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Original Article

Expression of silent information regulator 1 in chronic rhinosinusitis and regulatory effects of inflammatory factors

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Abstract: We aimed to investigate the expression of silent information regulator 1 (Sirt1) in chronic rhinosinusitis (CRS) and the regulatory effects of inflammatory factors. The mucosal epithelial tissues of the nasal ethmoid sinus were collected from 30 patients with CRS from March 2017 to March 2019, and tissues from patients undergoing functional rhinoplasty were included as a control group. H&E staining and immunohistochemistry were performed to detect the histopathologic changes in the nasal mucosa and the expression of Sirt1. Epithelial cells in the control group were extracted from the ethmoid sinus mucosa and cultured *in vitro*. After the cells were treated with 0, 1, 10, and 100 ng/mL interleukin-5 (IL-5) and interferon-gamma (IFN-γ) for 24 h, qRT-PCR and western blotting were carried out to detect the mRNA and protein expressions of Sirt1. Nasal mucosal tissues of the control group were complete in structure, whereas large quantities of inflammatory cells infiltrated in nasal mucosa of the CRS group. Compared with the control group, the CRS group had significantly decreased protein and mRNA expression levels of Sirt1 ($P<0.05$), which significantly declined with increasing concentrations of IL-5 and IFN-γ ($P<0.05$). Thus, expression of Sirt1 in the nasal mucosa tissues of CRS patients is decreased, and inflammatory factors can reduce such expression in a dose-dependent manner. Sirt1 may participate in the inflammatory stress process of CRS.

Keywords: Silent information regulator 1, chronic rhinosinusitis, inflammatory factor

Introduction

Chronic rhinosinusitis (CRS) with nasal polyp (CRSwNP) and CRS without nasal polyp (CRSsNP) are two subtypes of CRS, with main clinical complaints of dizziness, nasal congestion, nasal discharge, or suppuration, which seriously influence people's normal work and study [1]. An epidemiologic study revealed that the incidence rate of CRS has increased annually [2]. CRS is a progressive inflammatory disease of mucosa in the upper respiratory system, with a complex pathogenesis. Bacterial infection, abnormal secretion of mucosal proteins, and action of inflammatory mediators can lead to CRS, and interferon-gamma (IFN-γ) and interleukin-5 (IL-5) are common pathogenic factors [3]. Silent information regulator 1 (Sirt1), as a member of the Sirtuin protein family, is a histone deacetylase which exerts crucial effects on cell proliferation, differentiation, apoptosis, autophagy, and senescence [4-6]. In osteoarthritis of the temporomandibular joint, the expression of Sirt1 is closely associated with inflammatory stress in synovial tissues. Sirt1 can significantly relieve the damage to mouse alveolar epithelial cells caused by paraquat poisoning-induced pneumonia stress [7]. However, the effect of Sirt1 in epithelial cells of the nasal sinus mucosa upon CRS has never been reported hitherto. This study investigated the expression of Sirt1 in the nasal sinus mucosa upon CRS and the influence of inflammatory mediators on the expression of Sirt1, aiming to provide a theoretical basis for the clinical treatment of this disease.

Materials and methods

Tissue samples

The epithelial tissues of the nasal ethmoid sinus mucosa were collected from 30 patients who received surgical treatment by nasal endoscope in our hospital from March 2015 to September 2018. The CRS patients were divided into a CRSsNP group and a CRSwNP group. In CRSsNP group (n=10), there were 6 males and 4 females aged 19-56 years old, with an average age of 43 years. CRSwNP group (n=10) consisted of 7 males and 3 females at the age of 22-58 years old, with a median age of 45 years. Specimens in the control group were collected from 10 patients undergoing functional rhinoplasty. Patients in the control group included 5 males and 5 females aged 22-48 years, with a mean age of 44 years, and they had no CRS history, allergic rhinitis, or history of asthma or inflammatory diseases in the respiratory tract. No statistically significant differences were detected in the gender and age among the three groups. Bronchial asthma, diabetes complications, acetylsalicylic acid intolerance, nasal sinus fungus, bacterial or viral infection, pregnancy, malignant tumors, nasal immotile cilia syndrome, and immune deficiency syndrome were not detected by sinus CT in all the included patients. Also, all patients received nasal endoscopic surgery for the first time, and were not administered hormone or antihistamine drugs prior to operation [8]. The patients in this study were diagnosed according to the standards of the European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS) 2012 [9]. Moreover, all the patients were informed of the relevant research contents and signed an informed consent. This study was approved by the Ethics Committee of our hospital (approval No. 2019-NIS-012).

Reagents and apparatus

The main reagents included paraformaldehyde (Nanjing Zhuopu Biotechnology Co., Ltd., China), Dulbecco's modified Eagle's medium (DMEM, Gibco, USA), hematoxylin and eosin (H&E) staining kit (Thermo Fisher Scientific, USA), trypsin (Amresco, USA), and immunohistochemical and western blotting kits (Thermo Fisher Scientific, USA).

The main apparati included CO_2 cell incubator (Shel Lab, USA), cell culture plates (Costar, USA), qRT-PCR system (Applied Biosystems, USA), ultraclean bench (Beijing Liuyi Biotechnology Co., Ltd., China) and laser confocal fluorescence microscope (Leica, Germany).

Observation of nasal mucosal tissues by H&E staining

A total of 10% of ethmoid sinus mucosal epithelial tissues were independently taken out from each group, fixed with 10% paraformaldehyde, and subjected to H&E staining. Then the histopathologic changes of the nasal mucosa were observed under an optical microscope.

Detection of Sirt1 protein expression in nasal mucosal tissues by immunohistochemistry

A total of 10% of ethmoid sinus mucosal epithelial tissues were taken out, conventionally fixed, embedded, and sliced. Afterwards, the tissues were deparaffinized with xylene and ethanol with gradient concentrations, retrieved in citrate buffer, soaked in 3% hydrogen peroxide at room temperature for 10 min and blocked. Subsequently, the slices were incubated with diluted primary antibodies at 4°C overnight, rinsed on the next day, incubated with secondary antibodies at room temperature for 30 min and washed. Next, an appropriate amount of DAB was added for 2-5 min of reaction which was terminated by deionized water. Then the slices were counterstained with hematoxylin, dehydrated by ethanol in gradient concentrations, and soaked in xylene and dried, followed by mounting with neutral resin. The experimental results were observed under the microscope and recorded. Yellow or brownish yellow particles in the cell membrane and cytoplasm were the criteria for a positive reaction. A total of 10 fields of view (400×) were randomly selected from each slice, and the staining area and intensity were scored with 100 cells as a counting unit according to the following scoring criteria. 0 point: staining area ≤10%, 1 point: staining area >10-25%, 2 points: staining area >25-50%, 3 points: >5-

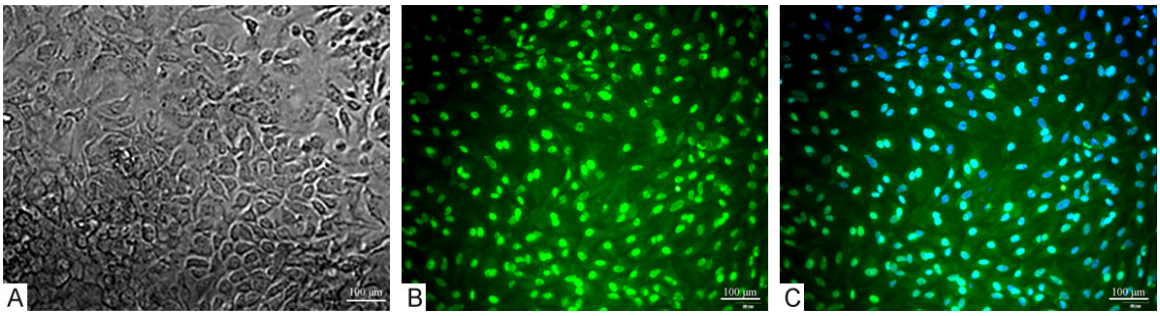

Figure 1. Identification of primary nasal mucosal epithelial cells (magnification: ×400). A: Cell morphology under an inverted phase contrast microscope; B: Cytokeratin AE1 immunofluorescence staining of cells; C: AE1 immunofluorescence staining of cell nuclei.

75%, and 4 points: staining area >75%. The cells unstained were recorded as 0 points, and those stained pale yellow as 1 point, yellow as 2 points and brownish yellow as 3 points. If the sum of the two scores ≥3 points, the staining results were positive, otherwise they were negative. The slices were read in a double-blind fashion, scored separately, and finally summarized uniformly by two pathologists. Disputed scores were reviewed and revised by more than two experienced pathologists to obtain the final results.

Extraction and in vitro culture of nasal mucosal epithelial cells

A total of 10% of epithelial tissue of the nasal ethmoid sinus mucosa was taken out from the control group, washed with sterile normal saline 3 times, maintained in a mixed solution of trypsin (0.25%) and EDTA (0.01%) at 4°C for 15-20 h, and centrifuged at 1,000 rpm for 3 min to remove the supernatant. Then the precipitate was washed with phosphate-buffered saline (PBS) 3 times, and the cells were suspended with 200 mL of DMEM and Ham's F-12 medium containing 10% fetal bovine serum at a ratio of 3:1. Afterwards, the cells were routinely cultured in an incubator with 5% CO_2 at 37°C.

Identification of primary nasal mucosal epithelial cells

When the monolayer cells grew to nearly complete fusion, they were inoculated to 96-well culture plates at a concentration of 1×10^5/mL, and placed in an incubator with 5% CO_2 at 37°C for 24 h. After the morphology of primary nasal epithelial cells was observed under an inverted microscope, the cells were washed with PBS twice and fixed by 4% paraformaldehyde, followed by membrane permeation using 0.1% Triton X-100 for 15 min and blocking with BSA for 30 min. Cytokeratin IgG antibody (AE-1) diluted at 1:100 was added for incubation at 4°C overnight [10]. On the next day, secondary antibodies were added for incubation at room temperature for 1 h. At last, DAPI was added for staining, and the results were observed under a fluorescence microscope and recorded through photography. Under an inverted phase contrast microscope, primary nasal mucosal epithelial cells adhered to the wall and grew well mostly in polygonal and fusiform shapes (**Figure 1A**). These cells were closely connected, showing a "stone pavement" growth trend, with no obvious multilayer. Subsequently, cytokeratin AE1 immunofluorescence identified that the epithelial cells had green fluorescence, and the stained nucleus displayed blue fluorescence, which confirmed that the extracted cells were epithelial (**Figure 1B** and **1C**).

Detection of Sirt1 mRNA expressions in nasal mucosal epithelial cells induced by different concentrations of IL-5 and IFN-γ by qRT-PCR

The cells were inoculated into 96-well culture plates at 1×10^5/mL and incubated in an incubator with 5% CO_2 at 37°C for 24 h. Following independent cell treatment with 0, 1, 10, and 100 ng/mL IL-5 and IFN-γ for 24 h, single cells were collected, respectively, and qRT-PCR was carried out to detect the mRNA expression of Sirt1 in nasal mucosal epithelial cells.

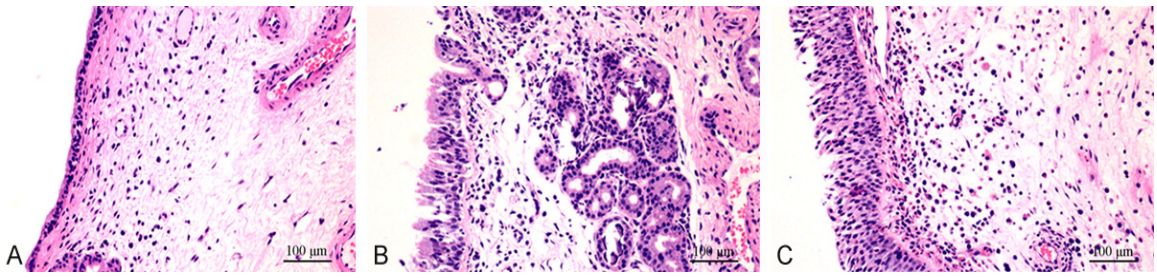

Figure 2. Pathologic changes of nasal mucosa tissues observed by H&E staining (magnification: ×400). A: Control group; B: CRSsNP group; C: CRSwNP group.

Upstream primer for Sirt1: 5'-CGTCGGCCACTGATTCTCAAA-3'; downstream primer: 5'-GGCAGGGGATCTCTTAGGTTC-3'.

Detection of Sirt1 protein expressions in nasal mucosal epithelial cells induced by different concentrations of IL-5 and IFN-γ by western blotting

The cell concentration was adjusted, and the cells were seeded into 96-well culture plates at 1×10^5/mL and incubated in an incubator with 5% CO_2 at 37°C for 24 h. After respective cell treatment with 0, 1, 10, and 100 ng/mL IL-5 and IFN-γ for 24 h, single cells were collected, and the protein expression of Sirt1 in each group was examined by western blotting. Total protein was extracted and subjected to SDS-PAGE. The product was transferred to a PVDF membrane that was then blocked with 50 g/L skimmed milk in TBST buffer at room temperature for 60 min and incubated with rabbit anti-human Sirt1 antibody (Abcam, USA; 1:300 diluted) for 1.5 h at room temperature. After washing, the membrane was incubated with HRP-labeled goat anti-rabbit IgG (Abcam, USA; 1:5000 diluted) at room temperature for 1.5 h, and then color-developed by ECL reagent. GAPDH was used as the internal reference, and UVP GelStudio processing system and Labworks 4.6 software were used to analyze the absorbance of target band. The ratio of Sirt1/AGAPDH was used to represent the relative expression of Sirt1.

Statistical analysis

All data were statistically analyzed by SPSS 26.0 software, and figures were plotted with Graphpad5.01 software. Immunohistochemical assay results were subjected to the rank sum test. The differences between three groups were compared by the Kruskal-Wallis test, and pairwise comparisons were performed by the Mann Whitney U test. The results under different stresses were subjected to analysis of variance. $P<0.05$ was considered significant.

Results

Pathologic changes of nasal mucosa tissues by H&E staining

Patients in the control group had a complete nasal mucosa tissue structure, clear and distinguishable pseudostratified columnar cilia that were uniform in thickness and orderly arranged, a small amount of fibrous connective tissue distributed in the submucosa, and uniformly arranged mucosal epithelial cells with a complete structure. However, patients in the CRSsNP group displayed disordered nasal mucosa cilia of different thickness, pseudostratified columnar and stratified squamous structures, obviously thickened epithelial matrix, remarkable edema in interstitial cells, infiltration by a large number of inflammatory cells, especially neutrophils, and notably increased fibroblasts and goblet cells. In addition, in the CRSwNP group, the epithelial cell structure of nasal mucosal tissues was incomplete, and even fell off. The number of goblet cells became larger. The basement membrane of mucosa was thickened. Infiltration of numerous eosinophils could be seen in the lamina propria of mucosa, and glands were markedly swollen with evidently reduced quantity. No nerve structure and no new blood vessels appeared in polyps (**Figure 2**).

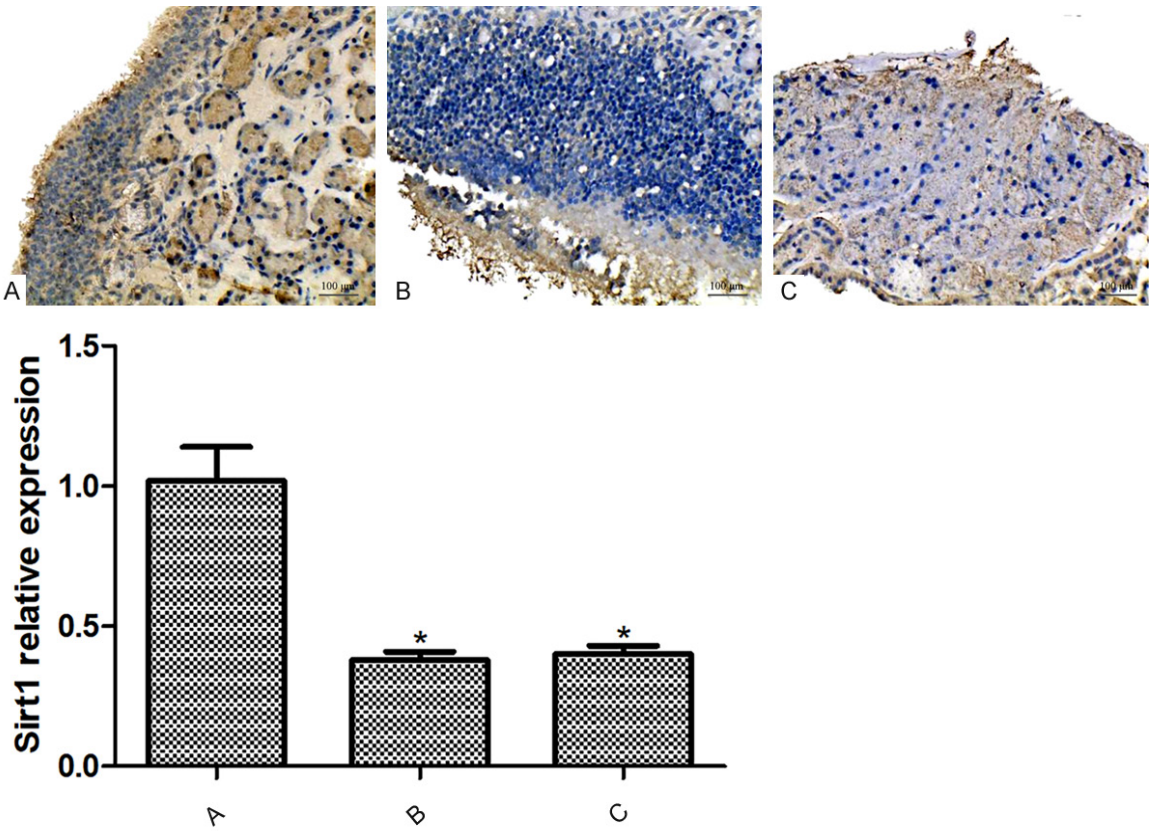

Figure 3. Sirt1 protein expressions in nasal mucosal tissues detected by immunohistochemistry (magnification: ×400). A: Control group; B: CRSsNP group; C: CRSwNP group.

Sirt1 protein expressions in nasal mucosa tissues detected by immunohistochemistry

Sirt1 was mainly expressed in the membrane and cytoplasm of normal nasal mucosal cells. Compared with the control group, CRSsNP group and CRSwNP group exhibited a significantly decreased expression of Sirt1 in the nasal mucosa ($P<0.05$). However, there was no statistically significant difference in the expression of Sirt1 in the nasal mucosa of patients between the CRSsNP group and CRSwNP group ($P>0.05$) (**Figure 3**).

Effects of IL-5 on Sirt1 protein and mRNA expressions in nasal mucosal epithelial cells

The effects of different concentrations of IL-5 on the protein and mRNA expressions of Sirt1 in the epithelial cells of nasal mucosa were examined by western blotting and PCR (**Figure 4**). Results demonstrated that the protein and mRNA expressions of Sirt1 declined significantly with the increase of IL-5 concentration ($P<0.05$), showing a dependence on IL-5 concentration.

Effects of IFN-γ on Sirt1 protein and mRNA expressions in nasal mucosal epithelial cells

Western blotting and PCR were employed to examine the effects of different concentrations of IFN-γ on the protein and mRNA expressions of Sirt1 in the epithelial cells of nasal mucosa (**Figure 5**). The protein and mRNA expressions of Sirt1 were reduced significantly with an increasing IFN-γ concentration ($P<0.05$), displaying dependence on IFN-γ concentration.

Discussion

Chronic rhinosinusitis (CRS) is an inflammatory disease of the upper respiratory system in mucosa of the nasal cavity and paranasal sinuses, which seriously influences the heal-

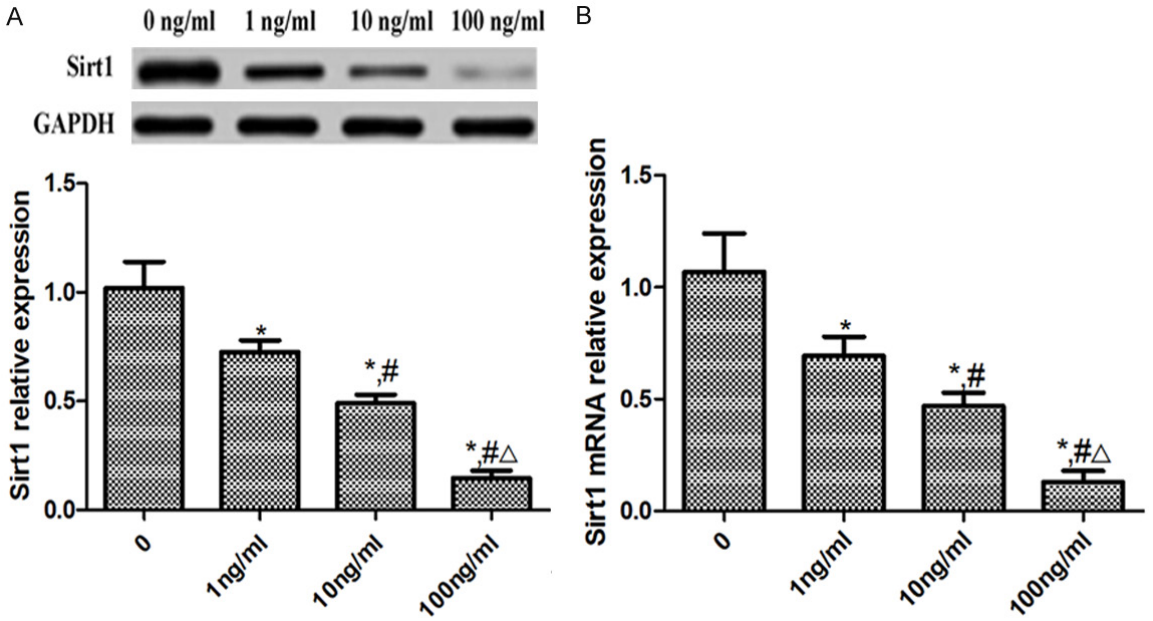

Figure 4. Effects of IL-5 on (A) Sirt1 protein and (B) mRNA expressions in nasal mucosal epithelial cells. Compared with 0 ng/ml, *P<0.05; compared with 1 ng/ml, #P<0.05; compared with 10 ng/ml, ΔP<0.05.

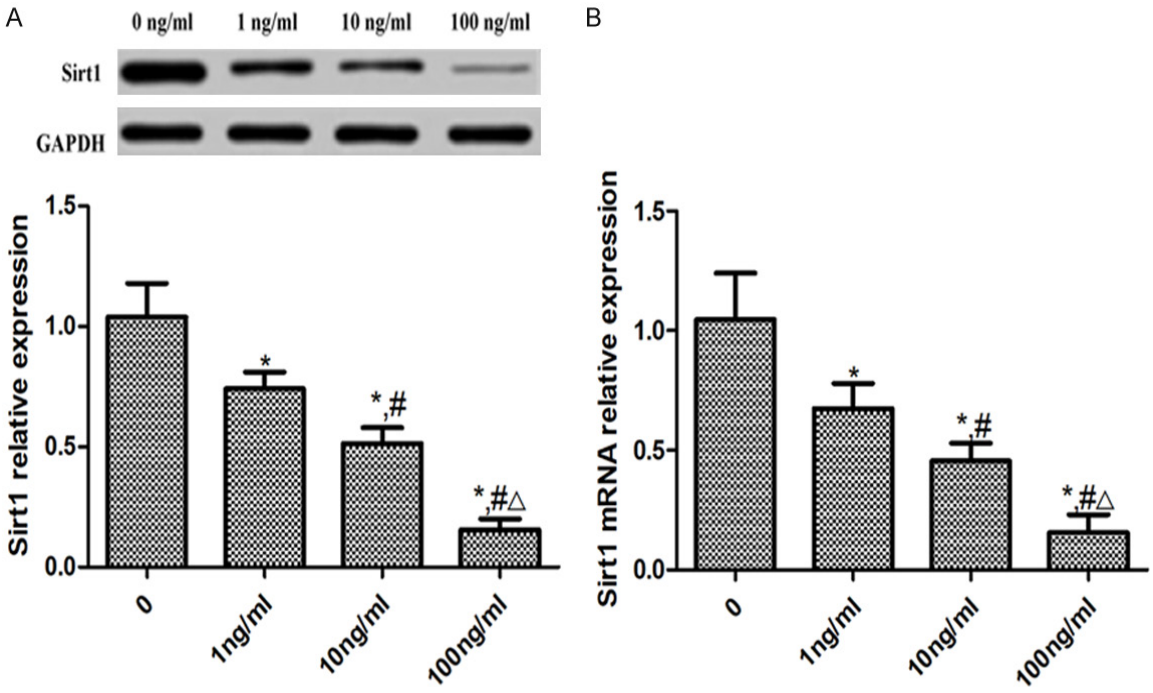

Figure 5. Effects of IFN-γ on Sirt1 protein and mRNA expressions in nasal mucosal epithelial cells. Compared with 0 ng/ml, *P<0.05; compared with 1 ng/ml, #P<0.05; compared with 10 ng/ml, ΔP<0.05.

th and life of patients. In spite of many hypotheses about the pathogenesis of CRS, such as allergic reaction, immune imbalance, and microbial membrane infection [11], the specific pathogenesis has not been fully clarified and there is no radical treatment. Inflammatory fac-

tors exert crucial effects on the pathogenesis and development of CRS, so exploring the role of inflammatory factors in the treatment of CRS is of great significance [12].

H&E staining is an important means to objectively evaluate the grade of CRS in patients at present [13]. Herein, H&E staining demonstrated that patients in control group had a complete nasal mucosal tissue structure, while those in CRSsNP group displayed disordered nasal mucosa cilia, infiltration of neutrophils, and notably increased fibroblasts and goblet cells. In addition, in the CRSwNP group, the number of goblet cells became larger, the basement membrane of the mucosa was thickened, infiltration of numerous eosinophilic inflammatory cells could be seen in the lamina propria of the mucosa, glands were markedly swollen with evidently reduced quantity, and no nerve structure and no new blood vessels appeared in polyps. The above observations verified that CRS is an inflammatory stress response of the nasal mucosa.

Moreover, CRS is caused by the synergistic effect of many physiologic factors [14]. Sirt1 is a deacetylase widely found in the nucleus. Sirt1 exerts vital regulatory effects on the physiologic processes of cells, including oxidative stress, autophagy, metabolism, aging and apoptosis, and inflammatory stress [15]. Ma et al. conducted *in vivo* research and discovered that upregulating the expression of Sirt1 could improve the ability to resist the proliferation of macrophage THP-1 and inhibit the expression of inflammatory factors induced by oxidized low-density lipoprotein [16]. Inoue et al. indicated that activating the Sirt1 signaling pathway could markedly suppress endotoxin-triggered inflammatory stress [17]. Chen et al. found that the targeted regulation of Sirt1 expression was able to relieve the symptoms of vascular tension meningitis [18]. In this study, the results of immunohistochemistry displayed that the expression of Sirt1 in the nasal mucosa of patients in CRS group dropped significantly in contrast to that of the control group. Therefore, it can be inferred that the abnormally expressed Sirt1 may affect the morphology and structure of nasal mucosal epithelial cells and play a pivotal role in the physiologic process of nasal mucosa and the pathologic process of CRS.

Inflammatory lesions are the most crucial pathologic changes to the CRS nasal mucosal epithelium [19]. IL-5, IFN-γ, and other cytokines are indispensable pro-inflammatory factors in the body's mucosal epithelium [20]. During inflammatory stress, IL-5 and IFN-γ secreted by the mucosal epithelium can amplify the inflammatory signal cascade and activate the expression of related inflammatory signaling pathways, resulting in the accumulation of a large number of inflammatory factors and aggravating the injury of epithelial cells. Sirt1 expression in the serum of patients with chronic obstructive pulmonary disease had an obvious negative association with the level of inflammatory factor pairs [21]. IL-5 and IFN-γ are active immunoinflammatory molecules in the nasal mucosal tissues of CRS patients, and their expressions in tissues can be used as markers for postoperative relapse of CRS [22]. In this study, after the primary nasal mucosal epithelial cells cultured *in vitro* were stimulated by different concentrations of IL-5 and IFN-γ, the mRNA and protein expressions of Sirt1 were detected using PCR and western blotting. It was discovered that the mRNA and protein expressions of Sirt1 declined significantly with an increase in inflammatory factor concentration, showing a dose dependence. Regardless, this study still has limitations. The roles of Sirt1 in the onset and progression of CRS and whether Sirt1 regulates the levels of inflammatory factors remain unclear. Further in-depth studies are in need to unravel the role of Sirt1 in CRS.

Conclusion

In summary, this study proves that the expression of Sirt1 in the nasal mucosa tissues of CRS patients is decreased, and inflammatory factors can reduce the expression of Sirt1 in a dose-dependent manner. Therefore, it can be inferred that Sirt1 may participate in the inflammatory stress process of CRS. However, the specific stress mechanism of Sirt1 in CRS needs further in-depth exploration.

Disclosure of conflict of interest

None.

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