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Original Article

Protective effect of SIRT6 against LPS-induced human dental pulp cell apoptosis via regulating Ku70 deacetylation

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Abstract: Dental pulpitis is the major reason resulting in the loss of dental body. Human dental pulp cells can provide nutrients and repair for dentin, thus to maintain normal structure and function of dental pulp tissues. Inhibitory protein Ku70 inhibits Bax-induced mitochondrial apoptosis pathway via binding to Bax, thus playing an important role in cell apoptosis. The deacetylation of Ku70 facilitates Bax binding. SIRT6 is a type of deacetylase belongning to Sirtuins familiy. Its role in regulating Ku70 deacetylation and further protection against apoptosis of dental pulp cells, however, is still unknown. *In vitro* culture of human dental pulp cells were randomly assigned into LPS (60 μg/ml) group, empty plasmid group, and SIRT6 group which received the transfection of SIRT6-over-expressing vectors. Real-time PCR and ELISA were used to determine SIRT6 expression, while MTT assay was applied to evaluate cell proliferation. Flow cytometry and co-immunoprecipitation were adopted to detect cell apoptosis and Ku70 deacetylation, respectively. Caspase 3 activity was then quantified by assay kit. SIRT6 expression was significantly decreased in LPS-treated cells, along with lower cell proliferation, elevated apoptosis, and enhanced caspase 3 activity ($P<0.05$ compared to control group). Overexpression of SIRT6 significantly facilitated cell proliferation and suppressed apoptosis, along with lower caspase 3 activity and de-acetylation of Ku70 ($P<0.05$ compared to LPS). SIRT6 exerts protective effects against the apoptosis of human dental pulp cells via regulating Ku70 deacetylation.

Keywords: SIRT6, Ku70, deacetylation, dental pulp cells, LPS

Introduction

Dental pulpitis is an inflammatory disease manifested as severe pains. It can cause the loss of dentin or affect other organs, making it a common disease in dental clinics [1, 2]. Human dental pulp cells locate within dental pulp cavity that provide nutrients and repairmen, and protect against external stimuli for dentin [3, 4]. Damage of dental pulp tissues makes dentin fragile, and may affect mastication functions. Therefore, the protection of dental pulp cells and alleviation of cell apoptosis benefit the maintaining of normal cell structure and function [5, 6].

Ku70 regulates Bax activation and inhibits Bax-induced mitochondrial apoptotic pathway via binding to Bax, thus playing a crucial role in cell apoptosis. The interaction between Ku70 and Bax is under the regulation of the acetylation status of Ku70 [7, 8]. As one member of silent mating type information regulation homology (Sirtuins) family, SIRT6 is widely distributed in animal. In contrast to other members, SIRT6 shows activities of both NDA^+-dependent and NDA^+-independent pathways [9, 10]. SIRT6 can repair DNA damage, regulate inflammatory response, participate in glucose and lipid metabolism, and is an important tumor inhibitory protein [11, 12]. The effect of deacetylase SIRT6 on Ku70 deacetylation, and further protective effects on the apoptosis of human dental pulp cells, however, remains unknown yet.

Materials and methods

Major reagent and equipment

Sodium pentobarbital and lidocaine were purchased from Zhaohui Pharm (China). α-MEM

medium, fetal bovine serum (FBS), and penicillin-streptomycin were purchased from Hyclone (US). DMSO and MTT powders were purchased from Gibco (US). Trypsin-EDTA digestion buffer was purchased from Sigma (US). P.g-LPS was purchased from Invitrogen (US). E. Coli DH5a and pCDNA3.1 plasmid were purchased from Roche (US). Caspase 3 activity assay kit was purchased from Jiancheng (China). Surgical instruments were purchased from Suzhou Medical Instrument (China). RNA extraction kit and reverse transcription kit were purchased from Axygen (US). Annexin V-PI double staining flow cytometry assay kit for cell apoptosis was purchased from BD (US). PVDF membrane was purchased from Pall Life Sciences (US). EDTA was obtained from Hyclone (US). Western blotting reagent was purchased from Beyotime (China). ECL kit was purchased from Amersham Biosciences (US). Rabbit anti-human Bax monoclonal antibody, rabbit anti-human Ku70 monoclonal antibody, anti-keratin monoclonal antibody, and mouse anti-rabbit horseradish peroxidase (HRP)-labelled IgG secondary antibody were obtained from Cell signaling (US). PCR amplification kit and product purification kit were purchased from Promega (US). ELISA kit for SIRT6 was purchased from R&D (US). Plasmid extraction kit and restriction digestion enzyme were purchased from Roche (US). SAB immunohistochemistry kit was purchased from Boster (China). FACS Calibur flow cytometry was a product of BD (US). Fluorescent quantitative PCR cycler (7700 Fast) was produced by ABI (US). Primers were designed by Primer 6.0 and were synthesized by Yingjun (China). Microplate reader was a product of BD (US). Other common reagents were obtained from Sangon (China).

Cultivation and identification of dental pulp cells in vitro

The third molar (dentes sapientiae) was collected from dental orthodontics surgery, in which iodine tincture and ethanol were used for sterilization to keep intact dental crown. Obtained dentin was rinsed in saline. After rinsed in PBS for three times, dental crown and root were incised along with groove to extract dental pulp. The pulp tissue was washed by α-MEM medium containing penicillin-streptomycin repeatedly, and then incised into 1 mm^3 cubes, which were layered at the bottom of 6-well plate. 2 ml DMEM medium (90% high glucose, containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin) was added to the plate for 37°C incubation in a chamber with 5% CO_2. The medium was changed every three days. Cells were passaged when reaching 60% confluence. Cell suspension was moved into 25 ml culture flask with medium changing every other day. Cells were passaged every 2~3 days. Dental pulp cells at logarithmic phase (2~8^{th} generation) were used for further experiment.

Immunohistochemistry

Dental pulp cells were mounted onto glass slides inside 6-well plate and fixed in 4% paraformaldehyde for 20 min. After treated with 0.1% TritonX-100 and H_2O_2, the cells were blocked by 5% bovine serum albumin (BSA). Anti-keratin primary antibody (20 μl) was added at 4°C and incubated overnight. On the next day, after washed by PBS, the cells were incubated in 20 μl secondary antibody at 37°C for 45-m. Reagent A and B (20 μl each) were added to develop the slide, and then the slide was observed under the microscope.

Cell treatment and group

Cultured dental pulp cells were randomly assigned into control group, LPS group (treated with 60 μg/ml LPS), empty plasmid group, and SIRT6 group (LPS treated cells transfected with SIRT6-overexpression vector).

SIRT6 overexpression vector construction and transfection

Based on sequence of SIRT6 gene, primers were designed (Forward, 5'-AGCTA GCTTT TCAGA CTGAT GTTGA G-3'; Reverse, 5'-GATCC TCAAC ATCAG TCTGA TAAGC TA-3'. PCR conditions were: 95°C 2 min, and 50°C 1 min, followed by 35 cycles each containing 94°C 30 s, 60°C 50 s and 72°C 35 s. PCR product purification kit was used to extract DNA from agarose gel. After ligated with pcDNA3.1 plasmid (3:1 ratio) at 4°C for 16 h, the recombinant SIRT6-pcDNA3.1 vector was transfected into competent cells. In brief, SIRT6-pcDNA3.1 plasmid or empty plasmid was mixed with 200 μl serum-free medium for 15-min incubation at room temperature. Lipo2000 dilution was mixed with plasmid dilutions for 30-min incubation at room temperature. Cultured cells were changed to

Table 1. Primer sequence

Target gene	Forward primer 5'-3'	Reverse primer 5'-3'
GADPH	ACCAGGTATCTGCTGGTTG	TAACCATGATGTCAGCGTGGT
SIRT6	AGCTAGCTTTTCAGACTGATGTTGAG	GATCCTCAACATCAGTCTGATAAGCTA

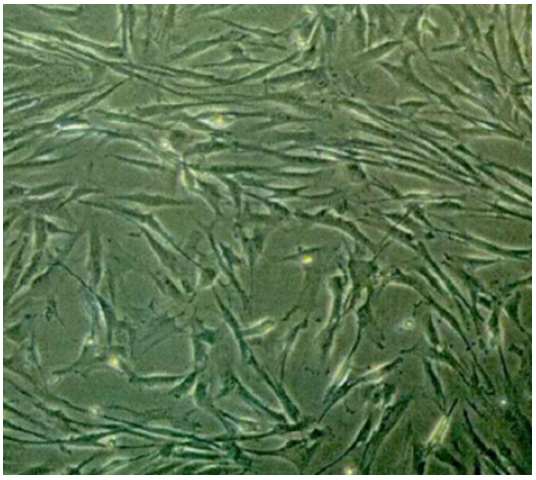

Figure 1. Light-field microscopic image of cultured human dental pulp cells (×200).

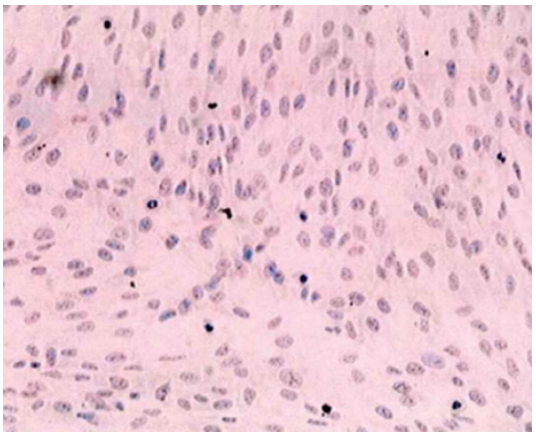

Figure 2. Immunohistochemistry staining of human dental pulp cells (×100).

serum and washed with PBS. 1.6 ml serum-free medium was then added, followed by 37°C incubation in a chamber with 5% CO_2 for 6 h. Serum-containing medium was then applied for 48 h continuous incubation in further study.

Real-time PCR

Trizol reagent was used to extract RNA from cells following manual instruction. Reverse transcription was performed to synthesize cDNA. Primers were designed by Primer 6.0 based on target gene sequence (**Table 1**). Real-time PCR was applied under the following conditions: 55°C for 1 min, followed by 35 cycles each containing 92°C for 30 s, 58°C for 45 s, and 72°C for 35 s. Data were collected by PCR cycler. GAPDH was selected as the reference. Fluorescence level was used to calculate CT value of all samples and standards. Standard curve was plotted for semi-quantitative analysis by $2^{-\Delta Ct}$ method.

ELISA for measuring SIRT6 secretion

Rat serum samples were kept in -80°C fridge. ELISA kit was used to detect the expression level of SIRT6 in rat serum following manual instruction of test kit. Optical density (OD) values at 450 nm wavelength were measured within 15 min of quenching. Linear regression function was obtained based on OD values of standard sample concentrations and respective OD values. Concentration of samples was then deduced from OD values.

MTT assay for cell proliferation

Dental pulp cells at logarithmic phase were inoculated into 96-well plate containing DMEM medium with 15% FBS at 5×10^3 per ml. After 24 h incubation, the supernatant was discarded. Cells were randomly assigned into control, LPS, empty plasmid, and SIRT6 groups as previously described. After 24 h incubation, 20 μl sterilized MTT was added into each well (N=3 at each time point). Supernatant was removed after 4 h, with the addition of 150 μl DMSO for 10 min vortex until complete dissolution of violet crystals. Absorbance value (A) at 570 nm was measured to calculate cell proliferation rate.

Caspase 3 activity assay

The activity of caspase 3 in cells was measured using test kit following manual instruction. Cells were digested by trypsin, and centrifuged for 5 min at 4°C. Supernatant was discarded with the addition of lysis buffer for ice incubation (15 min). The mixture was then centrifuged at 4°C for 5 min, followed by adding 2 mM Ac-DECD-pNA. OD values at 405 nm were measured to reflect caspase 3 activity.

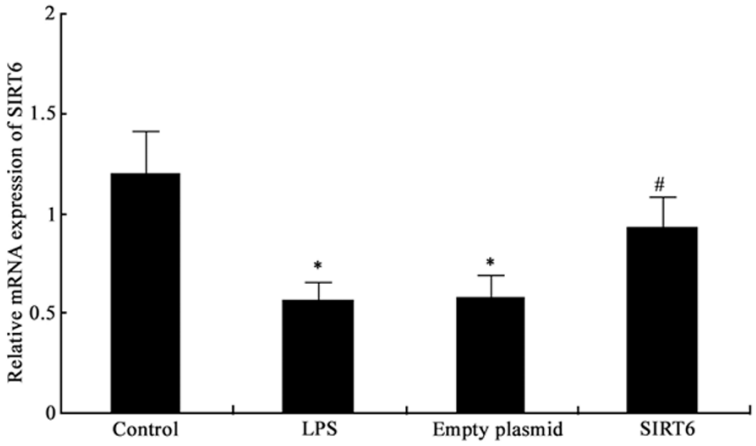

Figure 3. SIRT6 mRNA expression level in dental pulp cells. *, P<0.05 compared to control group; #, P<0.05 compared to LPS group.

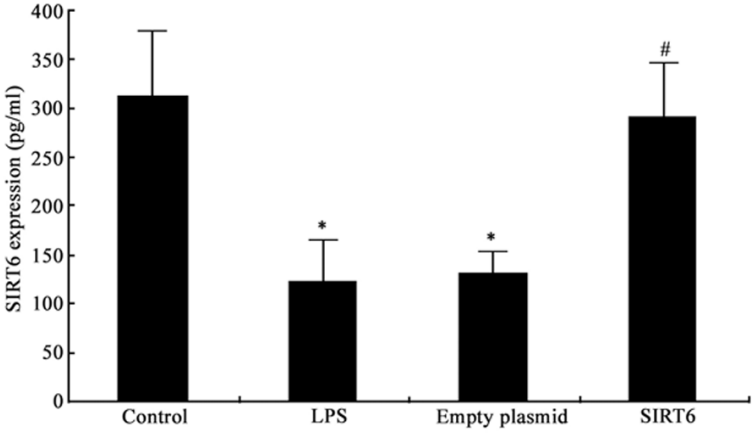

Figure 4. SIRT6 secretion in supernatant of human dental pulp cells. *, P<0.05 compared to control group; #, P<0.05 compared to LPS group.

Flow cytometry for dental pulp cell apoptosis

Dental pulp cells were digested and inoculated into 50 ml culture flask at 5×10^5/ml concentration and were randomly divided into four groups as abovementioned. After 48 h of transfection, cells were digested, counted, and collected (2×10^6/ml). Cells were rinsed in PBS by 1000 rpm centrifugation for 5 min and fixed in 75% cold ethanol at 4°C overnight. Ethanol was discarded, while cells were re-centrifuged in PBS at 1000 rpm centrifugation for 5 min. Cells were re-suspended in 800 μl PBS containing 1% BSA. 100 μg/ml PI dye (in 3.8% sodium citrate, pH 7.0) was added with 100 μl RnaseA (10 mg/ml) for 30 min avoid of light incubation at 37°C. Flow cytometry was applied, and data analysis was performed in FCSExpress 3.0 software.

Co-immunoprecipitation for Ku70 deacetylation

Proteins were firstly extracted from dental pulp cells. In brief, cells were lysed on ice for 15~30 min by lysis buffer. Cells were then ruptured by ultrasound (5 s, 4 times). The mixture was centrifuged at 10000 g for 15 min at 4°C. The supernatant was transferred to a new tube. Protein contents was quantified and stored at -20°C. Protein samples were added with 5 μl A/G agarose beads and 2 μg antibody, followed by lysis buffer to reaching 1 ml final volume. The mixture was homogenized for 3 h. Beads were rinsed in 1 ml lysis buffer for 3 times using 4°C centrifugation (3000 rpm) for 3 min. 50 μl SDS loading buffer was mixed with protein sampled for 10 min boiling denature. Samples after centrifugation were separated in 10% SDS-PAGE, and were transferred to PVDF membrane by semi-dry method. Non-specific binding sites were removed by 5% defatted milk powder for 2 h incubation. VEGF antibody (1:1000 dilutions) was added for 4°C overnight incubation. PBST was used to rinse the membrane, followed by dark incubation for 30 min at room temperature using goat anti-rabbit secondary antibody (1:2000). After PBST rinsing, chemiluminescent agent was added to develop the membrane for 1 min. X-ray was used to expose the film, which was scanned in protein gel image processing system and was analyzed in Quantity One software. Each experiment was repeated for four times (N=4) for statistical analysis to reveal the interaction between proteins.

Statistical processing

SPSS19.0 software was used to analyze all data, of which measurement data were presented as mean ± standard deviation. One-way

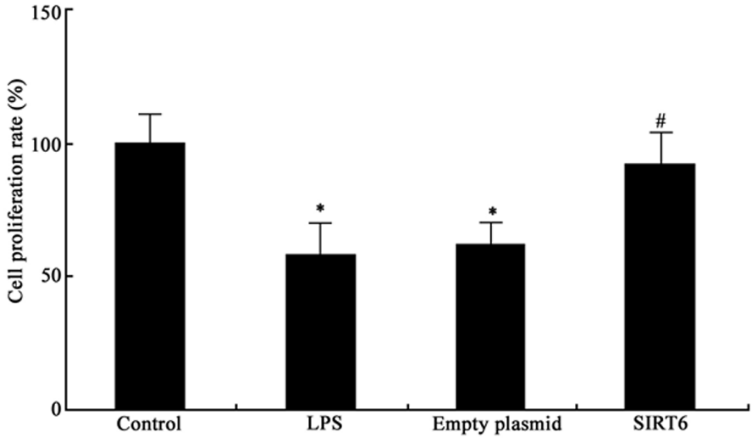

Figure 5. Effect of SIRT6 on proliferation of LPS-induced human dental pulp cells. *, P<0.05 compared to control group; #, P<0.05 compared to LPS group.

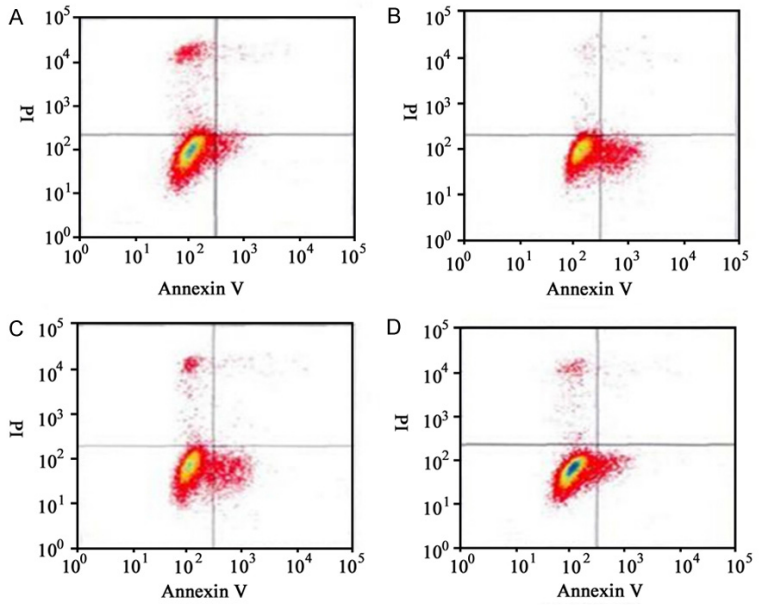

Figure 6. Human dental pulp cell apoptosis after SIRT6 transfection. A. Control; B. LPS; C. Empty plasmid; D. SIRT6.

analysis of variance (ANOVA) was used to compare means among multiple groups, followed by LSD test in comparison. A statistical significance was defined as P<0.05.

Results

Culture and identification of human dental pulp cells

In vitro cultured human dental pulp cells showed spindle or polygon shape in spiral-like arrangement. Cells exhibited abundant cytoplasm and centrally located nucleus (**Figure 1**). Immunohistochemistry staining against keratin revealed signal in nucleus instead of cytoplasm (**Figure 2**), suggesting dental pulp cells.

SIRT6 mRNA expression in human dental pulp cells

Real-time PCR was used to detect the expression alternation of SIRT6 mRNA in cultured human dental pulp cells. Results showed that in LPS-treated cells, SIRT6 mRNA level was significantly decreased compared to control group (P<0.05). The transfection of SIRT6 overexpression plasmid facilitated SIRT6 mRNA expression compared to LPS group (P<0.05, **Figure 3**).

SIRT6 secretion in cultured cells supernatant

ELISA was applied to detect the secretion of SIRT6 in the supernatant of cultured human dental pulp cells. Results showed significantly suppressed SIRT6 secretion in LPS treated cells (P<0.05 compared to control group). The over-expression of SIRT6 facilitated SIRT6 expression in supernatant of dental pulp cells compared to LPS group (P<0.05, **Figure 4**).

Effects of SIRT6 on proliferation of LPS-induced dental pulp cells

MTT assay was further adopted to detect cell proliferation after induction of LPS and transfection of SIRT6 plasmid. Results showed that LPS significantly inhibited the proliferation of dental pulp cells compared to control group (P<0.05). The transfection of SIRT6-overexpression plasmid on LPS-induced dental pulp cells facilitated cell survival compared to LPS group (P<0.05, **Figure 5**).

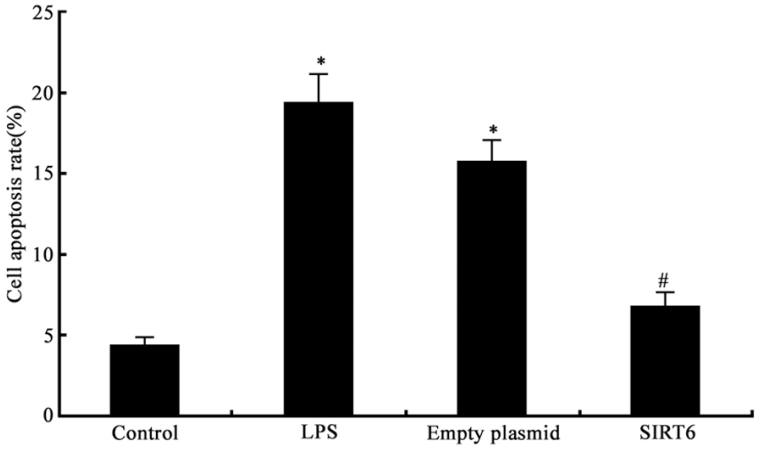

Figure 7. Effect of SIRT6 on LPS-induced apoptosis of human dental pulp cells. *, P<0.05 compared to control group; #, P<0.05 compared to LPS group.

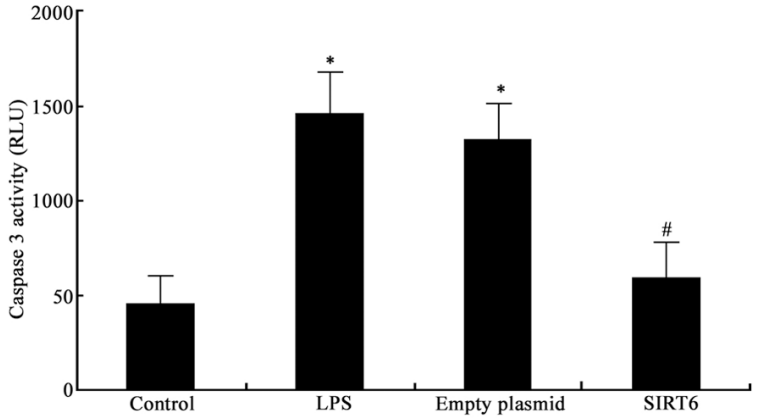

Figure 8. Effect of SIRT6 on LPS-induced caspase 3 activity in human dental pulp cells. *, P<0.05 compared to control group; #, P<0.05 compared to LPS group.

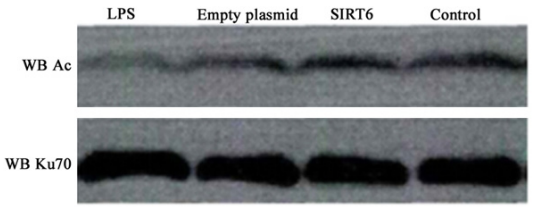

Figure 9. Effect of SIRT6 on deacetylation of Ku70 in LPS-induced human dental pulp cells.

Effect of SIRT6 on apoptosis of human dental pulp cells

Flow cytometry was used to analyze the apoptosis of LPS-induced human dental pulp cells after SIRT6 transfection. Results showed that LPS significantly facilitated cell apoptosis compared to control group (P<0.05). SIRT6-overexpression, however, inhibited LPS-induced cell apoptosis compared to LPS group (P<0.05, **Figures 6** and **7**). These results suggested that SIRT6 could enhance the proliferation of dental pulp cells via inhibiting LPS-induced cell apoptosis.

Effect of SIRT6 on caspase 3 activity of human dental pulp cells

Caspase 3 detection kit was used to determine the effect of SIRT6 on the caspase 3 activity in human dental pulp cells. Results showed that LPS significantly facilitated caspase 3 activity of dental pulp cells compared to control group (P<0.05). The overexpression of SIRT6 plasmid in LPS-induced dental pulp cells suppressed caspase 3 activity compared to LPS group (P< 0.05, **Figure 8**).

Regulatory effect of SIRT6 on deacetylation of Ku70 in LPS-induced human dental pulp cells

The effect of SIRT6 on deacetylation of Ku70 was measured by co-immunoprecipitation. Results showed that LPS induction weakened the interaction between Bax and Ku70 proteins. The transfection of SIRT6-overexpression vector in LPS-induced dental pulp cells enhanced such interaction (**Figure 9**). These results suggested that SIRT6 modulated Ku70 deacetylation, leading to Bax protein binding and the loss of apoptotic induction function.

Discussion

Dental pulpitis has complicated pathogenesis, among which bacterial infection is accepted as a major factor causing the disease. Bacterial infection leads to symptoms including tooth pain and dental pulp necrosis, and thus playing

an important role in the pathogenesis and progression of pulpitis [13, 14]. As the major component of Gram-negative bacteria on cell wall, endotoxin has certain lipid bilayer structure containing protein, LPS and phosphide. LPS is the dominant active ingredient of endotoxin and has multiple biological activities. It is the initiating factor for inflammation in immune response [15]. During the occurrence of pulpitis, LPS induced the release of large amounts of inflammatory factors, and brought toxicity on dental pulp cells directly or indirectly [16, 17]. High concentration of LPS can lead to inflammatory cascade reaction, during which bacteria are replicated in plasma, facilitating accumulation of LPS in blood circulation, thus breaking mitochondrial and lysosomal structure of cells, along with the disorder of glucose and energy metabolic disorder causing the progression of dental pulpitis [18].

Protein acetylation is an important protein modification pathway in maintaining cell homeostasis regarding histone modification and transcriptional factor regulation. Protein acetylation inside cytoplasm can regulate cell cycle and cell apoptotic proteins [19, 20]. SIRT6 is an important deacetylase. DNA repair protein Ku70 participates in transcriptional regulation and repair of DNA via specific binding. Its functions are under the regulation of histone acetylation and deacetylation [21]. In Bcl-2 family, Bax is a pro-apoptotic factor. As an important member of mitochondrial apoptotic pathway, Ku70 can regulate cell apoptosis via binding to Bax and inhibit Bax-induced mitochondrial apoptosis pathway. When Ku70 is undergone acetylation, its binding affinity with Bax is weakened and thus enhancing regulatory ability of Bax on cell apoptosis. On the contrary, deacetylation of Ku70 potentiated their binding affinity to inhibit Bax-induced cell apoptosis potency [11, 12]. This study showed elevated cell apoptosis after treating human dental pulp cells with LPS, as shown by higher caspase 3 activity, which further inhibited cell proliferation and survival, leading to lower SIRT6 expression. The overexpression of SIRT6 in LPS-induced human dental pulp cells significantly inhibited cell apoptosis by suppressing caspase 3 activity and facilitated dental pulp cell proliferation. Further mechanism study revealed the de-acetylation of Ku70 by over-expressing SIRT6 in LPS-induced human dental cells, for enhanced binding between Ku70 and Bax, leading to the suppression of Bax-induced cell apoptosis. Therefore, SIRT6 overexpression exerts protective effects on LPS-induced apoptosis of human dental pulp cells.

In summary, this study confirmed the protective effect against apoptosis of human dental pulp cells by SIRT6 via regulating Ku70 protein deacetylation. Our results provide new insights for searching drug target of dental pulpitis.

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Disclosure of conflict of interest

None.

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