MicroRNA let-7c-5p Suppressed Lipopolysaccharide-Induced Dental Pulp Inflammation by Inhibiting Dentin Matrix Protein-1-Mediated Nuclear Factor kappa B (NF-κB) Pathway In Vitro and In Vivo

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Background: Let-7c-5p is down-regulated in dental pulp tissues in inflammatory disorders. The microRNA (miR) molecule shows an anti-inflammation potential due to its direct regulation of dentin matrix protein-1 (DMP1), which promotes inflammation changes in dental pulp tissues. In the present study, the effect of let-7c-5p on lipopolysaccharide (LPS)-induced pulpitis was detected and the associated mechanism was explored.

Material/Methods: Dental pulp stem cells (DPSCs) were isolated from rat dental tissues, infected with let-7c-5p lentivirus particles, and subjected to LPS administration to induce inflammation. Then, the effect of let-7c-5p overexpression on LPS-induced impairments on DPSCs were detected and the mechanism was explained by focusing on the DMP1 expression and NF-κB pathway. The role of DMP1 in the anti-inflammation effect of let-7c-5p was assessed by incubating let-7c-5p-expressed DPSCs with DMP1 protein. The results of in vitro assays were verified in LPS-induced rat pulpitis models.

Results: LPS administration increased the production of IL-1β and TNF-α and decreased DPSCs viability by increasing the expression of DMP1 and activating NF-κB pathway. However, the induced expression of let-7c-5p relieved DPSCs from LPS-induced inflammation and suppressed DMP1 as well as NF-κB pathway. The incubation of let-7c-5p-expressed DPSCs with DMP1 protein blocked the effect of let-7c-5p. In in vivo experiments, the injection of let-7c-5p attenuated LPS-induced pulpitis by inhibiting DMP1-mediated NF-κB pathway.

Conclusions: Findings outlined in the current study demonstrated the dental pulp protecting function of let-7c-5p during LPS-induced inflammation, which was exerted by inhibiting the DMP1-mediated NF-κB pathway.

MeSH Keywords: Dental Pulp Diseases • MicroRNAs • Pulpitis

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Background

Bacterial infection after the development of caries or dental traumatic lesions will cause pulpitis when the pathogenic microbes penetrates into dental tubules and pulp tissues [1]. The disorder is termed as pulpitis and depends on root canal procedures for alleviation (http://www.aae.org; last accessed November 1, 2011). During the progression of pulpitis, the secretion of pro-inflammatory cytokines induced by bacterial irritants will alter the proliferation, migration, and differentiation behaviors of dental pulp stem cells (DPSCs). The cell type derives from mesenchymal-derived cells characterized by self-renewal and multi-lineage differentiation capability [2], which has been employed as a promising option for the tissue engineering and regenerative medicine. Previous studies have demonstrated that the restoration of DPSC functions in pulpitis can induce a form of tertiary dentin adjacent to injury sites and cell differentiation into odontoblast-like cells [3], showing the central role of DPSCs in maintaining the homeostasis of the dental pulp microenvironment.

With the accumulation of information regarding the microbial infection of dental pulp tissues, microRNAs (miRs) have been recently reported to be a key regulator involved in the response of DPSCs to inflammation [4,5]. MiRs are small (~20–22 nucleotides) noncoding RNA species that regulate diverse gene functions by binding to the 3’ untranslated region (UTR) of targeted mRNA members [6]. Multiple miR members have been reported to be dys-expressed in inflamed human pulps [7], including up-regulated ones that might be involved in the onset of pulpitis and down-regulated ones that might have treatment potential against pulpitis. The expression of miR let-7c-5p is reported to be suppressed in inflamed human dental pulp tissues [7]. Moreover, let-7c-5p contributes to osteogenic differentiation of human stromal mesenchymal stem cells [8], representing the ability of let-7c-5p to store the normal function of DPSCs.

Dentin matrix protein 1 (DMP1) is a non-collagenous protein essential for the mineralization of dentin and bone [9]. Generally, DMP1 is highly expressed in odontoblasts and bone osteocytes, while in osteoblasts and cartilage the expression of DMP1 is suppressed [10–12]. However, during pulpitis, the level of DMP1 increases, suggesting that DMP1 contributes to inflammatory responses in dental pulp tissues [13]. Furthermore, DMP1 is a direct target of let-7c-5p and the interaction between the 2 factors has been verified in normal dental pulp cells [9]. Based on the above information, the hypothesis of the present study was that the suppressed expression of let-7c-5p during pulpitis upregulates the expression of DMP1, thus contributing to the progression of inflammatory responses in dental pulp tissues and impairing the normal biological behavior of DPSCs.

In the present study, a series of in vitro and in vivo assays were performed to verify this hypothesis. The inflammatory response was induced in DPSCs using lipopolysaccharide (LPS). Then, the effect of let-7c-5p overexpression on the DPSC viability, expression of DMP1, and activity of inflammation-related signaling was assessed. Moreover, the expression of DMP1 was induced in let-7c-5p-overexpressed DPSCs to determine whether the effect of let-7c-5p on inflammation in DPSCs was exerted in a DMP1-inhibition-dependent manner. The data derived from in vitro assays were further verified in rat pulpitis models. Findings outlined in the current study show that let-7c-5p had an anti-inflammation effect in DPSCs by suppressing DMP1 function, which contributes to the amelioration of pulpitis.

Material and Methods

Antibodies and chemicals

Antibody against DMP1 (cat. no. GTX55589) was obtained from GeneTex (USA). Antibodies against IκBα (cat. no. #9242), phosphorylated IκBα (p-IκBα) (cat. no. #2859), IKKβ (cat. no. #8943), NF-κB subunit p65 (cat. no. #8242), and Histone H3 (cat. no. #4499) were purchased from Cell Signaling Technology (USA). Antibody against p-IκBα (cat. no. ab59195) was purchased from Abcam (USA). Secondary goat anti-rabbit (cat. no. A0208) IgG-HRP antibody was obtained from Beyotime Biotechnology (China). Antibody against β-actin (cat. no. bsm-33139M) was purchased from Bioss (China). Lipopolysaccharides (LPS) (cat. no. L8880) was purchased from Solarbio (China). Rat let-7c-5p agomir was obtained from GenePharma (China), Trizol (cat. no. RP1002), super M-MLV reverse transcriptase (cat. no. RP6502), and 2×Power Taq PCR MasterMix (cat. no. PR1702) were purchased from Bioteke (China). SYBR Green (cat. no. SY1020) was purchased from Solarbio (China). RIPA lysis buffer (cat. no. P0013B), Plasma and Nuclear Protein Extraction Kit (cat. no. P0027), and Protein Concentration Determining Kit using BCA method (cat. no. P0009) were purchased from Beyotime Biotechnology (China). 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) (cat. no. M-2128) was obtained from Sigma (USA). Recombinant Mouse DMP-1 Protein (cat. no. 4386-DM-050) was purchased from Sigma (USA). Enzyme-linked Immunosorbent Assay (ELISA) Kits for detection interleukin 1β (IL-1β) (cat. no. EK301B1/2) and tumor necrosis factor α (TNF-α) (cat. no. EK3821/2) were purchased from Multi Sciences (China).

Cell culture

Eight-week-old Sprague Dawley (SD) rats (Huafulang Bioscience Co. Inc., Beijing, China) were killed by i.p. injection of an overdose of pentobarbital sodium and dental tissues were collected. After removing the soft tissues, dentinal cartilage was dissected to expose dental pulp tissues. Then, the dental pulp tissues...
were cut into pieces and incubated with collagenase type I (3 mg/ml) and neutral protease (3 mg/ml) at 37°C for 20 min. The tissues were then filtered and collected via centrifugation at 309×g for 7 min, repeating these procedures until the tissues were fully digested. Cells were re-suspended and preserved at 37°C in an atmosphere consisting of 5% CO₂ and 95% air. The expression statuses of CD29, CD90, CD146, CD105, CD34, and CD45 were detected with immunofluorescence. All the assays with animals were performed following the Institutional Animal Ethics Committee and Guidelines for the Care and Use of Animals of Jilin University.

Lentiviral vector construction, virus infection, and cell administration

Lentivirus plasmid expression let-7c-5p (5'-UGAGGUAGUA GGUGUAGUUGU-3') were constructed and packaged by GenePharma (Shanghai, China). For infection, DPSCs and different lentivirus particles were co-plated into 1 well of 6-well plates at a ratio of 1: 50. Cells stably expressing let-7c-5p were selected with 3 μg/ml ampicillin. The infection efficiency was validated with reverse transcription real time polymerase chain reaction (RT-q-PCR). For induction of inflammatory response, cells were incubated with LPS (10 μg/ml) for 24 h. For validation of the role of DMP1 in the effect of let-7c-5p on DPSCs, the let-7c-5p-expressed DPSCs were further incubated with DMP-1 protein (10 ng/ml) for 6 h after LPS administration.

Immunofluorescence

DPSCs were seeded in 14-well chambers and fixed with 4% paraformaldehyde for 15 min before being permeabilized with 0.1% Triton X-100 for 30 min. After being incubated with 10% goat serum for 15 min at room temperature, cells were incubated with the primary antibodies at 4°C overnight. Following 3 washings using PBS, secondary Cy3-labeled antibody was added and incubated for 1 h at room temperature in the dark. After being washed with PBS, the cells were stained with 4, 6-diamino-2-phenyl indole (DAPI) for 5 min. The images were captured with a fluorescent microscope (BX53, Olympus, Japan) at 400× magnification.

RT-q-PCR

Total RNA in different groups was extracted using Trizol method. cDNA templates were achieved using super M-MLV reverse transcriptase from total RNA. The reaction mixtures of real-time PCR contained 10 μl 2×Power Taq PCR MasterMix, 0.3 μl SYBR GREEN, 0.5 μl of each primer (forward: 5'-GTGCAGGGTCCGAGGTATTC-3', backward: 5'-GCTTCGG 7c-5p, forward: 5'-CGTGCGGTGAGGTAGTAGGTT-3', backward: 5'-GTGGAGCTGGCAGGTATTC-3'); U6, forward: 5'-GCTTCGG 7c-5p, backward: 5'-GCTTCGG 7c-5p, forward: 5'-GTGGAGCTGGCAGGTATTC-3', backward: 5'-GTGGAGCTGGCAGGTATTC-3'), 1 μl cDNA template, and 7.7 μl ddH₂O. Thermal cycling parameters were set as followings: a denaturation step at 94°C for 2 min, followed by 40 cycles of amplification at 94°C for 15 s, 60°C for 15 s and 72°C for 15 s, then the signaling was detected using ExicyclerTM 96 (BIONEER, South Korea). The relative expression levels of the let-7c-5p were calculated using according to the formula of 2^{-ΔΔCt}.

MTT assay

Cell viability was detected using MTT assay and DPSCs in different groups were then incubated with 0.5 mg/ml MTT for another 4 h at 37°C. The supernatant was aspirated and 150 μl DMSO was added into each well. The cell viability was represented by the OD₅₇₀ value detected using a microplate reader (ELX-800, BOTEK, USA).

ELISA

The production of IL-1β and TNF-α in cell cultures were detected using specific ELISA kits according to the manufacturers’ instructions. Briefly, cells were lysed using ultrasound and total protein was collected via centrifugation at 2500 rpm for 10 min. For ELISA detection, 20 μl protein sample and 50 μl antibody were incubated at room temperature for 2 h. Then, the mixture was reacted with 100 μl TMB at room temperature for 20 min in the dark and the OD values at 450 and 570 nm was determined using a microplate reader (ELX-800, BOTEK, USA).

Western blotting

Total cellular protein in DPSCs or dental pulp tissues was extracted using 1% PMSF. Protein concentration was determined using a microplate reader and DPSCs in different groups were then incubated with 0.5 mg/ml MTT for another 4 h at 37°C. The cell viability was represented by the OD₅₇₀ value detected using a microplate reader (ELX-800, BOTEK, USA).

Rat pulpitis induction and let-7c-5p agonism administration

Six-week-old male SD rats were anesthetized with 50 mg/kg body weight pentobarbital sodium and 2 mm of mandibular left and right incisor crowns were removed by a diamond disc
to expose the pulp chamber. After disinfection with 70% ethanol, the coronal pulp chamber was exposed to a sterile paper point infiltrated with LPS (10 mg/ml, 1 ml) for 9 h. Let-7c-5p agomir (50 pM, 10 μl) was injected to the alveolar mucosa of rats. After pulpitis induction, rats were killed by i.p. injection with an overdose of pentobarbital sodium and dental pulp tissues were collected and preserved at –80°C.

Hematoxylin and eosin (H&E) staining

Dental pulp tissues were dehydrated using different concentrations of alcohol and vitrified in dimethylbenzene. Samples of different groups were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). The results were recorded under a microscope (DP73, OLUMPUS, Japan) at 200× magnification.

Immunohistochemistry (IHC)

Dental tissues were dehydrated using different concentrations of alcohol and fixed with dimethylbenzene for 30 min. After incubating in antigen retrieval buffers for 30 min, the sections were incubated with primary antibody for DMP1 (1: 100) at 4°C overnight. Secondary antibodies (1: 200) were added onto the tissues and incubated for 30 min at 37°C. Thereafter, DAB was then added and incubated for 3–10 min before the reaction was stopped. The sections were re-stained with hematoxylin and dehydrated. The results were detected using a microscope (DP73, Olympus, Japan) at 400× magnification.
Statistical analysis

Data are expressed as mean ± standard deviation (SD). One-way ANOVA and post hoc multiple comparisons with Duncan method were performed with a significance level of 0.05 (two-tailed P value). The statistical analyses were performed using GraphPad Prism version 6.0 software (GraphPad Software, Inc., San Diego, CA).

Results

Isolation and characterization of DPSCs

DPSCs were successfully generated in the current study: results of immunofluorescence assay showed that the cells were positive for CD90, CD105, CD29, and CD146 and were negative for CD34 and CD45, showing a typical surface antigen characteristic of DPSCs (Supplementary Figure 1) [2].

Let-7c-5p attenuated LPS-induced inflammatory responses in DPSCs

The DPSCs were infected with NC particles or let-7c-5p expression particles (Figure 1A) before incubation with 10 μg/ml LPS for 24 h to induce inflammation. As shown in Figure 1B, incubation of LPS increased the production of pro-inflammation cytokines compared with the Blank group (P<0.05), including IL-1β and TNF-α. However, infection of let-7c-5p lentivirus particles rescued DPSCs from the impairments of LPS administration and production of IL-1β and TNF-α was inhibited by let-7c-5p (Figure 1B).

Let-7c-5p increased the cell viability in inflammatory DPSCs

The induced inflammatory response suppressed the viability of DPSCs. As detected by MTT assay, DPSCs incubated with LPS had a lower level of OD\textsubscript{570} value in comparison to the Blank group (P<0.05) (Figure 1C), but in DPSCs overexpressing let-7c-5p, cell viability was increased to relatively normal level (Figure 1C), indicating the protective effect of let-7c-5p on DPSCs against inflammation.

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Let-7c-5p attenuated the LPS-induced inflammatory responses by inhibiting DMP1 expression and NF-κB signaling in DPSCs

The expression of DMP1 in DPSCs as well as NF-κB signaling was detected to demonstrate the mechanism driving the function of let-7c-5p. It was found that LPS administration increased the expressions of DMP1, p-IκBα, and p-IKKβ, and nuclear translocation of NF-κB p65, but decreased the level of IκBα (Figure 2), showing activation of the NF-κB pathway. The infection of let-7c-5p particles reversed the expression patterns of all indicators, as expected. The results preliminarily indicated that the effect of let-7c-5p in protecting DPSCs was exerted through a DMP1 and NF-κB-inhibition manner.

DMP1 inhibition mediated the inflammation suppressing function of let-7c-5p

To explicitly explain the role of DMP1 in the inflammation-suppressing effect of let-7c-5p, the let-7c-5p-expressed DPSCs were further treated with DMP1 protein. The results showed that the inhibited production of IL-1β and TNF-α by let-7c-5p was again induced by DMP1 (Figure 3A). DMP1 also impaired the let-7c-5p’s effect on DPSC viability, significantly decreasing the OD570 value when compared with the let-7c-5p group (Figure 3B).

Figure 3. let-7c-5p inhibited activation of the NF-κB pathway in a DMP1-inhibition-dependent manner. DPSCs with stable let-7c-5p expression were first incubated with 10 μg/ml LPS for 24 h and then subjected to 10 ng/ml DMP1 protein for 6 h. The production of IL-1β and TNF-α, the cell viability, and the expression of DMP1 and NF-κB pathway members were detected with ELISA, MTT assay, and Western blotting, respectively. Administration of DPSCs with DMP1 protein counteracted the effect of let-7c-5p on LPS-induced impairments in DPSCs, increasing the production of IL-1β (A) and TNF-α (B), decreasing cell viability (C), and activating NF-κB signaling. * P<0.05 vs. let-7c-5p group. Each assay was represented by 3 replicates.
Figure 4. *let-7c-5p* ameliorated LPS-induced pulpitis by suppressing DMP1-mediated NF-κB signaling in vivo. SD rats were first injected with NC or *let-7c-5p* agomir and then subjected to pulpitis induction using LPS. At 9 h after LPS administration, rats were sacrificed and the dental tissues were collected for H&E staining, immunochemistry, RT^2^-PCR, and Western blot analysis. LPS decreased the level of *let-7c-5p* in dental tissues and injection of *let-7c-5p* agomir restored the level (A). As shown by H&E staining, LPS induced neutrophil infiltration in dental tissues and *let-7c-5p* agomir inhibited the LPS-induced neutrophils infiltration (B). LPS also increased the expression and distribution of DMP1 (C, D), which further induced the activation of NF-κB signaling (D). However, increased expression of *let-7c-5p* by agomir injection effectively inhibited the LPS-induced activation of DMP1/NF-κB signaling transduction. * P<0.05 vs. Blank group. * P<0.05 vs. LPS group. Arrows indicate neutrophil infiltration detected by H&E staining. Scale bar for H&E staining, 100 μm. Scale bar for immunochemistry, 50 μm. Each assay was represented by 6 replicates.
Regarding NF-κB signaling, the administration of DMP1 increased the expressions of DMP1, p-IκBα, and p-IKKβ, and nuclear translocation of NF-κB p65 but decreased the level of IκBα even in the presence of let-7c-5p overexpression (Figure 3C).

**Let-7c-5p attenuated the LPS-induced pulpitis by inhibiting DMP1/NF-κB signaling in SD rats**

Rats were first injected with let-7c-5p agomir (Figure 4A) and subjected to pulpitis induction by LPS. The results of H&E staining showed a higher level of neutrophils infiltration in the LPS group when compared with the Blank group (Figure 4B), showing the induction of inflammation in dental pulp tissues of rats. Similar to the results of in vitro assays, the expression and distribution of DMP1 (stained dark brown in IHC) was expanded in dental pulp tissues by the LPS administration (Figure 4C, 4D), which led to the activation of NF-κB signaling (Figure 4D). In rats pre-injected with let-7c-5p agomir, the effect of LPS was diminished; the lower level of neutrophil infiltration was accompanied by the suppressed activity of DMP1/NF-κB signaling (Figure 4D), further confirming the DPSC-protecting effect of let-7c-5p during pulpitis.

**Discussion**

The role of miR in regulating inflammatory response is complex in that a single miR member can have diverse targets. Regarding the interaction between miRs and inflammation in dental pulp tissues, different studies have identified hundreds of dys-expressed miR molecules, of which let-7c-5p has been proposed as an anti-inflammation factor during the development of inflammatory responses in dental pulp tissues [13], suggesting the possibility that the factor participates in the development of inflammatory responses in dental pulp tissues. Given the interaction between let-7c-5p and DMP1, it is reasonable to hypothesize that the anti-inflammation function of let-7c-5p depends on the inhibition of DMP1 function. Therefore, let-7c-5p overexpression DPSCs were subjected to DMP1 protein incubation. Higher DMP1 levels counteracted the anti-inflammation effect of let-7c-5p, increasing the production of pro-inflammatory cytokines, and decreasing DPSC viability. Moreover, we found that the administration of DMP1 activated NF-κB signaling, partially explaining the mechanism driving the effect of let-7c-5p on dental pulp inflammation, we focused on the function of DMP1. It was previously reported that DMP1 is a direct target of let-7c-5p [9] and DMP1 is induced during pulpitis [13]. The molecule is also involved in the regulation of mineralization of bone and dentin [9]. The deficiency of DMP1 causes hypomineralization and dental deformity in model animals [26]. However, the over-production of DMP1 is associated with augmented inflammatory response in dental pulp tissues [13], suggesting the possibility that the factor participates in the development of inflammatory responses in dental pulp tissues. Given the interaction between let-7c-5p and DMP1, it is reasonable to hypothesize that the anti-inflammation function of let-7c-5p depends on the inhibition of DMP1 function. Therefore, let-7c-5p correlated with the anti-inflammatory effect of let-7c-5p, increasing the production of pro-inflammatory cytokines, and decreasing DPSC viability. Moreover, we found that the administration of DMP1 activated NF-κB signaling, partially explaining the pathway through which DMP1 participates in the inflammatory responses in dental pulp tissues. These results help explain the mechanism through which let-7c-5p inhibits the NF-κB pathway, but further research is needed to clearly define the interaction between DMP1 and the NF-κB pathway.

**Conclusions**

**let-7c-5p** was shown to protect dental pulp cells against LPS-induced inflammation and the effect was exerted by inhibiting DMP1-mediated NF-κB pathway activation. However, a full elucidation of the role of DMP1 in the inflammation of dental pulp tissues is needed to elucidate the downstream pathway.
transducing the anti-inflammation effect of let-7c-5p and to identify novel therapeutic targets for treating dental inflammatory disorders.

**Conflict of interest**

None.

**Supplementary Figure**

*Supplementary Figure 1.* Identification of DPSCs with immunofluorescence. DPSCs were isolated from dental tissues of SD rats and the molecular surface antigen markers in the cells were analyzed with immunofluorescence. The results showed that cells were positive for CD90, CD105, CD29, and CD146 while negative for CD34 and CD45. Magnification, 400×.
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