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MiR-31 aggravates inflammation and apoptosis in COPD rats *via* activating the NF-κB signaling pathway

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Abstract. – OBJECTIVE: To study the effect of micro ribonucleic acid (miR)-31 on rats with chronic obstructive pulmonary disease (COPD) by activating the nuclear factor-κB (NF-κB) signaling pathway.

MATERIALS AND METHODS: A total of 36 Sprague-Dawley rats were randomly divided into normal group (n=12), model group (n=12) and miR-31 mimics group (n=12). The rats were fed normally in normal group. In model group, the COPD model was first established, followed by intervention using normal saline. In miR-31 mimics group, the COPD model was also first established, followed by intervention using miR-31 mimics. The expression of NF-κB was detected *via* immunohistochemistry. Protein expressions of B-cell lymphoma-2 (Bcl-2) and Bcl-2 associated X protein (Bax) were determined through Western blotting. Serum levels of interleukin-6 (IL-6), IL-18 and tumor necrosis factor-α (TNF-α) were measured *via* enzyme-linked immunosorbent assay (ELISA). Moreover, the apoptosis was examined *via* terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, and the relative expression of miR-31 was detected by means of quantitative polymerase chain reaction (qPCR).

RESULTS: The immunohistochemistry results showed that the positive expression of NF-κB was significantly higher in the other two groups than that in normal group ($p<0.05$), while it was also remarkably higher in miR-31 mimics group than that in model group ($p<0.05$). The results of Western blotting revealed that the relative protein expression of Bax significantly increased, while that of Bcl-2 notably declined in the other two groups compared with those in normal group ($p<0.05$). Similarly, the relative protein expression of Bax was upregulated, while that of Bcl-2 was distinctly reduced in miR-31 mimics group compared with those in model group ($p<0.05$). It was found *via* ELISA that the model group and miR-31 mimics group had evidently higher levels of IL-6, IL-18 and TNF-α than those in normal group ($p<0.05$), while miR-31 mimics group also had prominently higher levels than those in model group ($p<0.05$). In addition, according to the TUNEL assay, the apoptosis rate remarkably increased in the other two groups in comparison with that in normal group ($p<0.05$), while it remarkably rose in miR-31 mimics group compared with that in model group ($p<0.05$). Finally, a significantly higher expression of miR-31 was observed in the other two groups than that in normal group *via* qPCR ($p<0.05$), and such a higher expression was also found in miR-31 mimics group than that in model group ($p<0.05$).

CONCLUSIONS: MiR-31 aggravates inflammation and apoptosis in COPD rats by activating the NF-κB signaling pathway.

Key Words:

Chronic obstructive pulmonary disease, MiR-31, NF-κB signaling pathway, Inflammation, Apoptosis.

Introduction

Chronic obstructive pulmonary disease (COPD) is a common respiratory disease mainly characterized by airflow limitation and progressive dyspnea, which often leads to respiratory functional limitation in patients, endangering their life and health[1,2]. Due to atmospheric pollution and environmental degradation with the development of social industrialization, the morbidity rate of COPD has risen year by year. As one of the important pathological reactions during the pathological process of COPD, the persistent inflammatory response caused by respiratory airflow limitation leads to inflammation infiltration of lung tissues, which further aggravates the post-injury repair[3,4].

During the pathological process of inflammatory response in COPD, the nuclear factor-κB (NF-κB) signaling pathway is an important signal transduction pathway that possesses vital effects

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on regulating inflammation and apoptosis[5,6]. The NF-κB signaling pathway is continuously activated in the remission of persistent inflammatory infiltration. By stimulating the release of inflammatory factors, the activated NF-κB signaling pathway thus aggravates inflammatory infiltration and causes massive apoptosis in tissues. As a result, the damage is exacerbated, forming a vicious circle.

Micro ribonucleic acid (miR)-31 is an essential member of the miRNA family, which plays an important role in regulating various physiological and pathological reactions in the body[7,8]. Moreover, miR-31 regulates cell proliferation, differentiation, inflammation and apoptosis *via* regulating multiple downstream signaling pathways. This study aims to explore the effect of miR-31 on COPD rats through activating the NF-κB signaling pathway, and further clarify its mechanism.

Materials and Methods

Laboratory Animals

A total of 36 specific pathogen-free Sprague-Dawley (SD) rats aged 1 month old were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. [license No.: SCXK (Shanghai, China) 2014-0003]. The rats were fed with normal feed and sterile filtered water every day in the Laboratory Animal Center under a 12/12 h light-dark cycle, room temperature and regular humidity. This investigation was approved by the Animal Ethics Committee of Shaanxi Provincial People's Hospital Animal Center.

Laboratory Reagents and Instruments

Lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, MO, USA), miR-31 mimics (MCE, Monmouth Junction, NJ, USA), primary antibodies: anti-B-cell lymphoma-2 (Bcl-2) antibody, anti-Bcl-2 associated X protein (Bax) antibody and anti-NF-κB antibody (Abcam, Cambridge, MA, USA), secondary antibodies (Abcam, Cambridge, MA, USA), immunohistochemistry kit, enzyme-linked immunosorbent assay (ELISA) kit, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) apoptosis kit, and quantitative polymerase chain reaction (qPCR) kit (Vazyme, Nanjing, China), optical microscope (Leica, Wetzlar, Germany), and fluorescence qPCR instrument (ABI, Applied Biosystems, Foster City, CA, USA).

Animal Grouping and Treatment

The above 36 SD rats were divided into normal group (n=12), model group (n=12) and miR-31 mimics group (n=12) using a random number table. The rats were adaptively fed in the Laboratory Animal Center for 7 d before experiments.

The rats were fed normally in normal group. In model group, the COPD model was first established, followed by intraperitoneal injection of normal saline. In miR-31 mimics group, the COPD model was also first established, and the rats were given intraperitoneal injection of 3 μM miR-31 mimics. After intervention for 3 consecutive days, the samples were taken.

Establishment of COPD Model

After successful anesthesia *via* intraperitoneal injection of 7% chloral hydrate (5 mL/kg), the rats were fixed firmly. 200 μL of LPS solution (1 mg/mL) was injected into the airway using a laryngoscope. Then, the rats were erected, so that the LPS solution could completely enter the lung tissues. After daily injection of LPS for 6 consecutive weeks, the COPD model was established successfully.

Sampling

After successful anesthesia, the alveoli of rats were harvested first. Then, the lung tissues were directly taken from 6 rats in each group, washed with normal saline and stored in the Eppendorf (EP) tube at -80°C for later Western blotting, qPCR and ELISA. For the remaining 6 rats in each group, the thoracic cavity was cut open to expose the heart, and 400 mL of 4% paraformaldehyde was perfused from the left auricle. Then, the lung tissues were taken and fixed in 4% paraformaldehyde, used for immunohistochemistry and TUNEL assay.

Immunohistochemistry

The paraffin-embedded tissues were sliced into 5 μm-thick sections, flattened in warm water at 42°C, and prepared into paraffin sections. Then, the sections were soaked and routinely deparaffinized in xylene solution and gradient alcohol, placed in citric acid buffer, and heated in a microwave for 3 times (3 min/time, braised for 5 min each time) for complete antigen retrieval. After the sections were rinsed, the endogenous peroxidase blocker was added dropwise for 10-min reaction. Then, the sections were rinsed again and sealed with goat serum for 20 min. After the goat serum was discarded, the anti-NF-

κB primary antibody (1:200) was added for incubation in a refrigerator at 4°C overnight. On the next day, the sections were washed, reacted with the secondary antibody for 10 min, fully rinsed and reacted with streptavidin-peroxidase solution for 10 min. Color development was achieved using diaminobenzidine (DAB). Finally, the nuclei were counterstained with hematoxylin, and the sections were sealed and observed.

Western Blotting

The cryopreserved lung tissues were lysed with lysis buffer, subjected to ice bath for 1 h and centrifuged in a centrifuge at 14,000 g for 10 min. Then, the protein was quantified using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). The absorbance of protein was detected using a microplate reader and the standard curve was plotted, based on which the protein concentration was calculated. After protein denaturation, the protein in tissue samples was separated *via* sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and the position of the Marker protein was observed. The electrophoresis was terminated when the Marker protein reached the bottom of the glass plate in a straight line. Then, the protein was transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), sealed with the sealing buffer for 1.5 h and incubated with the anti-Bax (1:1000) and anti-Bcl-2 (1:1000) primary antibodies and secondary antibodies (1:1000). After the membrane was rinsed, the image was fully developed in the dark using the chemiluminescent reagent for 1 min.

TUNEL Apoptosis Assay

The paraffin-embedded tissues were sliced into 5 μm-thick sections, flattened in warm water at 42°C, and prepared into paraffin sections. Then, the sections were soaked and routinely deparaffinized in xylene solution and gradient alcohol, reacted with TdT solution in the dark for 1 h and incubated with deionized water for 15 min to terminate the reaction. Subsequently, the endogenous peroxidase was inactivated with hydrogen peroxide, and the working solution was added dropwise for reaction for 1 h, followed by color development using DAB solution. Finally, the sections were washed, sealed and observed.

ELISA

According to the instructions of the ELISA kit, the samples were loaded and added with standards, biotinylated antibody working solution and enzyme-linked working solution, and the plate was washed. Finally, the absorbance value was measured at 450 nm using a microplate reader.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) was used for statistical analysis in this study. Enumeration data were expressed as mean ± standard deviation. The *t*-test was used for the data in line with normal distribution and homogeneity of variance. The corrected *t*-test was performed for the data in line with normal distribution and heterogeneity of variance, and non-parametric test for the data not in line with normal distribution and homogeneity of variance. Comparison between groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). Rank sum test and chi-square test was adopted for ranked data and enumeration data, respectively. $p<0.05$ considered to be statistically significant.

Results

Immunohistochemistry Results

As shown in Figure 1A, cells stained dark brown expressed positive expression of NF-κB. The positive expression of NF-κB was lower in normal group than that in the other two groups. Higher positive expression of NF-κB was seen in miR-31 mimics group compared with that in model group. Statistical results of the positive expression of NF-κB in the three groups were shown in Figure 1B.

Relative Protein Expressions of Apoptosis Genes Detected Via Western Blotting

As shown in Figure 2A, the protein expression of Bax was lower and that of Bcl-2 was higher in normal group than the other two groups (Figure 2B). In addition, the relative protein expression of Bax was raised notably, while that of Bcl-2 was reduced distinctly in miR-31 mimics group compared with those in model group, and the differences were statistically significant ($p<0.05$).

ELISA Results

The other two groups had evidently higher levels of interleukin-6 (IL-6), IL-18 and tumor necrosis factor-α (TNF-α) than those in normal

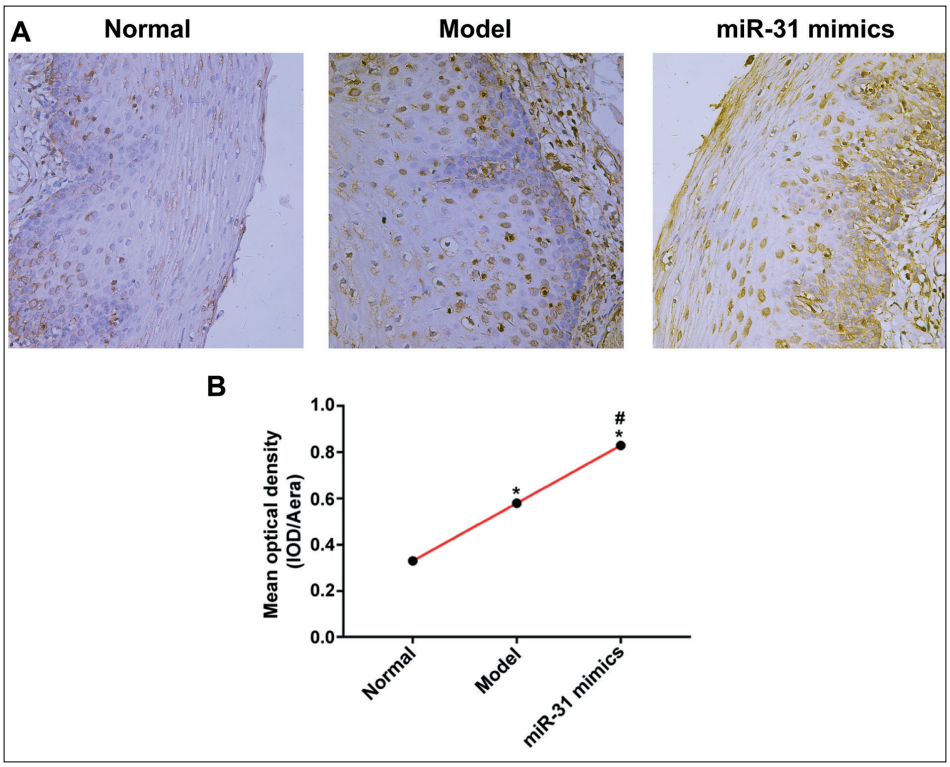

Figure 1. Immunohistochemistry (magnification: 200×). Note: **A,** Immunohistochemistry. **B,** Mean optical density of positive expression in each group. $^{*}p<0.05$ *vs.* normal group, $^{\#}p<0.05$ *vs.* model group.

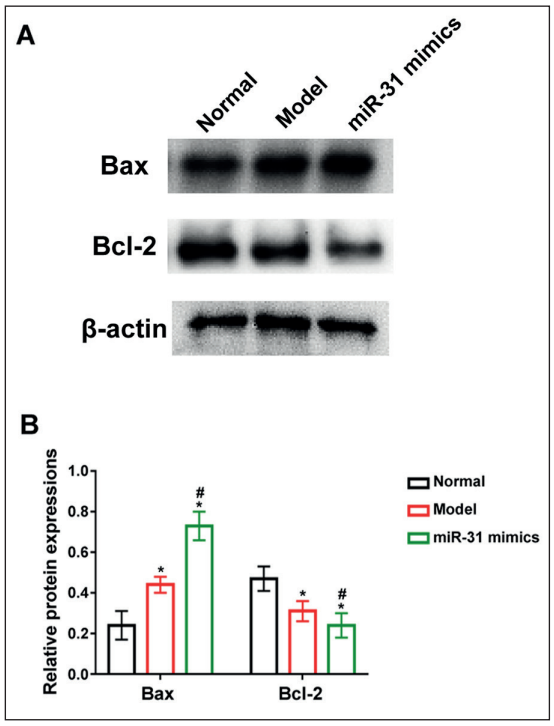

Figure 2. Protein expressions detected *via* Western blotting. Note: **A,** Western blotting. **B,** Relative protein expressions in each group. $^{*}p<0.05$ *vs.* normal group, $^{\#}p<0.05$ *vs.* model group.

group, and there were statistically significant differences ($p<0.05$). Besides, miR-31 mimics group also had prominently higher levels of IL-6, IL-18 and TNF-α than those in model group, and there were statistically significant differences ($p<0.05$) (Figure 3).

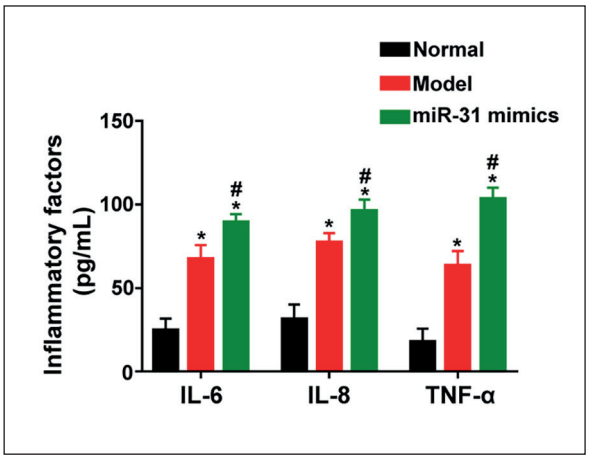

Figure 3. Relative levels of inflammatory factors in each group. Note: $^{*}p<0.05$ *vs.* normal group, $^{\#}p<0.05$ *vs.* model group.

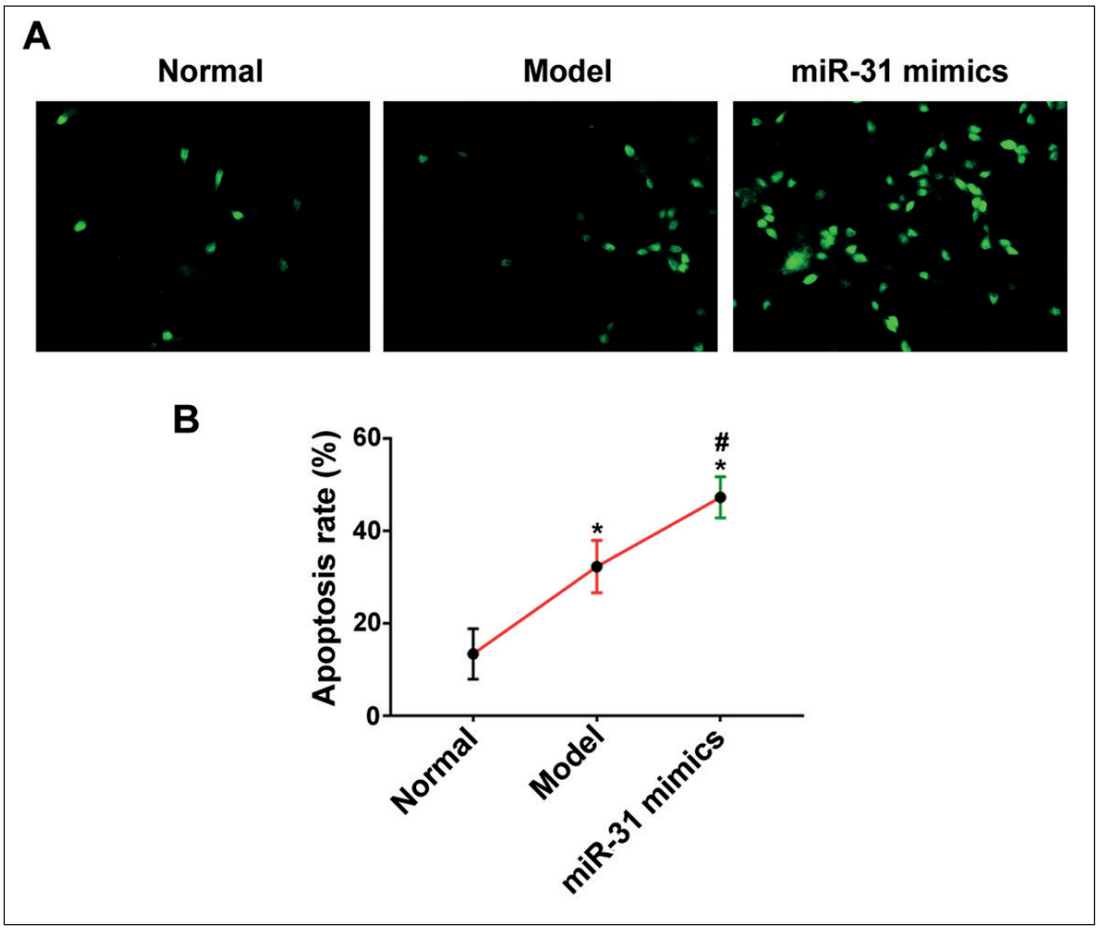

Figure 4. Apoptosis detected *via* TUNEL assay. Note: **A,** TUNEL assay. (magnification: 200×) **B,** Apoptosis rate in each group. $^{*}p<0.05$ *vs.* normal group, $^{\#}p<0.05$ *vs.* model group.

Apoptosis Rate Detected Via TUNEL Assay

The apoptotic cells were stained dark brown. There were fewer apoptotic cells in normal group than the other two groups (Figure 4A). The apoptosis rate remarkably increased in model group and miR-31 mimics group compared with that in normal group, displaying statistically significant differences ($p<0.05$). It remarkably rose in miR-31 mimics group compared with that in model group, displaying a statistically significant difference ($p<0.05$) (Figure 4B).

QPCR Results

The mRNA level of miR-31 was much higher in miR-31 mimics group and model group than that in normal group, and the upregulated trend was much more pronounced in miR-31 mimics group ($p<0.05$) (Figure 5).

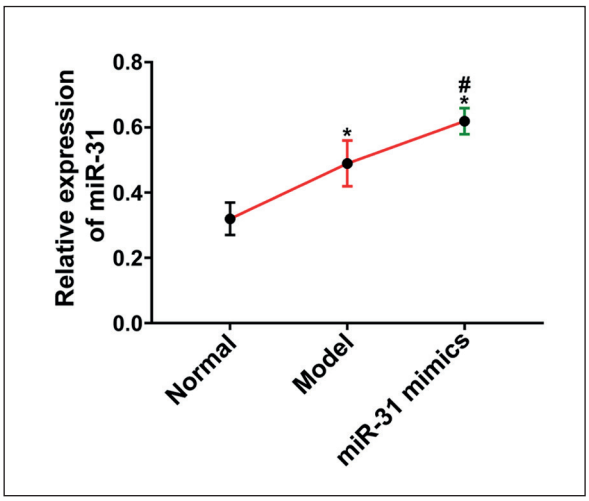

Figure 5. Relative expression of miR-31 in each group. Note: $^{*}p<0.05$ *vs.* normal group, $^{\#}p<0.05$ *vs.* model group.

Discussion

COPD frequently occurs in middle-aged and elderly people, mainly characterized by persistent airflow limitation and dyspnea. As a result, pulmonary heart disease and cardiopulmonary dysfunction will be further induced, seriously endangering patients' health and reducing quality of life. How to effectively alleviate the COPD-induced persistent airflow limitation and dyspnea, and clinical symptoms of COPD patients have always been a difficulty in clinical treatment[9-11]. It has been found[12,13] that inflammatory response is one of the most common pathological reactions, as well as an important one in COPD, which can cause inflammation infiltration of lung tissues, thus resulting in structural and functional changes in tissues. In addition, the long-term inflammatory infiltration can cause structural damage to airway tissues, and a large amount of mucus is released. As a result, developed airway tissue fibrosis ultimately leads to airway wall thickening, mucus hypersecretion and lung tissue fibrosis, which are responsible for persistent airflow limitation and dyspnea in COPD patients[14-16]. Therefore, the inflammatory response plays an important pathological role in COPD. Currently, it is believed[17-19] that the NF-κB signaling pathway is one of the vital signaling pathways in the body, as well as an essential one regulating inflammation and apoptosis. Moreover, the NF-κB signaling pathway can be activated by a large number of cytokines and inflammatory factors. Then, free NF-κB with transcriptional activity can enter the nucleus and bind to its transcription factors, thus activating the expressions of downstream substances (Bax, Bcl-2, IL-6, IL-8, TNF-α, etc.) that are related to apoptosis and inflammation. Besides, Bax (a pro-apoptotic factor) and Bcl-2 (an anti-apoptotic factor) are crucial factors in the apoptotic pathway, both of which interact with each other to keep the normal apoptosis level in the body[20]. In this study, Bax was upregulated and Bcl-2 was downregulated in lung tissues of COPD rats. Increased levels of inflammatory factors (IL-6, IL-8, and TNF-α) were also observed. It can be seen that there are severe inflammatory response and apoptosis in lung tissues of COPD rats. Meanwhile, the protein expression of NF-κB (a key substance in the NF-κB signaling pathway) remarkably increased, which may be one of the causes of severe inflammatory response and apoptosis in lung tissues of COPD rats. MiR-31 is one of the critical miRNAs that exerts an important regulatory effect on multiple downstream signaling pathways. In this study, miR-31 was able to promote the inflammatory response and apoptosis in lung tissues of COPD rats. Overexpression of miR-31 was believed to stimulate the inflammatory factors (IL-6, IL-8, and TNF-α) and apoptosis by activating the NF-κB signaling pathway.

Conclusions

Briefly, the novelty of this study was that miR-31 aggravates inflammation and apoptosis in COPD rats through activating the NF-κB signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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