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RESEARCH ARTICLE

Effects of miRNA-155 on Inflammatory Response and Autophagy Upon Pulpitis Through the NLRP3 Signal

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ABSTRACT

Pulpitis refers to the inflammation of dental pulp tissues caused by infection with dental caries. We aimed to evaluate the effects of micro ribonucleic acid (miR)-155 on inflammatory response and autophagy upon pulpitis *via* the nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3) signal. Forty rats were randomly assigned to negative control (NC), pulpitis model (PM), anti-miR-155, and anti-miR-155+diethyldithiocarbamate (DDC) groups (n=10). Primary human dental pulp cells were divided into NC, lipopolysaccharide (LPS), anti-miR-155, and DDC groups. Compared with the PM group, the IL-1β, TNF-α, and MDA levels and pulp necrosis rate decreased, while the SOD activity was enhanced in the anti-miR-155 group (P<0.05). Compared to the NC group, the positive expressions of LC3B and Beclin1 and the protein expressions of NLRP3 and Caspase-1 significantly rose in the PM group (P<0.05). Compared with the PM group, the protein expressions of NLRP3 and Caspase-1 significantly decreased, and the positive expressions of LC3B and Beclin1 increased in the anti-miR-155 group (P<0.05). Compared with the anti-miR-155 group, the DDC group had significantly enhanced activity of dental pulp cells, up-regulated mRNA levels of IL-1β, TNF-α, NLRP3, and Caspase-1, and decreased mRNA levels of LC3B and Beclin1 (P<0.05). Suppressing miR-155 expression can relieve inflammatory response and promote autophagy.

Keywords: Autophagy, Inflammatory response, MicroRNAs, Pulpitis

INTRODUCTION

Pulpitis refers to the inflammation of dental pulp tissues mainly caused by infection with dental caries [1]. Pulpitis can cause serious damage to the blood circulation of the teeth, resulting in pulp necrosis, dentition defect, and even tooth loss due to nutritional imbalance. The dental pulp tissue is surrounded by non-tenacious peripheral hard tissues, so the circulation of blood and lymph tissues is poor, leading to uncontrollable pulp inflammation. Besides, long-term severe pain poses a threat to the quality of life of patients [2]. At present, the diagnosis and treatment of pulpitis are still challenging. Pro-inflammatory cytokines can relieve the inflammation of dental pulp tissues, exerting apparent therapeutic effects on pulpitis [3]. The nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3) inflammasome is composed of different endogenous proteins that recognize danger signals and mediate various inflammatory responses [4]. The expression of NLRP3 in the dental pulp tissues of rats with pulpitis obviously increases [5]. Thus, NLRP3 may play a crucial role in the occurrence and development of pulpitis, but whether it is involved in the therapeutic mechanism of pulpitis has rarely been reported. Autophagy is a main protein degradation pathway in eukaryotic cells. As a key defender and metabolic function regulator, autophagy plays an important role in inflammation and infectious diseases [6]. Autophagy can promote the injury repair process [7], but the influence of autophagy on tissue repair after pulpitis-induced injuries remains largely unknown. Micro ribonucleic acids (miRNAs), as endogenous single-stranded non-coding RNAs, can bind messenger RNAs (mRNAs) to inhibit translation, thereby regulating numerous biological activities [8]. MiRNAs play an indispensable role in immune responses [9]. Therefore, they are involved in various infectious oral diseases, including dental caries and periodontitis. In addition, miR-155 shows a high expression in the periodontal ligament of

patients with chronic periodontitis [10], but the expression and mechanism of action in pulpitis are still unclear.

Therefore, this study aimed to evaluate the effects of miR-155 on the inflammatory response and autophagy of rats with pulpitis through the NLRP3 signal.

Material and Methods

Laboratory Animals

The animal experiments have been approved by the animal ethical committee of our hospital (ethical approval number: GYSKLL-KY-20220407-02), and great efforts have been made to minimize their suffering. A total of 40 SPF-grade male Sprague-Dawley rats, weighing 180-200 g, were purchased from Zhejiang Vital River Laboratory Animal Technology Co., Ltd. [China; Production License No. SCXK (Zhejiang) 2019-0001]. The rats were raised in an animal room at a temperature of 22-25°C and a relative humidity of 50-55%, with a 12/12 h light/dark cycle. They were allowed to eat and drink freely and adapted to the new environment for one week.

Reagents and Apparatus

The reagents used in this study included miR-155 inhibitor (anti-miR-155) (Shanghai GenePharma Co., Ltd., China), hematoxylin and eosin (HE) staining kit, bicinchoninic acid (BCA) protein kit, and enzyme-linked immunosorbent assay (ELISA) kit (Shanghai Sangon Biotech Co., Ltd., China), antibodies against NLRP3 and Caspase-1 (Abcam, USA), and NLRP3 activator diethyldithiocarbamate (DDC). The apparatus applied in this study included a freezing microtome and an inverted microscope (Leica, Germany), electrophoresis apparatus (Beijing Liuyi Instrument Factory, China), and membrane transfer system and a gel imaging system (Bio-Rad, USA).

Grouping and Establishment of a Rat Model of Pulpitis

The 40 rats were randomly divided into negative control (NC) group, pulpitis model (PM) group, anti-miR-155 group, and anti-miR-155+DDC group (n=10/group). Except for NC group, rat pulpitis models were prepared in the other three groups according to a previous reference [11]: The rats were anesthetized by intraperitoneal injection of 2% pentobarbital sodium and fixed on the operating table in the supine position. The upper jaw of the rats was opened with forceps to fully expose the maxillary molars, the first and second maxillary molars on the left side were disinfected with alcohol, and then a small hole was drilled in the maxillofacial region with a high-speed turbine until the red part of the dentin near the pulp was seen. Next, the pulp cavity was opened with a reamer, washed, and wiped dry. Then the cotton swab was soaked with complete Freund's adjuvant and placed in the pulp cavity for 30 min. Afterwards, the dental pulp cavity was observed after it was cleaned and dried. The appearance of congestion and abscess in the pulp cavity represented a successfully established pulpitis model.

The anti-miR-155 group was injected with miR-155 inhibitor (80 mg/kg) *via* the tail vein, and the anti-miR-155+DDC group was given NLRP3 activator DDC (300 mg/kg) by gavage 1 h after treatment. Meanwhile, the NC and PM groups were intraperitoneally injected with the same amount of normal saline. The intervention for each group was performed once a day for five consecutive days. Subsequent experiments were carried out 21 d after the intervention.

Detection of Levels of Interleukin-1 beta (IL-1β) and Tumor Necrosis Factor-alpha (TNF-α) in Serum and Levels of SOD and MDA in Dental Pulp Tissues by ELISA

After 21 d, the rats were anesthetized by ether inhalation. Then 2 mL of abdominal aortic blood was taken and centrifuged at 4°C and 3000 rpm for 15 min, after which the supernatant was aspirated. Then ELISA kit was utilized to measure the levels of pro-inflammatory cytokines IL-1β and TNF-α in serum according to the kit's instructions. In addition, the activity of superoxide dismutase (SOD) and the content of malondialdehyde (MDA) in dental pulp tissues were detected by ELISA.

Detection of Pathological Changes of Dental Pulp by HE Staining

After the above detection, the rats in each group were sacrificed by cervical dislocation. The left and right maxillary bones were immediately separated, and the maxilla and maxillary molars were taken out, fixed in paraformaldehyde, decalcified, and embedded in paraffin. The pathological sections with a thickness of 4 μm were made by a freezing microtome. Next, the tissue sections were stained with hematoxylin and eosin and mounted with neutral resin. Later, the pathological changes of the dental pulp in each group were observed under a microscope. Finally, the pulp necrosis rate was calculated based on the formula: pulp necrosis rate (%) = length of necrotic tissue/total length of root canal × 100%.

Detection of Autophagy in Dental Pulp Tissues by Immunohistochemical Staining

The sections prepared in 1.5 were blocked with antigen retrieval and serum at 37°C for 1 h, respectively, and incubated with droplets of working solutions of primary antibodies against LC3B and Beclin1 overnight at 4°C. The tissue sections were rewarmed, washed with phosphate-buffered saline (PBS) three times, and dripped with goat anti-rabbit IgG-labeled secondary antibody. After washing with PBS again for 5 min (3 times in total), the tissue sections were observed under the microscope, and

the distribution of positive cells was analyzed by ImageJ software.

Determination of Expressions of NLPR3 and Caspase-1 in Dental Pulp Tissues by Western Blotting

The molars of rats in each group were cut into pieces and ground into powder in liquid nitrogen. The powder was collected in an EP tube and lysed with RIPA lysate to extract total proteins from dental pulp tissues according to the protein kit instructions. After loading, electrophoresis, membrane transfer, and blocking, the tissue sections were incubated with primary antibodies (1:100 diluted) overnight, with β-actin as the internal control. Next, the HRP-labeled secondary antibody was added for the incubation of the tissue sections for 1 h at room temperature. In the end, ImageJ software was utilized to analyze the gray-scale values of proteins.

Primary Culture of Human Dental Pulp Cells

Fresh extracted third molars or orthodontic teeth were collected from patients aged 12-20 years old in our hospital. The human experiments have been approved by the medical ethical committee of our hospital, and written informed consent has been obtained from all patients. The teeth were washed with PBS containing 10% penicillin-streptomycin. The crowns were split with an osteotome. Then the dental pulp was taken out on the sterile operating table and washed twice with a medium containing the double-antibody solution. Later, the tissues were cut into pieces, spread in a 25 cm^2 culture flask, and cultured with a mixed culture medium (10% fetal bovine serum and 1% double-antibody solution) in a conventional incubator (37°C, 5% CO_2). After passage, the 3^{rd}-5^{th}-generation cells were taken for subsequent experiments.

Detection of Cell Viability by CCK-8 Assay

The cells were inoculated in a 96-well plate, and different cultures were added after the cells adhered to the wall. The cells were divided into NC group, lipopolysaccharide (LPS) group (cultured with 1 mg/L LPS), anti-miR-155 group (cultured with miR-155 inhibitor), and DDC group (cultured with DDC). The cells in each group were incubated for 1 d, 3 d, 5 d, and 7 d, respectively. During the detection, the cells in each well were incubated with 10 μL of CCK-8 solution for 2 h, and the absorbance at 450 nm was measured using a microplate reader.

Detection of mRNA Levels of IL-1β, TNF-α, NLRP3, Caspase-1, LC3B, and Beclin1 in Dental Pulp Cells by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The dental pulp cells were extracted from each group (grouping in 1.9), and lysed with TRIzol to extract total RNA according to the kit's instructions. Total RNA was reversely transcribed into cDNA according to the kit's instructions. The primers were listed below: IL-1β: forward: 5''-AAGACAAGCCTGTGTTGCTGAAGG-3' and reverse: 5'-TCCCAGAAGAAAATGAGGTCGGT C-3', TNF-α: forward: 5'-CTTCTCATTCCTGCTCGTGG and reverse: 5'-GCTACGGGCTTGTCACTCG-3', NLRP3: forward: 5'-CAGCGATGAAGACGCGAGAG-3' and reverse: 5'-AGAGATATGGCACGAAAGCTATCCA-3', Caspase-1: forward: 5'-ACTGCTACACCTGTTGCGCC TCA-3' and reverse: 5'-CTGCCGACGCAGGAAATTC-3', LCB3: forward: 5'-CAGGTTGCCTAGCAGAGGTC-3' and reverse: 5'-GGCATGGACCAGAGAAGTCC-3', Beclin1: forward: 5'-TATAGCAAAGCCCTGCCG-3', and reverse: 5'-AACTGTGCCACAAGCATC-3', GAPDH: forward: 5'-CAACTCCCTCAAGATTGTCAGCAA-3', and reverse: GGCATGGACTGTGGTCATGA. The primers were transiently centrifuged and mixed well with deionized water to prepare a 100 μmol/L stock solution. The forward and reverse primers were diluted to a final concentration of 10 μmol/L. PCR reaction conditions were set as follows: pre-denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 30 s, and annealing at 66°C for 30 s. At last, the expression level was analyzed by $2^{-\Delta\Delta CT}$.

Statistical Analysis

GraphPad Prism 8.0 software was employed for the analysis of experimental data. All measurement data were expressed as mean ± standard deviation (X±Sx). Repeated measures analysis of variance (ANOVA) and one-way ANOVA were conducted to compare the data among different groups, and the LSD-*t* test was performed for the pairwise comparison of data among groups. $P<0.05$ was considered statistically significant.

Results

Compared with the NC group, the levels of IL-1β, TNF-α, and MDA significantly rose, while the activity of SOD was significantly decreased in the PM group ($P<0.05$). Compared with the PM group, the levels of IL-1β, TNF-α, and MDA decreased, while the SOD activity was enhanced in the anti-miR-155 group ($P<0.05$). In comparison with the anti-miR-155 group, the anti-miR-155+DDC group had significantly increased levels of IL-1β, TNF-α, and MDA and decreased SOD activity ($P<0.05$) *(Fig. 1)*.

In the NC group, the dental pulp morphology was normal. In the PM group, dental pulp tissues were seriously injured, the dentin and fibroblasts were arranged in disorder, most of the cells were significantly changed or even necrosed, and a large number of inflammatory cells were infiltrated. The pulp necrosis rate was (81.36±2.47%) in the PM group. In the anti-miR-155 group, only small quantities of inflammatory cells were observed in the blood vessels

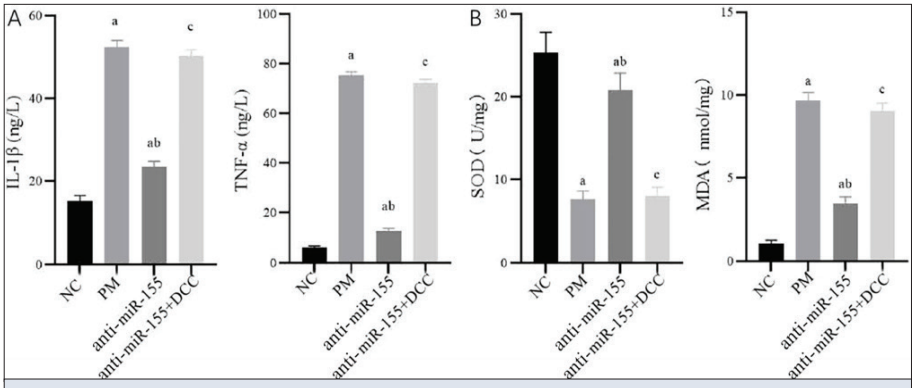

Fig 1. Levels of IL-1β and TNF-α in serum and levels of SOD and MDA in dental pulp tissues. A: Serum IL-1β and TNF-α levels. B: Levels of SOD and MDA in dental pulp tissues. [a]P<0.05 *vs.* NC group, [b]P<0.05 *vs.* PM group, and [c]P<0.05 *vs.* anti-miR-155 group

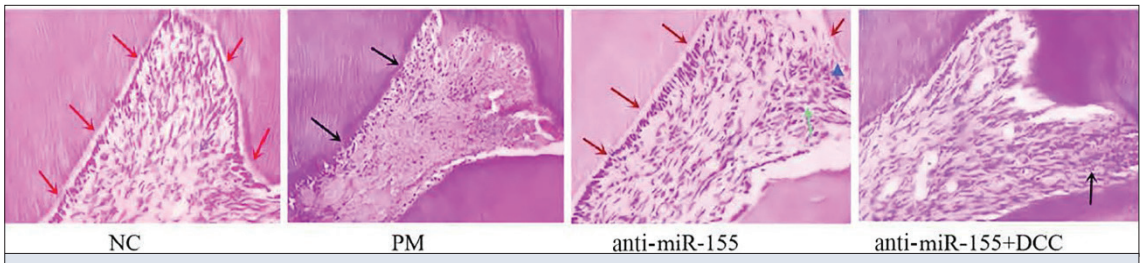

Fig 2. HE staining results of dental pulp tissues (×400). *Red arrows* indicate odontoblasts, *purple arrows* indicate dental pulp cells, *green arrows* indicate the round inflammatory cell aggregation, *blue triangles* indicate vasodilatation and hyperemia, and *black arrows* indicate nucleus fragmentation, cell necrosis, and residual nuclei

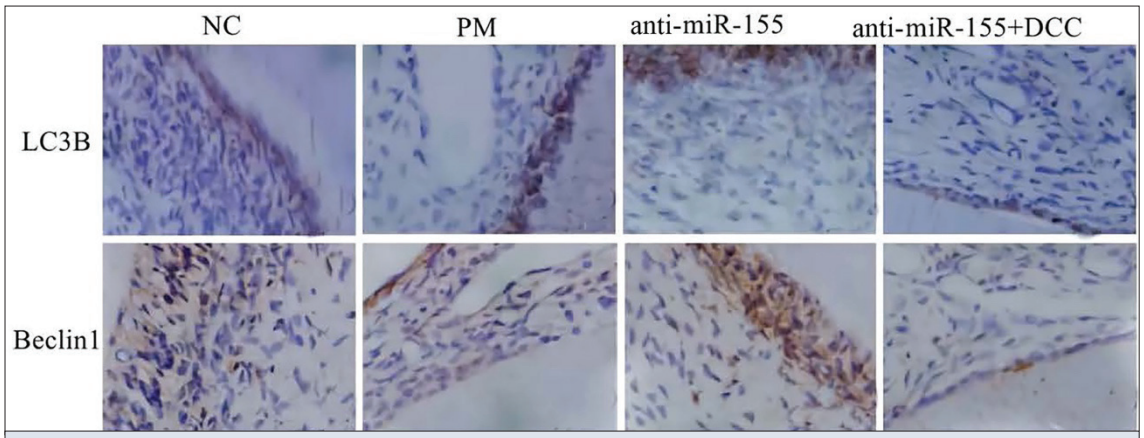

Fig 3. Immunohistochemical staining results of LC3B and Beclin1 expressions in dental pulp tissues (×400)

around the perforating point, which significantly reduced compared with those in the PM group. The pulp necrosis rate was (4.31±1.02%). In the anti-miR-155+DDC group, the cells in the pulp perforation were disordered, and the inflammatory cells gathered around the hyperemic pulp vessels. In addition, the infiltration of inflammatory cells was significantly aggravated in the anti-miR-155+DDC group compared with that in the anti-miR-155 group. The pulp necrosis rate was (75.42±2.26%) in the anti-miR-155+DDC group *(Fig. 2)*.

In the NC group, LC3B and Beclin1 were lowly expressed in dental pulp tissues. In the PM group, the expressions of LC3B and Beclin1 in dental pulp tissues increased, which were observed in the odontoblast layer near the pulp foramen. The anti-miR-155 group had significantly higher positive expressions of LC3B and Beclin1 in dental pulp tissues than those of the PM group, and the positive expressions were widely observed in the odontoblast layer and fibroblasts in the cell layer of all dental pulp tissues. Moreover, the expression levels of LC3B and Beclin1 in the anti-miR-155+DDC group were significantly lower than those in the anti-miR-155 group *(Fig. 3)*.

Compared to the NC group, the protein expressions of NLRP3 and Caspase-1 significantly rose in the PM group

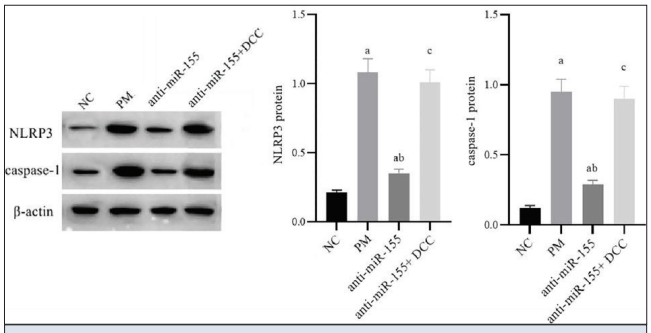

Fig 4. Protein expressions of NLPR-3 and Caspase-1 in rat dental pulp tissues. [a]P<0.05 *vs.* NC group, [b]P<0.05 *vs.* PM group, and [c]P<0.05 *vs.* anti-miR-155 group

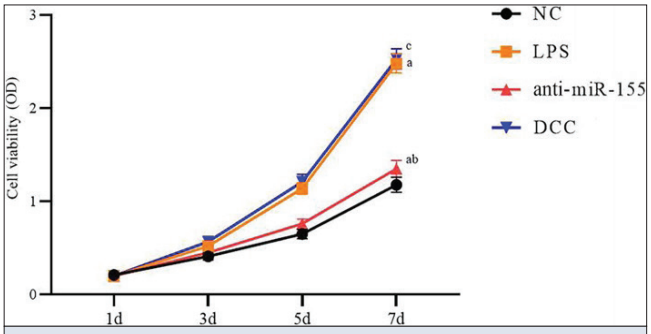

Fig 5. Dental pulp cell viability. [a]P<0.05 *vs.* NC group, [b]P<0.05 *vs.* LPS group, and [c]P<0.05 *vs.* anti-miR-155 group

(P<0.05). Compared with the PM group, the protein expressions of NLRP3 and Caspase-1 in dental pulp tissues significantly decreased (P<0.05). Additionally, the protein expressions of NLRP3 and Caspase-1 in the anti-miR-155+DDC group were significantly higher than those in the anti-miR-155 group (P<0.05) *(Fig. 4)*.

In comparison with the NC group, the activity of dental pulp cells was significantly enhanced in the LPS group (P<0.05). In comparison with the LPS group, the activity of dental pulp cells was significantly reduced in the anti-miR-155 group (P<0.05). Compared with the anti-miR-155 group, the DDC group showed significantly enhanced activity of dental pulp cells (P<0.05) *(Fig. 5)*.

In comparison with the NC group, the mRNA levels of IL-1β, TNF-α, NLRP3, Caspase-1, LC3B, and Beclin1 were significantly raised in the LPS group (P<0.05). Compared to the LPS group, the mRNA expressions of IL-1β, TNF-α, NLRP3, and Caspase-1 significantly declined, and the mRNA expression levels of LC3B and Beclin1 increased in the anti-miR-155 group (P<0.05). Compared with the anti-miR-155 group, the DDC group had significantly up-regulated mRNA levels of IL-1β, TNF-α, NLRP3, and Caspase-1, and decreased mRNA levels of LC3B and Beclin1 (P<0.05) *(Fig. 6)*.

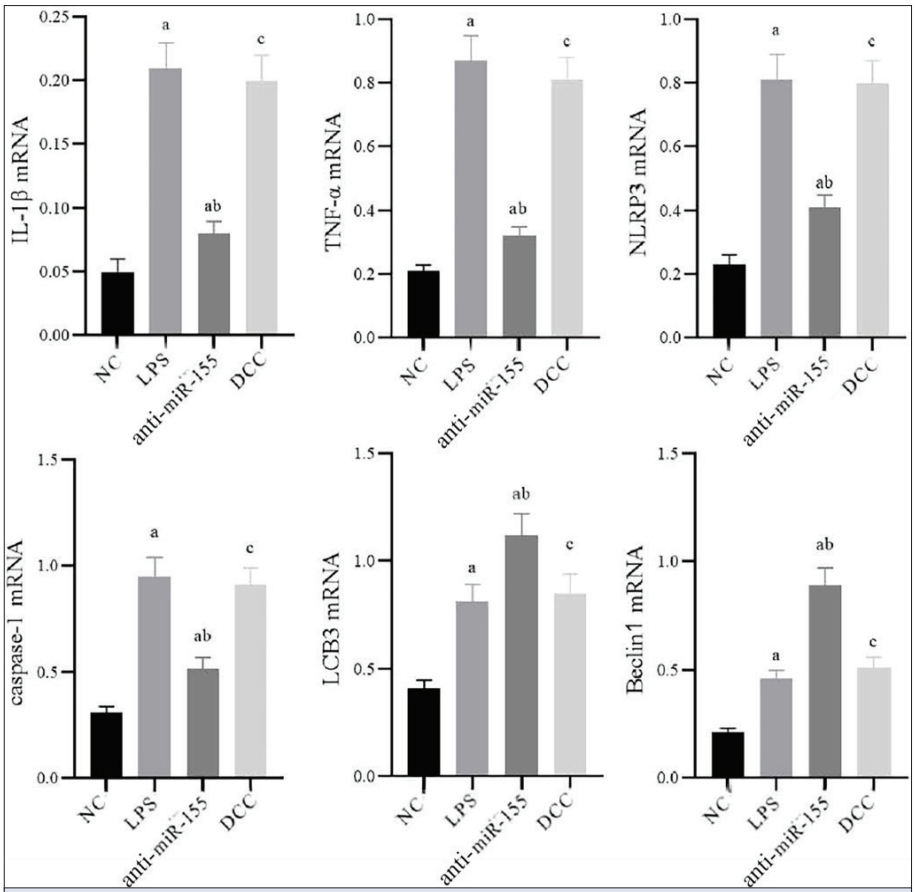

Fig 6. mRNA levels of IL-1β, TNF-α, NLRP-3, Caspase-1, LC-3B, and Beclin-1 in dental pulp cells. [a]P<0.05 *vs.* NC group, [b]P<0.05 *vs.* LPS group, and [c]P<0.05 *vs.* anti-miR-155 group

Discussion

Pulpitis is a common inflammatory disease in dental pulp tissues. External trauma-induced pulp exposure and bacterial infection facilitate inflammatory responses, thereby aggravating the pulp injury [12], so inhibiting inflammatory responses may be a measure for treating pulpitis. Dental pulp tissues are composed of a variety of cells, including odontoblasts and fibroblasts. Odontoblasts are located in the outermost layer of dental pulp tissues. They can express various pro-inflammatory cytokines such as IL-1β and TNF-α through injured dental caries, secrete large quantities of chemokines, accumulate a large number of dendritic cells, and participate in the regulation of immune responses [13,14]. In the case of pulpitis, the expressions of pro-inflammatory cytokines IL-1β and TNF-α are up-regulated, which are involved in the immune response of pulpitis [15]. In this study, in the rats with pulpitis, the dental pulp tissues were significantly injured, and numerous inflammatory cells were infiltrated. The activity of SOD in dental pulp tissues decreased, the level of MDA increased, and the pulp necrosis rate and the levels of IL-1β and TNF-α in serum rose significantly. Taken together, severe inflammation and oxidative stress occurred in dental pulp tissues in the case of pulpitis.

MiR-155 can regulate biological processes such as development, differentiation, activation, proliferation, and homeostasis of T cells, B cells, and macrophages, and exert crucial effects in inflammation, immunodeficiency diseases, and autoimmune diseases [16]. Besides, miR-155 can facilitate the occurrence and development of periodontitis, and its expression in periodontal tissues can be elevated by inflammation [17]. In this study, the levels of IL-1β, TNF-α, and MDA reduced, the SOD activity was enhanced, and the pathological injury of dental pulp tissues was significantly mitigated in the rats treated with miR-155 inhibitor, which indicated that suppressing miR-155 expression inhibited the inflammation and oxidative stress in rats with pulpitis. Liu et al.[18] confirmed that the miR-155 level was up-regulated in periodontal ligament stem cells under an inflammatory environment, and the knockdown of miR-155 facilitated osteogenic differentiation. Additionally, Li et al.[19] reported that the expression of miR-155 was up-regulated in the tissues of mice with pulpitis, and treatment with miR-155 inhibitor significantly alleviated the symptoms.

Autophagy is a key mechanism for maintaining cellular homeostasis. Autophagy is weak under normal conditions, but increases under the stimulation by starvation, oxidative damage, invasion of pathogenic microorganisms, *etc.* Autophagy activity is enhanced in mature dental pulp cells and can mediate odontoblast development. LC3, located on the surface of autophagic vesicles and the autophagic vesicle membrane, can participate in the formation of autophagosomes. The LC3 family has five members, including LC3B and LC3B2. Among them, LC3B is involved in the whole process of autophagy, and it is always located on the autophagosome membrane, which is positively correlated with the number of autophagic vacuoles. Currently, LC3B expression is commonly used to observe autophagy in clinical practice. The results of this study demonstrated that the expressions of LC3B and Beclin1 in dental pulp tissues increased in the rats with pulpitis, and their expressions were observed in the odontoblast layer near the pulp foramen. Additionally, the expressions of LC3B and Beclin1 in the rat dental pulp tissues significantly rose in the rats treated with miR-155 inhibitor, and they were distributed in the odontoblast layer and fibroblasts in the cell layer of all dental pulp tissues, which suggested that suppressing miR-155 expression facilitated autophagy in the dental pulp tissues of rats with pulpitis and thus participated in tissue repairing.

The NLRP3 inflammasome is essential in the innate immune defense. Injured or stimulated by exogenous stimuli, the pulp-dentin complex can trigger the defense activities and responses to external stimuli. The activated immunomodulatory pathway involving NLRP3 can activate Caspases-1, and the activated Caspases-1 participates in the immune inflammatory responses by regulating the synthesis and release of IL-1β, which can mediate the occurrence and development of pulpitis [20]. In this study, the protein expressions of NLRP3 and Caspase-1 in dental pulp tissues were significantly elevated in the rats with pulpitis, which were down-regulated through inhibiting miR-155 expression. Likewise, the expressions of NLRP3 and Caspase-1 in dental pulp cells significantly declined in the rats treated with miR-155 inhibitor, so the immune signaling pathway involving NLRP3 may be the mechanism of pulpitis-induced inflammation. To confirm this postulation, NLRP3 activator DDC was used to inhibit miR-155 expression. It was found that DDC reversed the inhibitory effect exerted by the inhibition of miR-155 on the inflammatory responses of dental pulp tissues and cells in rats with pulpitis, thereby aggravating pulpitis-induced inflammatory responses. Nevertheless, this study is limited. The number of animals was small, and only rats were tested. Further *in vitro* and *in vivo* experiments are still in need to verify our findings.

In conclusion, suppressing the miR-155 expression can inhibit the inflammatory response in rats with pulpitis, promote autophagy, and alleviate dental pulp tissue injury and clinical symptoms, probably by inhibiting the NLRP3 signal.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author (S. Guo) on reasonable request.

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Competing Interests

There is no conflict of interest.

Authors' Contributions

YJ and SG designed this study and significantly revised the manuscript; XZ performed this study and wrote the manuscript.

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