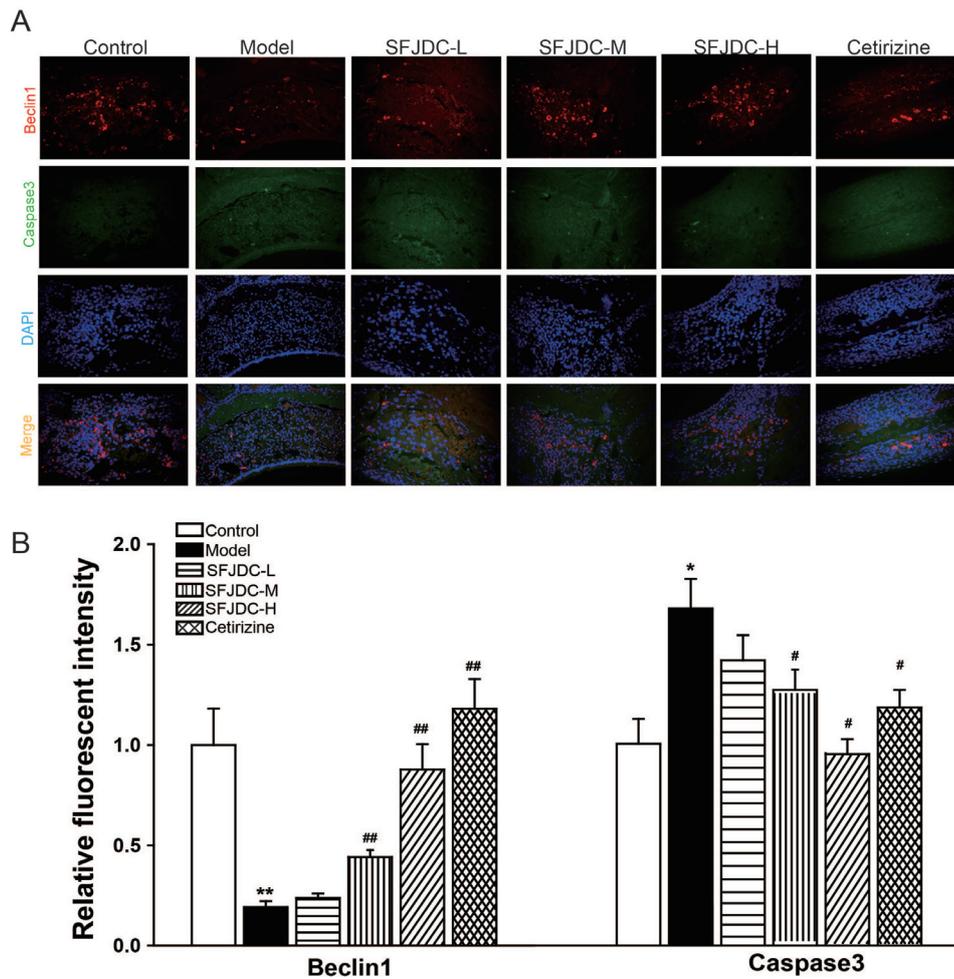


Figure 5. SFJDCs rescued impaired autophagy and levels of apoptosis in OE tissue. (A): Immunofluorescent staining of beclin1 and cleaved-caspase3 in OE tissue. **(B):** Bar graph of levels of beclin1 and cleaved-caspase3 expression in OE tissue. Data are representative of at least three separate experiments. (* $p < 0.05$, ** $p < 0.01$ vs. control group; # $p < 0.05$, ## $p < 0.01$ vs. model group).

by mediating autophagy, apoptosis, and the release of inflammatory factors.

In conclusion, this study revealed that SFJDCs alleviate OVA-induced AR in a rat model by mediating levels of autophagy and apoptosis and by suppressing the release of inflammatory factors. Thus, SFJDCs might serve as an alternative medication to inhibit inflammation in OE and lung tissue, and SFJDCs have great potential for widespread clinical use.

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Statement of Ethics

Animal care procedures were approved by the Institutional Animal Care and Use Committee (IACUC)

at Anhui Medical University in accordance with the guidelines of the National Institutes of Health.

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